The mechanisms underlying convergent evolution in the plumage patterns of birds

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This dissertation is submitted for the degree of Doctor of Philosophy

Declaration

This dissertation is my own work and contains nothing that is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text. This dissertation does not exceed the prescribed 60,000 word limit as prescribed by the Degree Committee.

Thanh-Lan Gluckman

Summary

Convergent evolution is a central theme in biology. Birds are an ideal system to examine the mechanisms underlying convergent evolution. Although bird patterning is diverse, within-feather patterns have repeatedly converged on the same four types: mottled patterns, scales, bars and spots. Other avian patterns occur, e.g. stripes, but are rare. In my thesis I examine the four main mechanisms underlying convergent evolution in plumage patterns: evolutionary genetics, evolutionary development, natural selection for signaling and camouflage. Japanese quail (*Coturnix japonica*) is a model system in developmental biology. Examining the developmental basis of pattern formation using molecular techniques, the dorsal patterning of embryonic quail is likely due to activation of the melanocortin-1 receptor, which is a highly conserved pathway in vertebrates. I examined whether a reaction-diffusion based theoretical model of pattern formation may predict developmental constraint in two groups that have different lifestyles and spectacular patterns: waterfowl (Anseriformes) and gamebirds (Galliformes). Tracing the evolutionary trajectory of pattern evolution with Bayesian comparative modeling there was evidence for developmental constraint in pattern evolution. Adaptive explanations may also result in convergence. Cuckoo-hawk mimicry has been demonstrated in the common cuckoo (*Cuculus canorus*) and the Eurasian sparrowhawk (*Accipiter nisus*), but may be prevalent in Old World cuckoos. Randomly selecting a parasitic cuckoo from each genera of Old World cuckoos and <8 sympatric raptors, I quantified their barred patterns using digital image analysis and found that parasitism can explain convergent evolution in the patterns of parasitic cuckoos and raptors. Patterns may have evolved due to ecological selection. Examining the patterns of 80% of all avian species worldwide, I found that habitat does not predict patterning, and that all four patterns are found in all habitats. These results demonstrate that the mechanisms of convergent evolution are diverse, and that development and natural selection have contributed to pattern evolution.

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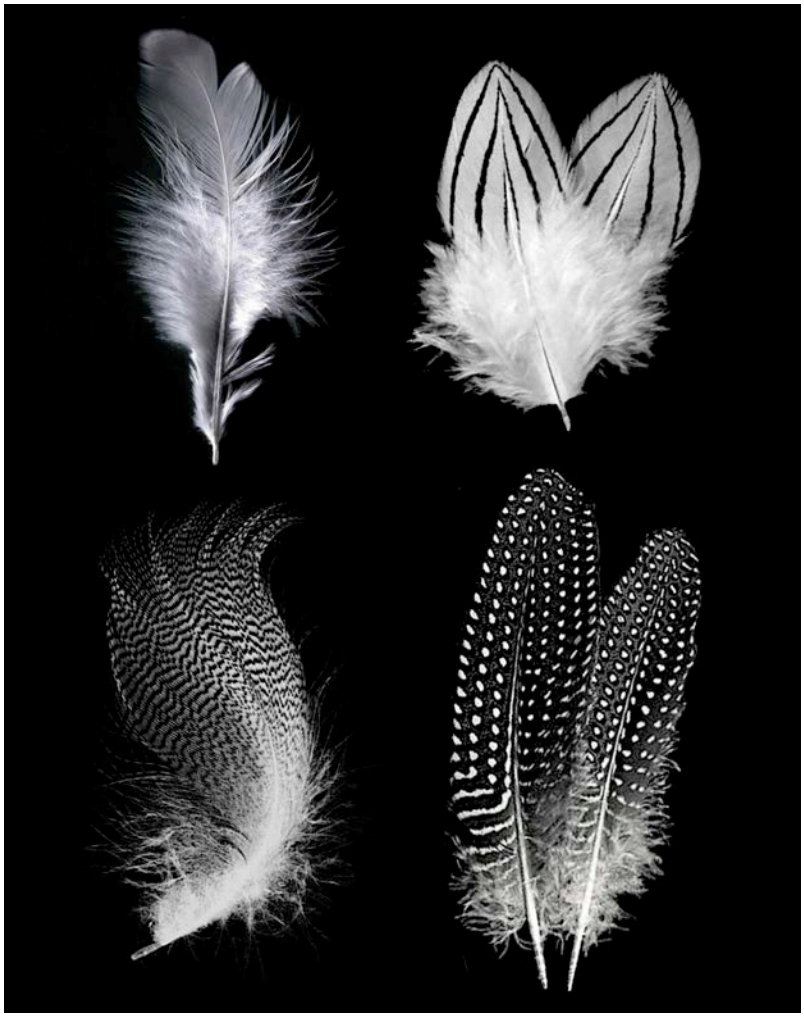


"*To create is to recombine*" (François Jacob, 1977).

The mechanisms underlying convergent evolution in the plumage patterns of birds

Summary

The remarkable diversity of the plumage of birds has captivated observers since ancient times. From the time of Charles Darwin and Alfred Wallace, avian phenotypes are classically considered to have evolved due to natural selection. Within-feather patterns, such as bars and spots, frequently occur in birds. In spite of the diversity in avian phenotypes, bird plumage patterns have predominantly converged on just four types of patterns: mottled, scaled, barred and spotted patterns. Other patterns have evolved in birds but are comparatively rare, e.g. stripes, squares, and triangles. The main mechanisms underlying convergent evolution in bird plumage patterns are currently unknown. The prevailing view is that plumage patterns function in camouflage and/or communication. However, that just four patterns have repeatedly evolved in birds implies that there may be developmental constraint in plumage pattern evolution. Developmental constraint as an evolutionary force is gaining momentum with proponents advocating an equivalent importance to natural selection. The relatively new field of evolutionary developmental biology (evo-devo) focuses on the means by which developmental processes may bias the evolution of morphological diversity. Evo-devo has the possibility to enhance our understanding of diversity by combining empirically derived principles of cellular and tissue morphogenesis to inform evolutionary processes. Natural selection in bird plumage has been studied widely. However, the majority of studies have focused on uniform patches of colouration, whereas the number of studies on plumage patterns is comparatively few. The main mechanisms underlying convergent evolution span the new field of evolutionary development, via modularity and developmental constraint, as well as neo-Darwinian theory, which spans natural selection for signaling as well as camouflage. In my PhD thesis I investigate how each of these major themes has contributed to convergent evolution in the plumage patterns of birds.

Introduction

The plumage of birds has captured the imagination since at least the time of Aristotle (384-322 BC) and has caused many an observer to ponder the existence of a diversity in the phenotypes of animals (Hunter 1780; Davies 2011). In particular, Charles Darwin and Alfred Russell Wallace emphasized the importance of natural selection in the evolutionary history of avian plumage. However, Darwin and Wallace broadly disagreed in key aspects of natural selection. Darwin emphasized the importance of extravagant traits such as the bright spotted train of the male peacock as a result of sexual selection (Darwin 1871), whereas Wallace stressed the importance of less brightly coloured "drab" plumage for camouflage (Darwin and Wallace 1858). Later, Wallace would deviate from his strictly adaptationist approach to argue that bright coloration may not in fact be adaptive, but may result from aspects of development: "Colour may be looked upon as a necessary result of the highly complex chemical constitution of animal tissues and fluids" (Wallace 1889).

Since Darwin and Wallace, studies of natural selection have attracted a wide range of research attention in many animal groups, and birds in particular have been studied widely (Andersson 1994). Melanin-based pigmentation is of particular interest as the mechanism of melanin synthesis is highly conserved in vertebrate animals. In birds, melanin can be uniform or spatially variable within feathers, and a range of hypotheses have been proposed to explain the function and evolution of avian melanin based traits (e.g. Prum and Williamson 2002; Majerus and Mundy 2003; Roulin et al. 2003; Mundy et al. 2004; Mundy 2005; Hill and McGraw 2006a; Hill and McGraw 2006b; Gluckman and Cardoso 2010; Emaresi et al. 2013; Emaresi et al. 2014). Recently, both empirical and macroevolutionary approaches have begun to demonstrate that our understanding of the process of natural selection is not always as imagined. For example, some studies have failed to find sexual selection on sexually dimorphic traits nor heritable genetic variation, that are key components of natural selection theory (e.g. Griffith, Owens, and Burke 1999; Hadfield et al. 2006; Westneat 2006).

The theory of Darwinian evolution is based on the principles of variation, heredity and natural selection (Darwin and Wallace 1858; Darwin 1868; Darwin 1869; Darwin 1871; Wallace 1889). At the time of the inception of Darwinian evolution, the modern founder of the science of genetics, Gregor Johann Mendel, had begun unraveling the rules of heredity (Mendel 1866) but his works remained largely unnoticed until their rediscovery in 1900 (Henig 2001). Genetics explained variation and heredity and its incorporation into Darwin's theory of evolution resulted in what is now known as the modern synthesis (Huxley 1942; Kutschera and Niklas 2004), which is the prevailing paradigm in evolutionary biology. In this current union of ideas, natural selection is regarded as the main mechanism of change that acts on the phenotype of populations of species' in the context of their environment. Under this model of evolution, the direct units that selection is acting upon are the genes involved in phenotypic change. For example, the rate of amino acid change in the melanocortin-1 receptor (*MC1R*) locus in birds is correlated with sexual dichromatism in galliform birds (Nadeau, Burke, and Mundy 2007) and changes at this locus are also involved in polymorphism which is a sexually selected trait in some avian species (Mundy et al. 2004). However, these same traits may be correlated with body size across birds, which implies a link between evolution and development in melanin-based avian plumage (Riegner 2008).

The field of evolutionary developmental biology (evo-devo) is a relatively new field of biology that studies the evolution of developmental processes and its contribution to morphological diversity. Evo-devo focuses on developmental plasticity, modularity in evolution and the regulatory mechanisms by which genes can be selectively turned on and off (West-Eberhard 2003; Klingenberg 2008). Seemingly disparate organisms may use the same genes, but these genes can be regulated differently between organisms to produce variation in dissimilar aspects of morphology. For example, bone morphogen proteins (BMPs) function in diverse aspects of morphology such as teeth and teeth suppression in mice and birds (Chen et al. 2000), beak morphology (Fritz et al. 2014) and human melanocortin systems (Giraldi et al. 2011). In the field of evo-devo, empirical genetic approaches applied at the level of cell and tissue morphogenesis are used to illuminate general principles of

development (Haag and Lenski 2011). For example, there are similarities in the developmental pathways that produce variation in mouse and avian phenotypes (Nadeau et al. 2008; Manceau et al. 2011) which is consistent with the idea of molecular economy, where evolution alters developmental processes to create novel structures from old or existing gene networks.

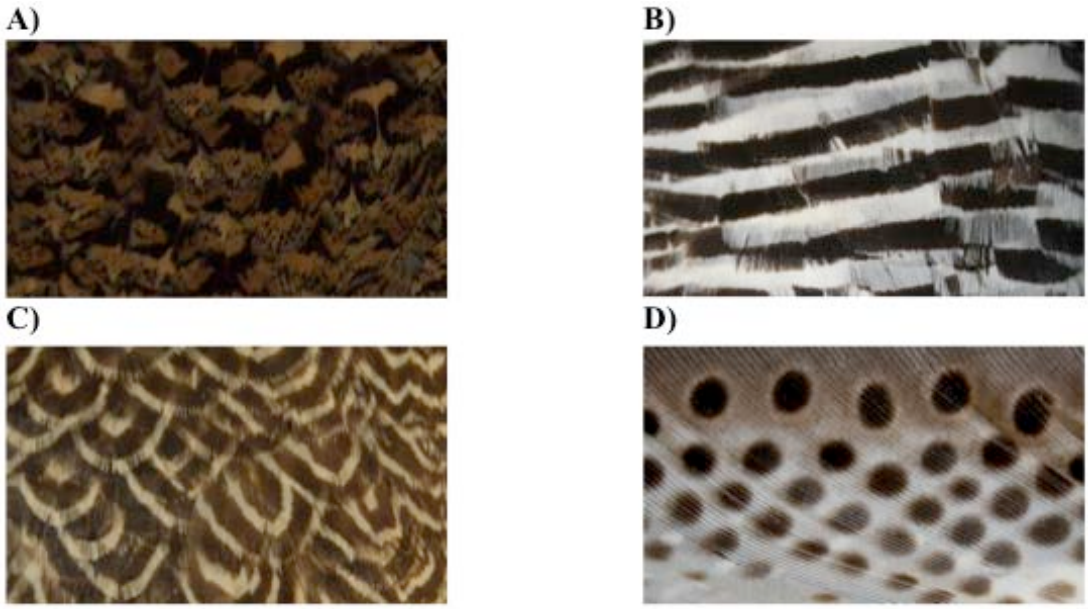


Fig. 1.1. The prevalent within-feather patterns of birds. In spite of the diversity of avian phenotypes, avian plumage patterns have repeatedly converged on the same four patterns: Irregular - a) mottled plumage in a female sharp-tailed grouse (*Tympanachus phasianellus*); Regular - b) barred plumage in a male Andean goose (*Chloephaga melanoptera*), c) scaled plumage in a male falcated duck (*Anas falcata*), d) spotted plumage in a male great argus (*Argusianus argus*). Figure taken from Marshall and Gluckman, *in review.*

Visual patterns, such as bars and spots, are common throughout the animal kingdom and are composed of a motif (sub-pattern) that is reiterated within and/or between feathers to create a patch of patterning (Fig. 1.1) (Prum and Williamson 2002; Kenward et al. 2004; Riegner 2008; Gluckman 2014). In birds, within-feather patterns are formed via differential control of melanin (Hill and McGraw 2006b) and

have predominantly converged on the same four motifs: mottled, scaled, barred, or spotted (Fig. 1.1). There are few other regularly repeating patterns in birds with the exception of stripes, checkered patterning, and triangles (Fig. 1.2), which are relatively rare across the class Aves (T-L. Gluckman *unpublished data*). Therefore, birds can make other types of patterns but instead repeatedly converge on the same four motifs. It is currently unknown why bird plumage patterns have repeatedly converged on these patterns and this is the subject of this PhD thesis.

A)  B) 

Fig. 1.2. Plumage patterns that appear to only occur in one species. A) The breeding checkerboard plumage pattern on the dorsal surface of the common loon (*Gavia immer*), and B) the triangles in the breast of the male Wood duck (*Aix sponsa*).

The mechanisms by which closely and distantly related species converge on the same phenotype are a central theme in evolutionary biology. Traditionally, the mechanisms underlying a similarity in animal phenotypes have been split into parallelism and convergence on the basis of whether the species' in question are closely or distantly related (Brakefield 2006; Arendt and Reznick 2008). Under parallelism, closely related species evolve a similar phenotype due to similar mechanisms. For example, the observed patches of melanin based pigmentation in leaf warbler species of the genus *Phylloscopus* can be explained by developmental changes (Price and Pavelka 1996). In contrast, convergence has historically been applied to similar phenotypes in distantly related species arising from different

genetic mechanisms, for example, winged flight in birds, bats and insects.

However, it is becoming clear that there is no consistent way of defining convergence when mechanisms are taken into account (Brakefield 2006). For example, the same genetic mechanism responsible for melanism in birds has also been implicated in lizards, cats and woolly mammoths (Majerus and Mundy 2003; Mundy et al. 2004; Arendt and Reznick 2008). Additionally, whereas the same locus is responsible for pale coloration in some populations of beach mice (*Peromyscus polionotus*), in other populations of the same species the mechanism underlying pale colour is unknown (Hoekstra et al. 2006). Here I use "convergent" as a flexible term to describe the independent evolution of similar phenotypic traits (Arendt and Reznick 2008).

Research into convergent evolution via evo-devo and natural selection is required to understand how these two processes lead to the evolution of similar phenotypes. The evolutionary developmental biology of bird plumage has largely focused on the growth and formation of the feather itself (e.g. Noramly and Morgan 1998; Prum 1999; Chen and Chuong 2000; Fliniaux, Viallet, and Dhouailly 2004; Jiang et al. 2004) rather than within-feather patterning (but see Prum and Williamson 2002). An understanding of the dynamics of within-feather patterning could clarify the function and evolution of this little studied but common avian plumage trait. The prevailing view of the function and evolution of plumage patterns has remained largely rooted in the perspective of adaptationist ideas, but has seldom received research attention. A broad scale analysis of bird plumage patterns from an evolutionary and macroevolutionary perspective is overdue.

In my thesis, I attempt to understand the evolutionary developmental biology of within-feather patterns and natural selection for patterning to examine how each contributes to convergent evolution. In the first half of my thesis I examine the developmental mechanism of patterning and whether the development biology of pattern formation may shape plumage evolution. In the second half of my thesis I focus on how natural selection has shaped plumage phenotypes to converge on the

same patterns for signaling and/or camouflage as per Darwin and Wallace's hypotheses, respectively. In this introductory chapter, I discuss what is known of the evolutionary developmental biology of within-feather patterns and natural selection on bird plumage. I then discuss how I will study these processes in birds.

"*We can see why characters derived from the embryo should be of equal importance with those derived from the adult, for a natural classification of course includes all ages*" (Darwin 1869).

Evo-devo of plumage patterns

Building avian phenotypes

Plumage coloration can vary over the body and is coordinated into species-typical phenotypes by individual patches containing up to thousands of feathers (Prum and Dyck 2003). For example, the feathers of the peacock's (*Pavo cristatus*) train are modified covert feathers from the upper tail that are pigmented with spots whereas the wing feathers have barred patterns. Patches of feathers that covary in coloration generally correspond to seven feather tracts (pterylae) over the body, which vary little in anatomical position between birds (Fig. 1.3; Lucas and Stettenheim 1972).

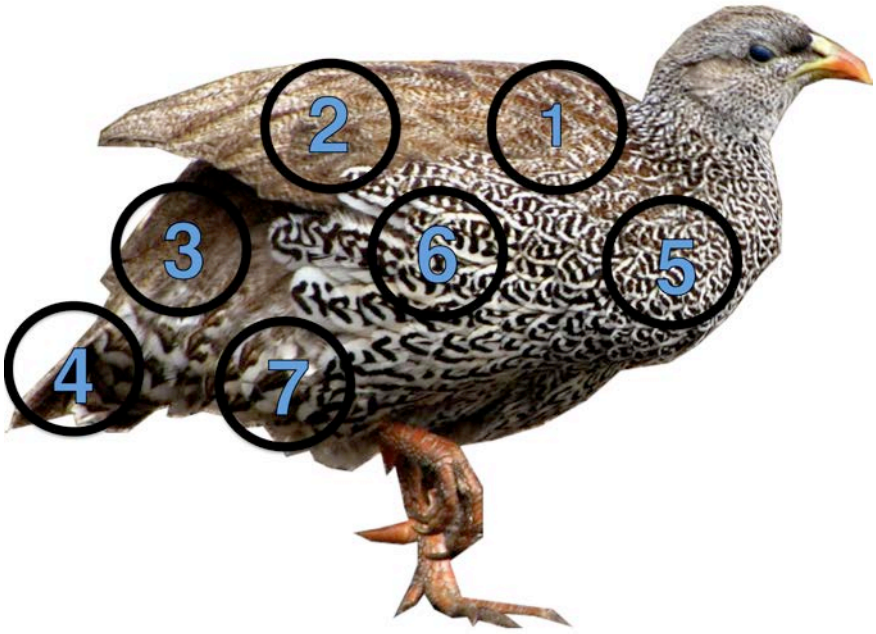


Fig. 1.3. The seven major individual feather tracts in birds. Field guide terminology, and the corresponding feather tracts (pterylae; Lucas and Stettenheim 1972) are as follows: a) Nape: interscapular tract, b) Wing (scapular, wing coverts, tertials, primaries and secondaries): humeral tract, upper marginal coverts of prepatagium and upper wing covert tract, c) Rump and uppertail coverts: dorsopelvic tract and dorsal caudal tract, d) Tail: upper major tail covert, upper median tail covert and rectrices tract, e) Breast: ventral cervical tract, f) Flanks or side: pecterosternal or pectoral tract, g) Vent and undertail coverts: abdominal or lateral and medial abdominal tracts. The species illustrated is the Natal francolin (*Pternistes francolinus*).

Plumage patterns appear to be determined by feather follicle-level processes (Takeuchi et al. 1996; Prum and Williamson 2002; Yoshihara et al. 2011; Oribe et al. 2012; Yoshihara et al. 2012). The potential to form feather follicles is distributed throughout the ectoderm (Harris, Fallon, and Prum 2002). Numerous interactions between the epithelium and mesenchyme signal pteryla formation (Chuong 1993; Prum 1999; Chuong et al. 2000; Dhouailly et al. 2004; Lin et al. 2013). Epithelial-mesenchymal recombination in quail-chicken chimeras shows that the dermal cells of the ventral and dorsal surface have different origins: the dorsal dermis is of neural crest origin whereas the ventral dermis originates from lateral plate mesoderm

(Dhouailly et al. 2004; Fliniaux, Viallet, and Dhouailly 2004; Lin et al. 2006).

From what is currently understood of plumage development, covariation of feather pigmentation within patches is indicative of local shared developmental mechanisms (Lucas and Stettenheim 1972; Prum and Dyck 2003; Lin et al. 2006; Wagner, Pavlicev, and Cheverud 2007). In contrast, it is unknown whether the development of pigmentation within one patch of plumage influences the evolution of pigmentation in another patch of plumage. For example, the pink-eared duck (*Malacorhyncus membranaceus*), has barred plumage on the flanks and the breast. Perhaps barred plumage evolved on the breast first, and the mechanism of pattern formation was subsequently recruited by the flanks, or vice versa. An interesting issue is whether plumage pattern evolution is segmented and follows an anteroposterior gradient or whether plumage pattern evolution is constrained to a dorsoventral gradient because the dermal cells of the ventral and dorsal surface are of different origins. At the level of the whole body, evolution of a novel plumage pattern may occur within the same patch or involve recruitment from other patches, or other modules, and might therefore appear relatively unconstrained. These considerations indicate that there may be modularity within a larger developmental hierarchy from the level of individual patches, to regions, and possibly over the whole body.

Building a feather

Feathers vary in type and coloration between chick, juvenile and adult phases (Fig. 1.4), as well as over the body and between the sexes. For example, chicks hatch with neoptile natal down feathers that are unbranched structures whereas adult plumage is predominantly comprised of pennaceous barbed feathers interspersed with neoptile down feathers (Prum 1999; Oribe et al. 2012). Both neoptile and pennaceous feathers are complex branched keratin structures. The entire plumage is replaced by moulting at least once a year

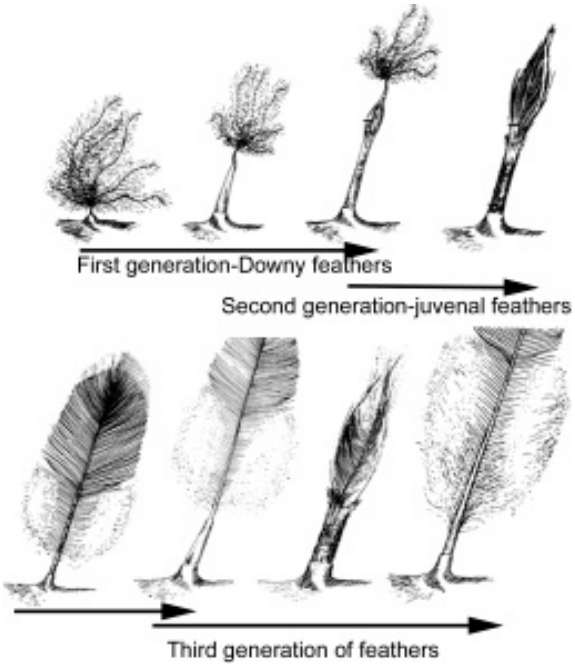


Fig. 1.4. Feathers vary in type between chick, juvenile and adult phases (Yu et al. 2004). During embryogenesis, and at hatching, chicks have unbranched feathers known as down feathers. During the first juvenile moult, barbed pennaceous feathers emerge and are similar in structure but smaller in comparison to the adult feathers that are depicted here are as the third generation of feathers.

The development of neoptile feathers begins early in embryogenesis and is indicated by a thickening of the ectoderm by the elongation of epidermal cells, forming the epidermal placode (Noramly and Morgan 1998; Jiang et al. 1999). An aggregation of dermal cells forms below the epidermal placode that subsequently develops into the feather germ and follicle. Elongation of the placode leads to the formation of a cylinder of dermis enclosed in a tubular epidermal structure, which is the first feather germ, or short bud. The basal epidermis of the feather bud invaginates forming the feather follicle (Prum and Dyck 2003). From epidermal differentiation of the feather bud, barb ridges of the first natal down are formed.

In adulthood, feathers continue to grow from feather follicles but change dramatically in shape. Similar to the development of neoptile feathers, nutrients for the pennaceous feather are delivered by the feather pulp, located between and under the feather follicle (Fig. 1.5). In contrast with neoptile feathers, the developing barbs at the anterior side of the feather follicle of pennaceous feathers fuse to create the rachis. The individual barb ridges of the feather are formed by the keratin producing cells of feathers (keratinocytes) which take up melanin pigmentation, and is a central component of within-feather pattern formation (Prum and Williamson 2002; Prum and Dyck 2003).

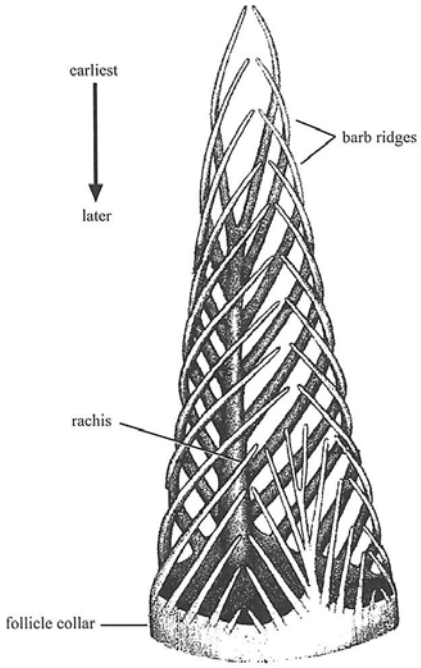


Fig. 1.5. Helical pennaceous feather growth taken from Lucas and Stettenheim (1972). A unique aspect of avian integument structure is the geometry of helical feather growth, which is an important consideration in within-feather pattern formation.

Melanin synthesis and deposition

From what is currently known, melanin is the most important type of pigment

involved in within-feather patterning (Hill and McGraw 2006a). The same types of melanin are synthesised in mammals and birds: eumelanin, resulting in dark brown or black coloration, and pheomelanin, resulting in yellow to buff brown (Richardson, Hornbruch, and Wolpert 1989; Takeuchi and Takahashi 1998; Tobita-Teramoto et al. 2000; Hill and McGraw 2006b). In addition, white coloration is due to an absence of melanin (Mundy et al. 2004). Within-feather patterning is predominantly comprised of combinations of eumelanin, pheomelanin and an absence of pigmentation. Alternate combinations do occur, such as in the barred plumage of the budgerigar (*Melopsittacus undulatus*), which is additionally pigmented with psittacofulvins that are specific to Psittaciformes, but these alternate combinations are rare (Hill and McGraw 2006b; T-L. Gluckman *unpublished data*).

The melanins of feathers are synthesised endogenously and are derived from melanocyte pigmentation cells. Feather melanocytes are of neural crest origin. From the dermal pulp of the feather germ, melanocytes migrate into the tubular epidermis of the developing feather (Fig. 1.6). The organelles within melanocytes that are responsible for the synthesis of melanin are melanosomes. Melanocytes transfer melanosomes to the feather keratinocytes via pseudopodia, and melanosomes are transferred into the keratinocytes by phagocytosis (Lucas and Stettenheim 1972; Prum and Williamson 2002). The melanocyte-specific enzyme tyrosinase (TYR) catalyses the first steps of both eumelanin and pheomelanin synthesis. Eumelanin synthesis additionally requires the tyrosinase-related protein 1 (TYRP1) and DOPAchrome tautomerase (DCT) whereas pheomelanin appears to require few other proteins, but additionally requires cysteine (Ito, Wakamatsu, and Ozeki 2000; Hill and McGraw 2006b; Dessinioti et al. 2009; Galván, Ghanem, and Møller 2012). Each type of melanin has its own particular type of melanosome – eumelanosomes for eumelanin, and pheomelanosomes for pheomelanin – and it is currently thought that only one type of melanosome is contained within each melanocyte. Therefore, melanocyte type and distribution in feathers is a key component of within-feather pattern formation. However, little is known of the mechanisms underlying the spatial distribution of melanocytes.

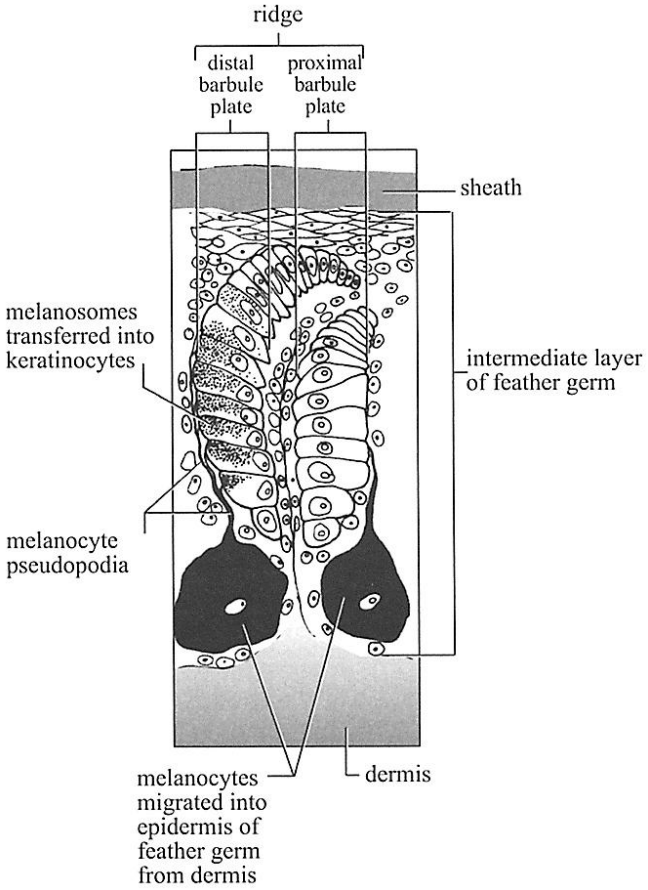


Fig. 1.6. Melanosome transfer of keratinocytes during feather formation taken from Prum and Williamson (2002). The differential uptake of melanosomes into feather keratinocytes is of crucial importance in within-feather patterning.

Melanin type synthesized in melanocytes is due to activity of the melanocortin-1 receptor (MC1R) which is a G protein coupled receptor on the cell surface (Ling et al. 2003; Takeuchi, Takahashi and Okimoto 2003) (Oribe et al. 2012). The *MC1R* locus is important in the evolution of avian coloration, and changes at this locus can have a widespread effect on melanin based coloration in birds (Ollmann et al. 1998; Kerje et al. 2003; Mundy et al. 2004; Roulin 2004; Mundy 2005; Nadeau 2006; Nadeau, Minvielle, and Mundy 2006; Nadeau, Burke, and Mundy 2007; Ducrest, Keller, and Roulin 2008; Dorshorst and Ashwell 2009; Vidal et al. 2010; Emaresi et al. 2013). Expression of eumelanin and pheomelanin is due to

differential stimulation of MC1R, which regulates the cyclic adenosine monophosphate (cAMP) signaling pathway. MC1R is activated by the binding of melanocortin peptides to MC1 receptors on the cell surface. Melanocortin peptides are generated by post-translational processing of the pro-opiomelanocortin peptide (POMC) that is encoded by the *POMC* gene (Benjannet et al. 1991; Takeuchi, Teshigawara, and Takahashi 1999; Yoshihara et al. 2011).The most common melanocortins that have a function in animal pigmentation are the adrenocorticotropic hormone (ACTH) and α-MSH (Takeuchi et al. 2003; Takahashi et al. 2006; Roulin et al. 2011; Yoshihara et al. 2011; Roulin and Ducrest 2013). Binding of melanocortin peptides to MC1R elevates intracellular cAMP levels which in turn stimulates the synthesis of eumelanin. Conversely, the agouti signaling protein (ASIP) acts as an inverse agonist of MC1R lowering intracellular cAMP levels leading to pheomelanin synthesis (Nadeau et al. 2008; Oribe et al. 2012; Yoshihara et al. 2012). ASIP has been implicated in mammalian (i) dorsoventral patterning, (ii) temporal-specific regulation of pigmentation during hair growth leading to banding patterns (Bultman, Michaud, and Woychik 1992; Vrieling et al. 1994; Barsh 1996; Fontanesi et al. 2010; Kaelin et al. 2012), and (iii) inhibition of melanocyte differentiation (Aberdam et al. 1998; Linnen et al. 2009). In chicken and quail, *ASIP* may function in dorsoventral patterning as well as within-feather patterning (Nadeau et al. 2008; Yoshihara et al. 2012).

Two mechanisms of within-feather pattern formation have been demonstrated: melanocyte distribution/differentiation and pigment-type switching via the melanocortin-1 receptor (MC1R). For example, the distribution of melanocytes can lead to bars and spots in certain breeds of chicken (Lin et al. 2013). The second mechanism acts via differential stimulation of the MC1 receptors (Yoshihara et al. 2012).

Models of within-feather pattern formation

The proposed organizing mechanism for within-feather pattern formation in birds is reaction-diffusion, based on Alan Turing's (1952) original proposition

describing morphogenesis (Turing 1952; Oster and Alberch 1982; Oster et al. 1988; Prum and Williamson 2002). Reaction-diffusion models are comprised of an activating morphogen, and an inhibiting morphogen, which is produced by the activator. The basis of the reaction-diffusion mechanism is that it interacts with a hypothetical gene product level, or morphogen, activating colour-specific enzymes (Oster and Alberch 1982; Oster et al. 1988). Reaction-diffusion models have been used to model patterns over the body in a variety of taxa including Lepidopterans and Zebras. For example, reaction-diffusion can accurately model animal patterning by incorporating the geometry and size of the domain on which the initiating reaction-diffusion mechanism resides, during embryonic development (Oster and Alberch 1982; Oster et al. 1988).

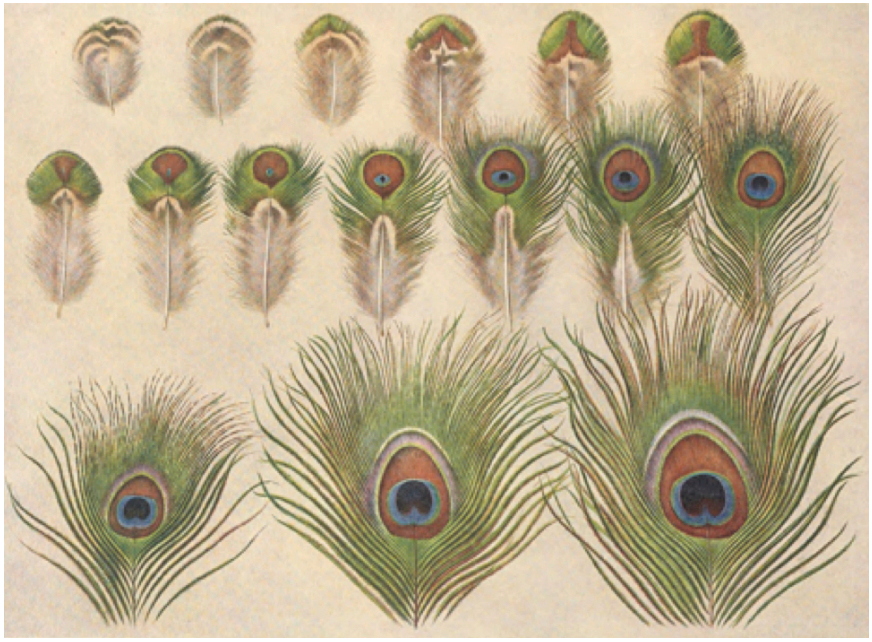
Reaction-diffusion models can accurately simulate pattern changes over the body. For example, where the body and the tail meet, “notch” markings are apparent on the tails of striped zebras whereas spotted cheetah’s have striped tails at the periphery (Oster et al. 1988). It is thought that these biological examples demonstrate developmental constraint in the mechanism of pattern formation: spotted animals can have striped tails but striped animals cannot have spotted tails. Although barred, or striped, bodies with spotted tails are rare in the avian world (T-L. Gluckman *unpublished data*), a few species exhibit this pattern distribution. For example, peacocks have barred wings and spotted upper tail covert feathers. However, patches of homogeneous colouration separate each patch of the peacock’s plumage patterns, thereby separating localised within-feather plumage patterning mechanisms. It is thought that where two adjacent domains with independent reaction-diffusion events meet, they will interact to produce a different pattern. For example, where the limbs of zebras meet the body, a scapular stripe occurs, which is often described as “chevrons” in avian literature (Oster and Alberch 1982; Oster et al. 1988; Oster and Murray 2005).

Domain size may also be important in animal phenotypes (Oster and Alberch 1982; Oster et al. 1988; Oster and Murray 2005). For example, a domain size that is too small or too large may result in an absence of patterning and a domain of

intermediate size is thought to facilitate reaction-diffusion based pigmentation patterns (Murray 1981). This is because when the spatial scale of the domain is restricted, the spatial structure between the morphogens cannot exist, but that when the spatial scale is large enough, the distribution of morphogens has the possibility to become uniform again. The repeating absence of patterns in large and small birds in avian morphospace supports these model based predictions (Murray 1981; Riegner 2008). However, reaction-diffusion models also predict a phenotype that shows variable pigmentation along the anteroposterior axis, e.g. white/black anterior section with a white/black posterior section. This is not observed in birds, which have pigmentation differences on the dorsoventral axis, e.g. countershading (Riegner 2008).

In contrast with models of pattern formation in animals, Prum and Williamson's (2002) reaction-diffusion model incorporates the unique considerations of feather growth. By modeling reaction-diffusion in the context of helical feather growth, Prum and Williamson (2002) were able to model the formation of all avian regular plumage patterns, i.e. scales, bars and spots, but not irregular mottled patterns (Fig. 1.1). This model makes explicit predictions as to which plumage patterns require the most stringent regulation: the regulation of scales is the least stringent, bars require a specific amount of morphogen, and spots have the most stringent formation parameters. This may have important implications for plumage pattern evolution as it implies that some patterns may be harder to make than others. In addition, perhaps the size of the patch of patterning may further constrain the mechanism of pattern formation, as some patterns may need smaller or larger feather tracts on which to form. Analyses of avian morphospace imply that with decreasing body size birds tend to evolve patches of black and white, then bars, then spots, before mottled plumage, and finally countershading (Riegner 2008). That avian morphospace correlates well with a transition in Prum and Williamson's model (2002) could reflect developmental constraint. However, the previous analysis of avian morphospace must be treated cautiously as scales are not included, and phylogeny is not accounted for in the statistical methods (Riegner 2008).

Reaction-diffusion based models provide testable hypotheses with which to explore plumage pattern development and evolution. Currently it is unknown if reaction-diffusion controls pattern formation within feathers. The few experimental manipulations of plumage patterns in poultry have shown that barred plumage patterns can be transplanted or altered by transplanting melanocytes from barred hosts into the feather ectoderm of chickens (Willier and Rawles 1938; Willier and Rawles 1940). It is potentially significant for reaction-diffusion models that MC1R is differentially stimulated by MSH and ASIP resulting in variation in pigmentation within-feathers, but it is currently unknown whether MSH and ASIP interact to form plumage patterns, and whether reaction-diffusion is the mechanism underlying pattern formation.



The development of the eyes on the tail-feathers of a peacock (Beebe 1922).

Developmental constraint

The possibility to evolve plumage patterns may be constrained by a difficulty in developing a particular kind of pattern. For example, spotted patterns may have the most stringent regulation parameters (Prum and Williamson 2002) and might be harder to make than others. Therefore, development may constraint plumage pattern evolution. Price and Pavelka (1996) proposed that the repeated evolution of similar avian phenotypes may be explained by developmental constraint with two possible consequences: 1) among-species diversity due to the magnification of slight differences in ancestors, 2) parallelism (convergence) (Price and Pavelka 1996). The former is a plausible explanation, resulting in minor variation to a particular ancestral motif. For example, ducks exhibit a diversity of barred plumage, e.g. thick bars in the pink-eared duck (*Malacorhyncus membranaceus*), as well as thin bars (vermiculated), as seen in the tufted duck (*Aythya collaris*). However, diversity due to the magnification of slight differences in ancestors does not explain the diversity of pattern types in closely related species such as bars, scales and spots in Anatidae, or recursion of the same patterns in distantly related families. Therefore, convergence due to developmental constraint may better explain similar plumage patterns in distantly related taxa, such as the barred plumage of cuckoos, woodpeckers and galliform species, and potentially that of closely related species.

Natural selection on plumage patterns

Natural selection acts on the phenotype of organisms and a heritable reproductive advantage may lead to the selected phenotype becoming prevalent in different populations depending on their environment, which includes conspecifics and predators (Darwin and Wallace 1858; Darwin 1869; Wallace 1889). Over time, the process of natural selection gives rise to populations that are adapted to their particular ecological niche. On the basis of an adaptive advantage animal visual signals should evolve to be effective for the function that they serve, which can be in

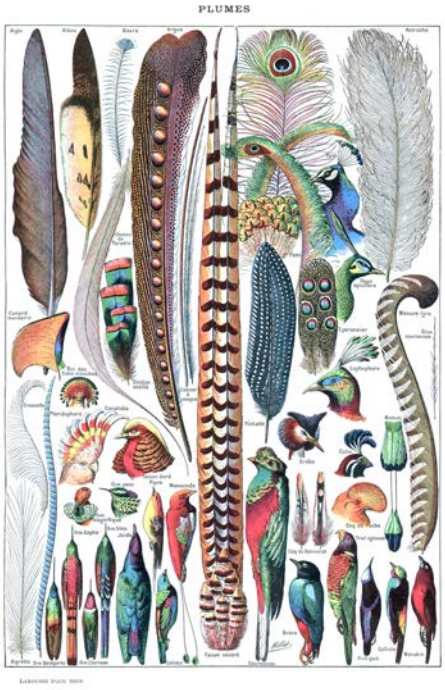
communication and/or camouflage (Endler 1978; Endler 1992; Bradbury and Vehrencamp 1998; Kenward et al. 2004). It is generally thought that patterning is beneficial because patterns allow animals to be less conspicuous against heterogeneous backgrounds. The general principle underlying the function of patterns in camouflage is that patterns resemble aspects of the background in order to evade detection by predators or prey. In the context of signaling, patterns must visually diverge from the background to stand out to conspecifics. For example, bold patterns on a plain background make an animal standout, having a similar pattern to the background allows the animal to blend in, whereas patterns that oppose the geometric pattern of the background are conspicuous (Bradbury and Vehrencamp 1998).

The communication signals of animals, especially sexually selected traits, are often visually striking and stand out from the background in which the animal is viewed (Andersson 1994). In contrast, the purpose of camouflage is to conceal the presence of an animal and this is often achieved by matching the background (Poulton 1890; Thayer 1909; Cott 1940). As a consequence of these opposing selection pressures there is a functional compromise between signaling and camouflage (Andersson 1994). A growing number of studies of animal behavior show that patterns can function in communication. In comparison, the number of studies of the function avian plumage patterns in camouflage is few, but the similarity of the patterns found in birds to other animal groups may be revealing of their protective benefit. For example, irregular pigmentation such as mottled patterns (Fig. 1.1) is common in many animal groups and has been shown to have a protective benefit in invertebrates (Thayer 1909; Cott 1940; Bradbury and Vehrencamp 1998; Stevens and Merilaita 2009a; Stevens and Merilaita 2009b).

Patterns as signals

In order for traits to serve as a social signal they must elicit a response in the receiver and in the context of sexual ornamentation, must be reliable indicators of

individual condition, such as through condition dependence (Andersson 1994; Bradbury and Vehrencamp 1998), or females that choose those ornaments benefit through indirect genetic benefits to their offspring and or improved access to resources (Darwin 1871; Fisher 1923). Empirical experiments demonstrate that patterns can elicit a social response in a range of species (e.g. Hasson 1991; Swaddle and Cuthill 1994; Roulin 1999a; Roulin et al. 2003; Bortolotti et al. 2006). For example, in a classical study of sexual selection and visual signalling, the number of eyespots on the tail/train of the male peacock (*Pavo cristatus*) is positively associated with mating success demonstrating that females select for males with more eyespots (Petrie, Halliday, and Sanders 1991). In addition, patterning can be a reliable indicator of individual condition. For example, the size of barred throat patterns is positively associated with body condition in female barred buttonquails (*Turnix suscitator*) (Muck and Goymann 2011) and adaptive sex-ratio biases are related to the plumage spottiness of parent barn owls (*Tyto alba*) (Roulin et al. 2010). Alternatively, pleiotropic effects, whereby one gene influences multiple phenotypic traits (e.g. via metabolic pathways), may also result in correlated responses to selection in melanin based traits (Ducrest, Keller, and Roulin 2008). For example, female eumelanin based traits in barns owls is correlated with offspring quality, life history attributes, physiology and morphology, and this interaction is thought to be due to pleiotropic effects of melanin production (Roulin 2004; Ducrest, Keller, and Roulin 2008; Emaresi et al. 2013).



Types de Plumes Larouss pour tous, Adolphe Philippe Millot (1907).

Barred plumage patterns are particularly common in many different species and may function in both communication and camouflage (Riegner 2008; Gluckman and Mundy 2013). For example, compared to irregular mottled patterns, barred plumage is more likely to evolve on the ventral surface of males at sexual maturity, which is a more likely location for avian communication (Gluckman and Cardoso 2010). In contrast with irregular mottled patterns, the regularity of barred patterns could perhaps act as a signal of individual quality by making irregularities within the pattern perceptually salient (Gluckman and Cardoso 2009). That regular patterns can facilitate communication of honest aspects of individual quality may select for repeated evolution of these types of patterns on the ventral surface of males (Gluckman and Cardoso 2010).

The function of barred plumage patterns appears to be diverse and an alternative function of this type of pattern may be in Batesian mimicry (Davies and Brooke 1988; Davies 2000; Payne and Sorensen 2005; Davies and Welbergen 2008; Davies 2011; Welbergen and Davies 2011). In Batesian mimicry, a harmless species (the mimic) mimics a harmful species (the model) in order to avoid detection (Bates

1862). The common cuckoo (*Cuculus canorus*) is thought to mimic the Eurasian sparrowhawk (*Accipiter nisus*) in order to evade detection while laying its parasitic egg in the nest of other host species. Its striking resemblance is primarily due to the barred plumage patterns that appear to be remarkably similar to its model. Moreover, barred plumage patterns evolved with parasitism in Old World cuckoos in five genera that implies that cuckoo-hawk mimicry may drive cuckoos to converge on the barred plumage patterns of their model raptor species (Kruger, Davies, and Sorenson 2007).

A camouflage function of plumage patterns

In contrast with a signal function, patterns could allow animals to be less conspicuous against heterogeneous backgrounds (Poulton 1890; Thayer 1909; Cott 1940). Wallace (1889) was the first to consider that less conspicuous coloration may have an adaptive function in concealment. This idea was later taken up by Thayer (1909), who observed that countershading might counterbalance the effects of an animals own shadow thereby concealing its form, and Cott (1940), who thought that animals might modify their colour and pattern to conceal their form.

In spite of the importance of camouflage in natural selection, understanding of the function of coloration and patterns in camouflage has progressed slowly since the time of Wallace, Poulton, Thayer and Cott. Recent years have seen a steady increase in the number of studies that test the function of colour and patterning in camouflage due to advances in techniques that allow the incorporation of the visual perspective of the receiver (Stevens and Merilaita 2009b). Most studies of camouflage are in non-avian animals and directly follow the hypotheses of the founding fathers of visual anti-predator defenses. However, the same patterns that have been studied in other animal groups, e.g. irregular mottled or stippled patterns as well as stripes and spots, are also exhibited in birds. Although there is some understanding of visual systems in predators, the main predators of any given avian species are often unknown. Given that there is some understanding of anti-predator defenses in other animals, it would seem that the best place to start in understanding

the camouflage properties of avian visual patterns, and whether camouflage drives convergent evolution in bird plumage patterns, is to review what is currently known of camouflage in other animal systems.

Pattern-based anti-predator defenses can be diverse in their form and the way that they facilitate the evasion of detection by predators. Some types of camouflage conceal animals while stationary, e.g. background matching and disruptive markings, and other types of camouflage prevent capture during movement, e.g. by motion dazzle and flicker fusion (Poulton 1890; Thayer 1909; Cott 1940; Bradbury and Vehrencamp 1998). Irregular mottled plumage patterns (Fig. 1.1) are likely to function in stationary camouflage, where the animal is still, because irregular patterns can generally match the patterning of one or several background types via background matching. Irregular patterns may also facilitate stationary camouflage by seeming to create false sets of edges to prevent recognition by predators via disruptive camouflage (Thayer 1909; Cott 1940; Bradbury and Vehrencamp 1998; Stevens and Merilaita 2009a; Stevens and Merilaita 2009b). For example, in the non-breeding plumage of ducks (e.g. *Aythya* and *Somateria*), it has been suggested that irregular patterning may facilitate the evasion of detection by predators during the flightless period accompanying wing moult (Hohman and Richard 1994; Hohman 1996), but this has not been empirically tested.

The other avian plumage patterns of scales, bars and spots (regular patterns) may facilitate the camouflage of animals as a second mode of camouflage during movement or flight via motion-dazzle and flicker-fusion (Thayer 1909; Jackson, Ingram, and Campbell 1976; Pough 1976; Endler 1978; Endler 1980; Brodie 1989; Brodie 1992; Brodie 1993; Madsen and Shine 1994; Lindell and Forsman 1996; Stevens and Merilaita 2009b; Scott-Samuel et al. 2011; Helversen, Schooler, and Czienskowski 2013; How and Zanker 2014). For example, experiments testing motion-dazzle using prey capture by human participants on computer screens have shown that striped/barred patterns make ‘capture’ of moving stimuli more difficult and produce more misdirected attacks than other forms of camouflage and coloration (Stevens et al. 2011; Helversen, Schooler, and Czienskowski 2013; How and Zanker

2014). The visual mechanism underlying the camouflage benefit of motion dazzle is currently unknown, however flicker-fusion may cause fatigue in the motion sensitive cells of the eye (Snowden 1998). Under flicker-fusion, regular patterns blur during movement and cannot be effectively perceived, for example adders (*Vipera berus*) that have regularly repeating patterns have higher survival rates (Jackson, Ingram, and Campbell 1976; Pough 1976; Endler 1978; Brodie 1989; Brodie 1992; Brodie 1993; Madsen and Shine 1994; Lindell and Forsman 1996). However, it is unknown whether this effect is via flicker-fusion or motion-dazzle.

Adaptation and convergent evolution

Although studies on the function of animal camouflage have been applied to many animal groups, the existing ideas on camouflage have not been applied to the function of bird plumage patterns. Studies of convergent evolution in plumage phenotypes have predominantly focused on patches of uniform coloration, in particular carotenoid coloration, with relatively fewer studies of melanin (Christidis, Schodde, and Baverstock 1988; Hackett and Rosenberg 1990; Price and Pavelka 1996; Kusmierski et al. 1997; Odeen and Bjorklund 2003; Andersson, Prager, and Johansson 2007; Bleiweiss 2007; Cardoso and Mota 2008; Jones and Kennedy 2008; Prager and Andersson 2010; Friedman, Kiere, and Omland 2011). The proposed adaptive mechanism of convergence in regional colouration has centered on ecology (Crochet, Bonhomme, and Lebreton 2000; Dumbacher and Fleischer 2001; Omland et al. 2006; Bleiweiss 2007; Weibel and Moore 2007), but several studies failed to find an ecological correlate (Omland 1997; Omland and Lanyon 2000; Omland et al. 2006). Additional suggested mechanisms of convergence are sexual selection (Omland and Lanyon 2000; Prager and Andersson 2010), and developmental constraint (Price and Pavelka 1996; Omland and Lanyon 2000; Majerus and Mundy 2003; West-Eberhard 2003; Prager and Andersson 2010). For example, examining the evolution of carotenoid coloration in widowbirds and bishops (*Euplectes* spp.), coupled with the underlying physiological mechanisms, Prager and Andersson (2010) found that the ancestral state of carotenoid based coloration is

yellow plumage, and that red coloration is a more derived state that has convergently evolved multiple times. However, convergent evolution in carotenoid based plumage in *Euplectes* is due to two different mechanisms: ketolase conversion or an increase in carotenoid concentration (Prager and Andersson 2010).

Conclusions

The prevailing view of plumage pattern function and evolution has focused on adaptationist ideas. There are multiple potential mechanisms underlying convergent evolution that are from the new field of evo-devo as well as the prevailing school of neo-darwinian theory. In my PhD thesis I explore the main mechanisms underlying convergent evolution, from evolutionary developmental biology, as well natural selection for signaling as well as camouflage in the plumage pattern of birds. To do this I used techniques spanning molecular biology, Bayesian comparative modeling, digital image analysis, and ecological selection.

Thesis outline

Chapter 2: An understanding of the molecular basis of within-feather pattern formation is lacking and in this chapter I investigate two mechanisms of pattern formation using molecular and developmental techniques.

Chapter 3: Previous studies indicate that there may be developmental constraint in plumage pattern evolution. Therefore, I investigated whether particular aspects of plumage pattern development may shape the direction of plumage pattern evolution in two ecologically different groups of birds using Bayesian comparative modeling.

Chapter 4: It has been demonstrated that plumage patterns can function in signaling in a number of species of birds. In Old World cuckoos, a signaling function may have been co-opted to facilitate deception via Batesian mimicry in many species of birds which I investigated using digital image analysis techniques. This chapter was

The mechanisms underlying convergent evolution in the plumage patterns of birds

published in Animal Behaviour (2013).

Chapter 5: The plumage patterns of birds have inspired many an observer to consider whether they may have evolved to facilitate concealment from predators. Given that patterns are viewed in the context of their habitat the natural extension of a camouflage benefit is that habitat selects for plumage patterns, which I investigated using ecological techniques in all birds worldwide in collaboration with Marius Somveille and Kate Marshall.

Chapter 6: In my final chapter I summarise my findings of the mechanisms underlying convergent evolution in the plumage pattern of birds, I discuss the implications of my results and highlight future areas of research that I will pursue.

Chapter 2: Activation or inhibition, *MC1R* and the developmental basis of within-feather pattern formation

Japanese quail (*Coturnix japonica*) chick.

Abstract

Pattern development is an important question in biology as it requires precise spatiotemporal control. Melanin pigmentation is highly conserved in vertebrates and is the basis of feather patterning. Due to shared ancestry with mice, the focus of avian pattern formation has been melanocyte distribution/differentiation and inhibition of MC1R, whereas activation of MC1R has been relatively understudied. Japanese quail is a model system in development biology and has a phenotype comprised of pheomelanin on the ventral surface, whereas dorsal feathers occur in alternating eumelanin and pheomelanin stripes as well as within-feather patterns. In this study, we explored the evolution of regulatory mechanisms underlying differential deposition of eumelanin and pheomelanin via inhibition (*ASIP* and its paralogue *AGRP*) and activation (*POMC*, *PC1* and *PC2*) of MC1R during quail embryogenesis for comparison with adult chicken and adult quail. *In situ* hybridization using *Sox10* revealed that melanocytes are found in all ventral and dorsal feather follicles (FFs) at E8 and E12. RT-PCR results demonstrated some similarity in *ASIP* alternatively spliced transcripts of adult quail and chicken, but also revealed four novel *ASIP* transcripts. *ASIP* transcripts were variably expressed over the ventral and dorsal surfaces. *In situ* hybridization revealed that *ASIP* is strongly expressed in ventral FFs in E8 and E12, but not dorsally. Additionally, there was no support for a role of *AGRP* in regulating pigmentation. *POMC* transcripts shared only some similarity with the chicken. *POMC* FF expression was strong at E8 and E12 in both ventral and dorsal FFs. *PC1* was not found within FFs. At E8 dorsoventral *PC2* FF expression was faint, but at E12, *PC2* was strongly expressed in ventral and dorsal FFs. Therefore, the pale-bellied phenotype of quail is due to inhibition of MC1R via ASIP*,* whereas *POMC* and *PC2* probably contribute to dorsal pigmentation and within-feather patterning. However, it is unknown how pigment-type switching to pheomelanin is controlled dorsally. Nevertheless, our study further supports the role of ASIP in quail pale-bellied phenotype, and that activation of MC1R via *POMC* and *PC2* may contribute to dorsal patterning.

Introduction

The developmental basis of plumage patterns is an interesting question, as it is the material that is shaped by natural selection, and the properties of developmental mechanisms have implications for the evolution of plumage phenotypes. Pigmentation patterns are common in mammals and birds, frequently differ between males and females, as well as adults and juveniles, and are derived from well-defined hierarchical developmental modules (Lu et al. 1994; Vrieling et al. 1994; Wilson et al. 1995; Jackson 1997; Krude and Grüters 2000; Prum and Williamson 2002; Prum and Dyck 2003; Steingrímsson, Copeland, and Jenkins 2006; Anistoroaei and Christensen 2007; Candille et al. 2007; Jackson et al. 2007; Fontanesi et al. 2010). The main focus of pattern evolution has been its functional significance in communication and camouflage, including background matching and aposematism (Ruxton, Speed, and Kelly 2004; Stevens and Merilaita 2009a; Allen et al. 2010; Gluckman and Cardoso 2010). Yet, the repeated convergence of these patterns within and between animal groups implies that the evolution of pattern phenotypes may be subject to developmental constraints (Murray 1981; Price and Pavelka 1996; Arendt and Reznick 2008). It is hypothesised that a Turing reaction-diffusion system of melanin synthesis may be responsible for within-feather patterning which relies on the combined action of two molecules (morphogens) (Prum and Williamson 2002). However, this has not been demonstrated empirically and it is currently unknown what these morphogens may be.

Pigmentation patterns can be comprised of patches of feathers that have the same pigmentation within feathers, or can be alternately pigmented with different types of melanin within-feathers creating visual patterns such as bars and spots. To produce within-feather patterns requires both spatial and temporal control of pigmentation. The only known type of pigmentation that can be precisely controlled by spatiotemporal mechanisms to produce within-feather patterns is melanin, which is solely produced by melanocytes. The developmental origins of feather-based melanin begin with neural crest cells that follow the dorsolateral migratory pathway before entering the ectoderm and developing feather follicles as melanoblasts. Melanoblasts develop into mature pigment forming cells, melanocytes, that

synthesise melanin in melanosomes and transfer them to keratinocytes via pseudopodia (Le Douarin and Kalcheim 1999; Prum and Williamson 2002).

Two mechanisms of within-feather pattern formation have been demonstrated: melanocyte distribution/differentiation and pigment-type switching via the melanocortin-1 receptor (MC1R). For example, the distribution of melanocytes can lead to complex patterns such as bars and spots, in certain chicken breeds (Lin et al. 2013). The second mechanism acts via differential stimulation of the MC1 receptors on the surface of melanocytes that determines the type of melanin synthesized: inhibition of MC1R leads to pheomelanin (pale yellow/red) whereas activation leads to eumelanin (black/brown) (Bilodeau et al. 2001; Tachibana et al. 2001; Gantz and Fong 2003; Hill and McGraw 2006b). The agouti signaling protein (ASIP) is an inverse agonist of MC1R which has been implicated in mammalian (i) dorsoventral patterning, (ii) temporal-specific regulation of pigment deposition during hair growth leading to banding patterns (Bultman, Michaud, and Woychik 1992; Vrieling et al. 1994; Barsh 1996; Fontanesi et al. 2010; Kaelin et al. 2012), and (iii) inhibition of melanocyte differentiation (Aberdam et al. 1998; Linnen et al. 2009). In chicken and quail, *ASIP* may function in dorsoventral patterning, within-feather patterning and sexual dichromatism (Nadeau et al. 2008; Oribe et al. 2012; Yoshihara et al. 2012). Due to the importance of *ASIP* in patterning in mice, coupled with the strong conservation of pigmentation genes in mammals and birds, previous studies on the mechanisms of feather patterning via differential stimulation of MC1R have focused on inhibition, whereas genes involved in activation have been largely overlooked (Yoshihara et al. 2011; Oribe et al. 2012; Yoshihara et al. 2012). The agonists of MC1R are melanocortin peptides. Melanocortins are a family of structurally related peptides that are generated by post-translational processing of the pro-opiomelanocortin peptide (POMC) that is encoded by the *POMC* gene (Benjannet et al. 1991; Takeuchi, Teshigawara, and Takahashi 1999; Yoshihara et al. 2011).

The development of plumage pigmentation in the Japanese quail (*Coturnix japonica*) is a classic model system (Willier and Rawles 1938; Rawles 1939; Willier and Rawles 1940; Richardson, Hornbruch, and Wolpert 1989; Richardson,

Hornbruch, and Wolpert 1990; Richardson, Hornbruch, and Wolpert 1991). Quail embryos develop dorsal stripes of dark and pale feathers, with differential pigmentation of eumelanin and pheomelanin within the dark feathers, and a pale belly consisting of pheomelanin-containing feathers. In adulthood, Japanese quail have pronounced patterns on the dorsal surface and retain a pale belly.

From early transplantation experiments between several host and donor species (e.g. quail, guinea fowl, and chicken breeds) it was thought that the capacity of a feather follicle to develop patterning was largely the property of the melanocyte (Willier and Rawles 1938; Rawles 1939; Willier and Rawles 1940). For example, the transfer of melanoblasts from donors with barred feather patterns into a non-barred host resulted in patterned feathers in the host, whereas the transfer of melanoblasts from a non-patterned donor into a patterned host did not result in patterns in the host (Willier and Rawles 1940). However, a recent landmark study demonstrated that within-feather patterning can occur either by alternating the presence/absence of melanocytes or by suppression of melanocyte differentiation which is likely due to extracellular factors (Lin et al. 2013). Although this represents an important step forward, Lin et al.'s (2013) study focused on plumage that is due to either black and/or white coloration whereas many plumage patterns, including that of quail, are largely due to differential deposition of eumelanin and pheomelanin.

Melanocyte development and differentiation is controlled by the endothelin B receptor (EDNRB), as well as its ligand endothelin 3 (EDN3), and the melanocyte initiation transcription factor (*MITF*). The EDNRB receptor and EDN3 can cause large changes in the phenotype of vertebrates and chickens (e.g. White leghorn) and quail (Panda mutation) due to impaired differentiation and survival of neural crest cells (Jackson 1997; Niwa et al. 2002; Miwa et al. 2006; Miwa et al. 2007; Saldana-Caboverde and Kos 2010; Kinoshita et al. 2014). Mutations at the *MITF* locus cause widespread changes in quail and chicken coloration e.g. blue in chicken and silver in quail (Mochii, Ono, et al. 1998; Niwa et al. 2002; Minvielle et al. 2010; Lin et al. 2013). Silver homozygotes in quail are entirely white, whereas heterozygotes retain a reduced amount of within-feather patterning. The major pathways that influence *MITF*

transcription are the mitogen-active protein kinase (MAPK) pathway, Wnt signalling pathway and cyclic adenosine monophosphate pathway (cAMP), all of which are known to play a role in pigmentation in mice (Steingrímsson, Copeland, and Jenkins 2006; Poelstra et al. 2014). MAP protein kinases have diverse functions in cell proliferation and gene expression (Cuadrado and Nebreda 2010). However, it is unknown whether this pathway affects pigmentation patterns in birds. Transcriptional activation via the Wnt signalling pathway also serves diverse functions, and regulates transcription of target genes (Mosimann, Hausmann, and Basler 2009). It appears that the Wnt signalling pathway is important in feather formation, however little is known of its function in avian pigmentation (Chang et al. 2004). Defective transport of melanosomes via mutations in the melanophilin gene (*MLPH*) within follicular melanocytes of vertebrates can cause phenotypic changes leading to an overall white or pale coloration (e.g. silver mutation) (Matesic et al. 2001; Ishida et al. 2006; Anistoroaei and Christensen 2007; Welle et al. 2009). In adult Japanese quail, mutations in *MLPH* result in a paler phenotype, including dorsal within-feather patterns, but the pattern is still present (Bed'Hom et al. 2012).

Melanin synthesis requires the melanocyte-specific enzyme Tyrosinase (TYR) to catalyse the first steps of the pathway. TYR catalyses the reaction of the amino acid tyrosine to dihydroxyphenylalanine (DOPA), and then catalyses the reaction of DOPA to form DOPAquinone (Ito, Wakamatsu, and Ozeki 2000). In chickens, mutations in the Tyrosinase gene have an albino phenotype (Tobita-Teramoto et al. 2000; Chang et al. 2006). Beyond these initial steps of melanogenesis, pheomelanogenesis and eumelanogenesis differ. Eumelanin synthesis requires tyrosinase-related protein 1 (TYRP1) and DOPAchrome tautomerase (DCT) (Ito, Wakamatsu, and Ozeki 2000). In contrast, pheomelanin appears to require few other proteins, but additionally requires cysteine for conjugation of dopaquinone (Hill and McGraw 2006b; Dessinioti et al. 2009; Galván, Ghanem, and Møller 2012). The main physiological reservoir of cysteine is the tripeptide glutathione (GSH) which is synthesised by glutathione S-transferase (GST) (Nataf et al. 1995).

A quail mutant, black at hatch (*Bh*), has a phenotype that lacks between

stripe and within-feather pigmentation on the dorsal surface (Shiojiri et al. 1999). Both *Bh* heterozygotes and homozygotes lack dorsal patterning, which is due to an increase in the distribution of eumelanin or pheomelanin, respectively. To elucidate the mechanisms underlying phenotypic differences between wildtype and *Bh* mutants, Niwa et al. (2002), examined the expression pattern of genes involved in melanocyte development and pigment production, e.g. the Melanoblast/Melanocyte early antigen marker (MelEM) and the Melanosomal matrix protein 15 (*Mmp115*). MelEM is an Alpha class subunit of GST and marks early melanoblasts that are committed to synthesize eumelanin (Nataf et al. 1995). *Mmp115* functions in melanosome production (Mochii, Agata, et al. 1988; Mochii, Takeuchi, et al. 1988; April, Jackson, and Kidson 1998). Most of the study genes involved in melanocyte development (*Mitf*, MelEm antigen, *Kitl*, *Kit* and *EdnrB2*) did not correlate with pigmentation patterns between wildtype and *Bh* mutants, except for the *MelEm* antigen. Therefore, *MelEM* may play a role in the dorsal pigmentation of quail, but its function in melanin synthesis is currently unknown (Nataf et al. 1995). Of the study genes involved in pigment production (*Dct*, *Tyrp1*, *Tyr* and *Mmp115*), *Dct* and *Tyrp1* were down-regulated in the melanocytes of homozygote *Bh* mutants. *Mitf* actively controls *Dct* and *Tyrp1* (Mochii, Mazaki, et al. 1998) and did not vary between wildtype or *Bh* mutants (Niwa et al. 2002). Therefore, the *Bh* locus may act upstream of *Dct* and *Tyrp1*, or the *Bh* locus might be involved in another signaling system that regulates the expression of eumelanogenic enzymes independent of *Mitf.*

Two G-protein coupled receptors are required for normal melanocyte function: EDNRB and MC1R (Jackson 1997). There is currently no evidence to suggest that the EDNRB locus may have a function in within-feather patterning (Kinoshita et al. 2014). However, mutations at the *MC1R* locus underlie large variation in melanin distribution over the body in a wide variety of mammals and birds, including both mutants and wild-type polymorphisms (Harding et al. 2000; Kijas et al. 2001; Theron et al. 2001; Eizirik et al. 2003; Kerje et al. 2003; Majerus and Mundy 2003; Doucet et al. 2004; Rosenblum, Hoekstra, and Nachman 2004; Mundy 2005; Hoekstra et al. 2006; Nadeau, Burke, and Mundy 2007; Pointer and Mundy 2008; Vidal et al. 2010).

MC1R is expressed on the plasma membrane of follicular melanocytes in mammals and birds and differential control of this receptor results in either pheomelanin or eumelanin. As mentioned earlier, binding of ASIP to MC1R leads to a decrease in intracellular cAMP, leading to pheomelanin production, whereas binding of the melanocyte-stimulating hormone (MSH) to MC1R increases cAMP production leading to eumelanin synthesis. However, basal activity of MC1R can vary between species. For example, in mice the absence of MC1R stimulation in a *POMC* mutant does not result in dramatic changes in eumelanin (Slominski et al. 2005). In contrast, activation of MC1R is required for eumelanin synthesis in human hair, because MC1R is expressed ten times less in humans than in mice (Krude et al. 1998; Steingrímsson, Copeland, and Jenkins 2006; Jackson et al. 2007). Therefore, in humans POMC is required for eumelanin pigmentation, whereas in mice it is not. However, mice require ASIP to produce the pheomelanin-containing pale-bellied phenotype. Chicken MC1R expressed *in vitro* has low basal activity (measured by cAMP production) in the absence of ligands (Ling et al. 2003). Comparing binding affinities of ligands to chicken and human MC1R, the chicken receptor has a much lower affinity than in humans, e.g. binding affinity of α-MSH to chicken MC1R is 363 nM compared to 0.210 nM in humans (Ling et al. 2004). Thus, avian MC1R is highly likely to require activation although the density of melanocyte receptors in avian melanocytes is currently unknown.

Two genes inhibit MC1R activity: the first to be discovered in birds was the agouti related protein (*AGRP*), followed by its paralogue *ASIP*. Early in vertebrate evolution, *AGRP* and *ASIP* arose by gene duplication. In the chicken, *AGRP* expression was first detected in skin tissue, before *ASIP* had been discovered, and is co-expressed with melanocortin peptides in the eye (Takeuchi, Teshigawara, and Takahashi 2000). AGRP is expressed in the nervous system of mammals and can have a paracrine effect on MCR function (Boswell, Li, and Takeuchi 2002; Klovins and Schiöth 2006; Nadeau et al. 2008). Given that AGRP can inhibit melanocortin receptors, it is possible that AGRP may play a role in inhibiting MC1R activity in avian pigmentation patterns, but this has not been investigated.

It has been reported that ASIP may function in phenotypic changes by inhibiting differentiation of melanoblasts (Aberdam et al. 1998; Linnen et al. 2009), e.g. Felidae, cattle, rabbit, quail and chicken (Eizirik et al. 2003; Girardot et al. 2005; Nadeau et al. 2008; Fontanesi et al. 2010; Oribe et al. 2012; Yoshihara et al. 2012). Unpigmented areas of plumage on the ventral surface of wings of Japanese quail chicks are DOPA-negative indicating an absence of melanoblast maturation (Richardson, Hornbruch, and Wolpert 1989). However, this only indicates whether pigment forming cells are capable of producing melanin, and does not indicate whether melanocytes are present or absent, and therefore, whether embryonic pale pigmentation in quail is due to melanocyte distribution and inhibited maturation. A more direct assessment of the expression of melanocyte distribution would be to trace neural crest cells within feather follicles. One such method is to use *Sox10* as a marker, for cells of neural crest origin (Cheng et al. 2000). Alternatively, pale pigmentation and within-feather patterning may be due to regulation of MC1R activity without effects on melanocyte distribution/maturation.

Different promoter sites of *ASIP* can lead to spatial and temporal control of pigmentation in laboratory mice and naturally occurring colour variants of the genus *Peromyscus* (Bultman, Michaud, and Woychik 1992; Vrieling et al. 1994; Fontanesi et al. 2010; Manceau et al. 2011). There are four murine *ASIP* alternatively spliced variants produced by two kinds of promoters – the distal promoter is ventral-specific and the other is a hair cycle-specific promoter that causes banded “agouti hairs” (Bultman, Michaud, and Woychik 1992; Vrieling et al. 1994; Manceau et al. 2011). In rabbits there are two *ASIP* alternatively spliced transcripts, each with a different promoter site (Fontanesi et al. 2010). Birds also have multiple *ASIP* alternatively spliced transcripts, with some evidence to suggest that they contribute to pattern formation and that the distal promoter site is also ventral specific (Nadeau et al. 2008; Yoshihara et al. 2012).

Nadeau et al. (2008) were the first to document *ASIP* gene expression in skin samples of adult quail, quail mutant *Yellow* (which has a pale phenotype) and the chicken (*Gallus gallus*). The expression of *ASIP* was significantly higher on the

ventral surface in both phenotypes and species examined (Nadeau et al. 2008). Three *ASIP* alternatively spliced transcripts were reported: 1a is expressed on quail dorsal and ventral surfaces but in the chicken 1a is ventral specific, expression of 1b and 1c is the same in quail and chicken, where the former is ventral specific and the latter is expressed on both the dorsal and ventral surfaces (Nadeau et al. 2008). High variability in the expression of *ASIP* transcripts between the ventral and dorsal surfaces of quail and chicken was interpreted as variation due to within-feather patterning. An additional four transcripts were subsequently reported in the chicken, that corresponded well with the previously described adult quail and chicken *ASIP* transcripts, and are transcribed over three promoter sites (classes) within chicken feather follicles: Class 1 consists of five transcripts that have E1S as the non-coding leader exon, whereas class 2 has E4 as the leader exon, and class 3 has E5 (Yoshihara et al. 2012). Of these seven transcripts E1S was consistently expressed and all classes of *ASIP* were found in chicken dorsal feather follicles. *In situ* hybridization revealed that *ASIP* is present in the dorsal feathers of adult chicken where pheomelanin is present, but not eumelanin that indicates that *ASIP* may have a role in pheomelanin synthesis. However, the relative abundance of the different transcripts was not described and no comparison was made with ventral plumage.

ASIP may also play a role in sexual dichromatism in chickens, and it was suggested that the most distal promoter (class 1) produces a pale-bellied phenotype that may have been conserved in mammals and birds (Oribe et al. 2012). Oestrogen treatment of adult male Okayama-Jidori chickens, that have slender barred ornamental feathers in the saddle, resulted in males molting into female like plumage, which consists of broader unbarred pennaceous feathers heavily pigmented with pheomelanin. Up-regulation of class 1 transcripts was correlated with this change in phenotype whereas the class 3 *ASIP* transcript was expressed on both surfaces, and class 2 could not be detected. However, barred patterns were still apparent in the female induced plumage in males, possibly because males were not castrated resulting in continued circulating testosterone. In addition, the electrophoretic figure for RT-PCR for class 1 mRNA showed a faint band on the dorsal surface of 1 week old chicks and 4 week old juveniles, albeit pale, and class 1 *ASIP* mRNA was also

found in dorsal feathers of the chicken (Yoshihara et al. 2012) demonstrating that class 1 is not ventral specific.

The *POMC* gene is thought to have co-evolved with the five melanocortin receptors (MC1R - MC5R) 500 million years ago (Dores and Baron 2011; Dores 2013). The receptors serve in a diverse array of physiological functions: MC1R - pigmentation, MC2R – steroidogenesis, MC3R – homeostasis, MC4R – regulation of food intake as well as sexual function, MC5R – sebaceous gland secretions (Jackson 1997; Kerje et al. 2003; Hoggard 2004; Boswell 2005; Boswell and Takeuchi 2005). The most common melanocortins implicated in pigmentation are adrenocorticotropic hormone (ACTH) and α-MSH (Takeuchi et al. 2003; Takahashi et al. 2006; Roulin et al. 2011; Yoshihara et al. 2011; Roulin and Ducrest 2013). Chicken ACTH contains 39 residues and the first 13 amino acids of ACTH contain the α-MSH peptide (Hayashi, Imai, and Imai 1991). The endoproteases prohormone convertase 1 (*PC1*) and prohormone convertase 2 (*PC2*) are responsible for cleavage of *POMC* products. *PC1* cleaves the pro-opiomelanocortin peptide to make ACTH as well as β-lipotropin whereas *PC2* cleaves pro-opiomelanocortin or ACTH to make α-MSH or desacetyl-α-MSH (Benjannet et al. 1991; Lu et al. 1994; Ling et al. 2004).

In vertebrates the main source of circulating MSH is the intermediate lobe of the pituitary, which is not well defined in humans and birds (Yoshihara et al. 2011). In humans, a lack of a well defined intermediate lobe of the pituitary may have led to the evolution of a cutaneous melanocortin system for integument pigmentation, or vice versa, and perhaps a similar situation has arisen in birds. ACTH and MSH coexist in the same cells in the cephalic half of the anterior lobe of the pituitary in the duck and chicken and have also been detected in chicken feather follicles (Yoshihara et al. 2011). In the chicken it has been found that ACTH binds with a higher affinity to MC1R than MSH and it has been proposed that ACTH may have a function in pigmentation patterns (Ling et al. 2004; Yoshihara et al. 2011).

In a study on extracellular MC1R ligands in the silky and Okayama-Jidori breeds of chicken, it was discovered that *POMC, PC1* and *PC2* are expressed within

adult feather follicles (Yoshihara et al. 2011). Clear positive signals were found for ACTH using dot-blotting, but MSH was only expressed at trace levels. Investigating patterns of expression in adult feather follicles, four alternatively spliced *POMC* transcripts were detected that result from two promoter sites (Class a and b), with and without a non-coding exon (Yoshihara et al. 2011). Class a transcripts were only expressed in feather follicles whereas class b transcripts were expressed in the pituitary, hypothalamus, and peripheral tissues, in addition to feather follicles. Given that strong positive signals were only found for ACTH, and that ACTH binds with a higher affinity to MC1R than MSH, the authors suggested that ACTH might have a function in plumage pattern formation. However, the results of this study are difficult to interpret given that two different breeds of chickens that have very different phenotypes were studied (white plumage vs. barred plumage) yet no comparisons were made among the breeds and it is unclear which results related to which breed. Nevertheless, this study is the first to demonstrate expression of an avian *POMC* gene with alternatively spliced transcripts in chicken feather follicles. In tawny owls (*Strix aluco*), it was suggested that *POMC/PC1/PC2* may mediate a correlation between melanin-based coloration and fitness (Roulin et al. 2011). Therefore, the loci involved in activation of MC1R may have an important contribution to within-feather patterns.

The previous studies of *ASIP* and *POMC* function in the patterning of chicken and quail did not control for developmental stage (Nadeau et al. 2008; Yoshihara et al. 2011; Oribe et al. 2012; Yoshihara et al. 2012). Embryonic development provides the opportunity to strictly control for developmental stage in the mechanism of pattern formation. In this study, we aimed to identify how melanocyte differentiation or MC1R activity may influence patterning in wild-type quail embryos. In particular, we wanted to test if the species-typical plumage patterning of embryonic quail is due to a) melanocyte differentiation, b) MC1R inhibition, or c) MC1R stimulation, at two stages of feather development. If plumage patterns are due to melanocyte distribution, the *Sox10* marker will show that melanocytes are not evenly spread within and between feather follicles on the dorsal surface and few melanocytes will be present in ventral feather follicles. In contrast, if plumage patterns are due to cAMP activity we would

expect the following: either *ASIP* or *AGRP* will be uniformly distributed in feather follicles on the ventral surface to control the pale-bellied phenotype and on the dorsal surface, *ASIP/AGRP* will be displayed between the dorsal stripes, and within-feathers that have banding patterns. In addition, we would expect *ASIP* cycle specific transcripts/promoter sites to be variable, whereas the ventral specific transcripts/promoter sites would be consistently expressed in all ventral samples. Finally, if *POMC/PC1/PC2* does have a role in pigmentation patterns it will be expressed within the feather follicles, and perhaps these genes will be expressed where *ASIP/AGRP* are not, thereby completing the study of *ASIP* and pheomelanin in the chicken by Yoshihara et al. (2012), or perhaps one gene may be constantly expressed and the other varies in a temporal-specific manner.

Materials and Methods

Fertilized wild-type quail (*Coturnix japonica*) eggs were obtained from commercial sources. Plumage pigmentation is first visible between 8-9 days, the equivalent of chicken stages 35-36, and is fully developed by 11-12 days, or chicken stage 41-42 (Hamburger and Hamilton 1992; Ainsworth, Stanley, and Evans 2010). Therefore, we harvested embryos at E8 and E12. As there can be variation in developmental stage between individuals, each embryo was checked to ensure that it was at the required stage of development. Embryos where the eyelid had begun to overgrow the surface of the eyeball and had some feather pigmentation on the dorsal surface, but not on the forehead and crown, were considered to be representative of E8. Embryos that had prominent pigmentation and white feather germs around the eye were considered representative of E12. Some of the E12 embryos did not have pigmentation on the feet, a key spotting character. However, all specimens had fully developed plumage and were considered representative of well-developed embryonic plumage appropriate for E12. Tissue and embryos were harvested in phosphate buffered saline (PBS) containing diethylpyrocarbonate (DEPC) treated-distilled water. Samples of ventral and dorsal epidermal tissue were dissected and stored separately in RNAlater at 4 degrees overnight for increased tissue penetration,

and then at -20 until RNA extraction. For RT-PCR, we sampled three embryos of developmental stage E8 and three embryos of developmental stage E12, and for *in situ* hybridization we sampled 2-3 individuals of each of these developmental stages.

RT-PCR analysis

Total RNA was extracted from each sample with an RNeasy mini-kit (Qiagen), with a final elution in 30µl of RNase free distilled water**.** RNA integrity, purity and concentration (RIN values) were quantified using a BioAnalyser (Agilent). First strand cDNA syntheses were conducted with 1-3µg RNA and 1µl of 150ng/µl N6 primer in a total volume of 10µl using Superscript RT II (Invitrogen) following the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR) was carried out to examine which transcripts were expressed in each stage of development on samples from the ventral and dorsal surface of each embryo. RT-PCR was performed in 25µl total reactions containing 1.0 unit BIOTAQ polymerase (Bioline), 2.5µl 10x reaction buffer, 0.75µl 1.5mM $MgCl_2$, 0.2µl 50mM dNTP, 1µl 10µM each primer and 1µl of first strand synthesis product. PCR reactions were performed in a DNA Engine (MJ Research, Watertown, MA), with the following cycling parameters: Heated lid 110°C, 94°C for 2 mins.; 40 x 94°C for 30 secs., 55-64°C for 30-60 secs., 72°C for 1 min; 72°C for 5 mins. PCR products were visualised on a 1% agarose gel. Sequencing was performed with Sanger sequencing on both strands using the PCR primers.

We searched for avian transcripts of our genes of interest on Genbank. Where possible, we used existing primers documented in other studies. To date, no study has demonstrated whether *AGRP* may have a function in avian phenotypes or that there are alternatively spliced transcripts of *AGRP* that have a function in plumage pigmentation. Therefore, we focused our sampling efforts

solely on the coding region of *AGRP*. Alternatively spliced transcripts and/or coding regions were amplified using published primers and primers designed from available quail and chicken *ASIP* and *AGRP* sequences from Genbank (Table 2.1; supplementary Table S2.1). Where available quail coding sequences were relatively short in comparison to the chicken, we designed primers using chicken transcripts to ensure increased sequence coverage, as these sequences are likely to be highly conserved between species.

During the course of amplifying alternatively spliced transcripts of *ASIP*, previously undocumented transcripts emerged: Novel 8a, Novel 8b, Novel 9, Novel 10, Novel 11. For these new alternatively spliced *ASIP* transcripts we designed reverse internal primers from the 3' end of the non-coding region to ensure full sequence coverage of the novel exons (Table 2.1). Subsequently, we conducted blast searches on Genbank to determine whether homologous sequences are present in the chicken genome.

Similar to our approach to factors inhibiting MC1R, to document the role of activation of MC1R in avian pigmentation we conducted blast searches on Genbank to determine whether there are alternatively spliced transcripts of *POMC*. Several *POMC* alternatively spliced transcripts may have a function in pigmentation in chicken (Yoshihara et al. 2011). In addition, there are several unpublished quail *POMC* alternatively spliced transcripts available on Genbank (Table 2.1). Therefore, we designed primers to amplify the coding region of *POMC* as well as all chicken and quail alternatively spliced transcripts. For *PC1* and *PC2*, there are no reported alternatively spliced transcripts that function in avian pigmentation. In addition, the previous study that demonstrated *PC1* and *PC2* expression in feather follicles focused solely on the chicken and did not demonstrate where these genes are being expressed within feather relative to pheomelanin and eumelanin. Therefore, we focused our efforts on testing whether *PC1* and *PC2* are expressed in quail feather follicles.

Sequences were aligned and edited using the SeqMan software (DNASTAR Inc., Madison WI, USA). Correspondence between quail and chicken alternatively spliced transcripts was examined by comparing and aligning sequences in Mega 6.06 (Tamura et al. 2013). Primers were subsequently optimized and redesigned as necessary. We used the housekeeping gene *β-actin* as a positive control for all RT-PCR reactions. Accession numbers of sequences from which the primers were designed, and amplicon size are listed in Table 2.1.

Table 2.1. Primers used for amplifying mRNA transcripts. The number of exons out of the total number of coding exons is provided as well as the expected sequence length, source of primers, and the accession number of each transcript.

Target sequence	Primer	Primer sequence	Non-coding exons (bp)	Coding exons (bp)	Expected amplicon length (bp)	Primer source	Accession
β-actin							
Coding sequence	ACT1F	TGCGTGACATCAAGGAGAAG	-	Exon 2/2: 250	250	Nadeau 2006	AB199913.1
	ACT1R	CAGGTCCTTACGGATGTCCA				Nadeau 2006	
ASIP							
Coding sequence	ASIPF2	TCATTTTCATGACAGTGGGATT	-	Exon 1-3/3: 465	450	Nadeau 2006	NM_001115079.1
	ASIPR2.0	CCTTAACATGTTCCTCATTAGGTTTA				Designed	
E1S	E1SF	TGAAAAGGAAGCAGAACCAGA	58	Exon 1-3/3: 465	523	Designed	AB518061.1
	ASIPR2.0	CCTTAACATGTTCCTCATTAGGTTTA				Designed	
E1L	E1LF	AGTTTTGGAGGTTCATTTCTAATGT	405	Exon 1-3/3: 465	870	Designed	AB518065.1
	ASIPR2.0	CCTTAACATGTTCCTCATTAGGTTTA				Designed	
E2	E2F	TAAACACATTGATGGCATTAACAA	64	Exon 1-3/3: 465	529	Designed	AB518062.1
	ASIPR2.0	CCTTAACATGTTCCTCATTAGGTTTA				Designed	
E3	E3F	GAAGCAGGCAGTCTTCTTGG	72	Exon 1-3/3: 465	470	Designed	AB518063.1
	ASIPR2.0	CCTTAACATGTTCCTCATTAGGTTTA				Designed	

E4	ASIPF8	CCAGCATTTTCATATTTTCTGGA	111	Exon 1-3/3: 465	576	Nadeau 2006	AB518066.1
	ASIPR2.0	CCTTAACATGTTCCTCATTAGGTTTA				Designed	
E5	E5F	TGAAATCAGTTGTGGCAGGAA	189	Exon 1-3/3: 465	654	Designed	AB518067.1
	ASIPR2.0	CCTTAACATGTTCCTCATTAGGTTTA				Designed	
Novel 8a	NewE1F	GTGTGGTTGTGATGGTGATGG	71	Exon 1/3: 239	310	Designed	TBA
	NewE1R	GGGAGATCTGGGAGGTTCATT				Designed	
Novel 8b	NewE1F	GTGTGGTTGTGATGGTGATGG	467	Exon 1/3: 239	706	Designed	TBA
	NewE1R	GGGAGATCTGGGAGGTTCATT				Designed	
Novel 9	NewE4F	GAGATCTTAAACAGCGCTGCA	372	Exon 3/3: 520	892	Designed	TBA
	E2_R	CAGCCTTAACATGTTCCTCATTA				Designed	
Novel 10	NewE1F	GTGTGGTTGTGATGGTGATGG	182	Exon 1/3: 239	421	Designed	TBA
	NewE1R	GGGAGATCTGGGAGGTTCATT				Designed	
Novel 11	NewE3F	TTTTTGGGAGCTGTTGTCCTC	184	Exon 3/3: 520	704	Designed	TBA
	E2_R	CAGCCTTAACATGTTCCTCATTA				Designed	
AGRP							
Coding sequence	AGRPF1	CCAGGACCATGCTGAAC	-	Exon 2/2: 449	449	Nadeau 2006	AB489990.1
	AGRPR	CAGGAAGATCAGCACCACCT				Designed	
POMC							
Coding sequence	POMCF	CTGGGGCTGCTGCTGCTGTGT	-	Exon 2/2: 717	717	Designed	NM_001031098.1
	POMCR	TGACCCTTCTTGTAGGCGCTTT				Designed	
Promoter A	APOMC	CCCATAAGCGACTTGCCTTC	169	Exon 2/2: 711	880	Designed	AB593424

	QER	CAGAGTCATCAGCGGGGTCT				Designed	
							AB593425;
A-2; B-2	POMC B-2	CTCTCCCCCTGCAGCATC	143	Exon 2/2: 711	854	Designed	AB593427
	QER	CAGAGTCATCAGCGGGGTCT				Designed	
Promoter B	BF	AGCGCTCCTCTGCAGTTTG	42	Exon 2/2: 711	753	Designed	AB593426.1
	BR	CAGAGTCATCAGCGGGGTCT				Designed	
T2	QEF2	ACTTCCAGCGTCTCCCAGAG	252	Exon 2/2: 711	963	Designed	AB620012.1
	QER	CAGAGTCATCAGCGGGGTCT				Designed	
T3	QEF1	GATTTCGGAGGCAAAGGATG	175	Exon 2/2: 711	886	Designed	AB620013.1
	QER	CAGAGTCATCAGCGGGGTCT				Designed	
PC1							
Coding				Exon 5-10/14:			
sequence	PC1F	CTACGCCAACTATGATCCAAGG	-	847	847	Designed	XM_003643060.2
	PC1R	TTTCCATCTTTTGGGATCAGC				Designed	
PC2							
Coding				Exon 9-11/12:			
sequence	PC2F	GGGAGGGAAAGGAAGCATCT	-	800	800	Designed	XM_419332
	PC2R	GGTCTTCTCCCCAAGTGTGTG				Designed	

Immunohistochemistry

Tissue preparation and procedures used DEPC-H_20 and DEPC-PBS as necessary. For *in situ* hybridization, whole embryos were fixed in modified Carnoy's (60% ethanol, 11.1% formaldehyde, 10% glacial acetic acid) for four hours, dehydrated in ethanol and cleared in Histosol (National Diagnostics). E12 embryos were cut along the sagittal plane to improve Histosol tissue penetration. Embryos were embedded in paraffin wax (Raymond Lamb) and transversely sectioned with a rotary microtome (Micron). E8 embryos were sectioned at 10µm, however due to the size difference and the extended time required to clear the E12 embryos in Histosol (3 weeks), the E12 embryos were more prone to crumbling and were sectioned at 14µm, which is still adequate for probe tissue penetration. Embryos were cut in serial sections and eight serial sections per embryo per slide were mounted in DEPC-H_20 on Superfrost® Plus slides (VWR), and dried overnight at 37°C. All probes were initially tested on a sub-sample of each embryonic stage to test whether optimization was required e.g. probe dilution, temperature of *in situ* hybridization, length of MABT washes, and temperature in which the colour reaction was applied. All experiments represent a minimum of two replicates of E8 and E12 for each probe and the experiments were considered representative of development where most sections in each slide showed the same result – sections with background or abnormal staining that covered most of a section were excluded from the final interpretation or repeated if necessary. For each round of *in situ* hybridization experiments, at least one embryo was represented twice, one had the sense probe, and other replicate had the anti-sense probe. Other than the probe applied, the slide with the sense probe was experimentally tested in the same way as the corresponding slide with the anti-sense probe, e.g. the colour reaction was applied for the same amount of time at the same temperature. The sense probe is provided alongside the anti-sense probe from the corresponding tissue from the same section.

Sox10 plasmids, containing the full-length chicken 2.2kb *Sox10* sequence, were gifts of M. Bronner (Caltech, Pasadena, CA) to C.V.J Baker. For all other probes, primers for the coding sequences used to generate each probe are listed in Table 1. PCR product was ligated into One Shot Top10 chemically competent cells (Invitrogen) using a PCR cloning kit (Qiagen). PCR products were incubated with

cloning reagents and the vector overnight at 4°C in a total volume of 5 µl using the following amounts: 2x Ligation Master mix = 2.5µl, PCR product = 2µl, pDrive cloning vector = 0.5µl. Cells were transformed and grown according to the manufacturers instructions (Invitrogen). Plasmids were extracted using alkaline denaturation and stored at -20°C until probe synthesis (Birnboim and Doly 1979). To examine the direction of the insert relative to the promoter sites, plasmids were sequenced with Sanger sequencing. Probes were linearised using standard restriction enzymes and transcribed with DIG RNA labeling mix (Roche). The *Sox10* anti-sense probe was linearized with HindIII and transcribed from T3. The *ASIP, PC1*, and *PC2* anti-sense probes were linearized with NotI and transcribed from SP6, and the sense (negative control) probes were linearized with PstI and transcribed from T7. The *AGRP* and *POMC* anti-sense probes were linearized with PstI and transcribed from T7, and the sense probe was linearized with NotI and transcribed from SP6.

In situ hybridization was performed on the paraffin embedded sections with Digoxigenin-labelled *Sox10, ASIP, AGRP, POMC, PC1* and *PC2* probes diluted in hybridization mix, separately. Each probe was hybridized overnight at 68-72°C in a Boekel slide incubator. After hybridization, slides were washed twice in 50% formamide, 50% 1xSSC and 0.1% Tween-20 at 65°C, then twice in MABT (0.1 M maleic acid, 150 mM NaCl, 0.1% Tween-20, pH 7.5) at room temperature. To block binding of nonspecific antibody, slides were incubated in 70% MABT, 20% natural sheep serum and 10% blocking reagent (Roche) for 2 hours. AP-conjugated anti-digoxigenin antibody (Roche) was diluted in this blocking solution at a dilution of 1/1500 and applied to slides, which were covered with parafilm and kept at room temperature overnight. Slides were subsequently rinsed in MABT 5 times for a minimum of 30 mins each. Slides hybridized with *POMC* probe were further rinsed in MABT overnight to reduce background. Slides were equilibrated in NTMT (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.1% Tween-20) twice for 10 mins each. To reveal colour, NBT-BCIP stock solution (Roche) was diluted in NTMT at a dilution of 1/1500 and applied to slides and covered with parafilm. The colour reaction of all probes, except for *POMC*, was performed at room temperature whereas the *POMC* colour reaction was conducted at 4°C.

The genes examined in our experiments have additional neuroendocrine functions as well as a putative function in pigmentation in the epidermis providing positive control of the anti-sense probe within sections (Takeuchi and Takahashi 1999; Takeuchi, Teshigawara, and Takahashi 2000; Ling et al. 2004; Nadeau et al. 2008; Yabuuchi et al. 2010; Roulin et al. 2011; Yoshihara et al. 2011; Saneyasu et al. 2013). Sections depicted in the figures are representative of staining between and within samples.

Results

Melanocyte distribution

Eumelanin is present in dorsal feather follicles at E8 but is more pronounced at E12 whereas pheomelanin is present in ventral feather follicles, with a small number of feather follicles in the flanks pigmented with eumelanin. *In situ* hybridization with *Sox10* revealed that melanocytes are present at E8 but that there is variation in distribution over the ventral and dorsal surface. On the ventral surface of E8 quail embryos, *Sox10* staining was apparent only in some feather follicles but was present throughout all feather follicles on the dorsal surface. In addition, *Sox10* staining was apparent in the epidermis on both ventral and dorsal surfaces (Fig. 2.1). At E12 *Sox10* was consistently expressed throughout feather follicles on both the ventral and the dorsal surface, as well as in the epidermis. In feather follicles at both stages of development, there was *Sox10* staining both where melanin is present, as well as where it is absent.

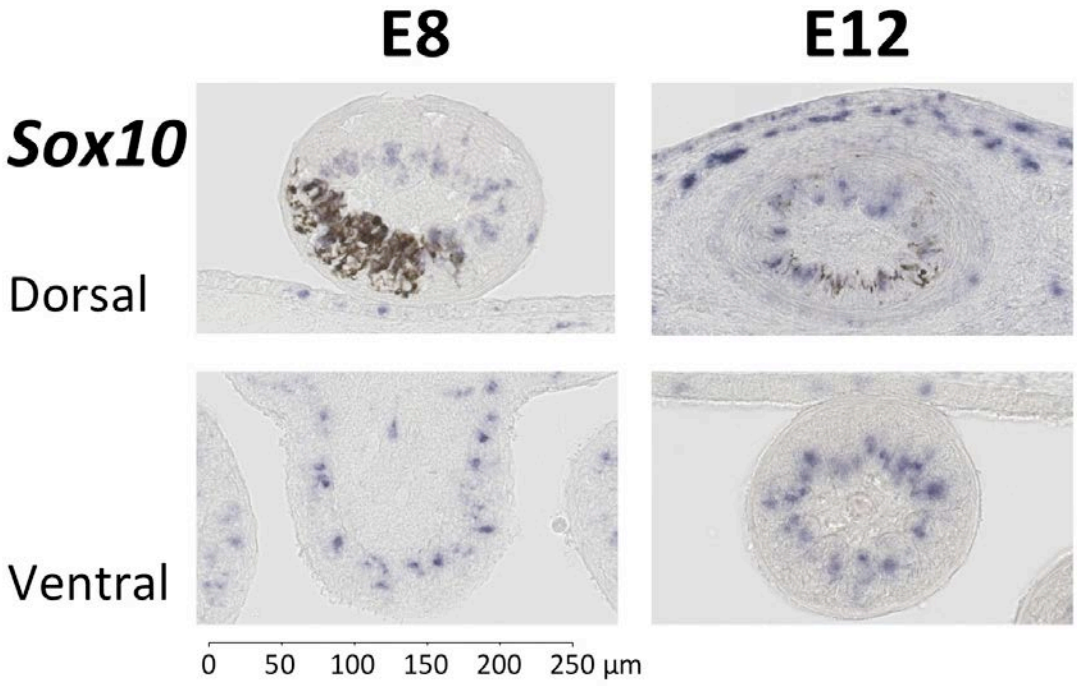


Fig. 2.1. Melanocyte distribution in Japanese quail feather follicles during embryonic development at E8 and E12. At E8 and E12 melanin is rarely present in ventral feather follicles (FFs) but is frequently observed in many FFs on the dorsal surface. Melanocytes are present within the epidermis and feather follicles. Staining of *Sox10* occurs in areas where individual barbs are developing in feather follicles on both the ventral and the dorsal surface of E8 and E12 quail embryos. The distribution of melanocytes is not restricted to areas pigmented with melanin but also occurs in areas where melanin is absent.

Genes inhibiting MC1R in melanocytes

We investigated 2 loci that inhibit MC1R: *ASIP* and its paralogue *AGRP.* Previously, it has been reported that adult quail have three *ASIP* alternatively spliced transcripts (1a, 1b, 1c), whereas the chicken has seven *ASIP* isoforms that are distributed among three promoter sites (classes) (Nadeau et al. 2008; Yoshihara et al. 2012). Aligning the non-coding regions of these *ASIP* isoforms reveals that there is close correspondence (Fig. 2.2). Quail transcript 1a, although short, matches chicken E1L, quail 1b matches chicken E3, and quail 1c matches chicken E4. However, neither 1a nor 1b is an exact match to the transcripts in chicken, as E1S is not alternatively spliced in either transcript. In contrast, 1c and E4, which are

The mechanisms underlying convergent evolution in the plumage patterns of birds comprised of only one non-coding exon, is an exact match.

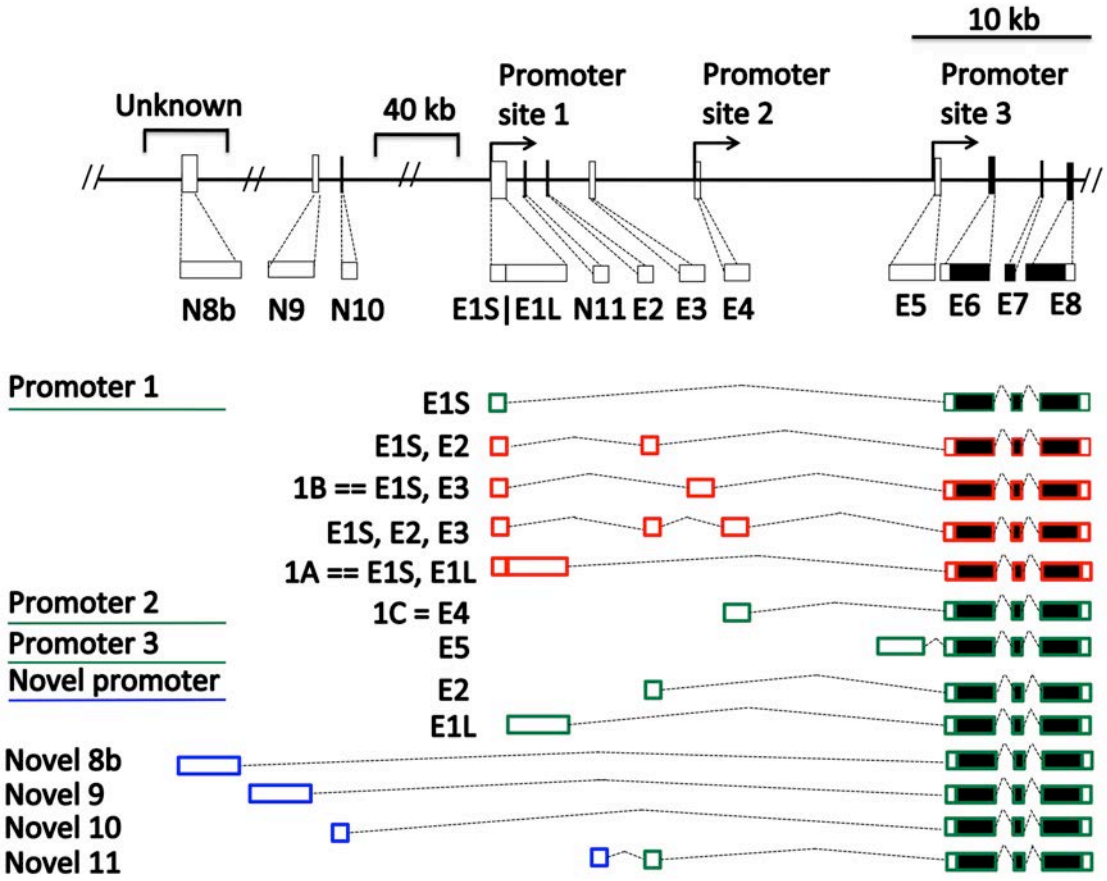


Fig. 2.2. Correspondence of *ASIP* alternatively spliced transcripts between embryonic Japanese quail, adult quail and chicken. Empty boxes represent non-coding exons and solid boxes denote coding exons. Chicken promoter sites are underlined in green, chicken transcripts begin with "E", and previously reported adult quail transcripts begin with "1" and are denoted with the corresponding chicken transcript with "==" (Nadeau et al. 2008; Yoshihara et al. 2012). Green lines and green boxes represent promoter sites and transcripts, respectively, that were successfully amplified in quail embryonic development, whereas red denotes transcripts that were not amplified. A blue line and blue empty boxes indicates a novel promoter site and novel alternatively spliced non-coding exons (begins with "N"), respectively, that have not been previously documented.

We report four new *ASIP* alternatively spliced transcripts (Fig. 2.3). Although we found 5 new alternatively spliced transcripts (Novel 8a, 8b, 9, 10, 11), we were only successful in sequencing Novel 8a once, which is similar in the 5' non-coding

sequence to Novel 8b. As such, we are uncertain if Novel 8a represents a new non-coding exon and discarded it from the subsequent results. The *ASIP* gene is located on chromosome 20. A refseq_genomic blastn search on Genbank against the *Gallus gallus* genome revealed that most of these novel transcripts are located upstream of *ASIP*. For Novel 8b, we did not find any significant similarity with genes on chromosome 20. Novel 9, 10 and 11 are located on the 5' side of the ASIP locus. The distance from the non-coding exons to *ASIP* is 40kb for novel 10 and 11 (Fig. 2.2, 2.3). Novel 11 is comprised of a new leader non-coding exon, alternatively spliced with the previously described non-coding exon 2 found in chicken, and is located between E1L and E2 (Yoshihara et al. 2012).

#Novel 8a; #Novel 8b

```
CAACGACTTGAAGTCAGGGCCGTCTTCTTGGCTACATGGCTGAG
CAACGAGCTACCTTCAGGTGGTGTGAGGCTGGCAATGGGGTGCAGATGGGGCCTCATATCTC
TGAGTAAATGGAACAGGCAGATGAGAAGGGCACAGCTGTGCTCTTCCACCTCAGCATGCTCC
CTGGACATCCATGCCTCTTGGCTGCCCACTAACCTCAGCTGGAGAGTGCACAGTGATTAATA
TTAGTGAATCTGATCTAAGCCTATTGCAAACCATTGATCTGAGCGGGCTGCACTGCTCCAAA
AATAGCCTCACAATATTACTGAGGCATAGCTCTTGGTGTGGTGAGCTGCTCAAGCTGCCTAA
TCACATACAAGACCCCAGGCTCTGTATGTTCTCAATGCTTTTTTCCTGTGACCACTGCTCAT
CACACTTGGCTACTGTGGGTTAATACAGCTATGTCATGTTCAGCCAAATCAGCTGTCACATC
AGCTCTCCATTT
```

#Novel 9

```
Cj CAGTGACTACATCTGGAAAAGAAAAAAGAAGAAACATGAAGATGACCATACGCCGTTGTA
   ||||||| ||||||||||||||||||  |||||||||||||||||| ||||| ||||||
Gg CAGTGACAACATCTGGAAAAGAAAAAA--AGAAACATGAAGATGACCGTACGCTGTTGTA

Cj CAGTGACTACATCTGGAAAAGAAAAAAGAAGAAACATGAAGATGACCATACGCCGTTGTA
   ||||||| ||||||||||||||||||  |||||||||||||||||| ||||| ||||||
Gg CAGTGACAACATCTGGAAAAGAAAAAA--AGAAACATGAAGATGACCGTACGCTGTTGTA

Cj TTTCCTTCTCAGATGAGTTATATTTTGAAAGCAGCAATAAAAAA-TTCTCAGATAAGGGA
   |||||||||||||||||||||||||||||||||||||||||||| ||||| | |||||||
Gg TTTCCTTCTCAGATGAGTTATATTTTGAAAGCAGCAATAAAAAACTTCTCGGGTAAGGGA

Cj TTGGAGAAGTGATGGATTTGTGGCAGCGGCTGTCTTTTGGTCCTTCGTTGACTGCTTTGG
   ||||||||||||||| |||||||||||||||||||||||||| | |||||  | |||
Gg TTGGAGAAGTGATGGAATTGTGGCAGCGGCTGTCTTTTGGTCCTTGGCTGACTATTCTGG
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Cj GGTTGA--ATTTTTCTTTTTAATGTTCACATGTGTGTGCCTTTTTTGGGGGAAATGGGGC
   ||||||   |||||||||||| ||||||||| |||||| |||||||| ||| |||||||||
Gg GGTTGATTTTTTTTCTTTTTGATGTTCACACGTGTGTACCTTTTTT-GGGAAAATGGGGC

Cj CAGATTCCCCCCTGGCTGAGAGTCTACAGCTTCTTAAAGGTGAAGGCTGTTCCAGT
   |||||||||||||||||||| ||||||||||||||||||||||||||||||||
Gg CAGATTCCCCCCTGGCTGAGAGGCTACAGCTTCTTAAAGGTGAAGGCTGTTCCAGT
```

#Novel 10

```
Cj TTGTGGCAGCGGCTGTCTTTTGGTCCTTCATTGACTGCTTTGGGGTTGA--ATTTTTCTT
   ||||||||||||||||||||||||||||   |||||  | |||||||||   ||||||||
Gg TTGTGGCAGCGGCTGTCTTTTGGTCCTTGGCTGACTATTCTGGGGTTGATTTTTTTTCTT

Cj TTTAATGTTCCACCATGTGTGTGCCTTTTTTGGGGGAAATGGGGCCAGATTCCCCCCTGG
   ||| ||||||   || |||||| |||||||| ||| ||||||||||||||||||||||||
Gg TTTGATGTTC--ACACGTGTGTACCTTTTTT-GGGAAAATGGGGCCAGATTCCCCCCTGG

Cj CCTGAGAGTCTACAGCTTCTTAAAGGTGAAGGCTG-TCCAGT
    ||||||| |||||||||||||||||||||||| ||||||
Gg -CTGAGAGGCTACAGCTTCTTAAAGGTGAAGGCTGTTCCAGT
```

#Novel 11 + exon 2

```
Cj TTCCAGGACCAGCAACTCTTATTTATAAATGCTATACAGCGTATATGTTAAGATTTATAC
   |||||| ||||||| |||||||||||||||| ||||| |||||||||| ||||||||||
Gg TTCCAGAACCAGCAGCTCTTATTTATAAATGTTATACCGCGTATATGTTCAGATTTATAC

Cj AAGATGTTTTCATGGTACGCATAGAATTCTGAACCCTGTAAACACACTGATGGCATTAAC
   ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Gg AAGATGTTTTCATGGTACGCATAGAATTCTGAACCCTGTAAACACATTGATGGCATTAAC

Cj AAGGACCAGATGTCAGTTGCCTTTCTCATAAAGAAAACAACAAG
   |||||||||||||||||||||||||||||| ||||||||||||||
Gg AAGGACCAGATGTCAGTTGCCTTTCTCATATAGAAAACAACAAG
```

Fig. 2.3. New *ASIP* alternatively spliced transcripts that have not been previously documented in the avian literature. Novel 8b had no significant similarity with the chicken genome. The first six base pairs of the 5' non-coding sequence of Novel 8a (base pairs written in grey) and Novel 8b is the same as indicated by an underline. We were only able to sequence Novel 8a once and given its similarity to Novel 8b, Novel 8a may not represent a new alternatively spliced transcript. Novel 9-11 (Cj:

Coturnix japonica) are aligned to the chicken genome (Gg; *Gallus gallus*). A vertical bar - | - indicates matching base pairs and a space indicates no match, whereas dashes indicate indels. Novel 11 contains a new leader exon alternatively spliced with the existing chicken non-coding exon 2 (Yoshihara et al. 2012) which is highlighted in grey.

RT-PCR analyses revealed that *ASIP* was always expressed on both the dorsal and ventral surfaces and in both stages of quail embryonic development (Table 2.2). Non-coding exon E3 as well as alternatively spliced transcripts that combine more than one non-coding exon, such as the leader exon E1S, could not be amplified despite multiple attempts with numerous primer pairs. Of the isoforms that we successfully amplified by designing primers based on previously reported transcripts, there was considerable variation in expression at both the temporal and spatial scale (Fig. 2.2; Table 2.1, 2.2; supplementary Table S2.1).

Exon E1S was consistently expressed on the ventral surface in both stages of development, but was only present on the dorsal surface at E12. Exon E1L was only present on the ventral surface at E8 and was not present later in development on either surface (Table 2.2). E2 was consistently present on the ventral surface at both stages of development, and was variable on the dorsal surface at E8 but was absent on the dorsal surface of E12. E4 was variable in expression on the dorsal surface early in development but was consistently present on the ventral surface. At E12, E4 was also consistently present on the ventral surface, but was not expressed on the dorsal surface. Similarly, E5 was consistently present on the ventral surface at both early and late stages of development, and was consistently present on the dorsal surface early in development, but in E12, E5 was variable in expression on the dorsal surface. For the primer pairs targeting E1S, E2 or E3, we did not find multiple bands in agarose gels, indicating that these exons do not appear to be alternatively spliced with other non-coding exons during quail embryonic development, as occurs in the chicken. Similar to the existing *ASIP* alternatively spliced transcripts there was variation in the expression of *ASIP* Novel 8b and Novel 10 within and between developmental stages. In contrast, *ASIP* Novel 11 and Novel 9 were expressed on both the ventral and dorsal surfaces in all embryos at stages E8 and E12 (Fig. 2.4; Table 2.2).

Table 2.2. Patterns of exon expression for *ASIP* and *AGRP* in embryonic quail at developmental stage E8 and E12 determined by RT-PCR. "+" = present, "-" = absent, "+/-" = variable expression.

Target sequence		**E8**	**E12**
β-actin	Dorsal	+	+
	Ventral	+	+
***ASIP* coding exons (6-8)**	Dorsal	+	+
	Ventral	+	+
E1S	Dorsal	-	+
	Ventral	+	+
E1L	Dorsal	-	-
	Ventral	+	-
E2	Dorsal	+	-
	Ventral	+	+
E4	Dorsal	+/-	-
	Ventral	+	+/-
E5	Dorsal	+	+/-
	Ventral	+	+
Novel 8b	Dorsal	+	+/-
	Ventral	+/-	+
Novel 9	Dorsal	+	+
	Ventral	+	+
Novel 10	Dorsal	+/-	-
	Ventral	+/-	+/-
Novel 11	Dorsal	+	+
	Ventral	+	+
***AGRP* coding exons (1-2)**	Dorsal	+/-	-
	Ventral	+/-	+/-

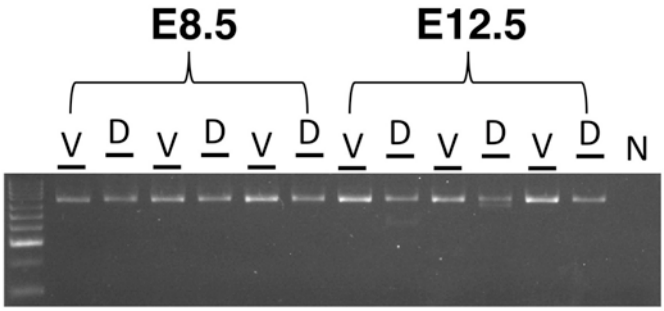


Fig. 2.4. mRNA expression, detected by RT-PCR, of the previously undocumented *ASIP* alternatively spliced transcript Novel 11 in Japanese quail. The dorsoventral surface is indicated with brackets and the developmental stage is above the gel lanes: V = Ventral, D = Dorsal, N = Negative control. RT-PCR results are provided for ventral and dorsal surfaces for three samples of each stage, followed by a negative control.

The second locus that can inhibit MC1R is the *ASIP* paralogue *AGRP*. As there is no previous evidence to suggest that *AGRP* may have a function in patterns, or that there are alternatively spliced transcripts that have a role in pigmentation, we focused on the expression patterns of the coding sequence. *AGRP* expression was detected a both developmental stages. Expression at E8 was variable on both the ventral and dorsal surfaces, whereas at E12, it was not expressed dorsally and had variable expression ventrally (Table 2.2).

In situ hybridization confirmed that *ASIP* is expressed early in development at E8 and continued into later stages of development at E12 (Fig. 2.5). In both early and late stages of development, *ASIP* is strongly expressed within the pulp of developing feather follicles on the ventral surface and many ventral cells surrounding the dermal pulp in the epidermis exhibit *ASIP* nascent transcription. Some feather follicles on the flanks of the ventral surface near or on the wing have eumelanin pigmentation and a minority of these feather follicles had some staining for *ASIP* in the feather pulp both where eumelanin is present as well as where it is absent (Fig. 2.6). In contrast, in feather follicles on the dorsal surface of E8 and E12 embryos, there was no staining for *ASIP* in the dermal pulp but there does appear to be faint nascent transcription within some cells of the feather pulp and the epidermis. The pattern of expression of

ASIP on the dorsal surface did not vary between feather follicles that are pigmented with eumelanin and those that are not pigmented.

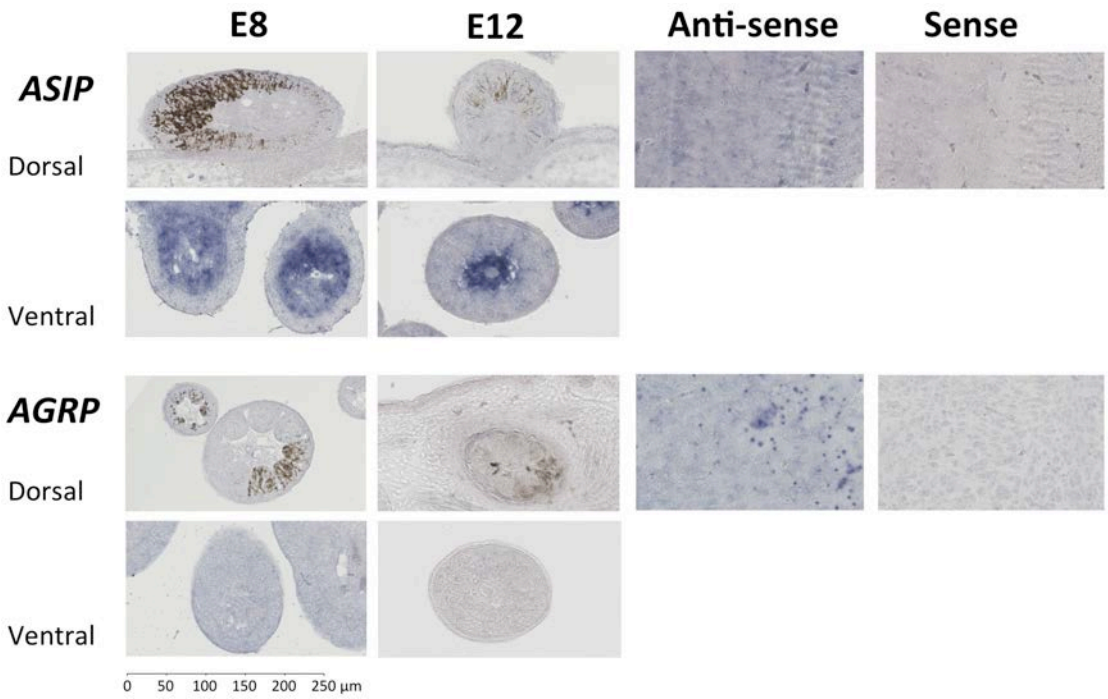


Fig. 2.5. *In situ* hybridization results for *ASIP* and *AGRP* in quail feather follicles at E8 and E12. *ASIP* is expressed in the dermal pulp of ventral feather follicles in E8 and E12 embryos, but is not visible on the dorsal surface. *AGRP* was not expressed in the feather follicles in early or late development, either ventrally or dorsally. Representative anti-sense and sense probes are shown in the spinal cord of E12 in *ASIP* and E8 in *AGRP*.

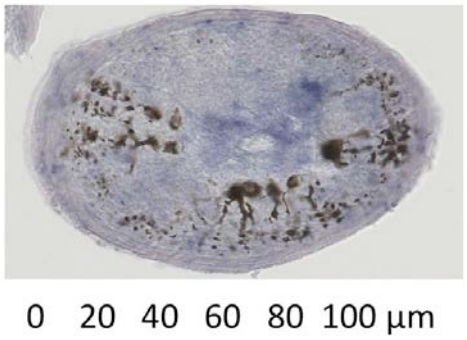


Fig. 2.6. *ASIP* expression in a feather follicle with eumelanin on the flank of an E12 Japanese quail embryo. In some feather follicles in the flank or on the wing of E12 embryos *ASIP* expression was observed with eumelanin pigmentation.

Staining of *AGRP* was less prevalent than *ASIP* (Fig. 2.5). Unlike *ASIP*, *AGRP* was not strongly expressed in the pulp of developing feather follicles on the dorsal or ventral surface, at either stage of development or in the epidermis. However, nascent transcription of *AGRP* was commonly observed on the ventral and dorsal surface of E8 as well as E12 in feather follicles that contain melanin as well as those that did not have melanin pigmentation (Fig. 2.7).

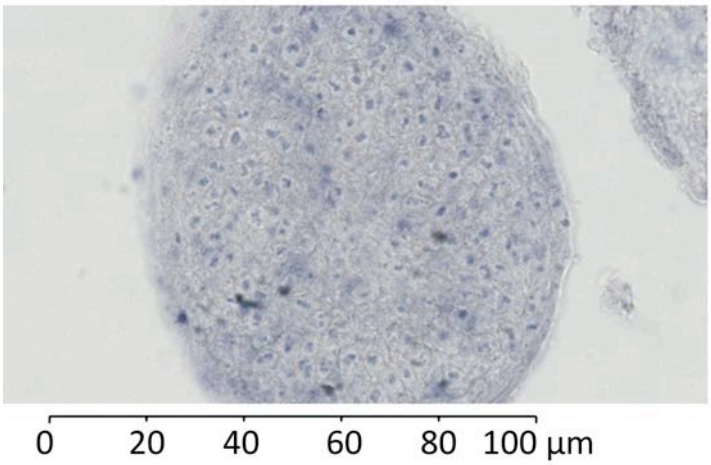


Fig. 2.7. Representative nascent transcription of *AGRP* in ventral developing feather follicles in E8 Japanese quail embryos. Similar staining was found in feather follicles on the dorsal surface of quail E8, as well as both the ventral and dorsal surface of quail E12, and the epidermis.

Genes activating MC1R in melanocytes

We investigated 3 loci that are involved in activation of MC1R: *POMC*, *PC1* and *PC2*. Yoshihara et al. (2011) previously described four alternatively spliced transcripts of *POMC* in chicken encompassing two promoter sites that are transcribed with and without an additional non-coding exon: A-1, A-2, B-1, and B-2. The chicken distal promoter site A is located 14kb upstream in another gene, *ANGPTL7*, whereas promoter site B is located within the *POMC* gene. In addition, three unpublished adult quail *POMC* alternatively spliced transcripts are available on NCBI: T1, T2, and T3 (Fig. 2.8; Table 2.1). Aligning these sequences, there is no correspondence between the chicken *POMC* A-1 and the quail transcripts. Chicken *POMC* B-1 corresponds well with quail T1 indicating that adult quail possess promoter site B. Chicken *POMC* A-2 and B-2 partially correspond with quail T2 and T3 as each possesses a chicken *POMC* non-coding exon but does not possess the corresponding chicken leader exon (Yoshihara et al. 2011). Finally, quail *POMC* T2 and T3 are similar in possessing a 5' quail non-coding exon, as well as the chicken non-coding exon, but T3 also possesses an additional 3' quail non-coding exon before the *POMC* coding region (Fig. 2.8).

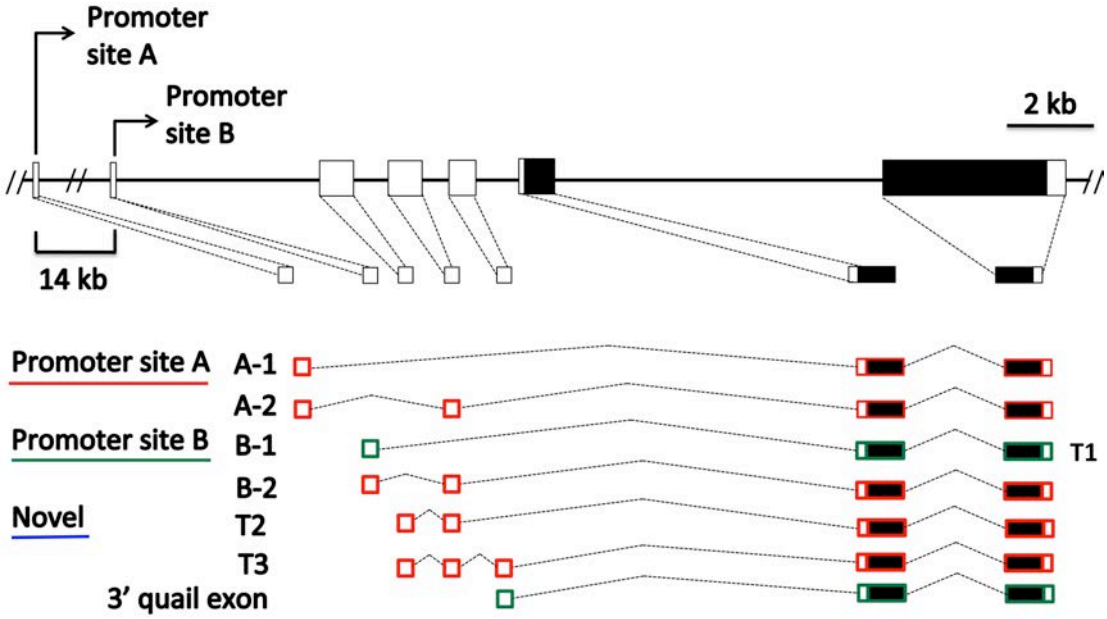


Fig. 2.8. Correspondence between alternatively spliced transcripts of *POMC* in embryonic Japanese quail and chicken. Adult chicken promoter sites and alternatively spliced transcripts are listed on the left, and the corresponding quail transcript is listed to the right of the transcript. Quail *POMC* transcripts that have no correspondence with chicken transcripts are listed with the novel promoter site (Yoshihara et al. 2011). Empty boxes represent non-coding exons and solid boxes denote coding exons. The non-coding exons that were amplified in quail embryonic development are represented with green, whereas red denotes the promoter site and non-coding exons that we were unable to amplify.

RT-PCR analyses revealed that the coding exons of *POMC* are always expressed on both the dorsal and ventral surfaces in quail E8 and E12 (Table 2.3). The chicken *POMC* non-coding exon *POMC* A-1 and quail T2 could not be amplified despite multiple attempts with numerous primer pairs (Fig. 2.8; Table 2.1; supplementary Table S2.1). Transcripts arising from promoter site B were present in almost all ventral and dorsal samples, at both E8 and E12, the exception being one dorsal sample in E8. The 3' quail non-coding *POMC* exon was also variable in its expression, as it was amplified in two samples from each of E8 and E12, and was expressed in both ventral and dorsal samples.

Table 2.3. Patterns of expression for *POMC* in quail at embryonic stage E8 and E12 determined by RT-PCR. "+" = present, and "+/-" = variable expression.

Target sequence		**E8**	**E12**
POMC	Dorsal	+	+
	Ventral	+	+
Promoter B	Dorsal	+/-	+
	Ventral	+	+
3' quail non-coding exon	Dorsal	+/-	+/-
	Ventral	+/-	+/-

In situ hybridization revealed that *POMC* is expressed in the epidermis and feather follicles on the dorsal and ventral surface of quail E8 (Fig. 2.9). In addition, nascent transcription of *POMC* is frequently observed throughout feather follicles on both ventral and dorsal surfaces, in early and late stages of quail embryonic development. Within feather follicles, *POMC* is apparent in the feather cuticle as well as the feather pulp and this pattern of expression is consistent on the ventral and dorsal surface, both where melanin is present and where it is absent. This pattern of expression is the same in E12 embryos.

The other loci that are involved in activation of the MC1R pathway are the endoproteases *PC1* and *PC2* that cleave *POMC* products to make ACTH and MSH, respectively. As there is no evidence to suggest that there are alternatively spliced transcripts of *PC1* and *PC2* that have a role in feather follicle pigmentation, we focused on the expression patterns of the coding sequence. RT-PCR revealed that *PC1* was present in nearly all samples on the ventral and dorsal surface at E8 and E12, the exception being one ventral sample from E8 (Fig. 2.9). Similarly, *PC2* was present in all dorsal and ventral samples, in both stages of development examined.

In situ hybridization demonstrated that there is nascent transcription of *PC1*

within the feather pulp and the feather cuticle on the ventral and dorsal surface in E8 quail embryos (Fig. 2.9). At E12 the low level of expression of *PC1* had not progressed further than nascent transcription. *PC2* is faintly expressed in feather follicles and the epidermis at E8 on both ventral and dorsal surfaces. At E12, *PC2* was also faintly expressed in the epidermis but there was strong expression of *PC2* within the feather pulp as well as the feather cuticle (Fig. 2.9). The patterns of expression were consistent in feather follicles over both the ventral and dorsal surface where melanin is expressed as well as where it is absent.

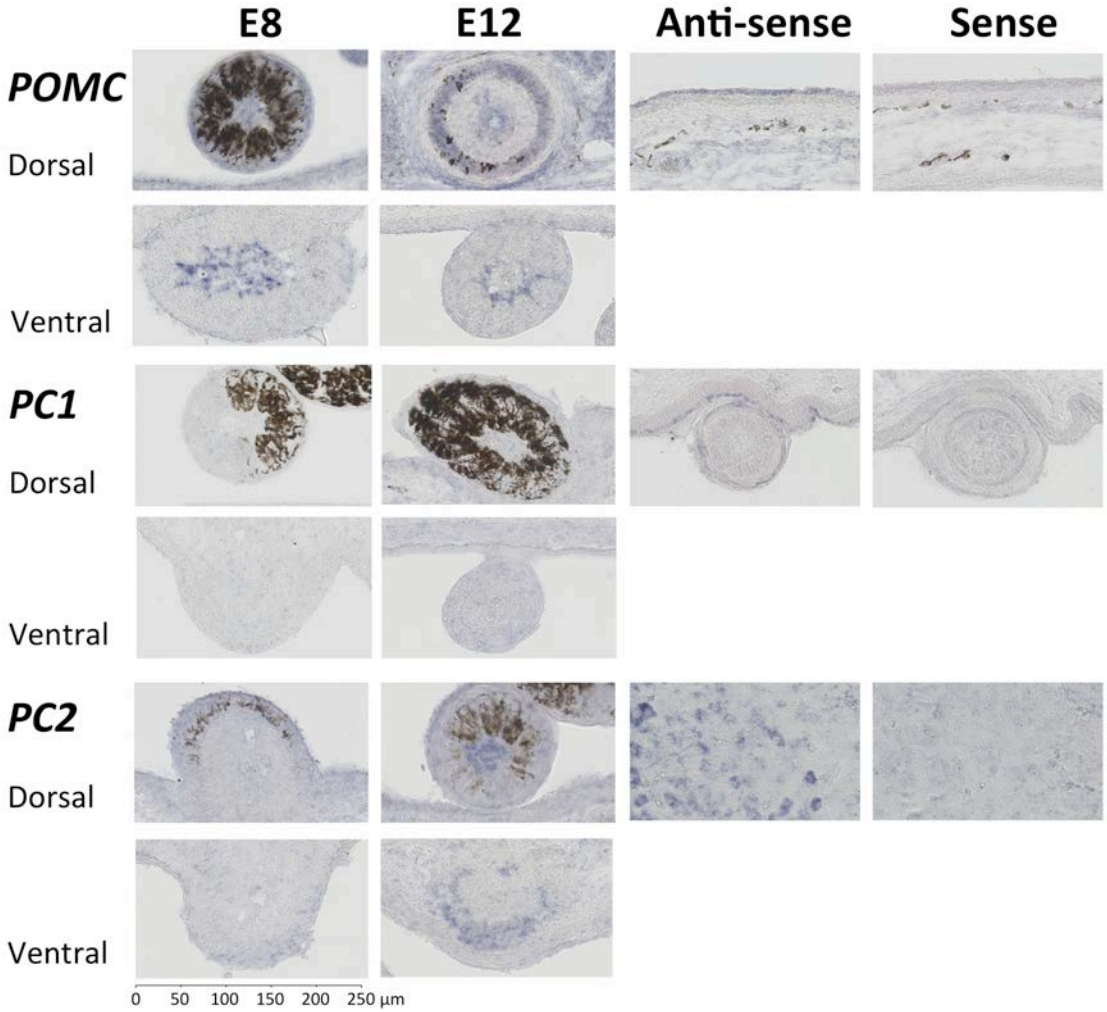


Fig. 2.9. *POMC*-, *PC1*- and *PC2*- Patterns of expression of *POMC*, *PC1* and *PC2* in feather follicles at E8 and E12. *POMC* is expressed in the dermal pulp of ventral and dorsal feather follicles in E8 and E12 embryos. Nascent transcription of *PC1* was observed in developing feather follicles but did not develop further at E12. Similarly, there is nascent transcription of *PC2* at E8 on both ventral and dorsal surfaces, but when feather development is more progressed (e.g. E12), *PC2* is strongly expressed

in feather follicles on both the ventral and dorsal surface. Representative anti-sense and sense probes are shown in the dorsal epithelium of E12 in *POMC* and *PC1*, as well as in the spinal cord of E12 in *PC2*.

Discussion

Our data provide some of the first evidence that extracellular ligands involved in MC1R activation and inhibition are involved in producing phenotypic variation over the ventral and dorsal surfaces in quail embryogenesis. *Sox10* staining demonstrated that the species-typical dorsal pigmentation in embryonic quail is not due to melanocyte distribution. We discovered novel quail *ASIP* transcripts, that have not been previously described that may indicate evolution in the mechanism of inhibition of MC1R (Nadeau et al. 2008; Yoshihara et al. 2012). Similar to previous studies, we found that the pale-bellied phenotype of quail is consistent with inhibition of MC1R via ASIP, and that this mechanism has been conserved between mammals and birds. In contrast with mice, dorsal temporal specific patterning within-feathers does not appear to be due to regulation of *ASIP*. Of the six *POMC* transcripts previously described, only one is conserved between quail and chicken whereas the other *POMC* transcript possessed a 3' non-coding exon that may be quail specific. Both of these transcripts showed variable expression over the ventral and dorsal surface. Nevertheless, the *POMC* coding region is expressed within dorsal and ventral feather follicles demonstrating that activation of MC1R may be required to produce eumelanin. In quail embryonic development, there is little expression of *PC1* within feather follicles whereas *PC2* is expressed strongly in later quail embryonic development. Therefore, it may be that *POMC* products are directly cleaved by *PC2* to make MSH. Together, this suggests that MC1R is differentially stimulated across the ventral and dorsal surface to create variation in phenotypes.

There was considerable variation in the expression of the seven previously described alternatively spliced transcripts of *ASIP*, between adult quail, chicken and embryonic quail (Nadeau et al. 2008; Yoshihara et al. 2012). Of these seven, only three transcripts were observed in embryonic quail, and only one of these three has previously been reported in adult quail (E4). In rabbits and mice, the distal *ASIP* promoter leads to ventral specific coloration, and the proximal promoter performs

temporal control of coloration during hair development (Bultman, Michaud, and Woychik 1992; Vrieling et al. 1994; Fontanesi et al. 2010; Manceau et al. 2011). Two of the three *ASIP* transcripts identified in adult quail have a leader exon of E1S, which corresponds to the chicken distal promoter, and these transcripts are expressed both dorsally and ventrally in adult quail. In embryonic quail, only one alternatively spliced transcript out of nine has a leader exon comprised of E1S (Fig. 2.3). Of the alternatively spliced sequences expressed in quail embryonic development, similar to the chicken but dissimilar to adult quail, the *ASIP* non-coding exon E1L is ventral specific but lacks the leader exon E1S. However, unlike adult quail and chicken, *ASIP* E3 could not be amplified in embryos (Fig. 2.3).

We report on four undescribed alternatively spliced transcripts of *ASIP*, one of which is alternatively spliced with E2 and three out of four of these transcripts are also on the chicken genome (Fig. 2.3, 2.4). The novel non-coding exon N9 is positioned next to E2, with which it is alternatively spliced, whereas two of the novel non-coding exons are located a further 40kb upstream of *ASIP* on chromosome 20 (N10 and N11) and the position of N8b is unknown. Annotation of the quail genome is likely to reveal a similarity in exon position in the genome and should improve the resolution of N8b. However, as our intention was to document the distribution of existing *ASIP* transcripts between embryonic quail, adult quail and the chicken, we did not perform 5' RACE that would clarify the number of *ASIP* transcripts, and which promoter sites initiate transcription. Nevertheless, our findings indicate surprising variation in the expression of *ASIP* alternatively spliced transcripts between embryonic quail, adult quail, and chicken breeds which could indicate that there is variation between developmental stages in quail, as well as evolution within the mechanism of the pale-bellied phenotype between different species/breeds of chicken that may be lineage specific. We anticipate that there may be further variation in *ASIP* transcripts in other species of birds.

The other locus we examined that may inhibit MC1R, *AGRP*, did not appear to influence ventral pigmentation or within-feather patterning (Fig. 2.5). RT-PCR results demonstrated that *AGRP* is variable in expression over the ventral and dorsal surface of developing quail embryos, which would be consistent with a temporal specific function (Table 2.2). Although nascent transcription of *AGRP* in E8 feather follicles was found, this did not progress further than nascent transcription at E12

(Fig. 2.7). If *AGRP* has a ventral specific function in inhibiting MC1R the expectation would be that it would have been expressed in all ventral samples, but this was not the case (Table 2.2).

In situ hybridization with the *ASIP* and *AGRP* probes failed to demonstrate expression of these genes on the dorsal surface of embryos, in contrast to RT-PCR results. This may be due to a difference in the sensitivity of these techniques (Fig. 2.7; Table 2.2). RT-PCR results demonstrated that *ASIP* is consistently expressed on both the dorsal and ventral surface of E8 and E12 embryos, yet *in situ* hybridization revealed that *ASIP* was strongly expressed within developing feather follicles only on the ventral surface of embryos of both developmental stages. Perhaps *ASIP* expression on the dorsal surface of quail embryos is important in pigmentation patterns but was not detected by *in situ* hybridization or perhaps *ASIP* has a more prevalent function in dorsal epidermis, which we did not control for in RT-PCR. However, a difference in the sensitivity between RT-PCR and *in situ* hybridization is unlikely to be the cause of a discrepancy between techniques in the case of *AGRP* given that the expression of the *AGRP* coding sequence was variable within and between samples (Table 2.2).

The gene involved in activation of MC1R, *POMC*, appears to have few alternatively spliced transcripts (Fig. 2.2, 2.8; Table 2.1, 2.3). Similar to *ASIP*, we found that *POMC* transcripts varied in their patterns of expression between quail embryonic development and the chicken. Of the *POMC* transcripts expressed, we did not find any that are dorsal specific. However, this may be due to a lack of detailed 5' information. A future study will investigate *POMC* alternatively spliced transcripts and promoter sites utilizing 5' RACE in quail. To definitively test whether MC1R activation can occur without POMC peptides, experimental downregulation of *POMC* is required, but given the low basal activity of MC1R in chicken in comparison with mice and humans (Jackson 1997; Ling et al. 2003; Ling et al. 2004) it is likely that *POMC* is required for activation. In addition, an important follow up study would be to examine the distribution of MC1R within feather follicles.

Our main finding, that *POMC* is transcribed in feather follicles on the dorsal surface of early and late quail embryonic development, indicates that the generation of within-feather patterning may require activation of MC1R. It has been reported that

ACTH has a higher binding affinity to MC1R than MSH (Ling et al. 2003). However, in the feather follicles of quail embryos in early and late stages of plumage development *PC2,* but not *PC1,* is expressed (Fig. 2.9). The patterns of expression of *PC2* correlates well with pigmentation in embryogenesis: both pigmentation and *PC2* are less prevalent at E8 whereas pigmentation and *PC2* are strongly expressed at E12 (Fig. 2.9). Therefore MSH, not ACTH, is probably responsible for activation of MC1R to produce dorsal pigmentation patterns in quail. In contrast chicken breeds express *PC1, PC2* and *POMC* within feather follicles (Yoshihara et al. 2011). However, the results of the chicken study are unclear given that the two breeds used in the study are apigmented (Silky chicken) and barred (Okayama-Jidori) and there was no comparison made between the patterns of gene expression between these phenotypes. Nevertheless, it is interesting that *PC1* is expressed within chicken feather follicles and this suggests differences in the colour mechanism between wildtype quail and chicken breeds. But it is unclear if these differences are representative of evolution or artificial selection in the chicken.

There are three avian MSH peptides, α-MSH, β-MSH and γ-MSH, which vary in similarity to human peptides (Ling et al. 2004). The amino acid residues of α-MSH are identical in human and chicken, whereas β-MSH varies in three positions, and γ-MSH is the most variable. It is thought that the ACTH peptide may have been an important ligand for all MCRs in the ancestral vertebrate but that during vertebrate evolution, the other MSH subtypes evolved specificity for MCR types. For example, α-MSH has evolved specificity for MC1R in humans. There are three forms of α-MSH: desacetyl-, monoacetyl-, and diacetyl-α-MSH and the MC1 receptors of chicken have a slightly higher affinity for the desacetylated peptide, which is the product that is directly cleaved from POMC peptides. Given that activation of MC1R may be required for melanin synthesis, similar to humans, perhaps the desacetylated α-MSH peptide functions in plumage pattern formation.

Our major finding, that *POMC* and *PC2* are expressed in feather follicles, contrasts with the emphasis of previous studies of within-feather patterning (Nadeau et al. 2008; Yoshihara et al. 2012; Lin et al. 2013). It is interesting that both *ASIP* and *PC2* are expressed within feather follicles of quail embryos on the ventral surface. It is currently unknown what concentrations of ASIP and MSH are found in the ventral feather follicles of quail embryos. The affinity of MC1R for ACTH/MSH is lower in

birds than humans and mice indicating that a significantly higher concentration of ligands would be required for MC1R activation (Ling et al. 2004).

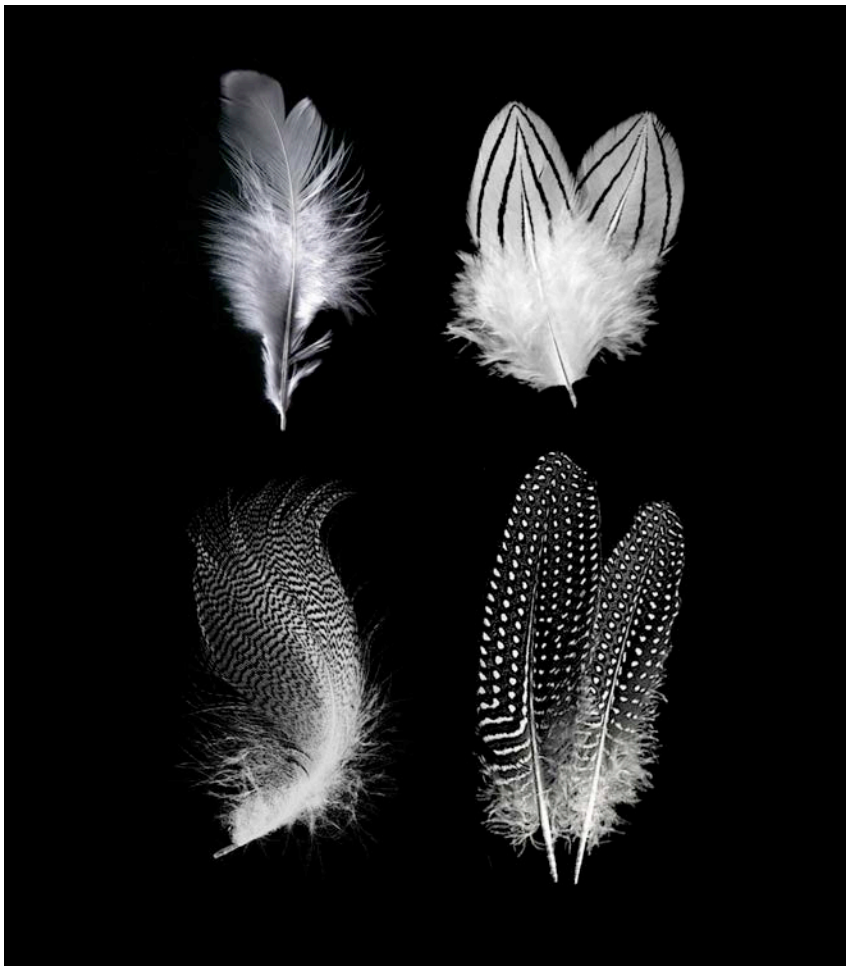
We showed that activation of MC1R may be required for melanogenesis on the ventral and dorsal surface, but it is unknown what mechanism may inhibit MC1R to create the species typical dorsal stripes and within-feather pigmentation. There are several possible candidate genes that downregulate MC1R activity. Alternatively, perhaps the β-defensin ligand may down-regulate cAMP in the dorsal plumage between stripe and within-feather patterns in quail. β-defensin is a member of a family of secreted peptides that are structurally similar to agouti. In vertebrates, β-defensin is highly polymorphic in sequence and copy number and has the potential for extensive cross-talk with the melanocortin system but its function in avian plumage coloration has not been demonstrated (Candille et al. 2007).

Several other candidate genes may inhibit MC1R to create quail dorsal stripes and within-feather patterning. In mice *Corin* appears to suppress the *ASIP* pathway and modulates temporal banding patterns. The *Corin* gene encodes a transmembrane protease that acts in the dermal papilla in mice (Enshell-Seijffers, Lindon, and Morgan 2007), but does not appear to have an effect in domestic and wild cats (Kaelin et al. 2012). Alternatively, attractin, a single-transmembrane-domain protein, interacts with melanocortin receptors to regulate energy metabolism and pigmentation (Gunn et al. 1999; Gunn and Barsh 2000). In mice, a lack of attractin produces little or no yellow pigmentation, and acts downstream of *ASIP* but upstream of MC1R, and prevents follicular melanocytes from responding to ASIP. Similarly, mice that lack intracellular ubiquitin ligase mahogunin also lack a pigmentary response to ASIP (He et al. 2003). Thus there are many different loci that could differentially inhibit the MC1R pathway to produce dorsal between stripe and within-feather patterning.

It is thought that plumage patterns may be a result of reaction-diffusion dynamics (Prum and Williamson 2002) but given that reaction-diffusion relies on an activator that syntheses its own inhibitor, it is unclear how the molecular mechanisms of pigmentation might relate to this model. Our data are consistent with previous reports that, similar to mice, *ASIP* is required to produce a pale-bellied phenotype in quail whereas, we found little evidence for a pigmentation function of *AGRP*. In

contrast with previous research on avian pigmentation, we showed that *POMC* and *PC2* are likely to play a greater role in pigment patterning than hitherto realised.

Chapter 3: Evolutionary pathways to convergence in plumage pattern phenotypes



The mechanisms underlying convergent evolution in the plumage patterns of birds

Abstract

Avian plumage is ideal for investigating phenotypic convergence because of repeated evolution of the same within-feather patterns. In birds, there are three major types of regular patterns within feathers: scales, bars and spots. Existing models of within-feather pattern development, suggest that scales have the simplest developmental mechanism, bars require more stringent regulation than scales, and spots have the strictest developmental parameters. We hypothesized that increasing stringency in the mechanism of pattern formation predicts the evolutionary trajectory of patterns, and hence scales evolve first, followed by bars and finally spots. Here, using Bayesian phylogenetic modeling we reconstructed pattern evolution in the most spectacularly patterned avian clades – aquatic waterfowl (Anseriformes) and terrestrial gamebirds (Galliformes). Independent analyses of seven feather patches reveal that spots evolve after bars and scales. However, bars evolve more frequently from an absence of patterns than scales, contradicting our predictions. Analyses of larger body regions support a dorsoventral axis of modularity. Over the whole body, many constraints are conserved, e.g. spots are derived. Overall there was remarkable similarity in the evolutionary trajectories of plumage pattern evolution in Galliformes and Anseriformes, suggesting that developmental constraint is similar in these two orders, despite large ecological differences.

The mechanisms underlying convergent evolution in the plumage patterns of birds

Introduction

Comparative studies are a powerful tool for understanding the underlying processes behind similarity in animal forms and have revealed that the mechanisms underlying convergent evolution are diverse as well as surprising (Parra-Olea and Wake 2001; Prud'homme et al. 2006; Arendt and Reznick 2008; Fritz et al. 2014). Phenotypic convergence may arise from similar selective regimes, but may also be at least partly explained by developmental constraint. This latter view is gaining momentum with proponents advocating an equivalent importance to natural selection (Maynard Smith et al. 1985; Gould 2002). Under developmental constraint, some phenotypes may be developmentally more readily accessible than others, thereby biasing evolution to follow particular pathways.

The spectacular plumage phenotypes of birds have been subject to considerable attention due to their diversity, functional significance and ease of study (Price and Pavelka 1996; Kimball and Ligon 1999; Chuong et al. 2000; Omland and Lanyon 2000; Badyaev et al. 2001; Price and Bontrager 2001; Harris, Fallon, and Prum 2002; Prum and Williamson 2002; Widelitz et al. 2003; Chang et al. 2004; Jiang et al. 2004; Harris et al. 2005; Badyaev and Landeen 2007; Pointer and Mundy 2008; Riegner 2008; Kimball, Mary, and Braun 2011). Plumage coloration can vary over the body and is coordinated into species-typical phenotypes by individual patches containing up to thousands of feathers (Prum and Dyck 2003). For example, the tail of the peacock (*Pavo cristatus*) has coloured spots whereas the wings have bars. Patches of feathers that covary in coloration generally correspond to feather tracts (pterylae), which vary little in anatomical position between birds, and are indicative of local shared developmental programs or modules (Lucas and Stettenheim 1972; Wagner, Pavlicev, and Cheverud 2007).

Plumage patches may be comprised of feathers of uniform coloration or feathers that are patterned. For example, the distinct patches of coloration on the crown and rump in the genus *Phylloscopus* are made up of uniform pigmentation, with little variation within individual feathers (Price and Pavelka 1996). In contrast, the patch of spotted plumage on the flanks of the male Zebra finch (*Taeniopygia guttata*) is due to regular within-feather patterning, which is composed of spatially variant pigmentation within the vane of each feather (Prum and Williamson 2002; Kenward

et al. 2004; Riegner 2008; Gluckman and Cardoso 2009; Gluckman and Cardoso 2010). While plumage coloration has been extensively studied, the evolution of within-feather patterning has received less attention despite abundant interspecific variation (Fig. 3.1) (Riegner 2008; Gluckman and Cardoso 2010).

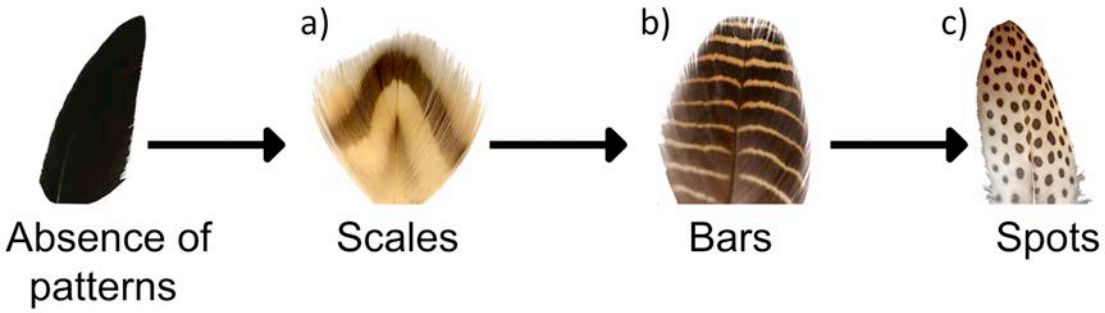


Fig. 3.1. The most frequent regular plumage patterns found in birds and the hypothesis of developmental constraint in plumage pattern evolution on the basis of increasing complexity. a) Scales - king eider (*Somateria spectabilis*), b) Bars - snow partridge (*Lerwa lerwa*), c) Spots - great argus (*Argusianus argus*). If there is developmental constraint in plumage pattern evolution on the basis of increasing stringency, then perhaps scales evolve from the ancestral state of uniform coloration, followed by bars, and finally spots. Images were taken at the University Museum of Zoology, the University of Cambridge, by T-L. Gluckman and are copyright of the University Museum of Zoology.

Within-feather patterning can be split into two types based on the distribution of pigmentation: irregular pigmentation (mottled plumage), where the vane is heterogeneously pigmented, and regular patterns, which are comprised of the same recurring motif (Gluckman and Cardoso 2010; Gluckman 2014). Regular within-feather patterns have largely converged on the same strikingly simple set of repeating geometric patterns in birds: scales - where the feather border is regularly pigmented with a different shade of melanin (Fig. 3.1a); bars - alternating bars of lighter and darker pigmentation perpendicular to the feather's axis (Fig. 3.1b); and spots - one or more regular spots of pigmentation within feathers (Fig. 3.1c) (Fig. 1 *in*

Prum and Williamson 2002). There are a few other regular motifs in birds, including chevrons and stripes, that are relatively rare, and two types of patterning that apparently occur in single species: checkered patterning in the common loon (*Gavia immer*), and triangles in the breast of the wood duck (*Aix sponsa*). This demonstrates that although other types of patterns are possible plumage patterns repeatedly converge on the same three motifs.

Bird plumage is predominantly pigmented with melanins and carotenoids (Hill and McGraw 2006b). Melanins are of key importance for feather patterning since they are the only pigment that can be differentially deposited in precise spatio-temporal sequence during feather growth to create within-feather patterns (Prum and Williamson 2002; Hill and McGraw 2006b). In contrast, carotenoid-based coloration is typically confined to feather tips and so only contributes to uniform patches of coloration over the body. In a comparative survey encompassing 90% of avian species worldwide there were no observed cases of plumage patterns where melanin appeared to be absent, and additional types of coloration in patterning (e.g. psittacofulvins and carotenoids) were rare (T-L. Gluckman *unpublished data*).

Studies of convergent evolution in plumage phenotypes have largely focused on patches of uniform coloration, in particular carotenoid coloration, with fewer studies of melanin (Christidis, Schodde, and Baverstock 1988; Hackett and Rosenberg 1990; Price and Pavelka 1996; Kusmierski et al. 1997; Omland and Lanyon 2000; Odeen and Bjorklund 2003; Hofmann, Cronin, and Omland 2006; Andersson, Prager, and Johansson 2007; Bleiweiss 2007; Jones and Kennedy 2008; Cardoso and Mota 2010; Prager and Andersson 2010; Friedman, Kiere, and Omland 2011). The proposed mechanisms underlying convergence in uniform coloration have focused on ecological explanations (Crochet, Bonhomme, and Lebreton 2000; Dumbacher and Fleischer 2001; Bleiweiss 2007; Weibel and Moore 2007), although several studies failed to find an ecological correlate (Omland 1997; Omland and Lanyon 2000; Omland et al. 2006). Additional suggested mechanisms of convergence are sexual selection (Omland and Lanyon 2000; Prager and Andersson 2010), and developmental constraint (Price and Pavelka 1996; Omland and Lanyon 2000; Majerus and Mundy 2003; West-Eberhard 2003; Prager and Andersson 2010).

The proposed mechanisms shaping the evolution of uniform plumage

coloration may also influence the evolution of regular within-feather patterning. In particular, ecological factors may select for convergence in phenotypes, for example to provide crypsis to evade detection by predators (Shine and Madsen 1994; Lindell and Forsman 1996; Marshall 2000; Stevens and Merilaita 2009b). Ecological selection for plumage patterns is likely to be dependent on habitat and how the patterns are perceived against their background (Bradbury and Vehrencamp 1998; Endler 1998). Therefore, selection for camouflage in avian phenotypes is likely to differ between species due to variation in habitat. However, different types of patterns are found in sympatric species of birds that occupy the same habitat demonstrating that ecological selection may not necessarily result in convergent evolution, e.g. the barred warbler (*Sylvia nisoria*) and the red-backed shrike (*Lanius collurio*) (Polak 2012). In addition, the same patterns are frequently found in avian orders that live in different habitats (Gluckman and Mundy 2013). For example, waterfowl, which are aquatic, frequently have barred plumage, but so do many non-aquatic birds, such as raptors.

In several species of birds, plumage patterns have a social function, such as the spots of the male peacock (*Pavo cristatus*) and the barred plumage of the red-legged partridge (*Alectoris rufa*) (Petrie, Halliday, and Sanders 1991; Bortolotti et al. 2006; Muck and Goymann 2011; Pérez-Rodríguez, Jovani, and Mougeot 2013). More broadly, barred patterns appear to have generally evolved for camouflage, but have also evolved under sexual selection on the ventral surface of males (a more likely location for signalling to conspecifics) and may function as a social signal in many species of birds (Gluckman and Cardoso 2010). As a communication signal, the same type of plumage patterns may converge on the same motif due to sensory exploitation or cognitive receiver biases. For example, perhaps barred patterns have frequently evolved under sexual selection because they are conspicuous to conspecifics (Gluckman and Cardoso 2009; Gluckman and Cardoso 2010). Additionally, barred plumage may have evolved as a specialised adaptation to facilitate cuckoo-hawk mimicry in Old World cuckoos (Gluckman and Mundy 2013). However, it is thought that signal evolution is likely to be habitat and context dependent, which can vary considerably between species (Bradbury and Vehrencamp 1998; Endler 1998).

Developmental constraint in uniform coloration has been studied in the genus

Phylloscopus by employing a Turing reaction-diffusion model (Price and Pavelka 1996). Turing reaction-diffusion systems form morphological patterns from the combined action of two molecules (morphogens), an activator and an inhibitor. Chemical gradients of the morphogens induce spatially explicit patterns that are controlled by four key parameters per morphogen: the rates of production, decay and diffusion, as well as the overall strength of the interaction (Turing 1952). Price and Pavelka (1996) showed that the evolution of patches of white plumage (unmelanized feathers) could be attributed to increasing and decreasing rates of morphogen production. For mammalian coloration, the application of Turing models could explain why mammals that have a spotted tail with a striped body are not found (Murray 1981), thus implying a form of developmental constraint, best considered as relative constraint (Price and Pavelka 1996).

In a landmark study, all regular within-feather patterns were successfully simulated with a reaction-diffusion based model (Prum and Williamson 2002). By modeling differential pigment uptake by keratinoctyes during feather development, Prum and Williamson found that regular plumage patterns could be produced by manipulating spatial and temporal periodicity. According to this model, the production of scales has a low rate of morphogen decay and is governed by spatial periodicity of melanin uptake. The production of bars requires a higher rate of morphogen decay resulting in temporal periodicity of melanin uptake. Notably, the formation of spots is distinct as it is comprised of simultaneous spatial and temporal differentiation and has the narrowest range of parameters (Fig. 6 *in* Prum and Williamson 2002)

From Prum and Williamson's model (2002), we hypothesised that the mechanism of within-feather pattern formation may bias the production of pattern variation during evolution in a stepwise order from decreasing to increasing stringency, thereby acting as a constraint. Our interpretation of this model of within-feather pattern formation is that scales have the least stringent conditions, bars have more stringent parameters than scales, and spots have the narrowest range of parameters. Therefore, our hypothesis of within-feather pattern evolution is that scales evolved first, followed by bars whereas spots are the most derived pattern (Fig. 3.1).

From what is currently understood of plumage development, covariation of

feather pigmentation within patches is indicative of local shared developmental mechanisms and hence patches are a logical focus to study evolutionary pathways (Fig. 3.2) (Lucas and Stettenheim 1972; Prum and Dyck 2003; Wagner, Pavlicev, and Cheverud 2007). In contrast, it is currently unknown whether the development of pigmentation within one patch of plumage influences the evolution of pigmentation in another patch of plumage, although it seems likely that this may be the case. For example, the pink-eared duck (*Malacorhyncus membranaceus*), has barred plumage on the flanks and the breast. Perhaps barred plumage evolved on the breast first, and the mechanism of pattern formation was subsequently recruited by the flanks, or vice versa. An interesting issue is whether such recruitment across patches may be more favoured within developmental compartments, or modules, which may in principle occur along the dorsoventral axis or anteroposterior axis. At the level of the whole body, evolution of a novel plumage pattern may occur within the same patch or involve recruitment from other patches, or other modules, and might therefore appear relatively unconstrained. Analysis of the whole body is confounded by the co-occurrence of multiple different pattern types, e.g. species in both Anseriformes (e.g. Hottentot teal, *Anas hottentoti*) and Galliformes (e.g. Elliot's pheasant, *Syrmaticus ellioti*) have separate patches with all four pattern phenotypes considered here: absence, scales, bars and spots. Scoring of patterns over the whole body thus necessitates prioritizing particular patterns over others.

These considerations lead to the development of a hierarchical approach in which we first consider evolution within patches, then evolution within regions, and finally evolution over the whole body. Evolutionary pathways within patches provide basic evidence for mechanisms occurring within a developmental unit. Directionality would demonstrate that some transitions are preferred over others and allow a direct test of our hypotheses. Similarity among patch models and particular regional models would indicate modularity in pattern development. Similarity at the level of the whole body would suggest an absence of other mechanisms at this level.

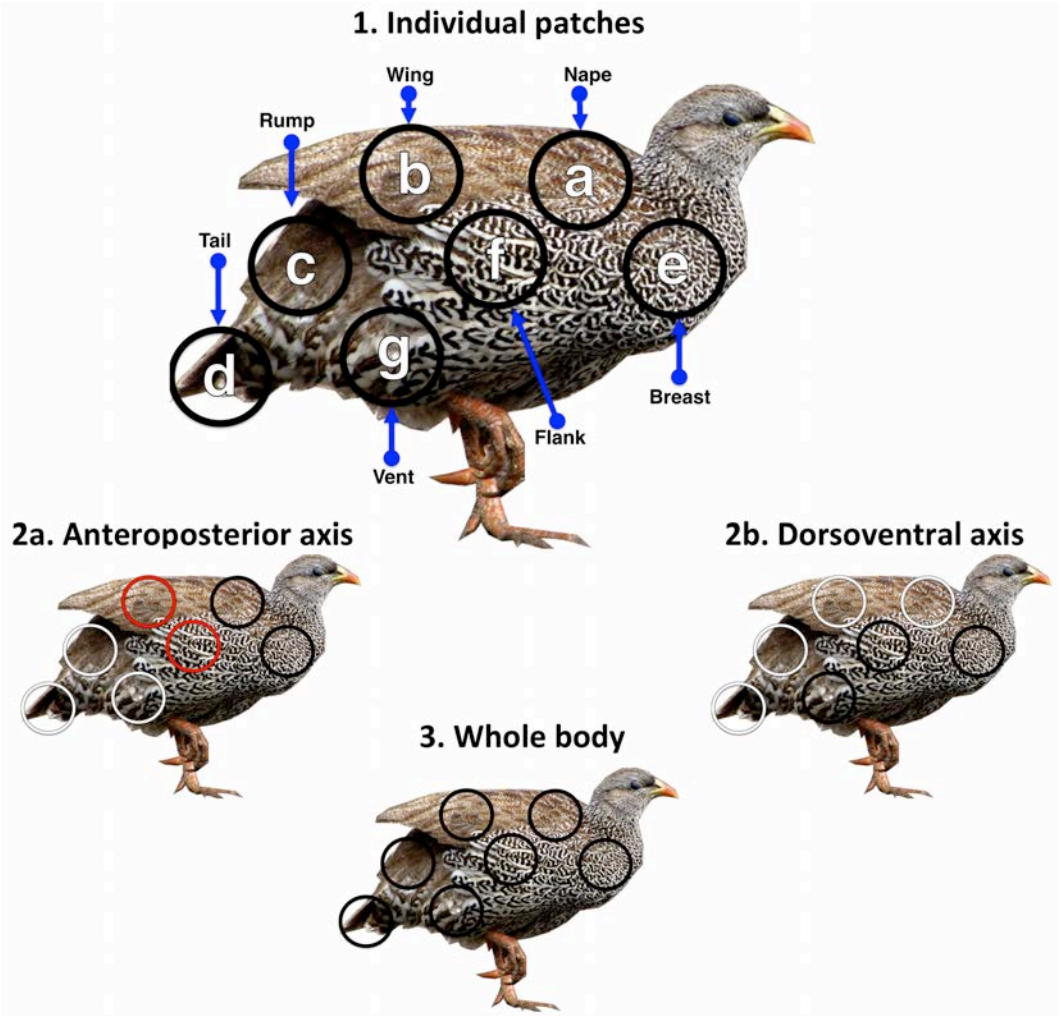


Fig. 3.2. The plumage patches sampled in this study and combinations of patches analysed to investigate regional modularity. (1) The seven individual patches. Field guide terminology, and the corresponding feather tracts (pterylae; Lucas and Stettenheim 1972) are as follows: a) Nape: interscapular tract, b) Wing (scapular, wing coverts, tertials, primaries and secondaries): humeral tract, upper marginal coverts of prepatagium and upper wing covert tract, c) Rump and uppertail coverts: dorsopelvic tract and dorsal caudal tract, d) Tail: upper major tail covert, upper median tail covert and rectrices tract, e) Breast: ventral cervical tract, f) Flanks or side: pecterosternal tract in Anseriformes or pectoral tract in Galliformes, g) Vent and undertail coverts: abdominal tract in Anseriformes or lateral and medial abdominal tracts in Galliformes. (2) In regional analyses, patches are grouped into regions that may represent developmental modules (similarly coloured patches form part of the same region), with either three regions arranged on an anteroposterior axis (2a) or two regions arranged on a dorsoventral axis (2a). (3) In the whole body analysis, all patches are analysed together. The species illustrated is the Natal francolin (*Pternistes francolinus*).

The avian orders with the most spectacular plumage patterns are the waterfowl (Anseriformes) and gamebirds (Galliformes), which together form a monophyletic group (Galloanserae) (Mindell et al. 1999; van Tuinen, Sibley, and Hedges 2000; Livezey and Zusi 2007; Morgan-Richards et al. 2008). Each order includes iconic examples of patterns such as the spotted plumage of the great argus (*Argusianus argus;* Galliformes*,* Fig. 3.1C). Anseriformes and Galliformes have dramatically different lifestyles, comprising waterbirds and landbirds, respectively (del Hoyo, Elliott, and Sargatal 1992), and are thus likely to be subject to a host of different selection pressures. Variation in selection pressure is ideal for testing our hypothesis of developmental constraint in these two orders because a similarity in developmental constraint should lead to similar evolutionary pathways. Here, we examine directionality in within-feather pattern evolution, using Bayesian phylogenetic modeling in Anseriformes and Galliformes separately, with patterning identified from museum skins. We traced pattern evolution in a hierarchical order to assess whether there may be generalities in these ecologically diverse orders and examine whether there is a) directionality in pattern evolution, b) whether the direction of evolution provides support for increasing complexity in within-feather patterning developmental mechanisms, c) whether convergence follows similar pathways in both orders, d) whether there is evidence for regional modularity, and e) whether global models of plumage pattern evolution differ from the developmental models of within patch and regional modularity.

Materials and Methods

Phylogenies

We searched the literature for published species level relationships. The best available phylogenies on the basis of species coverage, inclusion of both mtDNA and nuclear DNA, and inclusion of branch length information, are as follows: Anseriformes, 188 spp. (73%) (Gonzalez, Düttmann, and Wink 2009); Galliformes, 170 spp. (59%) (Kimball, Mary, and Braun 2011). Together these phylogenies cover

all families and 63% of extant species across the two orders (Howard, Dickinson, and Moore 2003). We collected plumage pattern information from each species (nominate subspecies where applicable) represented in these phylogenies from museum skins at the Natural History Museum at Tring and the University Museum of Zoology, Cambridge.

Data collection and coding

Current developmental evidence suggests that the default plumage phenotype in males and females in Anseriformes and Galliformes is the male plumage (Owens and Short 1995). Therefore, we collected plumage pattern information for the seven patches of plumage over the body for the males of each sample species (Fig. 3.2). We assigned the character state of each of the seven feather patches as scales, bars, spots, or an absence of patterns, following the description by Prum and Williamson (2002). Some species exhibit what appear to be longitudinal stripes along feathers, but on closer inspection are an angular version of scales with a central pigment patch, and were scored as scales, e.g. the breast and nape plumage of the vulturine guineafowl (*Acryllium vulturinum*) (Fi.g 1a in Prum and Williamson 2002). A small number of species in this study have chevron patterns – Anseriformes – 2 *spp.*, Galliformes – 5 *spp.*. Given that chevrons are rare in these orders and that they are similar to patterns made of bars, in that the borders do not meet to create a central pigment patch, we scored chevrons as bars (Fig. 1e and Fig. 6e *in* Prum and Williamson 2002).

For most species sampled, the type of within-feather patterning across the vane of each feather, as well as between individuals of the same species, was the same for each patch considered. However, in a rare number of cases there was variation between individuals. To focus on the most developmentally relevant patterns in these rare cases we recorded the pattern that covered the majority of the feathers, in the patch under consideration, and where relevant, the predominant pattern in the majority of individuals sampled. For example, in the Natal francolin (*Pternistes francolinus*), the feathers in the flanks can have both bars and scales (Fig. 3.2). In the example depicted, bars cover the majority of the feathers in the flanks, and this individual would have been assigned as having bars. However, in

most individuals of the sample population of the Natal francolin, scales predominantly covered most of the feathers in the flanks, and were considered representative of this species.

An additional type of pattern, mottled plumage, is present in many birds. It is currently unknown whether all mottled patterns can be considered homologous, or whether they may be classified into discrete types based on the size, shape and distribution of pigmentation across the vane of the feather. Therefore, mottled plumage was scored as unknown.

To summarize our findings of local pattern evolution within all seven patches of plumage, we report on the frequency with which each transition between patterns occurs out of the total number of patches. The most probable model of evolution could then be compared with our hypothesis as well as the full (null) model of evolution.

To investigate whether within-feather pattern evolution in one patch of plumage may precede and/or promote evolution of patterning in other patches, we conducted our analyses by combining patterns into body regions in three ways (Fig. 3.2). Each body region (e.g. ventral surface) was coded as the most derived pattern for each patch it contained as indicated by the summary model of local evolution. For example, if a body region consisted of patches containing both bars and spots, it was coded as having spots.

In Anseriformes, in the summary model of local evolution within patches there is no conflict in the order of transitions and the most derived pattern is clear. However, in Galliformes, bars evolve from an absence of patterns and the next pattern to evolve from bars could be either scales or spots (*see* Results). We took this uncertainty into account by examining each possible trajectory for each body region separately for comparison in Galliformes. For example, males of the satyr tragopan (*Tragopan satyra*) have scales on the flanks and vent, and spots on the breast. In the analysis of the ventral region of Galliformes, where the flanks, breast and vent are collapsed into one character, we compared whether assigning either scales or spots as the most derived character created conflict in the analysis.

Similar to the summary model of local evolution within-patches, we derived models of regional modularity by reporting on the frequency of each transition between plumage patterns out of the total number of regions. The mode of regional modularity that is most plausible should contain the same transitions as the summary model of evolution within-patches of plumage.

Finally, to derive a global model of plumage pattern evolution over the whole body, we used the same approach for scoring each species as used in the models of regional modularity, but incorporating information from all patches.

Modelling of plumage pattern evolution

We modeled plumage pattern evolution over the phylogeny to estimate which patterns evolve into one another, allowing us to derive a model of the probable evolutionary pathways to current plumage pattern phenotypes. Anseriformes and Galliformes have a wide distribution and live in different habitats, which may alter the evolutionary trajectory of each order (Gluckman 2014). Therefore, we examined each order separately to assess for similarity and differences in their evolutionary history. To estimate plumage pattern evolution in each order, we used the Reversible Jump Markov Chain Monte Carlo Multistate option in BayesTraits v.2 (Pagel, Meade, and Barker 2004; Pagel and Meade 2006).

Markov Chain Monte Carlo (MCMC) is based on the proposition that traits can repeatedly evolve between any possible state on any branch of the tree. To estimate the rate of change between states, the Markov chain samples the plumage patterns at the internal nodes of the tree, in proportion to their probability, which is conditioned on the values at the tips. New rate parameter values are proposed in successive steps in the Markov chain resulting in a posterior sample distribution of rate coefficients and ancestral states. Each model of pattern evolution rate coefficients is visited in direct proportion to its posterior probability in the sample distribution (Pagel, Meade, and Barker 2004). Given that there are four pattern states, which in turn offer many parameters that describe evolution between plumage patterns, we used Reversible Jump MCMC (RJMCMC).

RJMCMC integrates rate restrictions by searching the posterior distributions of model parameters to avoid over parameterization. As such, we allowed BayesTraits to propose transition rates of plumage pattern evolution without restriction (e.g. we did not constrain any rate parameters to equal 0 based on *a priori* predictions) thereby making the analysis conditional on the data rather than our hypothesis (Gluckman 2014). For example, we hypothesized that spots are the most derived pattern as a consequence of having the strictest developmental parameters and therefore do not evolve directly from an absence of patterns (Fig. 3.1). In transition rate models that support this hypothesis, a rate parameter between an absence of patterns and spots equals 0, and therefore does not occur. This allows both incremental and non-sequential changes to occur in any direction and avoids imposing potentially false hypothesis based predictions. By making the analysis conditional on the data, this approach uses the best data currently available to assess the most probable models of plumage pattern evolution.

Potential models of plumage pattern evolution visited by the Markov chain are distinct from the most probable model of plumage pattern evolution. The former describes the proposed models of plumage pattern evolution that make up the posterior sample distribution, whereas the latter is derived from statistically evaluating the posterior sample distribution. Each model of plumage pattern evolution is composed of a unique combination of transition rate parameters with values fixed to zero or are sampled as free parameters with positive values. Rate parameters fixed to zero were interpreted as an evolutionary transition that does not occur, and free rate parameters with a positive value were interpreted as evidence for an evolutionary transition that does occur. Therefore, qualitatively, each unique model of plumage pattern evolution is composed of transitions that do not occur, and transitions that do occur.

Null model testing and model comparisons were conducted by assessing the posterior distribution of unconstrained models. If there were no developmental constraint, such as where natural selection drives plumage patterns to evolve in any direction, forward and backward evolutionary transitions between all pattern states would occur – the full (null) model. Therefore, if there were no directionality in plumage pattern evolution, the full model would be visited more frequently than expected by chance. Conversely, if sequential or non-sequential evolutionary

transitions were more probable, then models with these transitions would be most probable. In assessing the models of evolution without constraining any transitions, each unique model of pattern evolution is compared with every other possible model of pattern evolution (statistical methods are described in the next section).

In BayesTraits we modeled the rates of plumage pattern evolution using a hyperprior with a gamma distribution defined by an empirical Bayes estimator (Pagel, Meade, and Barker 2004). For each analysis, we discarded the burn-in and the Markov chain was run until convergence across four independent runs (<1 lnHM). After convergence was reached, we thinned the sample distribution to remove autocorrelation. In analyses of within patch pattern evolution, we sampled 10,000 generations, per patch, per phylogeny. For the analyses of regional modularity, we sampled 2,000 generations, per region, per phylogeny. The average rate for each transition could not be averaged across feather tracts or body regions, as it is statistically incorrect. Instead, the average transition rates for each patch of plumage and body region are presented in the supplementary material.

Model priors and modelling parameters

The prior density on the free transition rate parameters were estimated using an empirical Bayes estimator (where the interval of the hyperprior is defined by the average and standard deviation of the maximum likelihood of all rate parameters) to reduce bias and uncertainty in choice of priors (Pagel, Meade, and Barker 2004). We used a hyperprior approach with a gamma distribution as our empirical Bayes estimator values had an intermediate range. The intervals were estimated for each analysis, for each patch of plumage, and for each region, in each group separately. For the analysis of independent evolution within patches of plumage, in each phylogeny, the Markov chain was run for 250 million generations sampling every $10{,}000^{th}$ generation (7 x 2 = 14 individual analyses). The first 120,000 generations of RJMCMC (burn-in) were discarded to ensure parameter space was sufficiently explored. For the analysis of regional modularity the Markov chains were less stable and were run for 500 million generations sampling every $50{,}000^{th}$ generation, discarding the first 150,000 generations as burnin (6 x 2 = 12 individual analyses). Each analysis of within-patches patches and modular feather regions, per order, was

run four times to ensure convergence had been reached within analyses as indicated by a stable harmonic mean of the log-likelihood that varied by <1 lnHM across all four runs. We checked for autocorrelation using the Ljung-box test statistic in SPSS v19.0 at lag 1 (IBM Corp.). A Ljung-box $P > 0.05$ was interpreted as indicating no autocorrelation. There was autocorrelation and we thinned the posterior sample distribution of models of plumage pattern evolution in each analysis, preserving the order of the models in which they were visited. For the analyses of both within patches and modular feather regions we thinned the sample distribution to every $100{,}000^{th}$ model resulting in a posterior sample distribution of 10,000 models (2,500 models per independent run) for the analyses of within patches, and 2,000 models (500 models per independent run) for the analyses of modular feather regions.

Statistical analysis

The most probable models of plumage pattern evolution, each with their own most probable ancestral state of patterning, are visited in proportion to their Bayesian posterior probability. To qualitatively summarize whether each transition probably does not occur, or occurs, and account for model variation and uncertainty in the posterior sample distribution of models proposed, we employed multimodal inference (Burnham and Anderson 2002; Gluckman 2014). To assess which models of plumage pattern evolution are visited more frequently than expected by chance, we compared the prior odds of seeing each model of plumage pattern evolution, with the posterior odds derived from the posterior sample distribution. Final comparisons were made using Bayes Factors. Given that there are four states of patterning in this analysis, twelve possible evolutionary transitions can occur, but each can also have a transition rate of zero. To account for the varying numbers of zero and non-zero transitions, we calculated the prior odds of encountering each unique model of plumage pattern evolution in the sample distribution. Prior odds were calculated using binomial numbers for the number of transitions that do not occur, as well as bell numbers for transitions that occur, combined (supplementary Table S3.1, S3.2; *see* Currie et al. 2010 for a detailed explanation of calculations used). To assess the most probable model of plumage pattern evolution, we derived a top model set using a threshold approach of a BayesFactor of >=2, which is considered positive evidence (Kass and Raftery 1995; Burnham and Anderson 2002).

Due to variation in the total collection of models sampled in the posterior distribution, some ancestral states as well as rate parameters vary widely in whether they are fixed to zero, or sampled as free parameters with positive values. Therefore, we investigated what ancestral states and free parameters were favored in the top model subset of the posterior distribution, accounting for sampling variation. The marginal probability (MP) per ancestral pattern, as well as the unique model of pattern evolution, was calculated from the entire posterior sample distribution. For example, the ancestral for each type of pattern MP = (models in which this pattern is ancestral/10,000), and for each unique model of plumage pattern evolution MP = (Unique model/10,000).

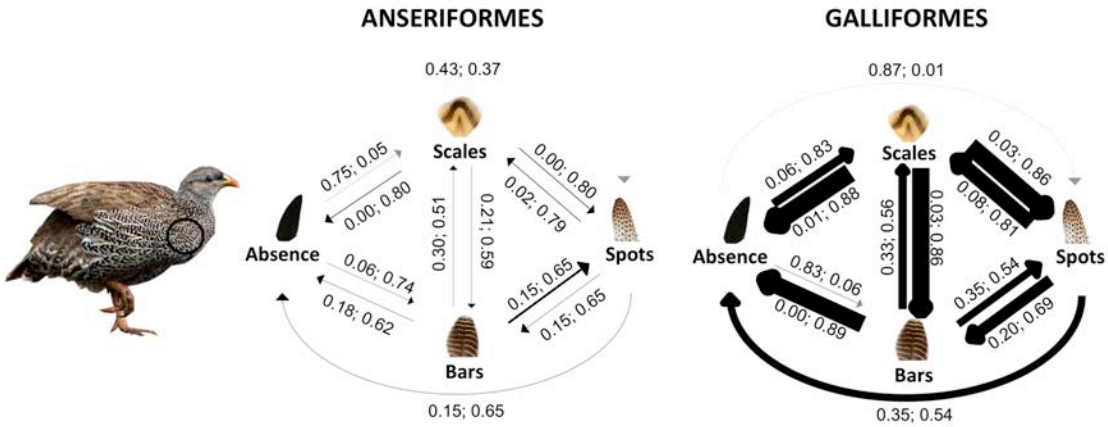


Fig. 3.3. The marginal probability of evolutionary transitions between plumage patterns in the feather tract of the breast in Anseriformes and Galliformes. Next to each transition is the marginal probability of a transition not occurring, followed by the marginal probability of it occurring. The width of the transition line is proportional to the rate of transition. Where the probability of a transition occurring is less than the probability of the transition not occurring, the transition line is grey indicating that it most probably does not occur. Conversely, where the marginal probability of it occurring is higher than not occurring, the transition line is black indicating that the transition probably occurs. The marginal probability of occurring and not occurring does not equal due to variation in the transitions represented in the top model set.

The final marginal probability was calculated by cumulatively adding the MP of models in the top model set for each ancestral state of patterning and for each

evolutionary transition where it does *not* occur, as well as where it occurs, for comparison (Burnham and Anderson 2002). For example, in the breast of the galliform birds, the marginal probability (MP) of an absence of patterning *not* being the most probable ancestral state is 0.00 in the top model set whereas the MP of an absence of patterning *being* the most probable ancestral state is 0.89 (Table 3.1). In addition, the MP of scales, bars and spots *not* being the most probable ancestral state is 0.89 versus 0.00 of *being* the ancestral state. Assessing a transition from an absence of patterns to spots, the MP of the transition rate parameter describing it as *not* occurring is 0.87 and its MP of being non-zero is 0.01 (Fig. 3.3). Together this shows that an absence of patterns is most probably the ancestral state in the breast of galliform birds, and a transition from an absence of patterns to spots most probably does *not* occur.

The marginal probability in the top model set accounts for variation in the entire posterior sample distribution, therefore the sum of the marginal probability of a transition *not* occurring and occurring rarely equals 1 as this requires every model in the posterior sample distribution to have the same result for that transition.

Results

Taxonomic distribution of patterns

All of the different types of regular plumage patterns were represented in the seven plumage patches, with the exception of spots on the rump, nape and tail, as well as bars on the tail in Anseriformes, and scales on the tail in Galliformes (Table 3.1; *see* supplementary Fig. S3.1 for a taxonomic distribution of plumage patterns). In all body regions (dorsoventral and anteroposterior), and over the whole body, all plumage patterns were represented in both orders. We first present analyses of plumage pattern evolution within individual patches, followed by regions and finally the whole body.

Evolution within patches of plumage

For individual patches of plumage that have more than two pattern states, there was variation in the number of unique models supported in the top model set (Table 3.1; supplementary Table S3.3). Plumage patches with only three pattern states (Anseriformes: nape, rump; Galliformes: tail) had less variation in the number of unique models in the top model set, than plumage patches with four pattern states. In the plumage patch with just two pattern states (Anseriformes: tail), only the full model was present in the entire posterior sample distribution. In contrast, in all models of patches of plumage with more than two pattern states, the full model was not visited more than expected by prior odds (Table 3.1; supplementary Table S3.3).

In six out of seven patches of plumage in Anseriformes, and in all patches in Galliformes, the ancestral plumage was an absence of patterns (Table 3.1; supplementary Table S3.3). Both the average probability and the MP supported an absence of patterns in three patches in Anseriformes, and across all patches in Galliformes. Four patches in Anseriformes have equivocal support - rump, breast, vent and tail. However, the MP, which integrates model support, unlike the average probability, indicated that pattern absence is the most probable ancestral plumage in the rump, breast and vent. In only the tail of Anseriformes was there an equivocal ancestral state, which is probably due to having only two pattern states – barring and an absence of patterns (Table 3.1; supplementary Table S3.3).

In all models of plumage evolution, except for the tail of Anseriformes, there was evidence of directionality as some transitions probably occurred and others did not (Fig. 3.4, supplementary Fig. S3.2). Examining the order of pattern evolution within-patches, bars evolve more frequently from an absence of patterns (Anseriformes: 4/6; Galliformes: 5/7) than scales (Anseriformes and Galliformes: 2/6) (Fig. 3.4). Indeed, in all models of plumage pattern evolution, the average transition rate from an absence of patterns to scales is low (supplementary Fig. S3.2). In both orders there are strong bidirectional transitions between scales and bars, and spots evolved more frequently from scales (Anseriformes: 5/5; Galliformes: 6/6) than bars (Anseriformes: 3/5; Galliformes: 5.5/6). The latter transition from bars to spots in Anseriformes includes equivocal transitions in two patches (supplementary Fig. S3.2). Finally, transitions from an absence of patterning to spots were rare and had

the lowest rate of transition where they occur (Anseriformes: 1.5/5; Galliformes: 1/7; supplementary Fig. S3.2). Therefore, within-patches the predominant order of plumage pattern evolution is bars, followed by scales and finally spots.

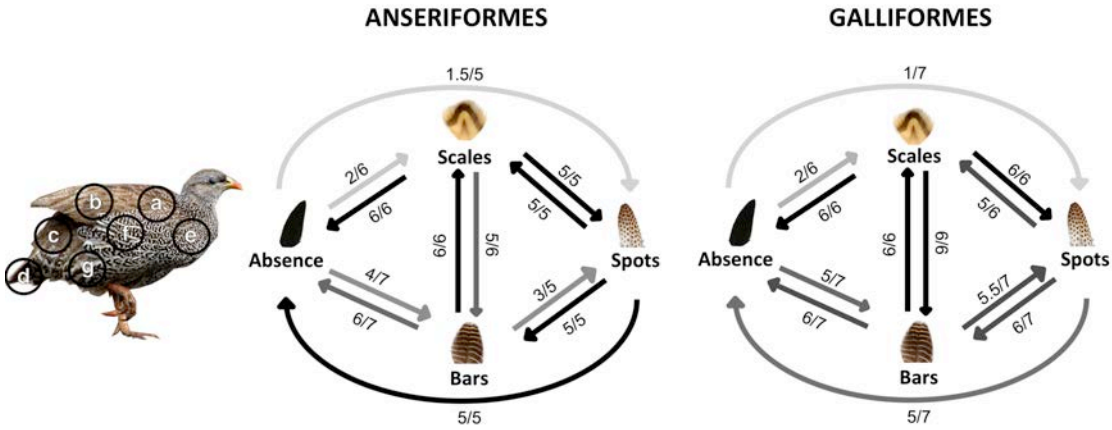


Fig. 3.4. The summary model of local evolution within-patches across all seven plumage patches in Anseriformes and Galliformes. Next to each transition is the number of plumage patches in which the transition occurs out of the total number of plumage patches. The total number of plumage patches can vary from the maximum (seven) because in some patches particular patterns do not occur e.g. in Anseriformes no species have evolved spots on the tail or the rump, so the total number of plumage patches in which it can evolve is five. Where transition lines have an intermediate value, e.g. 1.5/5 for a transition from absence to spots in Anseriformes, this indicates that the transition was equivocal in one of the models of pattern evolution within plumage patches. The weight of each transition probably occurring is represented on a scale of pale grey (occurs rarely) to black (occurs in every plumage patch possible).

Regional modularity in plumage pattern evolution

Pattern evolution across body regions showed variation in the number of unique models supported in the top model set (Table 3.1; supplementary Table S3.3). For all body regions in both orders, the full model was not visited more than expected by prior odds, and, in some cases, was not visited in the entire posterior sample distribution (Table 3.1; supplementary Table S3.3). In three out of six body regions in Anseriformes, and in all analyses in Galliformes, both the average

probability and the MP support an absence of patterning as the ancestral state of plumage (Table 3.1; supplementary Table S3.3). The source of uncertainty in the ancestral state of Anseriformes is in three body regions - middle, posterior and ventral - however, the MP for each supports pattern absence as the most probable ancestral plumage.

Incorporating uncertainty in the order of pattern evolution in Galliformes resulted in conflict in five transitions spanning four out of five body regions (Fig. 3.5; supplementary Fig. S3.3). There were varying degrees of similarity between the summary model of local evolution within-patches and all three modes of regional modularity. However, the mode of regional modularity that most resembled the summary model of within patch pattern evolution in both orders was a dorsoventral axis (Fig. 3.4, 3.5).

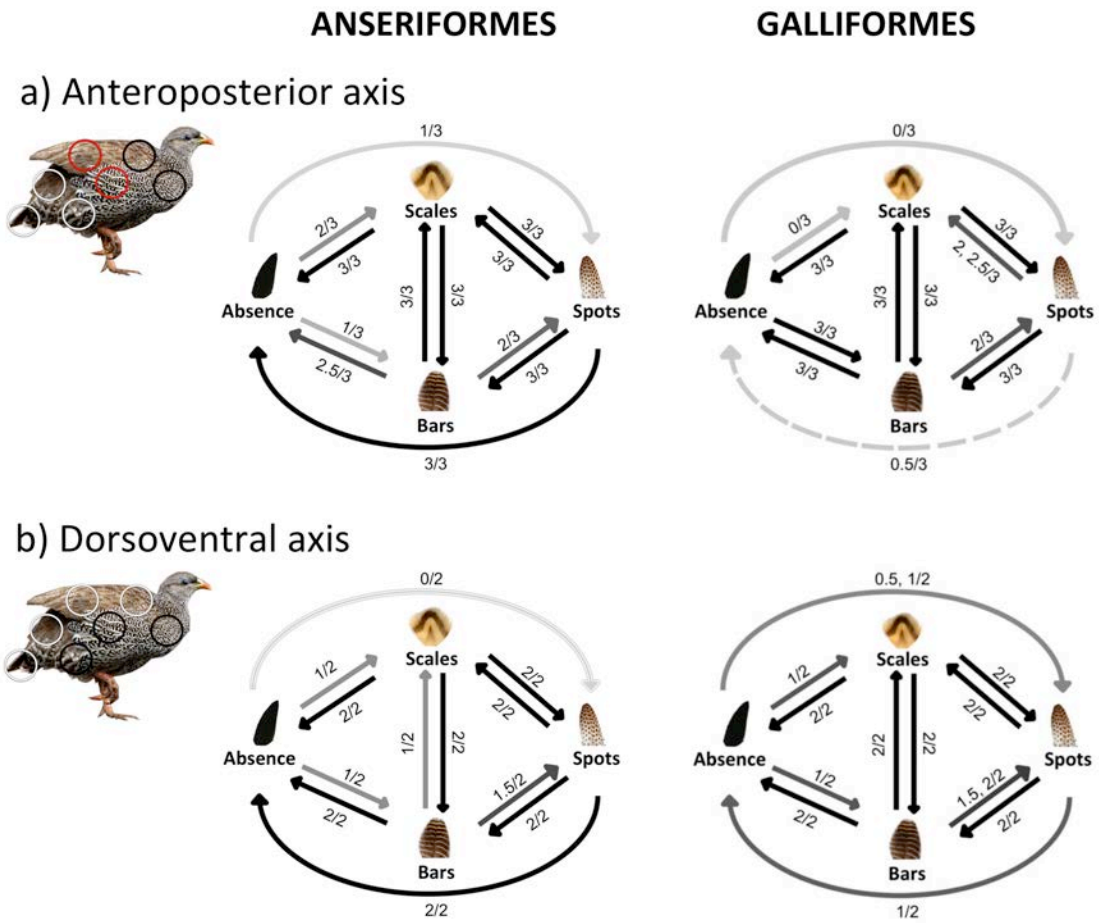


Fig. 3.5. Regional models of plumage pattern evolution in the proposed modes of modularity in Anseriformes and Galliformes. Next to each transition is the number of

body regions in which the transition occurs out of the total number of body regions for that mode of modularity. The weight of each transition probably occurring is represented on a scale of pale grey (does not occur) to black (occurs in every modular feather region for that analysis). A dashed grey line indicates an equivocal transition - where the marginal probability was equally in favor of not occurring and occurring (0.5).

Across both orders, there was disagreement between the summary model of within-patch evolution and the anteroposterior mode of modularity. In the Anseriformes anteroposterior model, scales evolve from an absence of patterns more frequently than bars, opposite to the summary of local patch evolution. Furthermore, in Galliformes a transition from spots to an absence of patterns is equivocal whereas in the summary model of location evolution it is a strong transition. In the dorsoventral axis model, in Anseriformes all transitions are qualitatively the same as the summary model of pattern evolution within-patches, the exception being a transition from scales to bars only occurring on one surface. In Galliformes, the dorsoventral axis model of pattern evolution does not conflict with the summary model of within-patch evolution (Fig. 3.4, 3.5). Together these results demonstrate support, for a regional dorsoventral axis of modularity.

Global model of plumage pattern evolution

The global models of plumage pattern evolution showed some important similarities with within-patch models. First, in both orders, a direct transition from an absence of patterns to spots probably does not occur. Second, there are strong bidirectional transitions between bars and scales in both orders (Fig. 3.6, supplementary Fig. S3.4). Third, in Galliformes bars evolve first. However, there were some differences between the global model and the summary model of evolution within-patches: in the Anseriformes global model, scales evolve first and the model lacks bidirectional transitions between bars and spots, while in Galliformes the whole body model does not have a transition from spots to an absence of patterns (Fig. 3.4, 3.6).

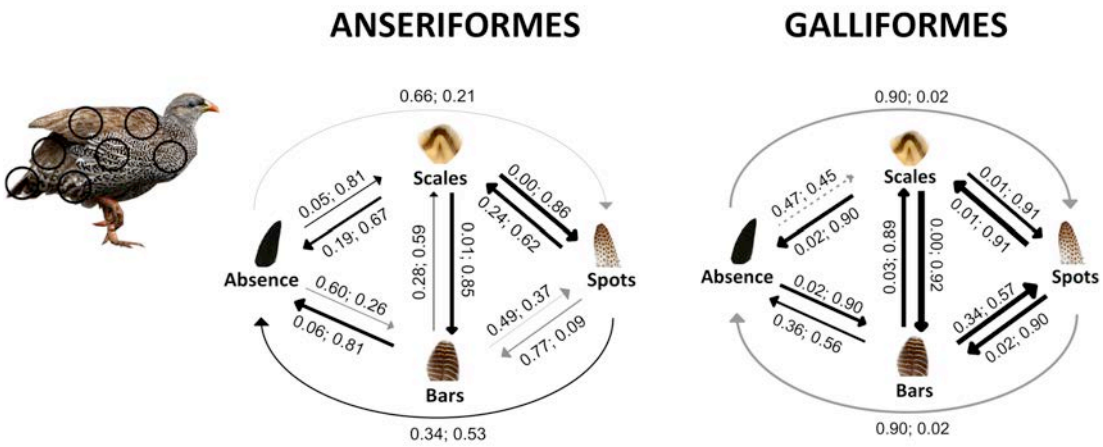


Fig. 3.6. The most probable evolutionary transitions between plumage patterns over the whole body in Anseriformes and Galliformes (where spots are derived – *see* Methods). Next to each transition is the marginal probability of a transition not occurring, followed by the marginal probability of it occurring. The width of the transition line is proportional to the rate of transition. A grey transition line indicates transitions that probably do not occur and black transition lines indicate transitions that probably occur. The marginal probability of occurring and not occurring does not equal 1 due to variation in the top model set.

Table 3.1. The frequency of the different types of patterns in the seven plumage patches over the body, the number of unique models in the entire posterior sample distribution as well as the top model set, and the average probability and marginal probability of the ancestral state of patterns, in Anseriformes and Galliformes.

								Ancestral state							
	Plumage pattern frequency within plumage patches					Posterior sample distribution			Average probability				Marginal probability		
	Absence of patterns	Scales	Bars	Spots	Mottled	Unique models	Top model set (BF>=2)	Full (null) model	Absence of patterns	Scales	Bars	Spots	Absence of patterns	Scales	Bars	Spots
Anseriformes (N = 118)																
Nape	78	16	19	3	2	464	257	118: BF = 0.08	0.31	0.25	0.20	0.24	0.02; 0.78	0.80; 0.01	0.79; 0.01	0.80; 0.00
Wing	105	5	5	1	2	698	454	57: BF = 0.04	0.27	0.26	0.23	0.23	0.11; 0.67	0.75; 0.03	0.70; 0.08	0.77; 0.01
Rump	100	12	4	-	2	15	4	1435: BF = 0.72	0.33	0.33	0.33	N/A	0.30; 0.38	0.38; 0.29	0.68; 0.00	N/A
Tail	109	7	-	-	2	1	1	All	0.5	0.5	N/A	N/A	0.50; 0.50	0.50; 0.50	N/A	N/A
Breast	74	14	12	13	5	379	218	207: BF = 0.14	0.25	0.27	0.22	0.26	0.44; 0.36	0.61; 0.19	0.77; 0.03	0.65; 0.15
Flanks	65	11	34	5	3	339	164	68: BF = 0.05	0.26	0.25	0.24	0.25	0.12; 0.76	0.88;	0.77;	0.88; 0.00

Vent	86	9	14	4	5	379	229	106: BF = 0.07	0.2	0.25	0.29	0.26	0.45; 0.34	0.00 0.72; 0.07	0.11 0.50; 0.29	0.70; 0.09
Galliformes (N = 170)																
Nape	75	18	29	10	38	229	121	11: BF = 0.01	0.72	0.13	0.11	0.05	0.01; 0.93	0.94; 0.00	0.94; 0.00	0.94; 0.00
Wing	81	14	13	9	53	475	292	40: BF = 0.03	0.66	0.14	0.13	0.07	0.00; 0.87	0.87; 0.00	0.87; 0.00	0.87; 0.00
Rump	83	11	25	8	43	305	178	12: BF = 0.01	0.8	0.09	0.08	0.03	0.00; 0.93	0.93; 0.00	0.93; 0.00	0.93; 0.00
Tail	93	-	24	8	45	36	7	454: BF = 0.20	0.78	N/A	0.09	0.13	0.08; 0.63	N/A	0.71; 0.00	0.63; 0.08
Breast	105	16	17	13	19	265	134	34: BF = 0.02	0.48	0.19	0.2	0.13	0.00; 0.89	0.89; 0.00	0.89; 0.00	0.89; 0.00
Flanks	79	21	29	14	27	399	235	101: BF = 0.07	0.69	0.16	0.09	0.06	0.05; 0.76	0.78; 0.03	0.80; 0.01	0.81; 0.00
Vent	139	6	11	4	10	312	191	10: BF = 0.01	0.48	0.2	0.23	0.09	0.00; 0.95	0.95; 0.00	0.95; 0.00	0.95; 0.00

Discussion

Studies of phenotypic convergence in bird plumage have mostly focused on coloration, although regular patterns within feathers are widespread across the class Aves. Our analyses suggest that the ancestral state of plumage is an absence of patterns, a consistent finding in Galliformes, but with some variability in Anseriformes (Table 3.1). Contrary to our predictions of pattern evolution, bars largely evolved first rather than scales. However, our analysis confirms that spots are derived from other pre-existing patterns (scales), and therefore, as hypothesized, reaction-diffusion based spatiotemporal differentiation may constrain spots to evolve from an absence of patterning by a minimum of two transitions (Fig. 3.1, 3.4). Our analyses also suggest that the potential for patches of plumage to develop patterning is not equally spread over the body. Instead there is regional modularity over the dorsoventral axis, but not the anteroposterior axis, such that evolutionary trajectories of patches within the same dorsoventral region are similar. This occurs in two avian orders that have very different lifestyles, illustrating the importance of development in evolution. Finally, the models over the whole body demonstrate that many mechanisms are conserved from the level of patches, including the highly derived nature of spots, but also highlight some interesting differences.

There is consistent support for directionality in plumage evolution at all levels of analysis, including all patches of plumage with more than two pattern states, all body regions and the whole body. (Fig. 3.4, 3.5, supplementary Fig. S3.2, S3.3). From an absence of patterning, bars predominantly evolved first, followed by scales, and finally spots, in both Anseriformes, and Galliformes (Fig. 3.4-3.6). The evolutionary trajectory of within-feather patterns demonstrates that with increasing complexity in the mechanism of pattern formation, different types of patterns become developmentally more accessible. These results are congruent with developmental constraint in this system, but the main pathway, that bars evolve first, does not follow our predictions and may indicate that the developmental basis of scales is more complex than that of bars.

Some support for a relative lack of mutational constraint on bar formation comes from genetic studies. Several independent mutations, both autosomal and Z-

linked, can lead to bars from an absence of patterning (Muscovy duck - Hollander 1968; chicken - Crawford 1990). In the best studied case, the sex-linked barred mutation in chickens, controlled by the *CDKN2A/B* locus, is associated with pale bands devoid of melanocytes (Hellström et al. 2010). Thus a different locus, *ASIP*, controls temporally-related patterning in mammalian hairs (Tamate and Takeuchi 1984; Bultman, Michaud, and Woychik 1992; Barsh 1996; Kaelin et al. 2012), and is a potential candidate for within-feather patterning. Mutations at this locus in quail affects bar width in individual feathers (N.I. Mundy and F. Minvielle *unpublished data*), and *ASIP* expression in developing chicken feathers is spatially variable (Oribe et al. 2012; Yoshihara et al. 2012). Thus the evolutionary origin of bars may be more straightforward than inferred from the reaction diffusion model. Currently, a large gap in our understanding is a plausible mechanism for how these loci are involved in a reaction-diffusion mechanism.

A transition from an absence of patterning to spots occurred in the flanks in both orders but in none of the other six patches. It has been demonstrated that spots can have a social function (Petrie, Halliday, and Sanders 1991; Kose, Mänd, and Moller 1999; Roulin 1999a; Roulin, Riols, and Dijkstra 2001) and sexually selected traits evolve quickly potentially masking a signal of constraint (Pomiankowski and Iwasa 1998). It therefore seems likely that this rare transition is a result of strong selection pressure circumventing developmental constraint.

Results from regional models showed that constraint was still present at this level of analysis, and that the summary model of patch evolution showed greater similarity to the dorsoventral axis than anteroposterior axis models, in both orders (Fig. 3.5). This provides evidence that the dorsoventral regions are behaving as hierarchical developmental units or modules, each composed of multiple patches evolving in a similar manner. The similarity was particularly strong for Galliformes, while in Anseriformes there was one major difference – the dorsoventral model lacks a transition from bars to scales in the ventral region (Fig. 3.3, 3.5).

The striking overall similarities between the summary model of local pattern evolution within-patches and the dorsoventral axis model of patterning imply an additional layer of developmental constraint in plumage pattern evolution. We currently know little about the interaction between pteryla formation and modularity in

phenotypes. Numerous interactions between the epithelium and mesenchyme are known to signal pterylae formation (Chuong 1993; Prum 1999; Chuong et al. 2000; Dhouailly 2004; Lin et al. 2006). Epithelial-mesenchymal transplant experiments in quail-chicken chimaeras show that there are important dorsoventral differences in integument development: the dorsal trunk dermis originates from the dermomyotome of the somites whereas the ventral dermis originates from lateral plate mesoderm (Mauger et al. 1982; Fliniaux, Viallet, and Dhouailly 2004). In addition, the feather dermal progenitors of the dorsal and ventral regions are specified by different signals (Lin et al. 2006). Therefore, the differential dorsoventral origins of dermis cells may have lasting developmental and evolutionary effects on the phenotype of birds.

As for patches and regions, the whole body models showed evidence for directionality in plumage pattern evolution. If recruitment of patterning mechanisms across local patches and regions were common, this would lead to more transitions occurring in the whole body model. However, evidence for this is limited. For Galliformes it is striking that the main features of the patch and dorsoventral models, including a stronger transition from an absence of patterning to bars than scales, occur in the whole body model. For Anseriformes, the picture is mixed – whereas a transition from absence of patterns to scales occurs in the whole body, the transition from absence to bars does not, even though this is strongly supported in the within-patch models. Most strikingly, in both orders, spots can only evolve from pre-existing patterns and not from an absence of patterns for the whole body. Given the evidence for sexual selection acting on spots, this reinforces the view that there is overall developmental constraint on spot development.

We have carried out a broad level analysis of two large sister taxa that are ecologically distinct, and the decision to analyse them separately receives *post hoc* justification from the finding of differences among them. It is of course possible that different transition rates of plumage patterns occur among different clades within these taxa. This is an interesting issue for future investigation. Although we analysed most extant species within Anseriformes and Galliformes (63% combined), 37% of species were excluded because of a lack of robust phylogenetic information. The effects of this on our results are unknown, but we note that the absence of these taxa is not different in principle to the absence of an unknowable number of extinct taxa in the dataset. Hybridization is common in both orders: an estimated 41.6% species of

Anseriformes and 21.5% species of Galliformes hybridize (Grant and Grant 1992). Hybridization can lead to rapid shifts in phenotype that could cause uncertainty in estimating model transitions. The effect of categorizing mottled plumage as missing data is also unknown. Given that mottled patterns do not appear to have a regular motif, categorizing these patterns is plagued by uncertainties. Having many categories for different types of mottled patterns would likely obscure a signal of directionality in evolution, whereas using a single category for a pattern that exists in many states, might overly constrain the model. Therefore, using a category of "unknown" is representative of what is currently known about plumage pattern formation, and using robust Bayesian based analyses based on multi-model inference should largely control for uncertainty (Pagel, Meade, and Barker 2004).

An issue for future consideration will be the potential influence of female patterning on the evolution of patterning in males. In both orders studied, there is sexual dimorphism in plumage patterns (Gluckman 2014), which is estrogen-dependent (Owens and Short 1995; Kimball and Ligon 1999). As a consequence, it was thought that elaborate coloration initially evolved in both sexes via genetic correlation (Lande 1980; Owens and Hartley 1998; Kimball and Ligon 1999; Kraaijeveld, Kraaijeveld-Smit, and Komdeur 2007). However, currently there is little evidence to suggest that there is genetic correlation in plumage pattern evolution between males and females in Anseriformes and Galliformes (Gluckman 2014). Hence the possibility that particular patterns evolve first in females and are later acquired by males remains, and will be considered in future studies.

Similar plumage patterns have evolved in many distantly and closely related species of birds (Riegner 2008; Stern and Orgogozo 2009; Gluckman and Cardoso 2010). We demonstrated directionality in plumage pattern evolution that is congruent with developmental constraint. Overall there was remarkable similarity in the trajectories of pattern evolution in Galliformes and Anseriformes, suggesting that the constraint is similar in the two orders, despite large ecological differences. As suggested by Price and Pavelka (1996) the role of natural selection may be "fine-tuning the appearance of the pattern, fixing and maintaining pattern elements at a given level of expression, and modifying behavioral and other features to maximize the patterns' utility" on the basis of the order that patterns evolve. We suggest that directionality in plumage pattern evolution may be caused by the underlying

dynamics of the developmental system of patterning, which may be of general significance to birds.

Chapter 4: Cuckoos in raptors' clothing, barred plumage illuminates a fundamental principle of Batesian mimicry

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Banded bay cuckoo
(*Cacomantis sonneratii*)

Japanese sparrowhawk
(*Accipiter gularis*)

The mechanisms underlying convergent evolution in the plumage patterns of birds

Abstract

A fundamental principle of Batesian mimicry is that it pays to look like a local harmful species that is recognizable to other local species (receivers). Mimicking an allopatric species confers no benefit, as it is not recognizable to local species. It is thought that the common cuckoo, *Cuculus canorus*, is a Batesian mimic of the Eurasian sparrowhawk, *Accipiter nisus*, predominantly via its barred plumage, which facilitates access to host nests to deposit eggs. Barring is widespread in five genera of Old World cuckoos, unlike nonparasitic cuckoos, and evolved after the evolution of parasitism. Although barred plumage is predominant in parasitic cuckoos, it is unclear whether it may have a widespread function in cuckoo-hawk mimicry. If widespread, there should be a visual similarity between all five genera of Old World parasitic cuckoos and sympatric raptors. In addition, given that it pays to look like a local harmful species, sympatry should predict the degree of similarity. We compared barred plumage from all five genera of parasitic Old World cuckoos and up to eight sympatric raptors using digital image analysis. Cuckoos predominantly matched most raptors for at least one pattern attribute. In addition, three out of five cuckoos closely resembled a sympatric raptor for all barred pattern attributes examined, and potential model species were not confined to sparrowhawks. Habitat did not appear to influence plumage pattern similarity in most species studied. Finally, the barred plumage of sympatric species was more similar in appearance than those in allopatry. Together this demonstrates that cuckoos look like a local harmful species, which is congruous with Batesian mimicry.

The mechanisms underlying convergent evolution in the plumage patterns of birds

Introduction

In Batesian mimicry, a harmless species mimics an unpalatable or a harmful one. For example, *Dismorphia* butterflies (mimics) vary their colour pattern according to the local species of toxic Neotropical *Heliconius* butterflies (models; Bates 1862). By looking like a familiar unpalatable model, the mimic avoids detection. Therefore, a fundamental principle of Batesian mimicry is that there is an advantage to looking like a local unpalatable or harmful species that is recognizable.

Parasitic cuckoos are an extraordinary example of mimicry with diverse strategies to trick hosts into rearing their young (Davies 2011). Some cuckoos closely mimic the eggs of their hosts (Brooke and Davies 1998; Moskát et al. 2008; Spottiswoode 2010; Stoddard and Stevens 2010), and others mimic host nestlings (Langmore et al. 2011). Studies of brood parasitism in cuckoos have predominantly focused on egg as well as chick mimicry, and have highlighted the drastic impact on host reproductive potential that has set the scene for a well-documented coevolutionary arms race (Davies, Brooke, and Kacelnik 1996; Davies 2000; Soler and Soler 2000; Grim 2006; Spottiswoode and Stevens 2011). However, given that parasitism begins with depositing eggs in a host nest, blocking access to the nest has the greatest potential to minimize reproductive costs (Moksnes et al. 2000; Davies and Welbergen 2009; Feeney, Welbergen, and Langmore 2012). As a consequence, brood-parasitic cuckoos appear to have evolved a range of strategies to gain access to host nests. For example, in the genera *Clamator*, *Eudynamys* and *Scythrops* it is reported that males elicit a mobbing response to distract hosts while females discreetly lay their eggs in host nests (Gaston 1976; Davies 2000). However, given the costs that hosts can impose on parasitic cuckoos, inconspicuousness should be favoured to evade detection (Davies and Brooke 1988; Davies and Welbergen 2008; Požgayová, Procházka, and Honza 2009; Langmore et al. 2011).

Brood-parasitic cuckoos dupe hosts into treating the parasite as if it is something it is not by looking like either a harmless species (aggressive mimicry) or a harmful species (Batesian mimicry). Aggressive mimicry is suspected among drongo cuckoos (*Surniculus* spp.) that form foraging flocks with multiple species of small passerines, and contribute to the flock by acting as a predator sentinel (Feeney, Welbergen, and Langmore 2012). Batesian mimicry in cuckoos has been

suspected since the time of Aristotle owing to their remarkable resemblance to raptors, especially *Accipiter* hawks (Wallace 1889). Their striking visual similarity is derived from their yellow eyes and legs, their size and shape, flight patterns and barred underparts (Honza et al. 2006; Davies and Welbergen 2008; Payne 2010; Welbergen and Davies 2011; Trnka, Prokop, and Grim 2012). In addition, it has been suggested that the rufous morph of the common cuckoo, *Cuculus canorus*, might represent mimicry of the Eurasian kestrel, *Falco tinnunculus* (Trnka and Grim 2013). Some species additionally possess polymorphisms in the colour of their barred plumage to thwart hosts that can see past the cuckoos' disguise. These polymorphic species are likely to possess multiple hawk-like features demonstrating that the host-parasite arms race probably also occurs in the adult phenotype of parasites (Thorogood and Davies 2012; Trnka and Grim 2013) and the alternative morph may represent frequency-dependent mimicry for an additional model, the Eurasian kestrel (Honza et al. 2006; Thorogood and Davies 2012; Trnka and Prokop 2012; Trnka and Grim 2013).

In Old World cuckoos, barred plumage evolved within the context of host-parasite coevolution, suggesting that it is an adaptive strategy to facilitate access to host nests (Kruger, Davies, and Sorenson 2007). This type of plumage pattern covers most of the ventral surface and is composed of within-feather alternating light and dark pigmentation, transversal to the feather's axis (Payne and Sorensen 2005; Bortolotti et al. 2006; Gluckman and Cardoso 2010; Payne 2010). Studies of Batesian mimicry in parasitic cuckoos have focused on the common cuckoo, which is thought to mimic the Eurasian sparrowhawk, *Accipiter nisus* (Davies and Welbergen 2008; Davies and Welbergen 2009; Welbergen and Davies 2011). Field experiments measuring host responses to models of the common cuckoo demonstrate that barring can constrain host aggression at close range and that polymorphisms in barred plumage coloration can thwart detection by hosts (Honza et al. 2004; Grim 2005; Moksnes et al. 2007; Thorogood and Davies 2012; Trnka and Prokop 2012; Trnka and Grim 2013). By looking like a harmful model, the common cuckoo can facilitate access to host nests in which to place its eggs and when hosts learn to discriminate, altering the phenotype can be a successful strategy. In Old World parasitic cuckoos (Cuculidae) 35 species out of 58 have barred plumage, which is also common in both sparrowhawks (*Accipiter*) and other raptor genera (Payne and Sorensen 2005; Christie and Ferguson-Lees 2010). Given that resembling a local

dangerous species has allowed the common cuckoo to flourish, Batesian mimicry via barred plumage patterns may be widespread in Old World cuckoos to facilitate access to host nests, and many types of raptors may be the dangerous model.

Fig. 4.1. Barred plumage patterns. Although barred plumage is a common and easily identifiable pattern, it can vary extensively as shown on the same scale in (a) *Cuculus saturatus* and (b) *Aviceda cuculoides*. Photographs of plumage within the figure are copyright of the Natural History Museum and were taken by Thanh-Lan Gluckman.

Barred plumage may have multiple functions in brood parasitism. It has been well documented that avian brood parasites monitor host nests discreetly from nearby perches (Alvarez 1993; Hauber and Russo 2000; Begum et al. 2011). At a distance, barred plumage may provide camouflage while the parasite is searching for and watching potential hosts, but when detected at close range it may contribute to hawk mimicry and constrain aggression; however, it has also been suggested that it may increase aggression by some hosts towards parasites (Honza et al. 2006; Welbergen and Davies 2008; Payne 2010; Welbergen and Davies 2011; Trnka and Prokop 2012). Barred plumage can vary extensively in size, spacing, contrast and

relative importance of the main marking size to the overall pattern (Fig. 4.1). If barred plumage patterns function in mimicry in brood-parasitic cuckoos, recognition of local raptors as predators by hosts (i.e. host discrimination and response) should drive a similarity in plumage pattern attributes to be geographically specific (Bates 1862; Briskie, Sealy, and Hobson 1992; Lindholm and Thomas 2000; Hale and Briskie 2007; Pfennig and Mullen 2010).

Materials and Methods

We sampled representatives of Old World parasitic cuckoos with barred plumage on the basis of taxonomy and geographical distribution. According to Howard and Moore (2003) there are six genera containing parasitic cuckoos with barred plumage: *Cuculus*, *Chrysococcyx*, *Eudynamys*, *Cacomantis*, *Cercococcyx* and *Scythrops*. The genus *Scythrops* has limited barring on the lower part of the ventral surface and, given its orientation when the bird is flying towards hosts, the barring is unlikely to function in mimicry; we thus removed this genus from further consideration. We focused our sampling efforts on cuckoos and raptors with a restricted distribution range in the tropics of Africa and Oceania (Asia and eastern Australia), where parasitic cuckoos are concentrated (Yom-Tov and Geffen 2005). North and South American species are not represented in this study as only a few brood-parasitic cuckoos are found on these continents and they do not have barred plumage (Payne and Sorensen 2005). To assess overlap of distributions we scanned maps from Raptors of the World (Christie and Ferguson-Lees 2010) and The Cuckoos (Payne and Sorensen 2005) for comparison by eye. For the purposes of this study it was important to establish approximate geographical range overlap, but not precise estimates of the extent of overlap. Therefore, we scored the overlapping distribution of raptors on the basis of up to 25%, 50%, 75% and 100% of that of cuckoos (Table 4.1). We aimed to sample all sympatric raptors for the cuckoos chosen. Where possible, most raptors that have a distribution overlap of greater than 0.25 were sampled. All raptors with the largest overlapping distribution category were sampled, except for *Hieraaetus kienerii*, which could not be sampled (Table 4.1). To ensure that these results are representative of geographical range overlap, we compared a subset of African species with a regional field guide and although there was some variation in the extent of overlap, both guides described sympatry for each

pair considered (supplementary Table S4.1; Sinclair and Ryan 2010).

In this study we aimed to sample each genus, while controlling for pattern similarity resulting from phylogeny. Therefore, we randomly selected a species to represent one barred parasitic genus from Africa and similarly a barred parasitic species to represent a separate genus from Oceania, for which there was a minimum of five undamaged museum skins at the Natural History Museum at Tring, U.K. An additional cuckoo from a separate genus was then selected that had an overlapping distribution with the first cuckoo selected, to test whether cuckoos from separate genera resembled the same raptor. The first randomly selected cuckoo representing each genus had an adequate number of undamaged specimens. This sampling design also allowed us to model whether sympatry and allopatry predict pattern similarity and dissimilarity by comparing species within and between geographical areas. Where species exhibited multiple subspecies, for example *Eudynamys scolopacea*, we sampled the nominate subspecies. One cuckoo species in this study, *Cuculus saturatus*, is polymorphic and we focused our sampling efforts on the primary (more frequent) morph (Voipio 1953). We sampled sympatric raptors with subspecies and polymorphisms in the same way.

Raptor species were selected on the basis of sympatry and the presence of barred plumage (Table 4.1). Only raptors that prey on live birds were included in the study - vultures were excluded as they are carrion eaters (supplementary Table S4.2). In some instances, raptors were illustrated with barred plumage in the books, but on inspection of specimens had little or negligible barring (i.e. barring only on the legs) and were thus removed from the study. In addition, some raptors could not be sampled owing to a lack of specimens and/or access (Table 4.1, species marked with an asterisk). This resulted in six raptors for the African cuckoos, and eight raptors for the Oceanian cuckoos, representing seven genera in total.

Table 4.1. Approximate distribution overlap between cuckoos and their respective raptor.

Species	*Eudynamys scolopacea*	*Chrysococcyx flavigularis*	*Cacomantis sonneratii*	*Cercococcyx mechowi*	*Cuculus saturatus*
Aviceda cuculoides	-	<1	-	<1	-
**Aviceda subcristata*	<0.25	-	-	-	<0.25
Aviceda leuphotes	<0.50	-	<0.50	-	<0.50
**Henicopernis longicauda*	<0.25	-	-	-	<0.25
Pernis ptilorhyncus	<0.50	-	<0.50	-	<0.50
**Pernis celebensis*	<0.25	-	-	-	<0.50
**Circaetus cinereus*	-	<0.25	-	<0.50	-
Polyboroides typus	-	<1	-	<1	-
**Circus assimilis*	<0.25	-	-	-	<0.25
Micronisus gabar	-	<0.25	-	<0.50	-
Accipiter trivirgatus	<0.50	-	<0.50	-	<0.50
Accipiter tachiro	-	<1	-	<0.75	-
Accipiter soloensis	<0.50	-	<0.50	-	<0.75
Accipiter fasciatus	<0.25	-	-	-	<0.25
Accipiter gularis	<0.50	-	<0.50	-	<0.75
Accipiter virgatus	<0.25	-	<0.50	-	<0.25
Accipiter ovampensis	-	<0.25	-	<0.25	-
**Accipiter melanoleucus*	-	<0.75	-	<0.75	-
**Accipiter meyerianus*	<0.25	-	-	-	<0.25
**Megatriorchis doriae*	<0.25	-	-	-	<0.25
Kaupifalco monogrammicus	-	<1	-	<1	-
**Harpyopsis novaeguineae*	<0.25	-	-	-	<0.25
**Hieraaetus morphnoides*	<0.25	-	-	-	<0.25
**Hieraaetus kienerii*	<0.25	-	<0.50	-	<0.75
Spizaetus nanus	<0.25	-	<0.50	-	<0.50
**Falco moluccensis*	<0.25	-	<0.25	-	<0.50
**Falco longipennis*	<0.25	-	-	-	<0.25

*Species not represented owing to a lack of access/intact specimens.

Currently, there is little information on how barred plumage functions in host-parasite interactions outside of the common cuckoo (Payne and Sorensen 2005). For example, it is unknown whether the male attracts a mobbing response from hosts while the female lays her eggs, or perhaps females approach the nest by themselves. Therefore, we sampled both males and females in species that have barred plumage (Table 4.2). Given that birds approach a nest facing forwards, it is more likely that the most important part of the patterning, where used in mimicry, is the ventral surface as well as the flanks. Therefore, we focused our sampling on these body regions, except in *Chrysococcyx flavigularis* for which we could sample only the ventral surface to avoid damage to the flanks/wing. Patterning can vary within barred plumage so we sampled from the top, middle and bottom of the ventral and left flank area of specimens, avoiding the brood patch, to assess pattern variation (Gluckman and Cardoso 2009).

Following Stevens et al. (2007) and Stoddard and Stevens (2010), we collected digital images of museum specimens with a Fujifilm IS Pro UV-sensitive camera with known spectral sensitivity, equipped with a quartz CoastalOpt UV lens (Coastal Optical Systems). Images were captured with a human pass filter (Baader UV/IR cut filter: 400-700 nm) as well as a UV pass filter (Baader U filter: 300-400 nm) at a standard distance (173 cm) at eye level with constant illumination provided by a Kaiser RB260 digital lighting unit. All images were linearized to control for a nonlinear response to changes in radiance prior to image analysis (Stevens, Parraga, et al. 2007). Current evidence shows that avian pattern perception occurs via achromatic (luminance) vision, which is encoded in the double cones (Jones and Osorio 2004; Osorio and Vorobyev 2005). To assess which visual system is most representative of hosts we collated information on host and prey species for cuckoos and raptors of these regions. Although the information available is limited, from what is recorded, the hosts and prey species of all cuckoos and raptors sampled are predominantly passerines (supplementary Table S4.2). The blue tit, *Cyanistes caeruleus*, has the best-studied visual system of any passerine (Hart and Hunt 2006; Hart et al. 2012) and has been widely used as a model for passerine vision in previous studies (Spottiswoode and Stevens 2010; Stoddard and Stevens 2010; Spottiswoode and Stevens 2011). Therefore, to account for passerine vision, all images were transformed from camera colour space to the relative photon catches of the double cones of a blue tit (Stevens et al. 2006; Stevens, Parraga, et al. 2007).

Table 4. 2. Female and male sample size for each cuckoo and raptor represented.

	Female	Male	Total
Cuckoos			
Eudynamys scolopacea	11	N/A	11
Chrysococcyx flavigularis	3	2	5
Cacomantis sonneratii	5	5	10
Cercococcyx mechowi	5	5	10
Cuculus saturatus	5	5	10
Raptors			
Aviceda cuculoides	4	3	7
Aviceda leuphotes	5	5	10
Pernis ptilorhyncus	N/A	4	4
Polyboroides typus	5	5	10
Micronisus gabar	5	5	10
Accipiter trivirgatus	5	5	10
Accipiter tachiro	5	5	10
Accipiter soloensis	5	N/A	5
Accipiter fasciatus	5	5	10
Accipiter gularis	10	10	20
Accipiter virgatus	4	5	9
Accipiter ovampensis	3	5	8
Kaupifalco monogrammicus	5	5	10
Spizaetus nanus	N/A	5	5

Barred plumage is a relatively simple pattern that consists of the same regularly repeating motif, which is found in many species of birds worldwide (Kenward et al. 2004; Riegner 2008; Gluckman and Cardoso 2009; Gluckman and Cardoso 2010). As such, there is likely to be an inherent degree of similarity in plumage pattern attributes. Therefore, we employed a granularity analysis to objectively quantify multiple aspects of patterning in the transformed images of each specimen, following methods employed to quantify the camouflage of cuttlefish as

well as mimicry in egg patterning (Barbosa et al. 2008; Chiao et al. 2009; Spottiswoode 2010; Stoddard and Stevens 2010; Spottiswoode and Stevens 2011). A granularity analysis is akin to early-stage visual processing, which breaks down information into different spatial scales depending on the receptive fields (Godfrey, Lythgoe, and Rumball 1987). To simulate this process, we fast Fourier transformed each image with seven octave-wide isotropic band-pass filters. Each filter size, or granularity band, captures information at different spatial scales where large filter sizes correspond to small markings with high spatial frequency, and small filter sizes correspond to large markings with low spatial frequency. Information from each spatial scale is represented by the granularity spectra for an image, from which aspects of patterning can be measured (Fig. 4.2).

Several absolute objective aspects of patterning were calculated from the granularity spectra (Fig. 4.2): the main marking size (the peak of the line), the importance of the main marking size to the overall pattern (proportion of power across all filter sizes that matches the peak value) and pattern contrast (overall amplitude of the spectrum across all spatial scales) (Stoddard and Stevens 2010). In addition, given that barred plumage is made up of a regularly repeating sub-pattern (Kenward et al. 2004; Gluckman and Cardoso 2009), we calculated the overall standard deviation of the main marking size over all spatial scales as a measure of pattern uniformity.

Barred plumage attributes can vary within patches of patterning (Gluckman and Cardoso 2009). To assess whether the pattern attributes measured in this study varied within species, we compared patterns from the ventral surface and flank area, as well as the top, middle and bottom of each surface sampled. As the results were qualitatively similar within and between patches of plumage patterns within species (supplementary Fig. S4.1), we averaged the total values for each patch per individual for the final analysis. To ensure that the age of the museum specimens did not affect the integrity of the pattern data (Doucet and Hill 2009), we correlated each measure of pattern with year of collection (when available) where there was a minimum of eight individuals to ensure that a low sample size did not bias the correlation, resulting in a sample size of 79 individuals. The data were not normally distributed so we report the median rather than the mean. To estimate robust confidence intervals, we used 1000 bootstrap bias-corrected and accelerated simulations (BCA) using the

bootstrap module in PASW 20 (supplementary Fig. S4.2) (Ruscio and T. Mullen 2012).

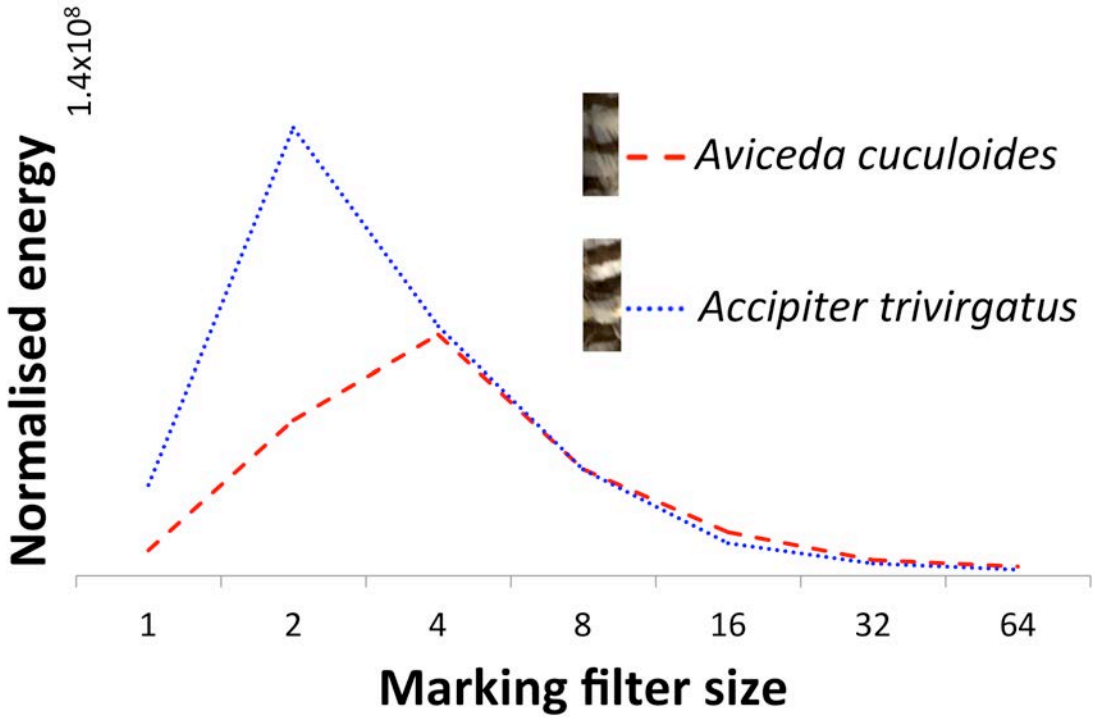


Fig. 4.2. Granularity spectra for an individual specimen of *Aviceda cuculoides* and *Accipiter trivirgatus* (representative samples of plumage are on the same scale). The granularity spectra of patterning are made up of the normalized energy at each filter size and indicate different aspects of patterning. For example, the granularity spectra peak at a marking filter size of two for *A. trivirgatus*, and four for *A. cuculoides*, which shows that *A. trivirgatus* has larger markings. The amplitude of the spectrum for the granularity spectra of *A. cuculoides* is much lower than that for *A. trivirgatus* indicating that *A. trivirgatus* has more contrast in its patterns than *A. cuculoides*. Photographs of plumage within the figure are copyright of the Natural History Museum and were taken by Thanh-Lan Gluckman.

To compare similarity between the barred plumage of each cuckoo-raptor pair, as well as assess the extent of overlap in pattern attributes, we used a

nonparametric measure of effect size: probability of superiority (PS), also known as stochastic superiority (Nakagawa and Cuthill 2007; Ruscio and Mullen 2012). PS is the probability that an individual from species A has a higher scoring pattern variable (i.e. main marking size) than an individual from species B. A PS value between 0.5 and 0.71 indicates that the sample populations are highly similar for the trait measured (Grissom 2004). We used the slightly increased threshold of 0.73 as it is still within the realm of biological importance (Nakagawa and Cuthill 2007). For example, a PS value of 0.6 for main marking size in a cuckoo-raptor pair indicates that the two species being assessed have plumage patterns that overlap in size and match well for this attribute. To have a high degree of similarity the cuckoo-raptor pair must match in all four pattern attributes measured.

Habitat may select for plumage pattern attributes to be similar in sympatric cuckoos and raptors to evade detection by prey and hosts alike. A posteriori, to examine whether habitat may be a confounding factor we compared the habitat of each species to assess its influence on cuckoo plumage patterns. If habitat shapes the plumage patterns of cuckoos, cuckoo-raptor pairs that have the highest number of overlapping habitat types should be most similar. Information on habitat was collated and tabulated for final comparison (Payne and Sorensen 2005; Christie and Ferguson-Lees 2010). No statistical tests were applied to these data owing to the small sample size per habitat type. Nevertheless, this represents the maximum available information for each cuckoo sampled and is indicative of the selection habitat may pose on plumage patterns.

Finally, to assess whether cuckoos look more like a local dangerous model than an allopatric raptor, we modeled sympatry/allopatry as a predictor of pattern similarity. As mentioned previously, barring is a ubiquitous pattern that is likely to exhibit a basic degree of similarity in birds that express this trait because it is a relatively simple pattern. However, it is unlikely that barred plumage will vary in all four measures described simultaneously as a result of the inherent simplicity of the pattern. Therefore, we measured the four-dimensional Euclidean distance between each specimen of cuckoo and every individual raptor in plumage pattern phenotypic space, which also accounts for within-species variation (Spottiswoode and Stevens 2011). A large Euclidean distance between individuals indicates dissimilarity, and a small distance indicates similarity. In addition, given that some similarity in patterning

was expected, and that we sampled multiple cuckoo and raptor species, as well as individuals within species, we modeled these data using generalized linear mixed modeling using the glmer function in the lme4 package in R (R Development Core Team 2012). To model whether geographical overlap predicts pattern similarity/dissimilarity, we compared the relative fit of sympatry and allopatry as a predictor variable for comparison with a model without a predictor variable: glmer(Euc_distance~Symp_Allopatric+(Raptor|Raptor_ID)+(Cuckoo|Cuckoo_ID)), glmer(Euc_distance~+(Raptor|Raptor_ID)+(Cuckoo|Cuckoo_ID)), respectively. Together this represents the hypothesis that cuckoos look more similar to a local sympatric raptor and the alternative, that geographical overlap does not fit the data well and that there may be an alternative reason for a similarity in barred plumage between cuckoos and raptors. Finally, we used the Akaike information criterion (AIC) and the evidence ratio statistic (ER), to assess which model is a better fit for the data as well as assess the magnitude to which the better fitting model explains the variation (Burnham and Anderson 2002; Symonds and Moussalli 2010). We used AIC rather than AICc as the number of observations was more than 40 times the number of explanatory variables (Anderson et al. 2001).

Results

There was no correlation between year of collection and any measure of patterning between or within species (main marking size: $0.277 < R < 0.622$, $P > 0.055$; proportion of power: $0.303 < R < 0.983$, $P > 0.116$; contrast: $0.340 < R < 0.771$, $P > 0.091$; marking size deviation: $0.342 < R < 0.821$, $P > 0.046$). Granularity spectra differed between most of the cuckoo species sampled although there was some similarity between *Cacomantis* and *Cercococcyx*. In addition, there was substantial variation among the granularity spectra of raptors sampled (Fig. 4.3). Cuckoos predominantly matched sympatric raptors for between one and three pattern attributes. In three of the five cuckoos, there was matching for all four pattern attributes with at least one raptor (Fig. 4.3, Table 4.3).

For most cuckoo species, there was no pattern matching in at least one raptor, except in the African *Chrysococcyx*, but for *Eudynamys*, there were two raptors with no pattern overlap (Fig. 4.3, Table 4.3). Two of the four closely matched

raptor species were *Accipiter* species. In addition, cuckoos closely resembled species of the genera *Aviceda* and *Polyboroides* (Table 4.3). There was variation in which pattern attribute was most matched across sympatric raptors: *Cercococcyx* matched most raptors for contrast, *Chrysococcyx* and *Cacomantis* predominantly matched raptors in main marking size and proportion of power, *Cuculus* matched most raptors for main marking size, whereas *Eudynamys* predominantly matched raptors for proportion of power (Table 4.3). In addition, there was no pattern matching between any allopatric cuckoo-raptor pair (Table 4.4).

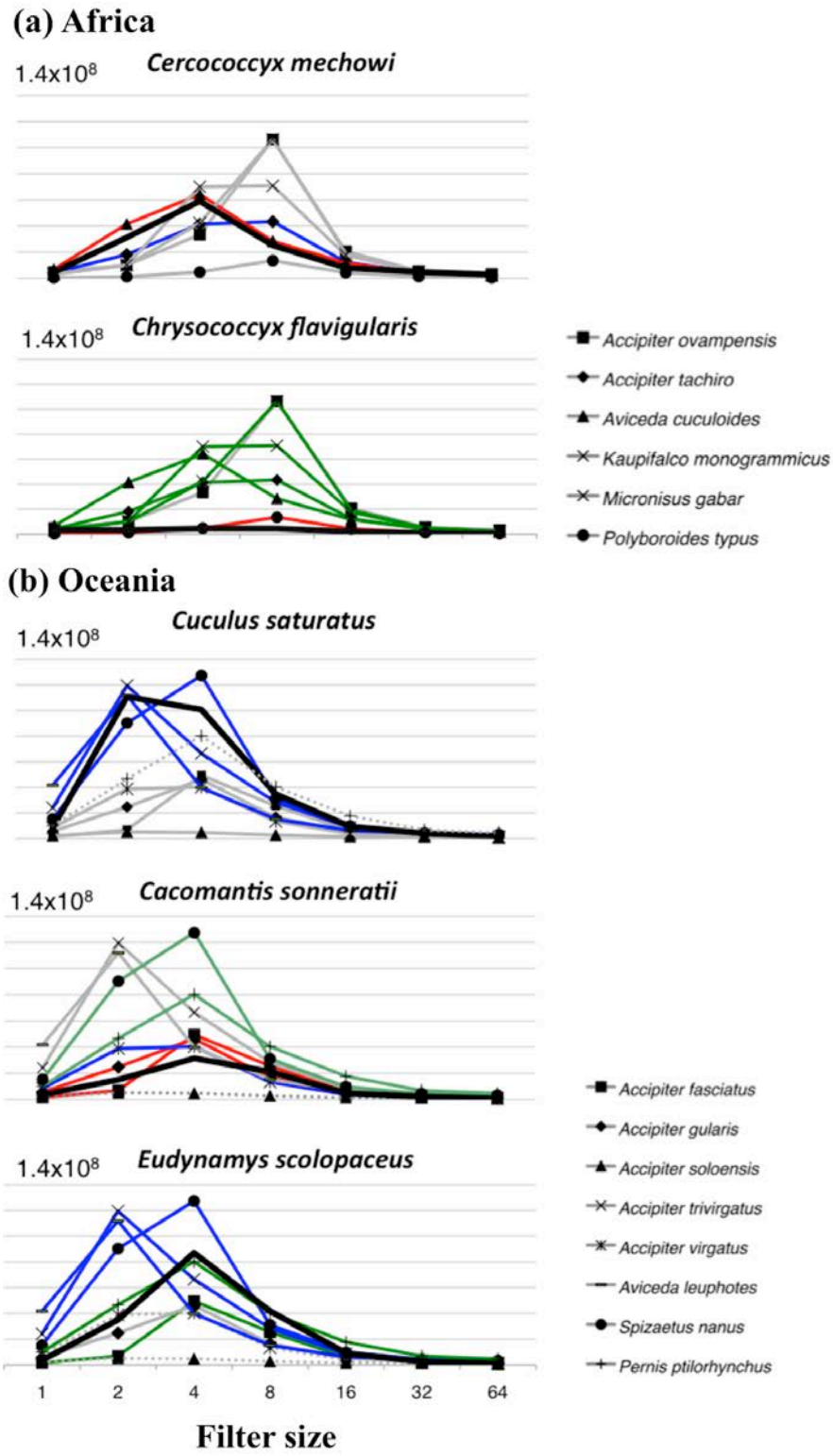


Fig. 4.3. Granularity spectra for cuckoos and sympatric raptors, with pattern matching in four attributes on the basis of the probability of superiority. Spectra are shown for each cuckoo and its sympatric raptors: (a) African spp.; (b) Oceanian spp. The black line indicates the cuckoo. A grey dotted line indicates no pattern matching over any

attribute, a solid grey line indicates matching for one attribute, green for two attributes, blue for three attributes and red for all four pattern attributes. The measures of probability of superiority are in Table 4.3, and the median and sample sizes used for these calculations are in Table 4.2.

The habitat types inhabited by each cuckoo and raptor were variable between species, as were the number of overlapping habitat types (Table 5, supplementary Table S4.3, S4.4). The number and type of habitats used by each pair predicted plumage pattern similarity only in *Cacomantis* and *Accipiter fasciatus* (Fig. 4.3, Table 4.5).

Table 4.3. Comparison of barring attributes of plumage patterns between each cuckoo and sympatric raptor on the basis of probability of superiority (PS).

Cuckoo	Sympatric raptor	Main marking size	Proportion of power	Contrast	Marking size deviation	Number of matching attributes
Eudynamys scolopacea	*Aviceda leuphotes*	1	0.63	0.61	0.6	3
	Pernis ptilorhyncus	0.65	0.98	0.65	0.8	2
	Accipiter trivirgatus	1	0.57	0.6	0.52	3
	Accipiter soloensis	0.9	1	1	1	0
	Accipiter fasciatus	0.66	0.67	0.97	0.93	2
	Accipiter gularis	0.81	0.63	0.94	0.91	1
	Accipiter virgatus	0.93	0.92	0.94	0.96	0
	Spizaetus nanus	0.82	0.54	0.7	0.7	3
Chrysococcyx flavigularis	*Aviceda cuculoides*	0.6	0.6	1	1	2
	Polyboroides typus	0.72	0.6	0.72	0.5	4
	Micronisus gabar	0.58	0.6	1	0.98	2
	Accipiter tachiro	0.52	0.6	1	0.98	2
	Accipiter ovampensis	0.75	0.6	1	1	1
	Kaupifalco monogrammicus	0.68	0.6	1	1	2
Cacomantis sonneratii	*Aviceda leuphotes*	0.82	0.56	0.96	0.97	1
	Pernis ptilorhyncus	0.58	0.93	0.9	0.6	2
	Accipiter trivirgatus	0.82	0.62	0.9	0.89	1
	Accipiter soloensis	0.87	1	1	1	0
	Accipiter fasciatus	0.7	0.52	0.6	0.54	4
	Accipiter gularis	0.54	0.65	0.55	0.65	4
	Accipiter virgatus	0.69	0.9	0.53	0.59	3
	Spizaetus nanus	0.6	0.58	1	1	2
Cercococcyx mechowi	*Aviceda cuculoides*	0.6	0.73	0.57	0.52	4
	Polyboroides typus	0.97	0.51	0.89	0.89	1

	Micronisus gabar	0.93	0.86	0.66	0.76	1
	Accipiter tachiro	0.75	0.66	0.57	0.61	3
	Accipiter ovampensis	0.99	0.98	0.73	0.78	1
	Kaupifalco monogrammicus	0.99	0.99	0.72	0.77	1
Cuculus saturatus	*Aviceda leuphotes*	0.65	0.9	0.72	0.65	3
	Pernis ptilorhyncus	0.97	1	0.94	0.98	0
	Accipiter trivirgatus	0.67	0.92	0.67	0.6	3
	Accipiter soloensis	0.65	1	1	1	1
	Accipiter fasciatus	0.97	0.7	1	1	1
	Accipiter gularis	0.71	0.87	0.98	0.98	1
	Accipiter virgatus	0.68	1	0.99	1	1
	Spizaetus nanus	0.63	0.83	0.63	0.4	3

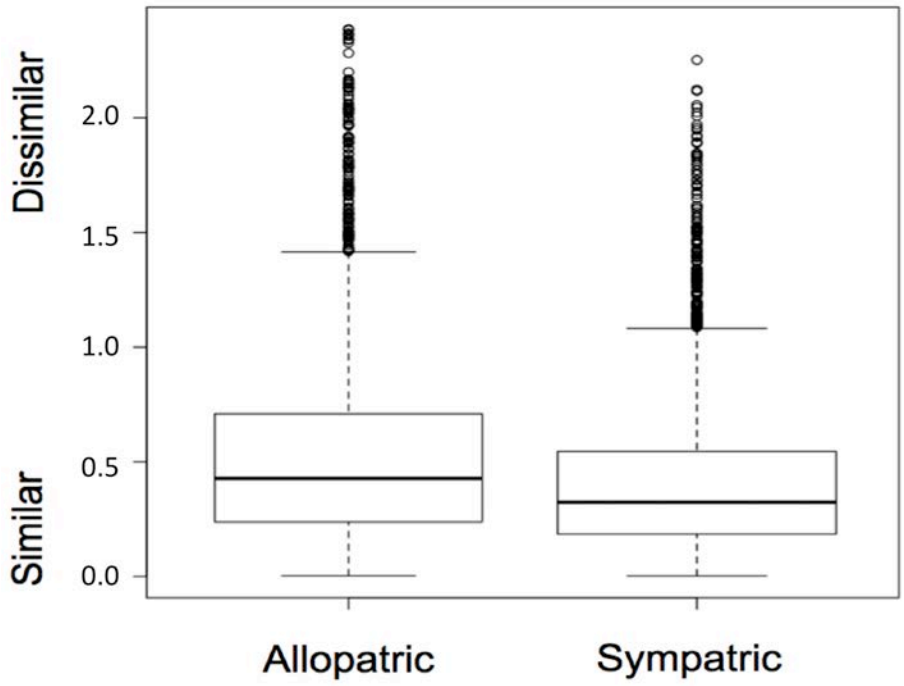


Fig. 4.4. Box plot of the Euclidean distance in four-dimensional plumage pattern space between each individual cuckoo and raptor. Allopatric and sympatric cuckoos and raptors are presented in separate box plots with their individual mean, interquartile ranges and outliers. The 25th to 75th quartiles are encapsulated in the box and dashed lines indicate the lower 25th and upper 75th quartile ranges.

Table 4.4. Comparison of barring attributes of plumage patterns between each cuckoo and allopatric raptor on the basis of probability of superiority (PS). A range of PS values of 0.5–0.71 indicates overlap and therefore a high degree of similarity, therefore a PS value of 0.5 - 0.73 was considered to have an effect (Nakagawa and Cuthill 2007).

Cuckoo	Allopatric raptor	Main marking size	Proportion of power	Contrast	Marking size deviation	Number of matching attributes
Eudynamys scolopaceus	*Aviceda cuculoides*	1.00	1.00	0.99	1.00	0
	Polyboroides typus	1.00	1.00	1.00	1.00	0
	Micronisus gabar	1.00	1.00	1.00	1.00	0
	Accipiter tachiro	1.00	1.00	0.99	1.00	0
	Accipiter ovampensis	1.00	1.00	1.00	1.00	0
	Kaupifalco monogrammicus	1.00	1.00	1.00	1.00	0
Chrysococcyx flavigularis	*Aviceda leuphotes*	1.00	1.00	1.00	1.00	0
	Pernis ptilorhynchus	0.99	1.00	1.00	1.00	0
	Accipiter trivirgatus	1.00	1.00	1.00	1.00	0
	Accipiter soloensis	1.00	0.97	0.99	0.97	0
	Accipiter fasciatus	1.00	1.00	1.00	1.00	0
	Accipiter gularis	1.00	1.00	1.00	1.00	0
	Accipiter virgatus	1.00	1.00	1.00	1.00	0
	Spizaetus nanus	1.00	1.00	1.00	1.00	0
Cacomantis sonneratii	*Aviceda cuculoides*	1.00	1.00	0.99	1.00	0
	Polyboroides typus	1.00	1.00	0.99	1.00	0
	Micronisus gabar	1.00	1.00	1.00	1.00	0
	Accipiter tachiro	1.00	1.00	0.99	0.99	0
	Accipiter	1.00	1.00	1.00	1.00	0

	ovampensis					
	Kaupifalco monogrammicus	1.00	1.00	1.00	1.00	0
Cercococcyx mechowi	*Aviceda leuphotes*	1.00	1.00	1.00	1.00	0
	Pernis ptilorhynchus	0.98	1.00	1.00	0.99	0
	Accipiter trivirgatus	1.00	1.00	1.00	1.00	0
	Accipiter soloensis	1.00	1.00	1.00	1.00	0
	Accipiter fasciatus	1.00	1.00	1.00	0.99	0
	Accipiter gularis	1.00	1.00	1.00	1.00	0
	Accipiter virgatus	1.00	0.99	0.99	0.99	0
	Spizaetus nanus	0.99	1.00	1.00	1.00	0
Cuculus saturatus	*Aviceda cuculoides*	0.99	1.00	1.00	1.00	0
	Polyboroides typus	1.00	1.00	1.00	1.00	0
	Micronisus gabar	1.00	1.00	0.99	1.00	0
	Accipiter tachiro	1.00	1.00	1.00	1.00	0
	Accipiter ovampensis	1.00	1.00	1.00	1.00	0
	Kaupifalco monogrammicus	1.00	1.00	0.99	1.00	0

Table 4.5. The number of overlapping habitat types between cuckoos and raptors. The number of habitats inhabited by each cuckoo is given after the species name.

Raptor	*Eudynamys scolopacea,* 13	*Chrysococcyx flavigularis,* 3	*Cacomantis sonneratii,* 7	*Cercococcyx mechowi,* 3	*Cuculus saturatus,* 7
Aviceda cuculoides	-	1	-	-	-
Aviceda leuphotes	6	-	5	-	2
Pernis ptilorhyncus	2	-	3	-	1
Polyboroides typus	-	1	-	1	-
Micronisus gabar	-	-	-	-	-
Accipiter trivirgatus	4	-	3	-	2
Accipiter tachiro	-	3	-	1	-
Accipiter soloensis	6	-	5	-	1
Accipiter fasciatus	6	-	5	-	1
Accipiter gularis	4	-	3	-	1
Accipiter virgatus	3	-	2	-	1
Accipiter ovampensis	-	-	-	-	-
Kaupifalco monogrammicus	-	1	-	1	-
Spizaetus nanus	3	-	3	-	1

Discussion

Previous work on the function of barred plumage in the common cuckoo revealed that it can reduce aggression in some hosts, which is congruent with Batesian mimicry (Davies and Welbergen 2008; Welbergen and Davies 2011; Thorogood and Davies 2012; Trnka and Prokop 2012; Trnka, Prokop, and Grim 2012; *but see* Honza et al. 2006). In Old World cuckoos, barring appears to have evolved with brood parasitism, but it was previously unknown whether this is perceptually relevant from the perspective of birds (Kruger, Davies, and Sorenson 2007). Although barring is common, it can vary greatly in a number of pattern attributes. Here, using objective digital image analysis we have shown that representative species from all five genera of barred Old World parasitic cuckoos have barring that resembles that of raptors. Of these five cuckoo species, three are remarkably similar to a sympatric raptor; the potential models came from the genus *Accipiter* as well as *Aviceda* and *Polyboroides*. This demonstrates that the putative mimic need not be constrained to the genus *Cuculus* in Old World cuckoos and the model can be from multiple raptor genera. Finally, we found evidence that distribution overlap predicts plumage pattern similarity, suggesting that cuckoos resemble sympatric raptors more than allopatric raptors. Together these results demonstrate that cuckoos from all five genera of barred parasitic Old World cuckoos resemble a local dangerous model, conforming to a fundamental principle of Batesian mimicry. Therefore, barred plumage may function in cuckoo-hawk mimicry in many more species than the common cuckoo.

Although barred plumage is an easily recognizable pattern, it can vary in multiple dimensions and the variation can be subtle (Fig. 4.3, supplementary Fig. S4.1). This underscores the importance of objectively quantifying patterns. Akin to other lines of the cuckoo-host coevolutionary arms race, if barred plumage broadly functions in cuckoo-hawk mimicry, there appears to be a range of mimicry strategies employed from matching multiple local models to specializing on one (Stoddard and Stevens 2010; Davies 2011). All cuckoos studied resemble a sympatric raptor, in some cases very closely (Fig. 4.3). Moreover, no allopatric cuckoo and raptor matched for any pattern attribute (Table 4.4). This indicates that Batesian mimicry, if indeed widespread, may be a well-developed strategy in the host-parasite arms race. There may not, however, be one clear strategy, similar to egg mimicry. For example,

C. flavigularis most closely resembles *Polyboroides typus*, whereas *C. saturatus* resembles multiple sympatric raptors over a range of pattern attributes (Fig. 4.3, Table 4.3). Perhaps in the former, hosts have evolved to become more discerning, whereas in the latter, cuckoo-hawk mimicry may be a relatively recent event akin to other stages of the host-parasite arms race (Stoddard and Stevens 2010; Davies 2011). *Eudynamys scolopacea* also resembles multiple raptors over a range of pattern attributes, which could reflect that males may use distraction displays to lure potential hosts from nests and perhaps in this system hosts are also less discerning than those of *C. flavigularis*. However, further study will be required to assess whether distraction displays are the predominant strategy in this species.

We should also consider that Batesian mimicry might have evolved over a long period of time and that the present overlap of populations may not be representative of the historical distribution of each species. Although the current distribution of each species may not describe where and with which raptors and hosts cuckoo-hawk similarity evolved, once plumage patterns evolve they appear to be evolutionarily labile, which indicates that they may be easy to modify in relatively short periods of time (T-L. Gluckman and N.I. Mundy *unpublished data*). Therefore, although current distribution is not indicative of historical distribution overlap, discrimination behaviour by hosts could drive plumage pattern similarity in short periods of evolutionary time (Thorogood and Davies 2012; Trnka and Grim 2013) and in this study we could only analyse the best of what is currently known about their overlap.

Hosts should be able to recognize broadly sympatric raptor species, which may not occur in precisely the same habitat on a finer scale (Pfennig and Mullen 2010). However, habitat may select for a similarity in barred plumage patterns (Thery 2001; Grim 2005; Endler 2007; Endler and Thery 2010). If this occurs, it would be expected that cuckoo-raptor pairs with the highest number of overlapping habitat types would be the most similar. However, this was not the case with the exception of *Cacomantis sonneratii* and *A. fasciatus* (Fig. 4.3, Table 4.4, 4.5, supplementary Table S4.3). These results, although crude in terms of characterization of habitat types, imply that selection for hawk mimicry, rather than camouflage, may be at least as important as habitat in driving the evolution of barred plumage in brood-parasitic cuckoos (Voipio 1953). Moreover, camouflage through barred plumage should

benefit both cuckoos and raptors, but similarly it should also benefit host and prey species, and other birds living in the same habitat. Yet many bird species sympatric with the study species do not have barred plumage, for example other raptors and passerine birds (supplementary Table S4.2). A link between barring and sedentary lifestyle in raptors has been proposed (Christie and Ferguson-Lees 2010), but there do not appear to be any studies showing statistical support for this, nor for a suggested link between barring and crypsis (Kruger, Davies, and Sorenson 2007). Future studies will need to characterize habitat attributes, or conduct field experiments, to elucidate the extent to which camouflage influences the evolution of barred plumage.

Alternatively, a similarity in phenotypes may arise from phylogenetic constraint or random matching (Grim 2005). Convergence from phylogenetic constraint is unlikely given that barring is more prevalent in parasitic cuckoos (35/58) than in nonparasitic cuckoos (4/83) and evolved after the evolution of parasitism in the Cuculidae (Kruger, Davies, and Sorenson 2007). Random matching is also unlikely given that allopatry/sympatry better predicted plumage pattern similarity/dissimilarity than chance alone (Fig. 4.4).

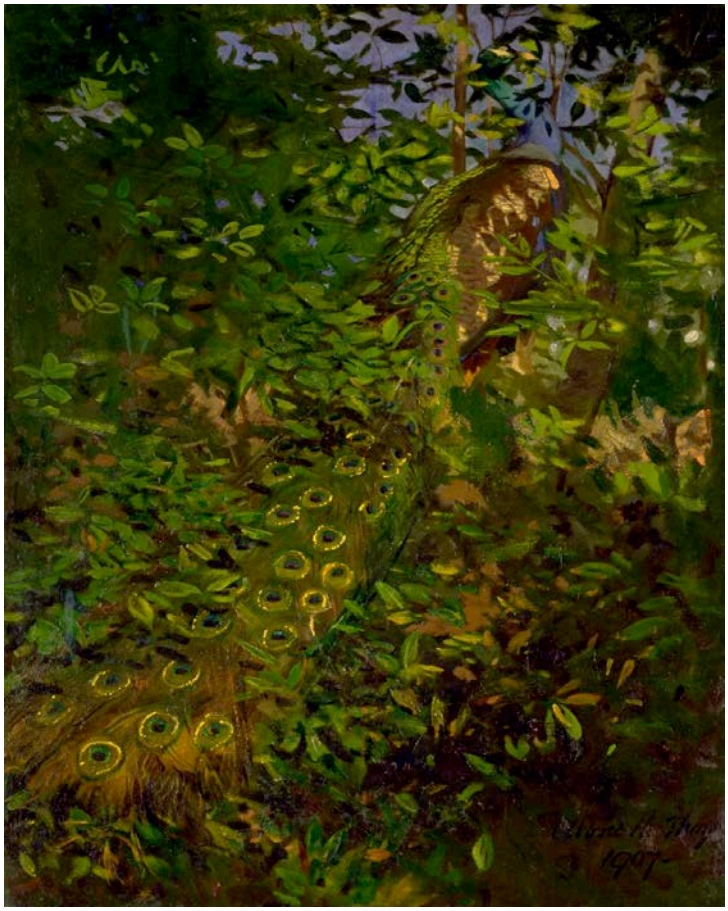
It has been suggested that variation in strategy is due to developmental constraint or progression in the arms race (Davies 2000; Stoddard and Stevens 2010). The current understanding of the mechanisms of plumage pattern formation is poor, and an added layer of complexity in cuckoo-hawk mimicry is that selection occurs via a third party, the host (Prum and Brush 2002). In this study we focused on barred plumage. However, other hawk-like features such as eye and leg coloration as well as polymorphisms are likely to represent important adaptations. Although these additional features may evolve via apostatic selection in some instances and selection for hawk mimicry in others, mimicry dynamics encompassing all features of hawk-like attributes is likely to be an important component of future research (Davies 2000; Thorogood and Davies 2012; Trnka and Prokop 2012; Thorogood and Davies 2013; Trnka and Grim 2013). In addition, this study encompasses only patterning but not colour, which will be investigated in a future study. Other important areas requiring investigation include selection on the host or prey to recognize barring as a threat in the systems we presented here. Our study has shown that there is a remarkable similarity of barred plumage in all genera of Old World barred parasitic

cuckoos and their sympatric raptors from the perspective of the host/prey. We hope this fosters further research beyond the common cuckoo and Eurasian sparrowhawk relationship.

Chapter 5: Ecological selection for bird plumage patterns worldwide

This project was conducted in collaboration with Marius Somveille and Kate Marshall, two current PhD students at the University of Cambridge. I conceived the project, collected all of the plumage pattern data, contributed to the literature review of the function of patterns in camouflage and communication, conducted all comparative testing as well as contributed some philosophical aspects of the ecological analysis. Marius designed and conducted the ecological analyses, and Kate contributed most of the literature review of the function of patterns in camouflage.

Supplementary Table S5.3 is supplied on a CD-ROM on the back cover of this thesis.



Abbott H. Thayer, Peacock in the Woods (1907).

The mechanisms underlying convergent evolution in the plumage patterns of birds

Abstract

It is thought that animal coloration matches the background in which it is viewed to minimize detection by predators. In particular, patterns, e.g. bars and spots, may function in camouflage because patterns are less conspicuous against heterogeneous backgrounds. Avian plumage patterns have converged on just four types: irregular - mottled patterns; regular - scales, bars and spots. Little is known about the camouflage function of avian patterns. To infer the potential utility of bird plumage patterns we conducted a literature survey of patterns in animals. Our literature survey revealed remarkable similarity in pattern-based anti-predator defences in aquatic and terrestrial, and vertebrate and invertebrate animals: irregular patterns facilitate concealment when motionless in cluttered backgrounds, whereas regular patterns (e.g. bars) prevent capture during movement and probably require open habitats for movement. However, regular patterns also function in communication and are likely to evolve on the ventral surface of males and to be independent of habitat. There are 16 types of habitats worldwide that vary in structural composition, e.g. closed habitats are cluttered whereas open habitats are less visually noisy, and perhaps habitat may explain convergent evolution in bird plumage patterns. Alternatively, similar patterns may have evolved due to shared ancestry. We considered adult plumage patterns in each sex, including breeding and non-breeding plumages, in 8008 spp. of birds, and juvenile plumages of 2603 spp., to examine whether patterns a) evolved due to shared ancestry, and b) whether habitat has selected for convergent evolution in plumage patterns. Shared ancestry predominantly explained <30% of pattern evolution. All four patterns are found in all combinations of age/sex/breeding/dorsoventral plumages in all 16 habitats of the world. The rare significant association between patterns and the eco-regions of habitats was dominated by all patterns together (7.65% eco-regions) in contrast with individual patterns (1.69% eco-regions). Significant associations between eco-regions and patterns predominantly occurred in <25% of 15/16 habitats worldwide and patterns were not clustered in particular habitats. The rare significant associations between eco-regions and irregular and regular patterns were found in both closed and open habitats. A lack of association of the ventral breeding plumage of males and habitats is congruent with sexual selection. Therefore, habitat does not predict convergence in bird plumage patterns at the macroevolutionary scale.

The mechanisms underlying convergent evolution in the plumage patterns of birds

Introduction

The prevailing view of signal evolution is that animal coloration matches the background in which it is viewed to minimize detection by predators (Wallace 1889; Poulton 1890; Bradbury and Vehrencamp 1998) but that sexually selected traits stand out (Darwin 1871; Andersson 1994). Animal phenotypes may be comprised of uniform coloration, such as the entirely black plumage of the common raven (*Corvus corax*), or spatially variant pigmentation, such as the barred and spotted patterns of the male Zebra Finch (*Taeniopygia guttata*). In comparison to uniform coloration, our understanding of the function of patterns is less well understood. It is generally thought that patterning is beneficial because patterns allow animals to be less conspicuous against heterogeneous backgrounds (Fig. 5.1). The general principle underlying the function of patterns in camouflage is that patterns resemble aspects of the background in order to evade detection by predators or prey. In the context of signaling, patterns must visually diverge from the background to stand out to conspecifics.

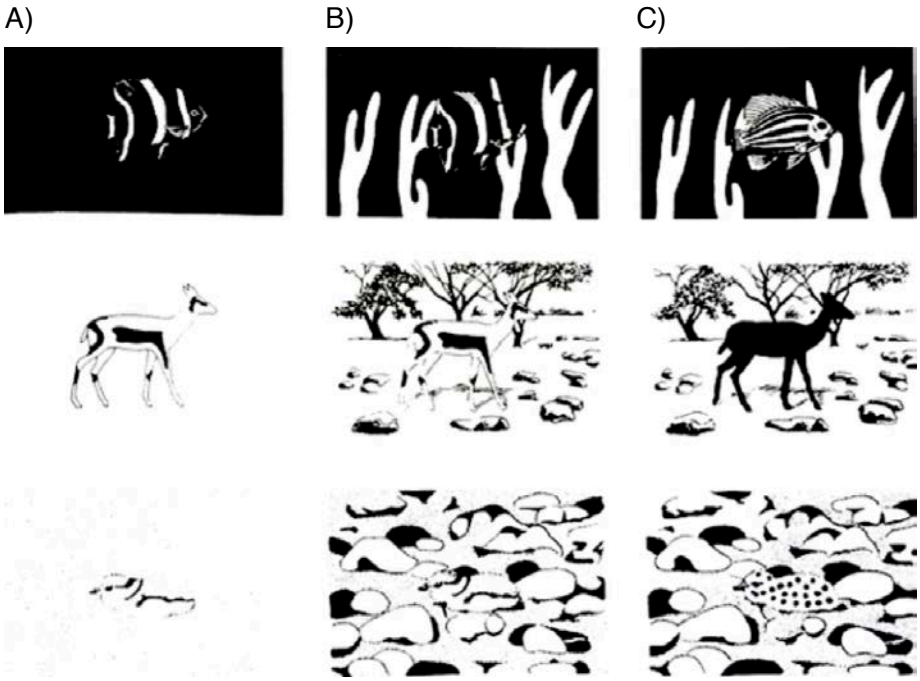


Fig. 5.1. The hypothesis of animal pattern strategies. Considering animal patterns in the context of habitat, patterns can A) stand out, B) conceal the animal if its pattern is similar to its background, and C) stand out if the pattern is dissimilar to its background pattern (Fig. 8.16 *in* Bradbury and Vehrencamp 1998).

Signal evolution theory predicts that a visual signal will evolve to be effective for the function it serves (Endler 1978; Bradbury and Vehrencamp 1998; Kenward et al. 2004). It is thought that animals exploit the visual patterning of their background, including vegetation and the substrate, to either camouflage themselves or to stand out to conspecifics. For example, bold patterns on a plain background make an animal standout, having a similar pattern to the background allows the animal to blend in, whereas patterns that oppose the geometric pattern of the background are conspicuous (Fig. 5.1). Birds are an ideal system in which to test ecological selection for patterns as they have evolved multiple pattern types (Fig. 5.2) and inhabit a wide variety of habitats on all major landmasses. In spite of the diversity of avian phenotypes, plumage patterns have repeatedly converged on the same four types: Irregular - mottled plumage where the vane is heterogeneously pigmented; Regular – scales where pigmentation follows the edge of the vane and may be concentric, bars which are made of alternating dark and light pigmentation transversal to the feathers axis, and spots where one or more spots pigment each feather (Fig. 5.2). Feather patterns mostly consist of combinations of neutral colours comprising brown, buff, cream, black and sometimes grey (T-L. Gluckman *unpublished data*), which are all formed from melanin, as well as white, which is due to an absence of melanin (Mundy et al. 2004). More striking coloration, including green, red and blue are infrequent in the patterns of birds.

Other types of patterns, such as the stripes of the vulturine guineafowl (*Acryllium vulturinum*), are comparatively rare. In addition, there is a pattern that is only found in one species of bird, the checkerboard pattern of the common loon (*Gavia immer*), and another pattern is found in less than ten species, the triangles in the breast of the male Wood duck (*Aix sponsa*) (T-L. Gluckman *unpublished data*). Therefore, birds can make other types of patterns but repeatedly converge on just the same four types (Fig. 5.2). Perhaps convergence in bird plumage patterns has arisen because these patterns are the most effective in camouflage and/or communication relative to the habitat in which each species' lives (Poulton 1890; Thayer 1909; Cott 1940; Endler 1978).

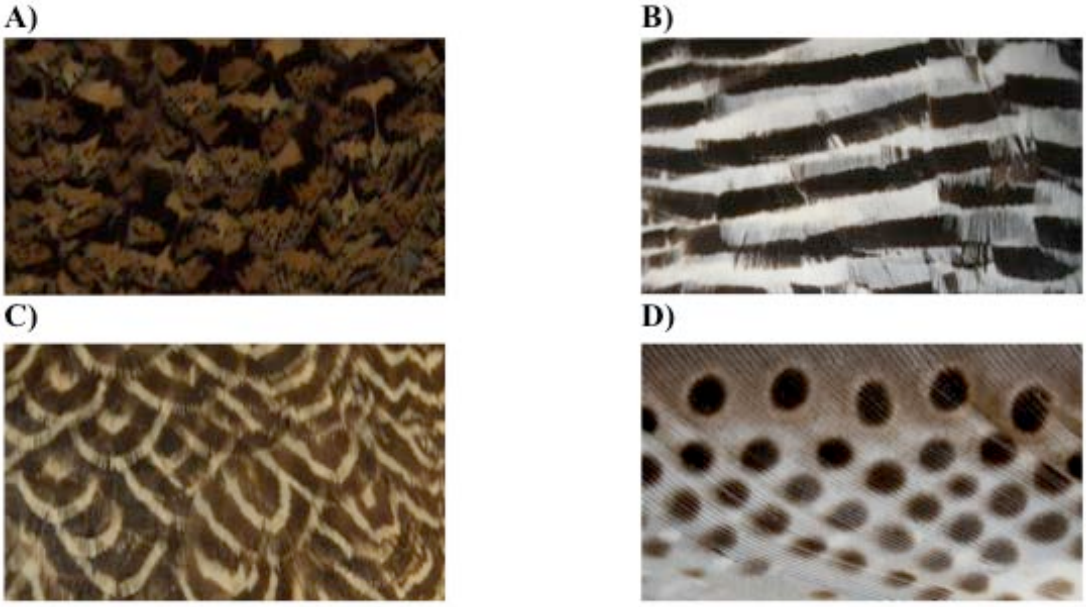


Fig. 5.2. The prevalent feather patterns of birds. In spite of the diversity of avian phenotypes, feather patterns have repeatedly converged on the same four patterns: Irregular - a) mottled plumage in a female sharp-tailed grouse (*Tympanachus phasianellus*); Regular - b) barred plumage in a male Andean goose (*Chloephaga melanoptera*), c) scaled plumage in a male falcated duck (*Anas falcata*), d) spotted plumage in a male great argus (*Argusianus argus*). Figure taken from Marshall and Gluckman, *in review.*

The main physical characteristics of visual signals are 1) intensity of the signal (brightness), 2) colour, 3) spatial characteristics, and 4) temporal variability of these three attributes (Bradbury and Vehrencamp 1998). Bird plumage patterns are dominantly composed of neutral colouration and current evidence suggests that texture (patterns) are detected by achromatic vision (Jones and Osorio 2004). Spatial and temporal characteristics of the pattern are likely to be important attributes in the context of the background in which they are viewed. For example, the relationship between spatial attributes of the pattern and the habitat changes in different combinations (Fig. 5.1) because pattern type can vary among juveniles and adults, and between breeding and non-breeding phenotypes (Björklund 1991; Gluckman and Cardoso 2010). Brightness and coloration are likely to have an important function in camouflage, but as a first attempt at ecological selection for plumage patterns we focused on the most prominent aspect of patterning: the spatial arrangement of pigmentation into the four types of patterning in relation to the spatial characteristics

of the background in which they are viewed (Fig. 5.2).

Currently, there is little empirical evidence for the function of bird plumage patterns. To assess the function of animal patterns, we conducted a broad survey of the literature spanning over 80 studies from vertebrates and invertebrates, including aquatic and terrestrial animals. Our literature review illustrates that the function of patterns is type and context dependent, but is nonetheless similar in many animal groups (Table 5.1; supplementary S5.1). Pattern-based anti-predator defenses can be subdivided into those that conceal while stationary and those that prevent capture during movement (Poulton 1890; Thayer 1909; Cott 1940; Bradbury and Vehrencamp 1998; Stevens et al. 2006; Mäthger et al. 2008; Stevens, Yule, and Ruxton 2008; Hanlon et al. 2009; Stevens and Merilaita 2009a; Stevens and Merilaita 2009b; Stevens et al. 2011). Patterns used in communication are likely to repeat a motif (sub-pattern, e.g. bars and spots) because repetition increases the likelihood that a signal will be received (Kenward et al. 2004). Accordingly, there is a growing body of evidence demonstrating that regular patterns function in communication (Hasson 1991; Petrie, Halliday, and Sanders 1991; Swaddle and Cuthill 1994; Omland 1996; Roulin 1999a; Roulin 1999b; Gluckman and Cardoso 2010; Muck and Goymann 2011).

Table 5.1. The number of species for which empirical, comparative and correlational evidence has demonstrated the function of irregular or regular patterns in camouflage and/or communication, spanning vertebrates and invertebrates, as well as terrestrial and aquatic species (see supplementary Table S5.1 for source studies). Table 5.1 and corresponding data taken from Marshall and Gluckman, *in review.*

	Irregular	Regular
Camouflage	8	7
Communication	1	7

It is likely that irregular patterns function in the camouflage of stationary animals as they appear to generally match the irregular pattern of one or several

background types (background-matching) and/or seem to create false sets of edges (disruptive camouflage) to evade detection by predators (Thayer 1909; Cott 1940; Endler 1978; Bradbury and Vehrencamp 1998; Stevens and Merilaita 2009a; Stevens and Merilaita 2009b). For example, it has been suggested that non-breeding plumage in ducks, which often consists of irregular patterning (e.g. the genera *Aythya* and *Somateria*), facilitates concealment of females during nesting and of both sexes during the flightless period accompanying wing moult (Batt 1992). Although there are few empirical studies demonstrating the role of irregular plumage patterns in the camouflage of birds, there is substantial evidence from studies in 8 species spanning amphibians, insects, crabs, cephalopods, and bird eggs (Table 5.1; supplementary Table S5.1). For instance, in colour-changing organisms, the irregular mottled, stippled and disruptive patterns of cuttlefish (*Sepia officinalis*) facultatively match their backgrounds for crypsis (Chiao, Kelman, and Hanlon 2004; Chiao, Chubb, and Hanlon 2007; Hanlon et al. 2007; Barbosa et al. 2008; Mäthger et al. 2008; Hanlon et al. 2009; Chiao et al. 2010; Chiao et al. 2013), octopuses exhibit irregular body patterns that imitate nearby background objects (Josef et al. 2012), and the colour-changing fiddler crab (*Uca vomeris*) exhibits irregular, mottled patterns under predation to be cryptic against their mud background (Hemmi et al. 2006). Thus, static camouflage patterns are likely to consist of irregular pigmentation. In birds irregular mottled plumage is common in many species (Fig. 5.2), and is more frequently found in females and juveniles in comparison to regular barred plumage patterns (Gluckman and Cardoso 2010).

Current evidence suggests that regular patterns, such as bars and spots, facilitate camouflage during movement and therefore function as a secondary defence during the escape of prey by exploiting specific features of receiver visual acuity, e.g. motion-dazzle and flicker-fusion (Thayer 1909; Pough 1976; Endler 1978; Endler 1980; Brodie 1989; Brodie 1992; Brodie 1993; Shine 1994; Lindell and Forsman 1996; Stevens, Hopkins, et al. 2007; Stevens, Yule, and Ruxton 2008; Stevens and Merilaita 2009b; Scott-Samuel et al. 2011; Allen et al. 2013; Helversen, Schooler, and Czienskowski 2013; How and Zanker 2014). The underlying mechanisms of motion-based camouflage can be explained by the repetitive nature of regular patterns whereby repetition causes perceptual lag or fatigue in vision based motion-sensitive cells (Snowden 1998) or illusions of motion (Stevens, Yule, and Ruxton 2008). Evidence for motion-based camouflage via regular patterning has

been demonstrated in at least 7 species comprising fish, snakes, mammals and cephalopods (Table 5.1; supplementary Table S5.1). For example, bars or stripes have been shown to reduce the ability of predators to estimate the speed and direction of moving prey via motion-dazzle, allowing prey to evade capture after being detected (Stevens, Yule, and Ruxton 2008; Scott-Samuel et al. 2011; Stevens et al. 2011; How and Zanker 2014). Regular patterns may also facilitate motion-based background matching via flicker-fusion camouflage, where patterns blend during movement to match the background (Jackson, Ingram, and Campbell 1976; Pough 1976; Endler 1978; Brodie 1989; Brodie 1992; Brodie 1993; Shine 1994; Lindell and Forsman 1996). Evidence for flicker-fusion comes from behavioural and survivorship correlations between regular colour patterns and anti-predator escape behaviour in garter snakes (*Thamnophis ordinoides*) (Brodie 1989; Brodie 1992; Brodie 1993).

However, regular patterns may have a dual function in visual communication with conspecifics at close range (Marshall 2000). Comparative and empirical experiments show that regular patterns function in communication in a range of species (Hasson 1991; Petrie, Halliday, and Sanders 1991; Swaddle and Cuthill 1994; Omland 1996; Roulin 1999a; Bortolotti et al. 2006; Gluckman and Cardoso 2010; Roulin et al. 2010; Muck and Goymann 2011). Out of the 14 species that exhibit regular patterns that have been investigated (Table 5.1), 7 use patterns in communication. Signal obstruction, via objects such as trees and branches in a forest, may select for regularly repeating patterns, because repetition of a message increases the chances that the signal is detected by their intended receivers (Kenward et al. 2004). A comparative study in birds showed that, compared to irregular mottled patterns, barred plumage is more likely to evolve on the ventral surface of sexually mature males – a likely location for visual communication (Gluckman and Cardoso 2010). Empirical evidence demonstrates that barred patterns are positively associated with body condition in female barred buttonquails (*Turnix suscitator*; Muck and Goymann 2011), and the barred flanks of the red-legged partridge (*Alectoris rufa*) appear to function as a social signal (Bortolotti et al. 2006), whereas spots on tail feathers of barn swallows (*Hirundo rustica*) appear to be a reliable signal of phenotypic quality in males (Kose, Mänd, and Moller 1999), and plumage spottiness in female barn owls (*Tyto alba*) appears to be a signal of mate quality (Roulin 1999b). Moreover, a classical study shows that the number of

eyespots on male peacock (*Pavo cristatus*) tails is positively associated with mating success (Petrie, Halliday, and Sanders 1991).

There is currently little evidence to suggest that irregular patterns may function in motion-based camouflage (supplementary Table S5.1). In addition, given that motion-dazzle or flicker-fusion camouflage strategies appear to be reliant on repetition of a regular motif to cause illusions of motion (Snowden 1998; Stevens, Yule, and Ruxton 2008) it seems unlikely that irregular mottled patterns function in motion-based camouflage. Therefore, the function of bird plumage patterns in camouflage and communication is likely to be linked to whether the pattern is irregular or regular (Table 5.1). The distribution of plumage patterns on the basis of age class (juvenile or adult), gender and on which surface the pattern is distributed can be indicative of function (Björklund 1991). Dorsal patterns are likely to function in camouflage in adults of both sexes, and juveniles, as shown in birds and lizards (Stuart-Fox and Ord 2004; Gluckman and Cardoso 2010; Garcia, Rohr, and Dyer 2013). In contrast, a communication function is likely to evolve on the ventral surface of males (Gluckman and Cardoso 2010). Such an arrangement provides a solution to antagonistic selection pressures in the form of signal partitioning where the signal is separated from the camouflage component, such as in *Bicyclus* butterflies and agamid lizards (Endler 1992; Stuart-Fox and Ord 2004; Oliver, Robertson, and Monteiro 2009), but is likely to be dependent on which background the animal is viewed against (Fig. 5.1).

Across the world the main visual difference between habitats is structural composition. Given the function of patterns (Table 5.1), and that patterns are viewed in the context of their environment (Fig. 5.1), pattern type and pattern location over the body may be associated with habitat. Static irregular camouflage patterns are harder to detect when viewed against more cluttered backgrounds with distractors and other moving conspecifics (Dimitrova and Merilaita 2009; Dimitrova and Merilaita 2012; Hall et al. 2013). Therefore, perhaps background-matching/disruptive camouflage occurs in more visually complex environments such as closed habitats, but probably not in open habitats such as aqueous environments and deserts (Fig. 5.1). The motion-based camouflage function of regular patterning has been demonstrated where prey/target is in motion (Stevens, Yule, and Ruxton 2008; Scott-Samuel et al. 2011; Stevens et al. 2011; How and Zanker 2014). Given that space is

required for movement, perhaps motion-based camouflage patterns are favoured in more open habitats such as sparse woodland (Fig. 5.1).

Similar plumage patterns may occur in closely related species due to phylogenetic relatedness rather than for adaptive reasons (Felsenstein 1985; Revell, Harmon, and Collar 2008). Therefore, we tested whether plumage patterns may have been retained due to shared ancestry in 8009 spp. of adult birds and 2603 spp. of juveniles. We then tested whether ecology selects for convergent evolution in bird plumage patterns. The majority of avian species across the world are land birds. A small number of avian species are marine (0.02% - 217 spp.) and their distribution, especially during the non-breeding season when marine birds are at sea, is not well known (The Nature Conservancy, http://maps.tnc.org/gis_data.html). In addition, their distribution is coarse and covers a wide area making inferences from these species difficult. Therefore, we focussed solely on land birds. A camouflage function is likely on both the ventral and dorsal surface of females and juveniles, and the dorsal surface of males (Houston, Stevens, and Cuthill 2007) whereas regular patterns that have a social function are likely to be displayed on the ventral surface of males (Gluckman and Cardoso 2010). Given that cluttered habitats may also select for regularly repeating patterns (Kenward et al. 2004), but that regular patterns may also stand out on uniform backgrounds (Fig. 5.1), male ventral patterns may be independent of habitat type. Based on our literature review and a current understanding of natural selection we expect to find a significant association between closed and open habitats with irregular and regular patterns, respectively (Table 5.1).

Methods and materials

Data collection

The best phylogenetic reconstructions for the class Aves are those by Jetz et al. (2012) on the basis of species coverage and breadth of data. 10,000 trees are available for this phylogeny, which is drawn from a single Bayesian analysis. To control for phylogenetic uncertainty we randomly selected 100 phylogenetic trees provided by Jetz et al (2012) on which to run our analyses. To collect plumage

pattern information we referred to field guides covering all major landmasses: North and Central America (Sibley 2000; van Perlo 2006), South America and Antarctica (De La Pena and Rumboll, 1998; Restall et al. 2007), sub-Saharan Africa and Madagascar (Langrand 1990; Sinclair and Ryan 2003), Europe, North Africa and Central Asia (Heinzel et al. 1995; Grimmett et al. 1999; Arlott 2007, 2009; Brazil 2009), East and South-East Asia (MacKinnon and Phillips 1993; Coates and Bishop 1997; Robson 2005), and Oceania (Beehler et al. 1986; Pratt et al. 1987; Simpson and Day 2004; Robertson and Heather 2005). Where multiple subspecies were present, we collected information on the nominate subspecies, resulting in a sample size of adults of 8008 spp.. Juvenile plumages are less frequently drawn in field guides resulting in a sample size of 2603 spp.

We scored the plumage of both sexes as well as juveniles of each study species (where applicable) for an absence of patterns, mottled, scaled, barred and spotted patterns on the ventral and dorsal surface separately (Fig. 5.2). Some species have multiple patterns on either the ventral and/or dorsal surface, e.g., the male zebra finch (*Taeniopygia* guttata) has barred patterns on the breast and spotted flanks. In such species, all of the different types of plumage patterns on each surface were scored and each pattern type was analysed separately. Where species exhibited variable patterns between molts we collected the breeding and non-breeding plumage given that there may be variation in selection pressure on the different types of patterns exhibited. Squares, triangles and stripes also occur within the feathers of birds, but are comparatively rare (e.g. stripes - 43 spp.), and were excluded from the analysis.

Measuring phylogenetic signal

The tendency of species to resemble related species, more than randomly selected species from a tree can be measured as phylogenetic signal (Pagel 1999). The best statistical approach to estimating phylogenetic signal is an area of ongoing debate but simulation studies indicate that with a large number of species Pagel's λ is the most robust test statistic (Münkemüller, Lavergne, and Bzeznik 2012). To examine whether plumage patterns have evolved due to phylogenetic inertia we estimated Pagel's λ in the package phylosig (Revell 2011) using 100 optimization

iterations in R (R Development Core Team 2012). In Phylosig, Pagel's λ is estimated as 0 or 1, where a value of 0 indicates that trait evolution is independent of the phylogeny, whereas 1 indicates a signal of phylogenetic inertia. We estimated phylogenetic inertia in each pattern, in each sex and age class, as well as breeding and non-breeding plumages, on the ventral and dorsal surface, over 100 randomly selected phylogenetic trees (*see* Data Collection; Jetz et al. 2012). We present the proportion of trees out of 100 in which $\lambda = 1$.

Ecological selection for plumage patterns

To examine whether habitat selects for plumage patterns at the global scale, we analysed the distribution of avian species across the main habitat types worldwide. We used a global dataset of the distribution of bird species which is described elsewhere (Somveille et al. 2013). Briefly, polygons representing the global distribution of 9783 non-marine bird species were obtained from BirdLife International and NatureServe (2012). Breeding distributions (polygons corresponding to the areas where a species is present during the breeding season) were estimated separately from non-breeding distributions (polygons where a species is present during the non-breeding season). Terrestrial eco-regions are defined as units of land with a distinct assemblage of natural communities and species, and are nested within biomes, which provide identification of habitats (Olson et al. 2001). To investigate how avian species are distributed in eco-regions, we used a global map of terrestrial eco-regions made available by The Nature Conservancy (http://maps.tnc.org/gis_data.html). After removing eco-regions that contained no avian species, 810 eco-regions remained. A species was classed as occurring in a given eco-region if its mapped range overlaps with any part of the eco-region. Given the coarseness of the species distribution data, this is not always true, but represents a good approximation of occurrence given the spatial resolution of the eco-regions (Hurlbert and Jetz 2007). Species richness was measured as the number of species occurring in a given eco-region (Figure 5.3a).

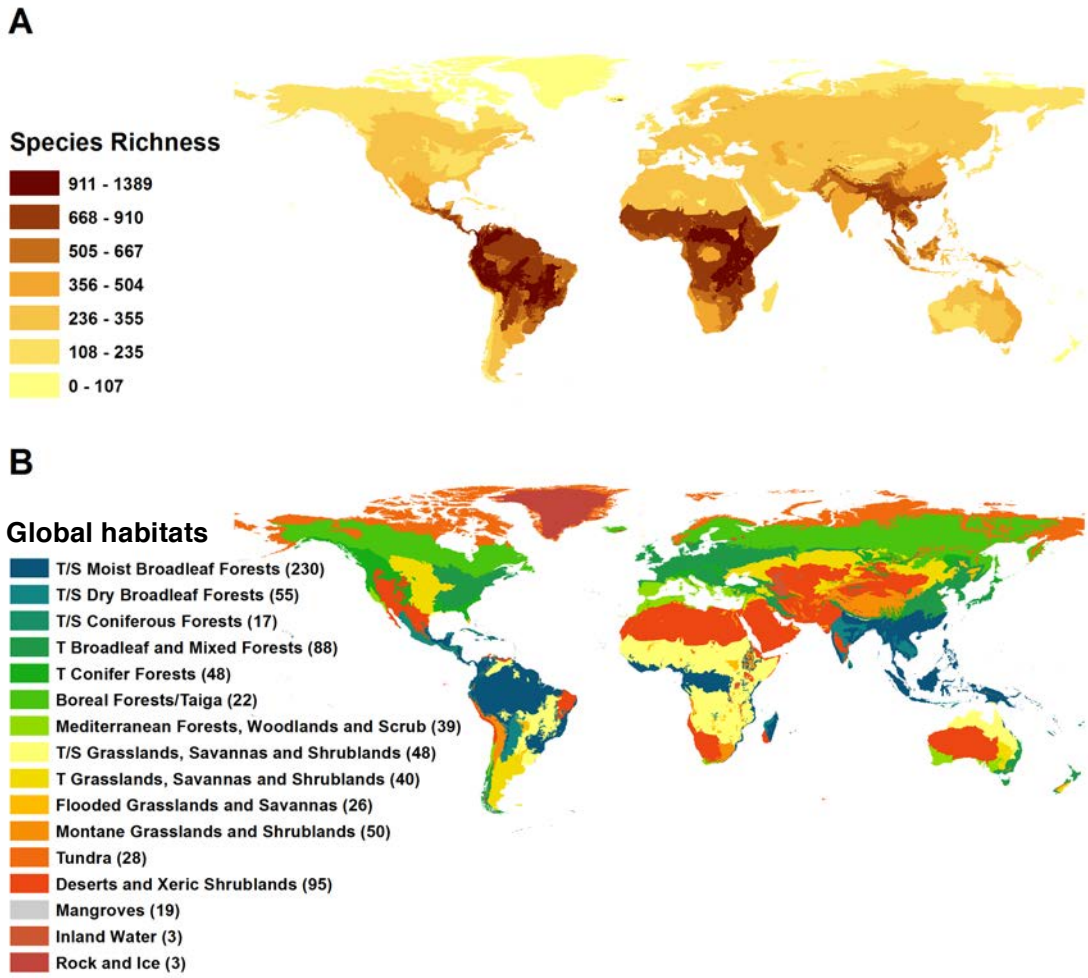


Fig. 5.3. Total avian species richness worldwide as well as the global distribution of terrestrial habitats containing avian species. A) The global distribution of avian species richness. B) Each habitat type encompasses multiple eco-regions (numbers in brackets). Each type of habitat may be classified as either closed or open as indicated by colour: green and blue = mostly closed; red, orange and yellow = mostly open. Mangroves are indicated with grey, as they cannot be classified as either open or closed. T = Temperate; and T/S - Tropical and Subtropical.

Across the world there are 16 major types of habitats as follows: 1) Tropical and subtropical moist broadleaf forests; 2) Tropical and subtropical dry broadleaf forests; 3) Tropical and subtropical coniferous forests; 4) Temperate broadleaf and mixed forests; 5) Temperate coniferous forests; 6) Boreal forests/taiga; 7) Mediterranean forests, woodlands and scrub; 8) Tropical and subtropical grasslands,

savannas and shrublands; 9) Temperate grasslands, savannas and shrublands; 10) Flooded grasslands and savannas; 11) Montane grasslands and shrublands; 12) Tundra; 13) Deserts and xeric shrublands; 14) Mangroves; 15) Inland water; and 16) Rock and ice. Each of these habitats occurs in multiple eco-regions. Given that a major hypothesis of this study is that irregular mottled patterns are associated with closed habitats whereas regular patterns, in particular barred patterns, may occur in more open habitats, we additionally categorized habitat type into open and closed. Closed habitats comprise habitats 1-7 described above which are dominated by dense forests. Open habitats are comprised of the other habitats (8-16) that typically consist of short vegetation that is often sparsely distributed. Mangroves cannot be easily classified as either closed or open and were removed from the comparison of closed and open habitats.

Each of the analyses was conducted for a subset of data comprised of each combination of patterns (all patterns, mottled, scaled, barred and spotted) and gender/age/body location class: male/female/juvenile, breeding/non-breeding, ventral/dorsal (herein subset). Each habitat occurs in multiple eco-regions (Fig. 5.3b). To determine whether habitat has an effect on the distribution of each subset of pattern data, we examined the geographical variation in the proportion of species that have a) any type of pattern, and b) each individual type of pattern. For each pattern data subset we compared the proportion of patterned species within each eco-region to the global proportion of patterned species. If the proportion within eco-regions was significantly above or below the global proportion this was interpreted as selection for or against patterning, respectively. However, if the proportion at the eco-region level was the same as the global level this indicated support for a uniform or random distribution of the subset of pattern data. The corresponding null expectations in our experimental approach are a) the eco-region has the same proportion of patterned species as the global proportion of patterned species, e.g. if half of the species of birds in the world are patterned then half of the species in the local eco-region will be patterned, b) the eco-region has the same proportion of species with a particular subset of pattern data as the global proportion of species with that pattern subset, e.g. if half of the patterned species of birds in the world have a mottled pattern then half of the species in the eco-region will be mottled.

To test whether the observed proportion of patterned species was

significantly higher or lower than the null hypothesis per eco-region we used a hypergeometric probability distribution around the null value in each eco-region. We obtained a cumulative probability distribution of the observed proportion of patterned species as well as an associated p-value for the observed value. If the p-value is significant and the cumulative probability is close to 0, this indicates that patterns appear less than expected by chance, and if the p-value is significant and the cumulative probability is close to 1, this indicates that patterns appear more than expected by chance. We allowed for some flexibility in variation of the cumulative probability and where the value was either <=0.02 or >=0.98, we considered this range of values as significant for selection against or for patterns, respectively. To correct for multiple testing, which can lead to pseudo-replication, we corrected the p-values of the eco-regions of each habitat using a multiple testing correction. We initially used a standard p-value significance threshold of 0.05 with a Bonferroni correction, which is the p-value threshold divided by the number of eco-regions. This correction can be overly stringent. For example, for 810 eco-regions this is 0.05/810 = 0.000062. Accordingly, a Bonferroni correction resulted in no significant results in any of our analyses (data not shown). Due to potential issues of power, we used a Benjamini-Hochberg correction per subset of patterns analysed, as it is the least stringent correction for multiple tests (Benjamini and Hochberg 2010).

Our analysis depended on comparing the local proportion of patterned species within an eco-region with the global proportion of patterned species. In a small number of eco-regions, there were no species with any type of patterning and we could not calculate the proportions for our test statistic. However, the geographic distribution of eco-regions that have a complete absence of patterned species may be important. Therefore, we present the cases where there are no avian species with any kind of patterning separately.

Results

Plumage pattern evolution due to shared ancestry

All types of plumage patterns are found in all combinations of females, males and juveniles, for breeding and non-breeding plumage on both the ventral and dorsal surface (Table 5.2). Mottled plumage is the most prevalent type of pattern in birds and is found on the dorsal surface more frequently than on the ventral surface in all sex and age classes, and is least frequent in males. Barred patterns are also common and are frequently found on the ventral surface of breeding adult males, but also in juveniles. In comparison, scaled and spotted patterns are less prevalent and are frequently biased towards the dorsal surface of adults and the ventral surface of juveniles. However, spotted patterns are also frequently found on the ventral surface of breeding males and females.

Table 5.2. The presence of plumage patterns in the class Aves (adults: 8008 species, juveniles: 2603 species). Plumage pattern information was collected from field guides covering all major landmasses – see Methods for references.

		Mottled			Barred			Scaled			Spotted		
Class	Season	Ventral	Dorsal	Both surfaces	Ventral	Dorsal	Both surfaces	Ventral	Dorsal	Both surfaces	Ventral	Dorsal	Both surfaces
Female	Non-breeding	860	1081	1508	533	679	539	163	269	163	127	291	314
Female	Breeding	864	1085	1514	500	624	978	162	202	321	300	157	427
Male	Non-breeding	689	954	1288	530	876	535	153	277	162	122	385	314
Male	Breeding	674	936	1261	514	478	975	161	156	320	305	104	427
Juvenile	Juvenile	873	914	1368	886	325	627	274	158	203	385	223	157

Table 5.3. The proportion of times that Lambda is estimated as 1 using 100 randomly selected phylogenetic trees for all types of plumage in 80% of avian species worldwide. In most cases, the proportion of times that $\lambda = 1$ was <0.3.

		Mottled			Barred			Scaled			Spotted		
Class	Season	Ventral	Dorsal	Both surfaces	Ventral	Dorsal	Both surfaces	Ventral	Dorsal	Both surfaces	Ventral	Dorsal	Both surfaces
Female	Non-breeding	0.28	0.26	0.28	0.25	0.33	0.20	0.28	0.29	0.27	0.22	0.22	0.20
Female	Breeding	0.39	0.28	0.29	0.27	0.23	0.24	0.22	0.29	0.27	0.28	0.34	0.66
Male	Non-breeding	0.32	0.25	0.21	0.26	0.26	0.18	0.29	0.20	0.28	0.25	0.22	0.17
Male	Breeding	0.25	0.30	0.29	0.21	0.24	0.23	0.26	0.28	0.35	0.31	0.23	0.28
Juveniles	Juveniles	0.23	0.27	0.25	0.27	0.27	0.32	0.28	0.21	0.22	0.24	0.28	0.26

For most of the 60 combinations of plumage pattern, sex, gender and dorsoventral patterning, $\lambda = 1$ in less than 30/100 randomly selected phylogenetic trees. Seven out of the 60 combinations of pattern/sex/age/breeding class, $\lambda = 1$ in over 40/100 phylogenetic trees, and only one combination, female breeding plumage comprised of spots where both ventral and dorsal surfaces were combined, $\lambda = 1$ in over 50/100 (0.66; Table 5.3).

Ecological selection for plumage patterns

All four plumage patterns are found in all habitats. Each individual combination of pattern type and age/sex/breeding/dorsoventral subset occurs within each of the global 16 habitats regardless of whether they are closed or open (Fig. 5.3; supplementary Table S5.2). For example, mottled and barred patterns occur on the dorsal surface of juveniles and breeding females in open habitats such as Inland water as well as Rock and ice in addition to closed habitats such as Temperate broadleaf and mixed forests as well as Tropical and subtropical coniferous forests.

In the analyses of an association between individual eco-regions and subset of plumage pattern data, the cumulative probability was 0 or 1 in 5.19% (414) and 53.02% (4229) of the 7976 individual analyses, respectively (eco-regions x subset of interest). 9.34% (745/7976) of the analyses had a significant association between plumage pattern subset and eco-region (where the cumulative probability was or close to 0 or 1 and the p-value was less than the adjusted significance threshold) (Table 5.4; supplementary Table S5.3 [CD-ROM]). Where there was a significant association with an eco-region, the results were dominated by a classification of all patterns together (7.65%) and an association with individual patterns was rare (1.23% + 0.01% + 0.39% + 0.06% = 1.69%).

Table 5.4. The patterns and number of eco-regions that is significantly different from the null expectation as well as the number of local patterned terrestrial species. All results are supplied in supplementary Table S5.3 [CD-ROM].

	Raw number of eco-regions that have significant results	Proportion of eco-regions that have significant results %
All patterns	610	7.65
Mottled	98	1.23
Scaled	1	0.01
Barred	31	0.39
Spotted	5	0.06
Total	745	9.34

An association between all patterns together and eco-regions spans 15 out of the 16 habitats of the world across all age/sex/breeding classes (Table 5.5). In all age/sex/breeding classes, all plumage patterns appeared to be influenced by habitat on the ventral surface less than the dorsal surface. However, this significant association was predominantly in <25% of the eco-regions of each type of habitat. In only 4 habitats the number of eco-regions with a significant association with all patterns was >25%, and two of these habitats are comprised of only three eco-regions.

Table 5.5. The habitats and eco-regions that deviate from the null expectation with all patterns in land birds. The number of eco-regions is given in brackets per habitat as well as the maximum percentage of eco-regions within habitats that have a significant association per pattern data subset. T = Temperate; and T/S - Tropical and subtropical.

Sex/Age	Male				Female				Juvenile	
Season	Breeding		Non-breeding		Breeding		Non-breeding		N/A	
Location	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal
Total eco-regions	78	43	67	17	111	54	106	51	44	33
Boreal forest/taiga (22) <18%	4	-	-	-	-	1	-	1	-	-
Desert and xeric shrubland (95) <5%	-	-	4	-	5	1	5	1	1	1
Flooded grassland and savanna (26) <12%	3	-	3	-	2	2	2	2	-	-
Inland water (3) <33%	-	-	-	-	-	-	-	-	-	1
Mangrove (19)	2	-	1	-	4	1	4	-	1	-
Mediterranean forest, woodland and scrub (39) <8%	3	-	1	-	-	-	-	-	-	-
Montane grassland and shrubland (50) <22%	7	3	8	-	11	2	11	2	4	4
Rock and ice (3) <33%	-	-	-	-	-	-	-	-	1	-
T broadleaf and mixed forests (88) <7%	6	3	5	1	4	3	4	2	4	3
Temperate conifer forest (48) <10%	5	4	4	2	3	2	3	2	3	1
T grassland, savanna and shrubland (48) <4%	-	-	-	-	-	2	-	2	-	2
T/S coniferous forest (17) <6%	1	-	1	-	1	-	1	-	-	1
T/S dry broadleaf forest (55) <16%	5	5	4	1	9	8	9	8	4	3
T/S grasslands, savannas and shrublands (48) <4%	1	-	1	-	8	3	8	3	4	2

T/S moist broadleaf forests (230) <26%	41	28	35	13	64	29	59	28	22	15
Tundra (28) 0%	-	-	-	-	-	-	-	-	-	-

Ecological selection for mottled, scaled, barred or spotted patterns

The individual patterns of mottled, scaled, barred and spotted rarely had a significant association with eco-regions (Table 5.4). In most cases, as the number of species increased, the number of eco-regions with a significant association decreased, e.g. where there is only one patterned species there are many eco-regions with a significant association, whereas few eco-regions had a significant association where the number of species >5 (Fig. 5.4). The rare cases of a significant association between patterns and eco-regions were not clustered in a particular plumage pattern subset and a particular habitat. For example, mottled breeding dorsal patterns in females occur more than expected by chance within the Amsterdam and Saint-Paul Islands temperate grasslands (cumulative $p = 1$, probability of cumulative $p < 0.000063$; supplementary Table S5.3 [CD-ROM]) that is one eco-region of the temperate grasslands, savannas and shrublands habitat. There are 39 other eco-regions within the temperate grasslands, savannas and shrublands habitat that have females with breeding dorsal mottled patterns and in none of these eco-regions does the species of this subset of data occur more (or less) than expected by chance.

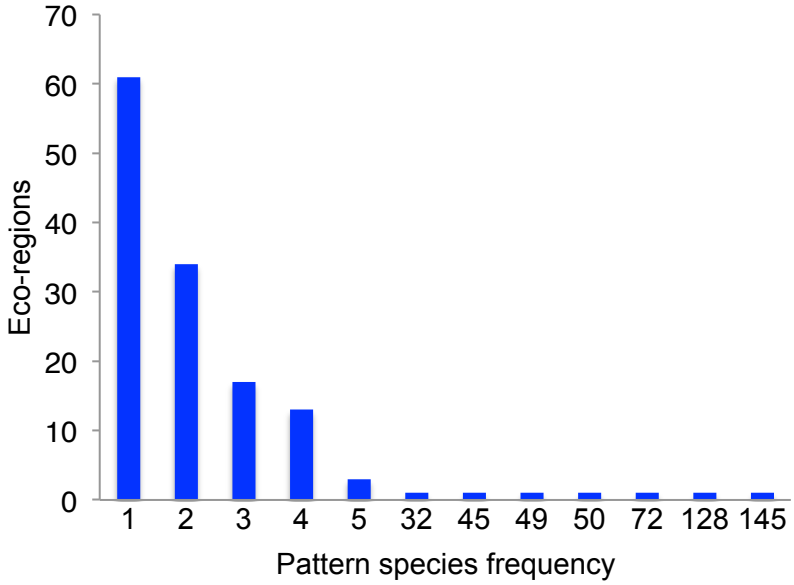


Fig. 5.4. The number of eco-regions that deviate from the null expectation with either mottled, scaled, barred or spotted patterns plotted against the local number of patterned species in land birds. Across all permutations of sex/age/breeding/dorsoventral class per pattern, there were few significant associations and where there was a significant association, it was predominantly where the number of species <=5.

A comparison of ecological selection between closed and open habitats

Based on the prevailing view of camouflage and communication, the patterns that should demonstrate an association with closed and open habitats (Fig. 5.1, 5.3) are mottled and barred patterns (Gluckman and Cardoso 2010). The distribution of both mottled and barred patterns, on the dorsal and ventral surface of breeding and non-breeding females, as well as juveniles, is similar in the eco-regions of closed and open habitats and rarely differed from the null hypothesis (Fig. 5.5; supplementary Table S5.3 [CD-ROM]). Similarly, the proportion of species where males have patterns on the ventral surface, but also on the dorsal surface, in the breeding season was similar and did not differ from the null hypothesis in both closed and open habitats.

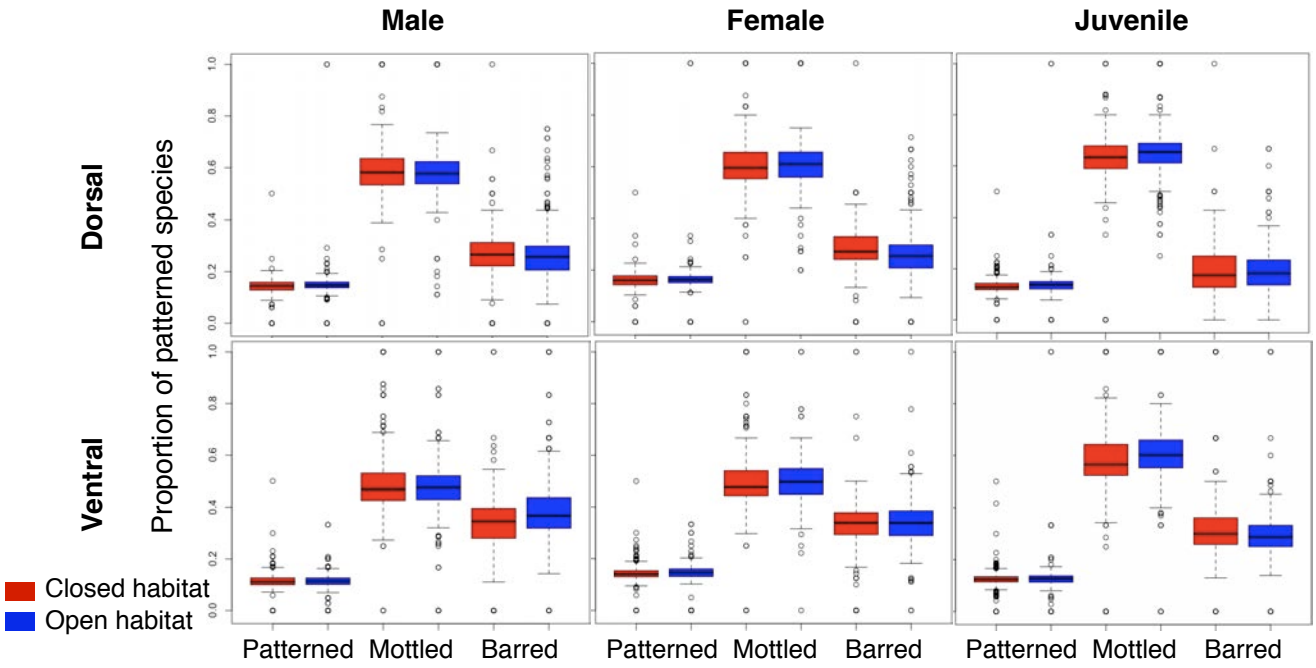


Fig. 5.5. Comparison of the proportion of species with mottled and barred patterns in breeding males and females, as well as juveniles, over the dorsal and ventral surface of land birds. Significant associations between mottled and barred patterns with eco-regions are rare (Table 5.4). The *Patterned* boxplots correspond to the proportion of patterned species in the pool of all avian species, the *Mottled* and *Barred* boxplots correspond to the proportion of these plumage patterns in the pool of patterned species for the indicated age/sex class. The boxplots in red correspond to closed forest habitat and the red boxplots correspond to open habitat. Mangroves (habitat 14; Fig. 5.3) do not easily fit into a closed or open classification and are not represented in this figure.

Mottled and barred patterns rarely had significant associations with eco-regions (Table 5.4) and the patterns of association did not vary between open and closed habitats or subset of data. For example, the dorsal mottled patterns of breeding females had a significant association with the Amsterdam and Saint-Paul Islands temperate grasslands eco-region which is found in open Temperate grasslands, savannas and shrublands as well as the Bermuda subtropical conifer forests eco-region which is found in closed Tropical and subtropical coniferous forests (Fig. 5.3; supplementary Table S5.3 [CD-ROM]). In addition, the dorsal barred patterns of breeding females had a significant association with the Ascension scrub

and grasslands eco-region which is found in open Tropical and subtropical grasslands, savannas and shrublands, as well as the San Félix-San Ambrosio Islands temperate forests eco-region which is found in the closed Temperate broadleaf and mixed forests habitat.

Finally, in 6 out of the 16 habitats worldwide there are 17 eco-regions that do not have avian species with any type of pattern (Fig. 5.3; Table 5.6). However, four of these habitat types have <=5 total species. In the other two habitats, few eco-regions had significant associations: Temperate and subtropical moist broadleaf - 6 eco-regions out of 230 (2.6%); Tundra - 2 eco-regions out of 28 (7.1%).

Table 5.6. Eco-regions that do not have any avian species with any type of pattern in land birds. T = Temperate; and T/S - Tropical and subtropical. The number of eco-regions per habitat type is given brackets.

Habitat	Eco-region	Avian species richness
T grasslands, savannas and shrublands (40)	Amsterdam and Saint-Paul islands temperate grasslands	1
	Ascension scrub and grasslands	3
T/S coniferous forests (17)	Bermuda subtropical conifer forests	2
T/S grasslands, savannas and shrublands (48)	St. Helena scrub and woodlands	1
T/S moist broadleaf forests (230)	Central Polynesian tropical moist forests	6
	Fernando De Noronha-Atol das Rocas moist forests	5
	Marquesas tropical moist forests	16
	Rapa Nui subtropical broadleaf forests	2
	Society Islands tropical moist forests	17
	Trindade-Martin Vaz Islands tropical forests	1
	Tuamotu tropical moist forests	18
	Tubuai tropical moist forests	8
	Western Polynesian tropical moist forests	8
Tundra (28)	Marielandia Antarctic tundra	5
	Maudlandia Antarctic desert	2
	Scotia Sea Islands tundra	8
	Southern Indian Ocean Islands tundra	6

Discussion

All types of plumage patterns have evolved in juveniles, females and males in non-breeding and breeding plumage, on both the ventral and dorsal surface (Table 5.1). In the majority of pattern types, shared ancestry only explained <30% of plumage pattern evolution (Table 5.3) indicating that convergent evolution in bird plumage patterns is unlikely to be predominantly due to phylogenetic relatedness. All types of patterns, in all age, breeding, sex and dorsoventral locations are found in all habitats (supplementary Table S5.2). Contrary to the existing hypotheses of the function of patterns in camouflage, the habitats in which patterns are viewed did not predict convergence in bird plumage patterns across either age or sex classes, breeding and non-breeding plumages, or the dorsoventral distribution of patterns (Fig. 5.4, 5.5; Table 5.4, 5.5). Congruent with the hypotheses of the function of plumage patterns in communication, habitat type did not predict the patterns found on the ventral surface of breeding males. However, the independence between plumage patterns and habitat was also found on the dorsal plumage of breeding males, as well as their non-breeding plumage. The rare significant associations between eco-regions and irregular and regular patterns were found in both closed and open habitats, irrespective of age/sex/breeding/dorsoventral class. In addition, there were few eco-regions where there are no patterned species and these were not clustered within a particular type of habitat (Table 5.6). Together this indicates that avian plumages appear to have converged on the same types of patterns independent of habitat (Fig. 5.5; Table 5.3).

Our analyses indicate that phylogenetic inertia has contributed <30% of plumage pattern evolution in most cases. Although this represents a minority of the overall number of trees tested, this result was similar across all pattern data subsets indicating that there was variation in the estimation of lambda across trees (Table 5.3). That the variation was relatively similar between pattern subsets indicates that the source of this variation may be due to adaptive radiation in a specific part of the avian tree of life (Jetz et al. 2012). However, the results from the large majority of phylogenetic trees in this analysis indicate phylogenetic uncertainty is unlikely to be the cause of convergent evolution in bird plumage patterns. The only subset of data where there appeared to be phylogenetic inertia, female breeding spotted patterns over the whole body, may not be representative given that in the separate analyses

of ventral and dorsal female breeding spotted patterns λ = 1 in 28/100 and 34/100 trees, respectively. The 100 phylogenetic trees in this analysis were randomly chosen to control for bias as well as represent the uncertainty in a current understanding of avian phylogenetic relatedness. Also, the package Phylosig used to compute Pagel's lambda is a binary statistic rather than continuous which may oversimplify the results, but given a limitation in the computational power required to run this analysis in other software packages such as BayesTraits, this approach represents the best available option.

Adaptive radiation may cause an erroneous inflation of the test statistic. Conducting analyses at the genus level can control for erroneously inflated results, but would require an adequate sample size of genera within eco-regions to avoid losing statistical power. The species level results, combined with the number of species present within eco-regions that had a significant result, indicated that a genus level analysis was not required (Fig. 5.4; Table 5.4). Most of the significant results for individual patterns was where there were <=5 species within an eco-region. This indicates that there may have been inflation of the test statistic due to a low number of species in these cases and may not be representative (Table 5.6).

Selection for any type of patterning was not clustered within eco-regions (Table 5.5). In addition, there did not appear to be an association between age, gender, breeding class or dorsoventral patterning with any type of habitat (Fig. 5.5; supplementary Table S5.3 [CD-ROM]). All of the different types of patterns were rarely found more or less than expected by chance on the ventral and dorsal surface of breeding and non-breeding females, as well as juveniles, per eco-region and per habitat (Table 5.4, 5.5; supplementary Table S5.3 [CD-ROM]). Similarly, all plumage patterns were found on the ventral and dorsal surface of the breeding and non-breeding plumages of males (p = > Benjamini-Hochberg corrected threshold; supplementary Table S5.3 [CD-ROM]). This could be due to low statistical power in our analyses. However, we used the least stringent correction possible to increase statistical sensitivity to ecological signal. In addition, regardless of the ecological techniques used, all of the different types of patterns, across all age, gender, breeding and dorsoventral classes are found in every type of habitat around the world, which is incongruent with camouflage-based predictions (Table 5.1; supplementary Table S5.2). Our analyses did not incorporate the extent to which a

pattern(s) may contribute to the overall phenotype of each avian species and as a result this analysis is necessarily coarse. However, this would not result in the prevalence of all pattern types in all habitats irrespective of age, gender, breeding/non-breeding and dorsal/ventral plumage.

We could not statistically calculate whether a complete absence of any type of patterning might be significantly associated with habitats (Table 5.6). However, the distribution of the number of eco-regions without any species with patterning was not biased towards a particular type of habitat. For example, Tropical and subtropical moist broadleaf forests are a closed type of habitat and Tundra is an open type of habitat and each of these habitats have the most number of eco-regions that have species with no patterns (Fig. 5.3; Table 5.6). In addition, both of these habitats frequently have patterned species in other eco-regions (supplementary Table S5.2, S5.3 [CD-ROM]). Therefore, these rare cases where patterns are absent are unlikely to be representative of selection against patterns.

If patterns evolved strictly for camouflage and/or signaling based on the way that the pattern is viewed in the context of its habitat, then it would be expected that patterns would be biased towards a particular gender and body surface. Under this expectation regular patterns in males should be independent of habitat on the ventral surface, but not necessarily on the dorsal surface, and patterning should have been biased towards the dorsal surface of females and juveniles, but were not (Fig. 5.1, 5.5; supplementary Table S5.2, S5.3). Comparing the mottled and barred patterns of breeding males and females as well as juveniles in closed and open habitats, there was little difference in their distribution between age and sex classes or the dorsal and ventral surface (Fig. 5.5). If patterns indeed make an animal stand out against its background then there should have been strong selection against regular patterns on the dorsal surface of females and juveniles in closed habitats, which was not the case. A lack of association in juvenile plumage patterns could be due to a low sample size (2603 *spp.*) but this would have resulted in either an absence of some types of plumage patterns, or an erroneous significance value of selection for or against some types of patterns in some habitats, which was not found (Table 5.2; supplementary Table S5.3 [CD-ROM]).

Based on previous research and hypotheses, irregular patterns would be

expected in cluttered environments and regular patterns would be expected in open environments (Table 5.1, supplementary Table S5.1). However, irregular patterns were found throughout all types of habitats, including less noisy open habitats where it would be expected that they would stand out, e.g. deserts (Fig. 5.1; supplementary Table S5.2, S5.3). Moreover, regular patterns were also found throughout all types of habitats, including more noisy and closed habitats where it would be difficult to invoke motion-dazzle/flicker-fusion camouflage through movement. Breeding females with regular patterns, which would be constrained to take care of nests, as well as juveniles, were also found in closed habitats, which is contrary to motion-dazzle/flicker-fusion camouflage which is dependent on movement (Fig. 5.5). Alternatively, perhaps similar to reef fish, regular patterns might become blurred at a distance and match the background (Marshall 2000).

A lack of association of the ventral surface of males with regular patterns, in particular barred patterns (Gluckman and Cardoso 2010), and habitats would be expected under sexual selection. However, visual signals should diverge in biological hotspots due to species recognition/sexual selection (Bradbury and Vehrencamp 1998). Habitat may influence whether the pattern stands out against its background, for example by standing out against a uniform background or opposing the geometric pattern of the background (Fig. 5.1). Other factors, such as whether the pattern is able to convey aspects of individual quality, would influence their evolution independent of the viewing background. This perhaps may explain why barred patterns have repeatedly evolved independently on the ventral surface of males (Gluckman and Cardoso 2010) (Fig. 5.5). Conceivably, the same forces of sexual selection/species recognition may have shaped the evolution of the other types of patterns in males, such as spotted patterns (Roulin et al. 2000; Roulin, Riols, and Dijkstra 2001; Petrie and Halliday 2008; Muck and Goymann 2011; Pérez-Rodríguez, Jovani, and Mougeot 2013).

Perhaps the use of different strata in the vegetation, e.g. ground-dwelling vs arboreal, may present different visual microhabitat backgrounds that alter the selection for pattern type. For example, the tops of trees are more open and may allow for more movement required for motion-dazzle/flicker-fusion regular patterns whereas ground-dwelling species may benefit from background matching irregular patterning. However, it is difficult to reconcile this idea with the distribution of all

pattern types, in all age/sex/breeding/dorsoventral, in habitats that do not have strata, e.g. desert and tundra. The role of specific behaviours, such as whether a species is gregarious, combined with habitat use, may explain our results. Perhaps there is less selection for camouflage patterns in gregarious species because they share social information to evade predation rather than rely on visual patterning. For example, Artiodactyls that are solitary are significantly associated with dense forest habitats (Caro 2005). However, information on gregariousness, breeding habits as well as specific anti-predator behaviours are lacking in many avian species of the world and could not be controlled for in this analysis. Data on avian anti-predator and social behavior as well as major predators per species would be an invaluable contribution to the literature.

Caro (2005) reports that although that there is a camouflage function in the patterns of many artiodactyl species, many studies did not appear to demonstrate an association with habitat. The reality is that we do not know how many studies have shown a negative result but remain unpublished. Perhaps a coarse granular approach to ecological selection for plumage patterns may explain our results. For example, many of the empirical studies that demonstrate a camouflage function in patterns conducted their analyses at short viewing distances and demonstrated an association at the scale of microhabitats (Table 5.1; supplementary Table S5.1). Therefore, perhaps an analysis at the habitat and eco-region scale contains too much variation in microhabitats. However, raptors, which are the main predators of birds, have excellent long range vision in many different types of habitats and it would seem unlikely that they would need short viewing distances to act as a selection pressure on the camouflage function of patterns (Tucker 2000; Christie and Ferguson-Lees 2010; O'Rourke et al. 2010).

An alternative explanation is that microevolution is too labile to be captured at a macroevolutionary scale, unless the adaptation of the pattern has a long evolutionary history. Perhaps a better approach would be to consider patterning in the context of a coevolutionary process between signaler and receiver (Thompson and Cunningham 2002; Thompson, Nuismer, and Gomulkiewicz 2002; Thompson 2005). In particular, an alternative hypothesis is that perhaps four patterns are enough to provide a camouflage benefit to a community of species. The previous empirical studies of camouflage predominantly examined the interaction between the

visual form of a species in the context of one or two background types. However, in reality species live in communities of other species, regardless of whether they are gregarious or social. Recently it was demonstrated that there may be frequency-dependent selection on alternative morphs of *Tetrix subulata* grasshoppers, where each morph has varying amounts of mottled patterns in varying colours. In this study it was demonstrated that when all morphs are present in a population, all morphs benefit by providing variation in the search image of predators (Karpestam, Merilaita, and Forsman 2014). In the case of birds, perhaps the four different types of patterns, in the context of a community of avian species that may also comprise uniform coloration without patterns, may benefit all member species. Although intraspecific polymorphism is quite different to interspecific variation, perhaps intraspecific polymorphism may be the first step to speciation leading to interspecific variation.

There are potentially three major reasons that may predict that the number of patterns increases with community size, assuming that phenotypes are mostly for crypsis: 1) More patterns reduces predation by increasing the number of predator search images, which may also favour polymorphisms within species, 2) niche differentiation via dietary specialisms and/or microhabitat use may lead to variation in the background in which predators view prey resulting in selection for different types of patterns, which may also have implications for polymorphisms, and 3) if some parts of the pattern phenotype are used in species recognition then there will be selection on patterns to diverge to facilitate recognition. In addition, it has been found that sexual dimorphism/monomorphism in avian phenotypes is not always indicative of breeding systems (Gluckman 2014) and perhaps these processes may also favour sexual dimorphism to reduce the chance of predator search images and/or facilitate gender recognition. Alternatively, all of these selection pressures may be operating on avian phenotypes simultaneously and this will be investigated in a future study.

It may also be the case that developmental constraint may explain repeated convergence of the four types of plumage patterns independent of function. For example, based on a theoretical model of reaction-diffusion based plumage pattern formation (Prum and Williamson 2002), the evolutionary trajectory of plumage in Anseriformes and Galliformes followed the same trajectories within and between plumage patches of plumage over the body (*see* Chapter 4). Given that Anseriformes and Galliformes are diverse in life history attributes, this result warrants further

investigation at the macroevolutionary scale. In addition, it has been demonstrated that some plumage patterns shift along a trajectory correlated with body size in most avian species of the world (Riegner 2008). However, these results should be treated with caution, as the analysis did not control for phylogeny. Nevertheless, these studies indicate that there may be developmental constraints in plumage pattern evolution that may have implications for natural selection. A future study will investigate whether allometric relationships may predict plumage pattern evolution in a controlled comparative framework.

Bradbury and Vehrencamp (1998) note that in regards to strategies for maximizing contrast in visual communication that “A serious problem… is that they are based on human perception”. Similarly, the predominant paradigm of the function of patterns in camouflage stem from a time when there were few, if any, objective approaches for quantifying patterning. Comparing the frequency of patterns per habitat type, we did not find that habitat predicts the presence of plumage patterns. The fact that all patterns are found in all habitats around the world is an intriguing result that implies that plumage pattern evolution does not conform to the prevailing views of selection for camouflage.

Chapter 6: Discussion and Conclusion

Concluding thoughts

In my concluding chapter I bring together the findings of each chapter and highlight possible avenues for future research. My work on the development of pigmentation in embryonic Japanese quail has implications for evolutionary genetics and evolutionary-development. The finding that activation of *MC1R* is important in avian melanin based plumage colouration demonstrates that there is variation in pigmentation dynamics between mice and birds. This finding highlights the importance of considering evolution, in addition to conservation, of the mechanism of pigmentation between vertebrates and birds. Activation of *MC1R* via *POMC* and *PC2* is a promising area of research in avian pigmentation dynamics and raises the possibility of directly investigating activation of *MC1R* in the developmental basis of plumage patterns in birds. Reaction-diffusion dynamics appear to have played a role in the evolution of within-feather patterning in Anseriformes and Galliformes and spots appear to be a derived trait. Given that patterns are known to have both a signaling and camouflage function, it may be that reaction-diffusion based systems have influenced the evolutionary developmental biology of trait diversity as well as speciation. Comparing the evolution and diversity of barred plumage patterns in parasitic cuckoos demonstrates that plumage patterns can be additionally refined for a specific signaling function. This underscores the importance of considering the visual perspective of the receiver as well as objective methods with which to analyse visual patterns. Although patterns are known to function in camouflage and communication, it is interesting rather than disappointing that ecology did not appear to predict plumage patterns. Perhaps this points to the reality that animals, regardless of whether they are social or not, live in a community of other animals which presents an exciting opportunity to re-examine what camouflage means, and also, whether evolutionary-development has had a further hand in plumage pattern evolution via allometric constraints.

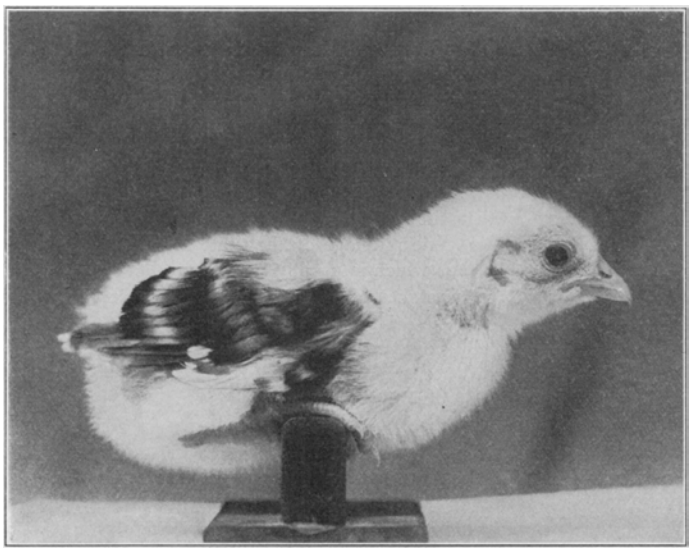
Developmental explanations

Japanese quail is a model system in understanding developmental mechanisms. An interesting feature of the developmental biology of pigmentation in quail is the variation in the patterns of expression between embryos and adults. This implies that there may be evolution in the developmental basis of phenotypes between different developmental stages within a species. Future studies could examine whether the novel *ASIP* alternatively spliced transcripts discovered here are expressed in adult quail, and whether similar patterns of variation in alternatively spliced transcripts might be found in other avian species. It would seem plausible that there may be variation between embryonic and adult stages given the different types of plumage involved (neoptile vs pennaceous) and variation in selection pressure at different life stages, e.g. juvenile and adult.

The finding that *ASIP* does not appear to be involved in dorsal patterns within-feathers demonstrates that there has been evolution in the developmental basis of phenotypes between mice and birds and highlights the importance of considering divergence in developmental mechanisms as well as conservation. Although the mechanism down-regulating dorsal within-feather and between-stripe pigmentation patterns in wildtype quail is currently unknown, there are several avenues of research (Walker and Gunn 2009; Walker and Gunn 2010). In particular, *Corin* affords an exciting prospect to examine divergence and conservation in the developmental basis of phenotypic evolution given that this loci, as well as *ASIP* and *MC1R*, explain variation in phenotypic evolution of two populations of *Peromyscus* mice (Manceau et al. 2010). This will be explored in the coming year.

The major finding of the developmental basis of embryonic wildtype quail pigmentation is that activation of *MC1R* appears to be required for eumelanin pigmentation. This is in contrast with previous findings because existing research on the developmental basis of plumage patterns has been on understanding developmental similarity based on shared evolutionary history between mice and birds (Nadeau et al. 2008; Oribe et al. 2012; Yoshihara et al. 2012) despite hints that *POMC* and *PC2* have a function in avian pigmentation (Yoshihara et al. 2011). However, there were dissimilarities between wildtype quail and chicken in that *PC1* was only expressed in feather follicles of chicken breeds. This may be indicative of

the effects of artificial selection rather than natural selection but *PC1* should nevertheless be tested alongside *POMC* and *PC2* on other wildtype avian phenotypes such as the northern bobwhite (*Colinus virginianus*) and the common quail (*Coturnix japonica*), and will be presented in a future study. There does however appear to be confusion in the literature as to the possibility that PC2 can directly cleave POMC (e.g. Takeuchi et al. 2003; Yoshihara et al. 2011), even though it has been previously reported that PC2 directly cleaves POMC in mice (Benjannet et al. 1991). An important future step will be to understand the biochemistry of *POMC*, *PC1* and *PC2* products in birds. In addition, 5' RACE of *POMC* transcripts may reveal promoter sites that are dorsoventral specific, and perhaps may be temporal-specific. Similarly perhaps there are *PC2* alternative splice transcripts that may also be dorsoventral/temporal specific and this will also be explored in future studies. These results have opened several new avenues of research into the rich tapestry of the evolutionary developmental biology of avian phenotypes. However, the organizing mechanism of within-feather patterning is currently unknown.



A 9-day old White Leghorn chick showing barred plumage on the right wing, produced by grafting limb-bud mesoderm from a Barred Plymouth Rock embryo (Willier and Rawles 1938)

Developmental constraint

Although it is unknown whether reaction-diffusion based mechanisms govern within-feather pattern formation, the significant similarity of the trajectory of plumage pattern evolution in two avian groups that have different life history attributes is predictable from a hypothetical model of pattern formation (Prum and Williamson 2002). This affirms the power of biologically relevant models. Coupling biological modeling with sophisticated comparative modeling techniques demonstrates the possibility to evaluate the relevance of hypothetical models of developmental dynamics before the time consuming task of confirming that the model itself represents reality. Of course, this is fraught with potential false positives/negatives, but given that biological modeling as well as comparative modeling can be undertaken in a fraction of the time required to test molecular and developmental biology hypotheses in live systems, this solution provides a promising preliminary step in examining the potential of developmental dynamics.

The similarity in the trajectory of plumage pattern evolution within and between patches in two different groups is remarkable and lends weight to "the following role for natural selection: fine-tuning the appearance of the pattern, fixing and maintaining pattern elements at a given level of expression, and modifying behavioral and other features to maximize the patterns' utility" (Price and Pavelka 1996). In spite of the strong directionality in plumage pattern evolution in Anseriformes and Galliformes, there were frequent and strong evolutionary transitions to other types of patterns, similar to other studies (Gluckman 2014). It would therefore seem that once plumage patterns have evolved, patterns are a labile trait that can adapt quickly to environmental changes.

This may have multiple important evolutionary consequences: i) if patterning has not evolved in an ancestral lineage then development may constrain trait evolution, ii) if patterning has evolved in an ancestral lineage of a species then it may be easier to adapt to changes in the environment, iii) together this implies that evolutionary-development may constrain or facilitate speciation. However, it is currently unknown whether this same evolutionary trajectory is the same in the most speciose part of the avian tree – Passeriformes. Additionally or alternatively,

allometric constraints may play a role in plumage pattern evolution (Riegner 2008) which may add an additional layer to mechanisms of plumage pattern speciation and evolution. A study examining whether plumage pattern evolution follows a similar evolutionary trajectory in all birds worldwide, whether evo-devo constrains or facilitates speciation, as well as whether there is an allometric relationship in avian pattern evolution (accounting for phylogeny and phylogenetic uncertainty) will be undertaken next year.

Adaptive explanations

Signal evolution in the barred plumage patterns of Old World parasitic cuckoos is apparent at both the macroevolutionary and microevolutionary scale (Kruger, Davies, and Sorenson 2007; Gluckman and Mundy 2013). In addition, parasitic cuckoos could mimic one or more models from multiple genera of raptors. This remarkable precision in sympatric similarity demonstrates that although it is possible that there may be developmental constraint in plumage pattern evolution, natural selection has adaptively modified this type of patterning to be parasite-specific. This has important implications for our understanding of signal evolution as a principle as it demonstrates that signals can be refined to be receiver specific, within a given type of signal. This underscores the importance of using objective based methods to examine visual traits.

The common cuckoo (*Cuculus canorus*) and the Eurasian sparrowhawk (*Accipiter nisus*)

The study of cuckoo-hawk mimicry was representative of five genera of parasitic cuckoos but there are many other barred parasitic cuckoos as well as barred non-parasitic species (Payne and Sorensen 2005). An interesting future study would be to investigate whether these variations in barred plumage patterns are indeed specific to parasitic cuckoos compared to non-parasitic species. Barred plumage patterns can additionally function in communication (Swaddle and Cuthill 1994; Bortolotti et al. 2006; Gluckman and Cardoso 2010) and melanin based traits have pleiotropic effects in the Tawny owl (Roulin 2004; Ducrest, Keller, and Roulin 2008; Emaresi et al. 2013). It would be interesting to examine whether barred plumage patterns can additionally function in communication in parasitic species, as well as non-parasitic species.

From a broader perspective, the prevailing view of the camouflage function of within-feather patterns did not predict where plumage patterns have evolved from a habitat perspective. In recent years there has been an increase in studies of the camouflage function of visual patterns in animals. It is an exciting finding that all pattern types are found in all habitat types across the world. This is congruent with the idea that patterns may fill visual niches, similar to ecological niches. To study this question would require examining all aspects of phenotypic diversity, including uniform colouration, in relation to habitat. In addition, as demonstrated in parasitic cuckoos, the same pattern may be refined in multiple ways, which may also add an additional layer of complexity. Therefore, such a study would require digital image analysis of all avian plumages worldwide, rather than scoring images from field guides. This type of question is timely given that the collection of avian plumages in the class Aves worldwide has begun using digital image analysis techniques, and is the part of a macroevolutionary project that I have begun managing.


Aegypodius arfakianus
Talegalla fuscirostris
Talegalla cuvieri
Talegalla jobiensis
Talegalla lathami
Alectura lathami
Leipoa ocellata
Macrocephalon maleo
Megapodius wallacei
Megapodius cumingii
Megapodius tenimberensis
Megapodius layardi
Megapodius pritchardii
Megapodius eremita
Megapodius reinwardt
Megapodius freycinet
Megapodius nicobariensis
Megapodius pritchardii
Megapodius forsteni
Megapodius decollatus

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Supplementary material

Table S2.1. Primer sequences tested for RT-PCR.

Target sequence	Name	Sequence
AGRP	AGRP F1	ACA CAG GAC CAT GCT GAA CG
AGRP	AGRP F2	GAC ATC GAG GCA GAG CGA CT
AGRP	AGRP R1	CCT CTG CGG CTC CAA TAA AG
AGRP	AGRP R2	CCT CTG CGG CTC CAA TAA AG
AGRP	AGRPF1	CCC AGG ACC ATG CTG AAC
AGRP	AGRPF1	CCC AGG ACC ATG CTG AAC
AGRP	AGRPF16	GTG GAC CAT GAG CCT CCT CT
AGRP	AGRPF17	CCA GCT CCC CAG CAC TAA G
AGRP	AGRPF4	ATG CTG AAC GTG CTG CTG
AGRP	AGRPR1	CCA CAT GGG AAG GTG GTG
AGRP	AGRPR16	GGA TTC TCC TCT TCC CAT CC
AGRP	AGRPR17	TGA GCA CAA TGG ACC TAT GG
AGRP	AGRPR4	AGG TGG TGC TGA TCT TCC TG
ASIP E2	ASIP E2_R	CAG CCT TAA CAT GTT CCT CAT TA
ASIP E3	ASIP E3R	AGT AAA CAC TGG CAG ATT GTC TGA
ASIP E4	ASIP E4R	CAG CCT TAA CAT GTT CCT CAT TAG GTT
ASIP NE1	ASIP NEW EXON 1F	GGG AGA TCT GGG AGG TTC ATT
ASIP	ASIP R	TTT GGG GGT GTC TTC AGT TC
ASIP	ASIP R2	CCT TAA CAT GTT CCT CAT TAG GTT TA
ASIP	ASIPF5	CCA ACA ATG AAA AGG AAG AAC C
ASIP	ASIPR5	GAT TTG GTT TAA CAC TTT GGG TTT
ASIP	ASIPF2	TCA TTT TCA TGA CAG TGG GAT T
ASIP	ASIPR6	TTT GGG GGT GTC TTC AGT TC
ASIP E1L	ASIP E1LF	TCT CCT CGG CTA TAT GGC TGA G
ASIP E1L	ASIP E1LF	AAG CCA GAA CTG GTG GTC AA
ASIP E1L	ASIP E1LF_2.0	AGT TTT GGA GGT TCA TTT CTA ATG T
ASIP E2	ASIP E2F	TCA TTT TCA TGA CAG TGG GAT T
ASIP E2	ASIP E2F_2.0	TAA ACA CAT TGA TGG CAT TAA CAA
ASIP E3	ASIP E3F	GAA GCA GGC AGT CTT CTT GG
ASIP E3	ASIP E3F	TGA AAA GGA AGC AGA ACC AGA
ASIP E4	ASIP E4F	GTT CTT TTG GCT CAG TGG TAT CTC A
ASIP E4	ASIP E4F	CCA GCA TTT TCA TAT TTT CTG GA
ASIP E5	ASIP E5F	TGA AAT CAG TTG TGG CAG GAA

ASIP NEW EXON 1	ASIP NEW EXON 1F	GTG TGG TTG TGA TGG TGA TGG
ASIP INTERNAL 1	INT1	AGC AGC TCC AGC CTT TCA TC
ASIP INTERNAL 2	INT2	TCT GGC GTA ACT GGA ACA GC
PC1	PC1 F	ACG GGC TGG AAA TTC AGG AT
PC1	PC1 R	AAC CCA AAT CGG CTG TTG AC
PC1	PC1F.1	CTA CGC CAA CTA TGA TCC AAG G
PC1	PC1F.2	GGG ACT CAC ACT GGG ACC TC
PC1	PC1R.1	TTT CCA TCT TTT GGG ATC AGC
PC1	PC1R.2	CCA AAT CCA AAT CGG CTG TT
PC2	PC2F	GGG AGG GAA AGG AAG CAT CT
PC2	PC2F.1	GCT GGG ATA CAC AGG GAA GG
PC2	PC2F.2	GGA GAG ACA TGC AGC ATC TGA
PC2	PC2R	GGT CTT CTC CCC AAG TGT GTG
PC2	PC2R.1	CAT AGC TGG CTT TGG CAT TG
PC2	PC2R.2	CCC AAC GCA GTG GAA TCT CT
PC2 X2	PC2_X2F.1	CAG CCG TCT ACA CCA ACC AG
PC2 X2	PC2_X2F.2	CCT TCG TCC TCC TCC TT
PC2 X2	PC2_X2R.1	TGA ATG TGG AGG CTA GGG TTG
PC2 X2	PC2_X2R.2	GGA AGC CCA GAC GTA GAT GC
PC2 X3	PC2_X3F.1	GGC ATT GCC AAG GTC AGA A
PC2 X3	PC2_X3F.2	CCT TCG TCC TCC TCC TCC TT
PC2 X3	PC2_X3R.2	GCC CAG ACG TAG ATG CTT CC
PC2 X3	PC2_X3 R	TGT AGC CAC ACC AGC CTC TG
PC2 X4	PC2_X4 F.3	GCA TTG CCA AGG TCA GAA GA
PC2 X4	PC2_X4 R	GGA AGC CCA GAC GTA GAT GC
POMC	POMCF	CTG GGG CTG CTG CTG CTG TGT
POMC	POMCF.1	GTA TCC CAA TGG CGT GGA TG
POMC	POMCR	TGA CCC TTC TTG TAG GCG CTT T
POMC	POMCR.1	CAG AGT CAT CAG CGG GGT CT
POMC	POMCR.2	CAT GGG GTA ACT CTC AGC CGA CT
POMC A PROMOTER	POMC A PROMOTER	CCC ATA AGC GAC TTG CCT TC
POMC A PROMOTER	POMC A PROMOTER.FV2	CCC ATA AGC GAC TTG CCT TC
POMC A PROMOTER	POMC A-1	CAC CCC TCG CCA GTA GGT T
POMC A PROMOTER R	POMC AF	CAA AGA ACT GAC CAT CCA CCA CAT T
POMC A PROMOTER R	POMC_AR.1	GGT ACG AGC CAC CAT CCT TC
POMC A PROMOTER R	POMC_AR.2	CCT CAC CCT CCT CCT CCT CT
POMC A PROMOTER R	POMC_AR.3	CCT CAC CCT CCT CCT CCT CT
POMC B PROMOTER	POMC B PROMOTER	GGA GAC GGG GAA GGT GGT

POMC B PROMOTER	POMC B-1	CAC CCC TCG CCA GTA GGT T
POMC B PROMOTER	POMC T1F.V2	GCC ACT GAG GCT GGA GTT TT
POMC B PROMOTER	POMC T1R	CCC CTC ACT GAC CCT TCT TG
POMC B PROMOTER F	POMC BF	GAA GGT GGT GGC TGC GCT CCA A
POMC B PROMTER	POMC T1	AGC GCT CCT CTG CAG TTT G
POMC CHICKEN NCE	POMC A-1F.V2	CAC CCC TCG CCA GTA GGT T
POMC CHICKEN NCE	POMC A-2	CTC AGG AGG GGC AGA AAT CC
POMC CHICKEN NCE	POMC A-2F.V2	CTC TCC CCC TGC AGC ATC
POMC CHICKEN NCE	POMC B-2	CTC TCC CCC TGC AGC ATC
POMC QUAIL NCE 1	POMC QE R	ATC TCC CTC CGG AAC TCC AT
POMC QUAIL NCE 1	POMC QEF.2	ACT TCC AGC GTC TCC CAG AG
POMC QUAIL NCE 1	POMC QEF.1	TCT CTT GCC TGT GGC TCT CA
POMC QUAIL NCE 2	POMC QEF.3	GAT TTC GGA GGC AAA GGA TG

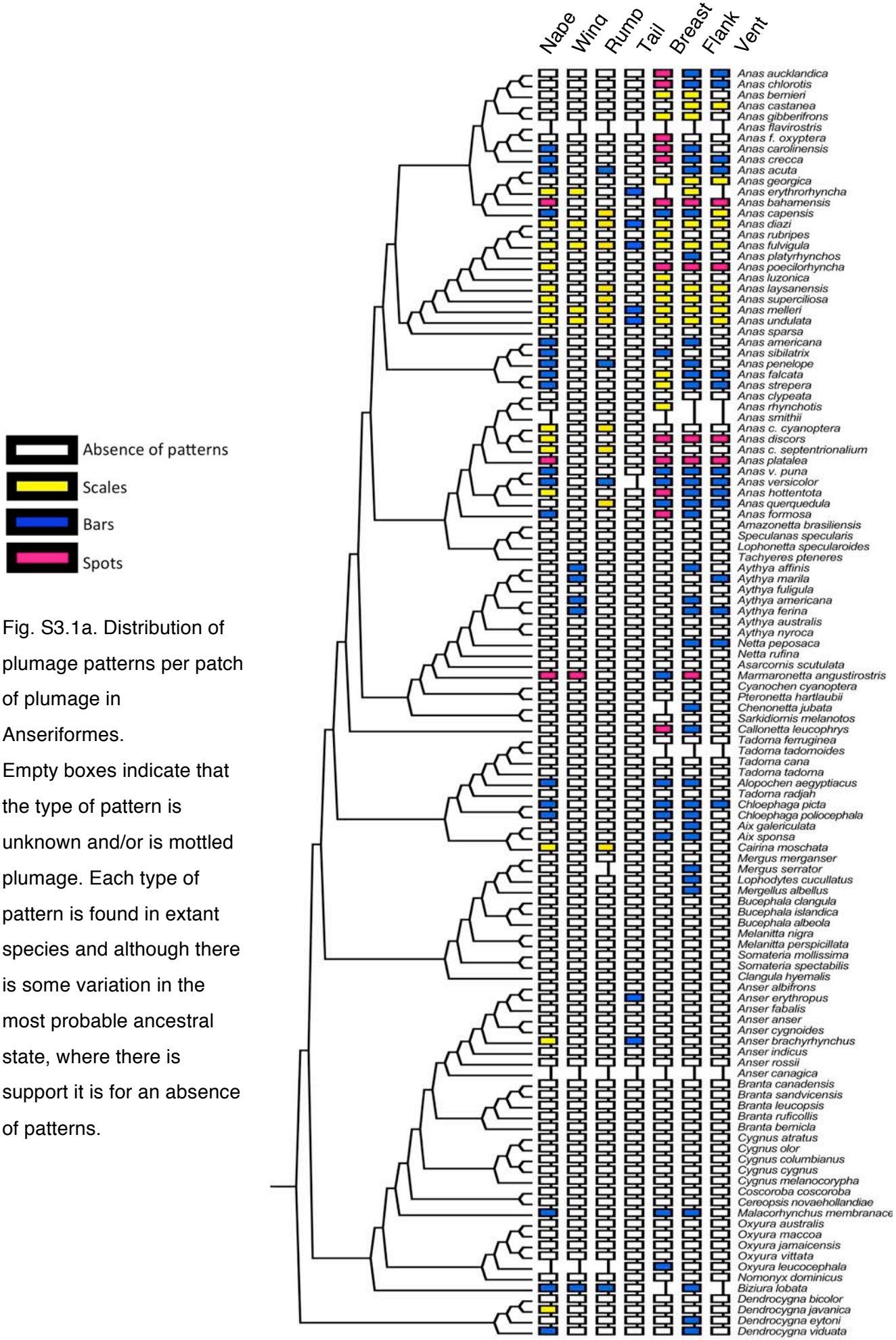
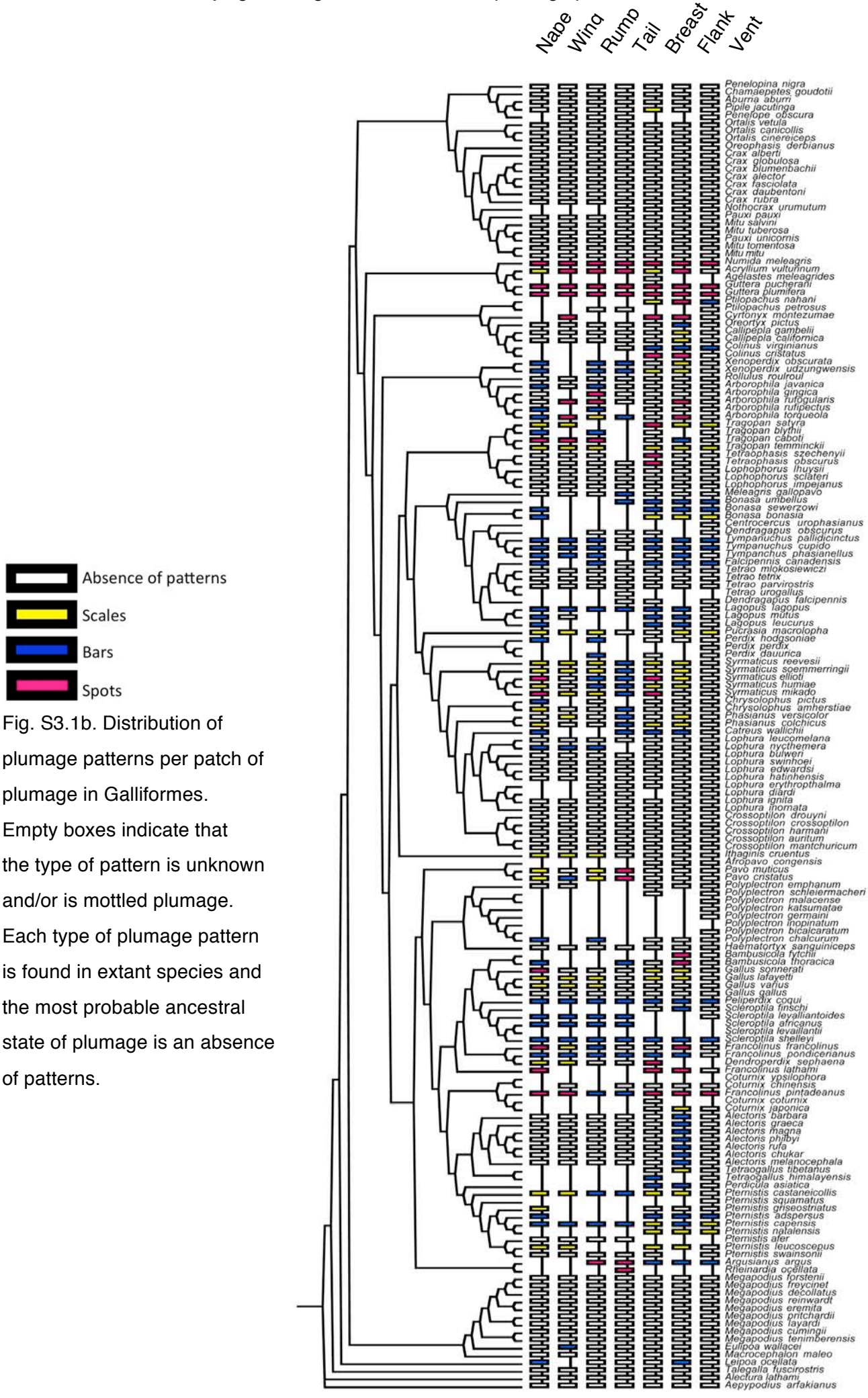


Fig. S3.1a. Distribution of plumage patterns per patch of plumage in Anseriformes.
Empty boxes indicate that the type of pattern is unknown and/or is mottled plumage. Each type of pattern is found in extant species and although there is some variation in the most probable ancestral state, where there is support it is for an absence of patterns.



Absence of patterns

Scales

Bars

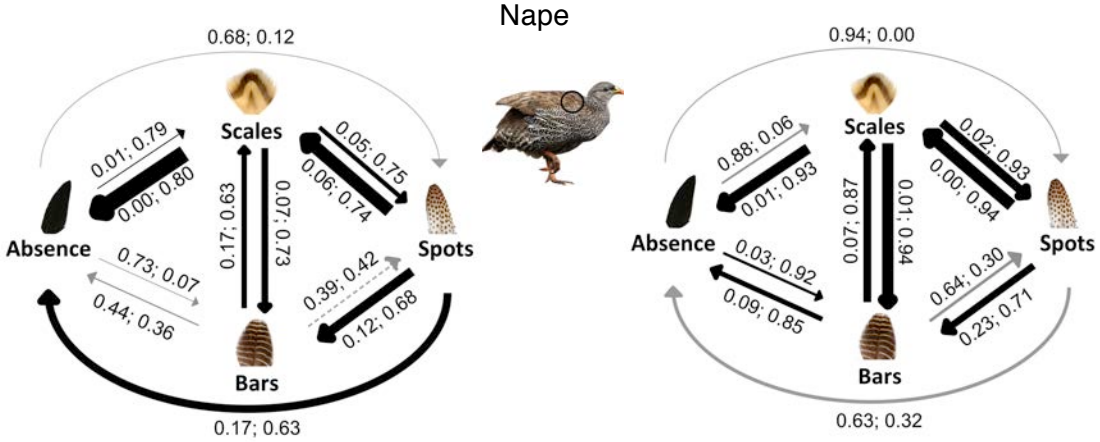
Spots

Fig. S3.1b. Distribution of plumage patterns per patch of plumage in Galliformes. Empty boxes indicate that the type of pattern is unknown and/or is mottled plumage. Each type of plumage pattern is found in extant species and the most probable ancestral state of plumage is an absence of patterns.

GALLIFORMES ANSERIFORMES

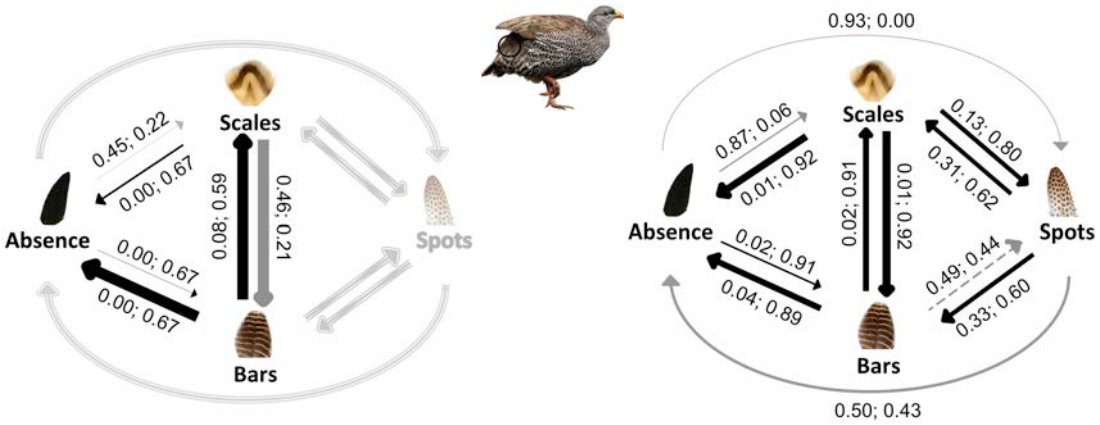
UPPERPARTS

Nape

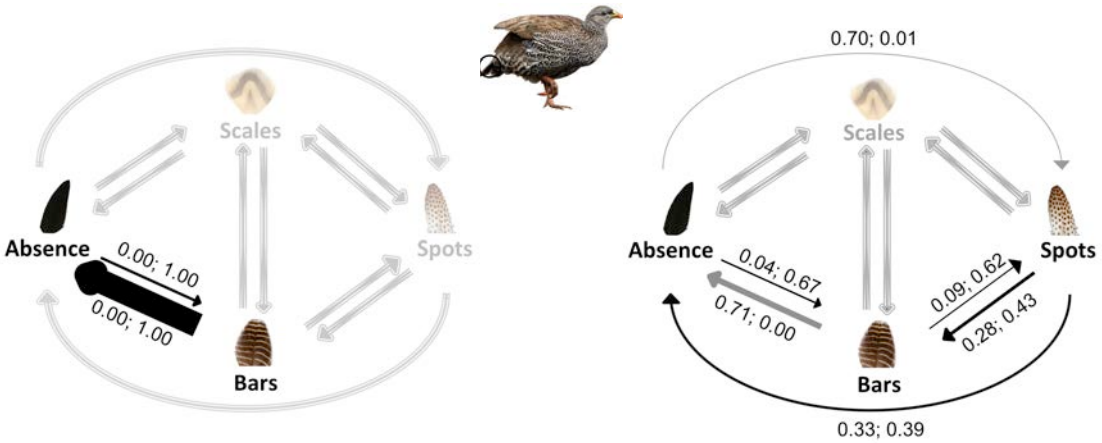


Wing

Rump

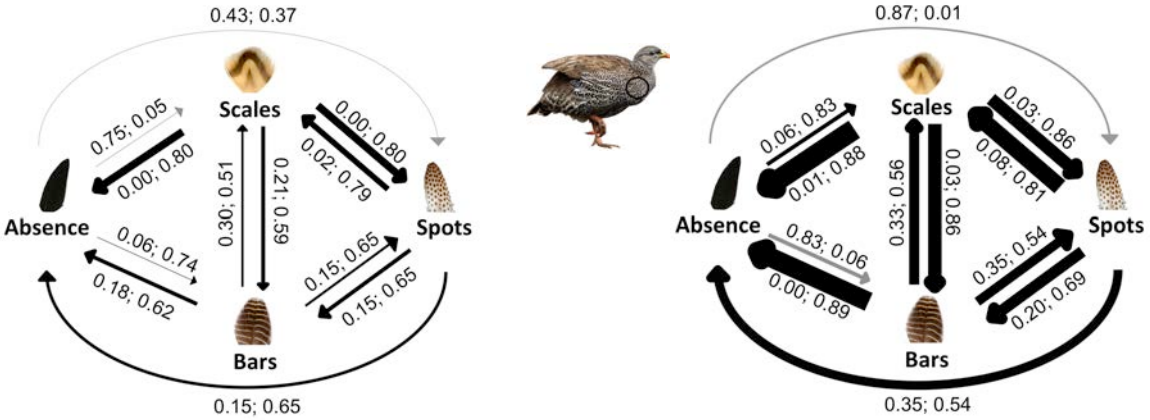


Tail

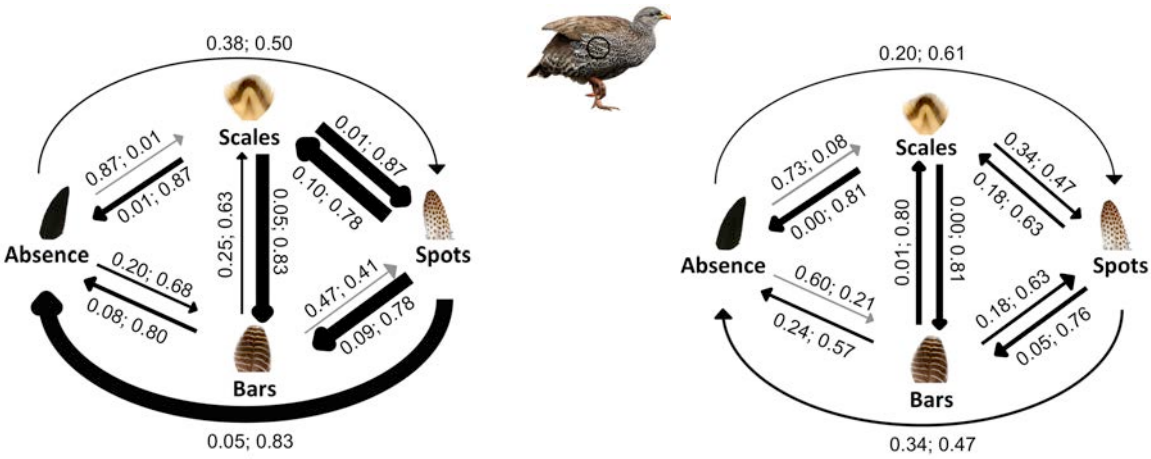


UNDERPARTS

Breast



Flank



Vent

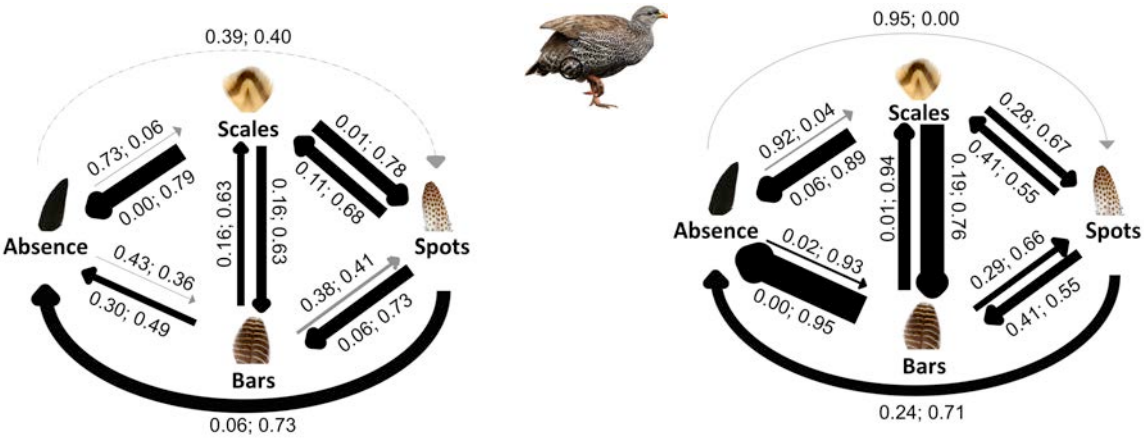


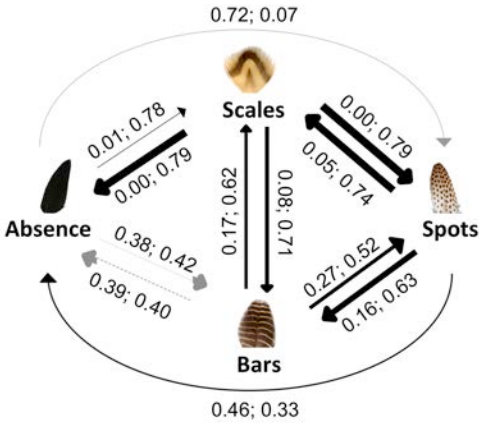
Fig. S3.2. Local pattern evolution within patches, in Anseriformes and Galliformes. The width of each evolutionary step is proportional to the average rate in the top model set. Beside each evolutionary step is the marginal probability of each transition not occurring, followed by the marginal probability of it occurring. Where the transition probably does not occur, the transition line is grey. Conversely, where the transition probably does occur, the transition line is black. Equivocal transitions, where the marginal probability is =< 0.05 difference between not occurring and occurring, are indicated by a grey dashed line.

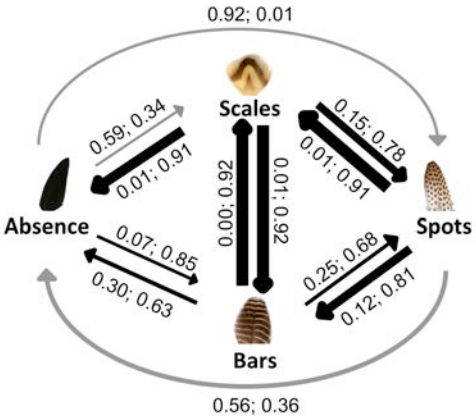
ANSERIFORMES GALLIFORMES

ANTEROPOSTERIOR AXIS

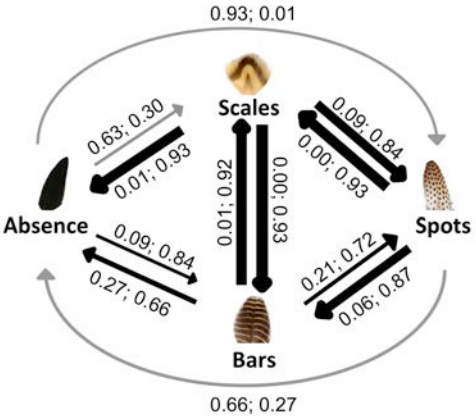
Anterior

Scales are derived

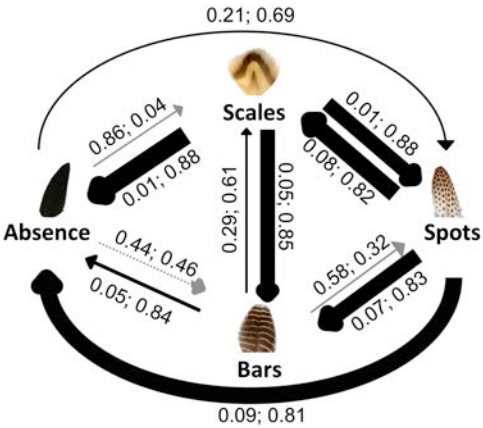


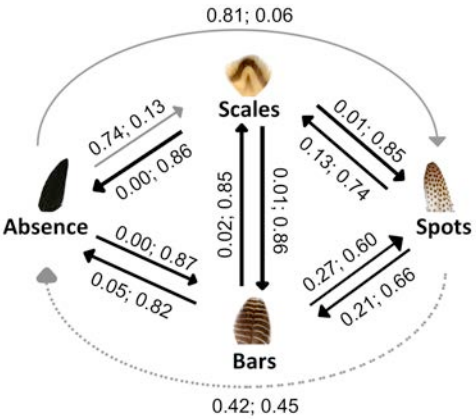


Spots are derived

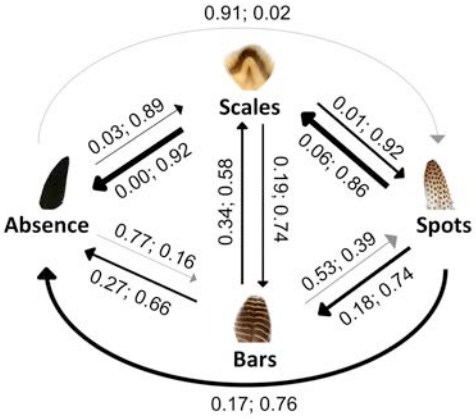


Middle

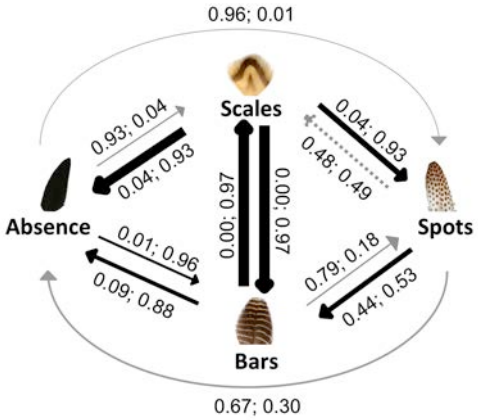




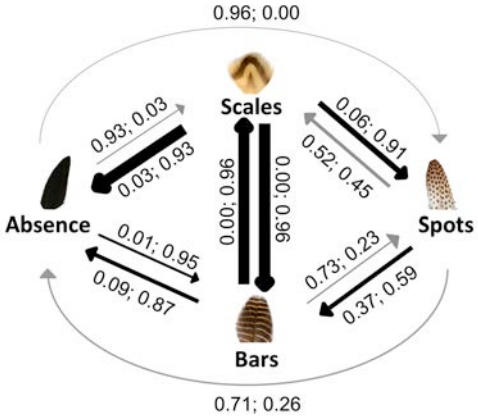
Posterior



Scales are derived

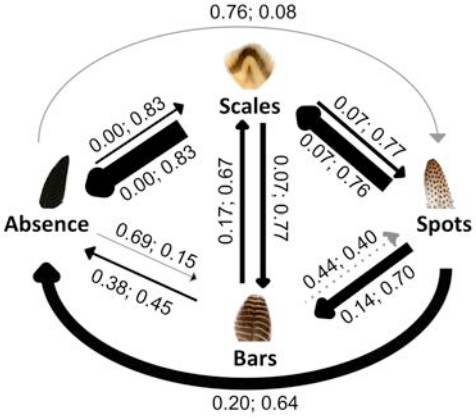


Spots are derived

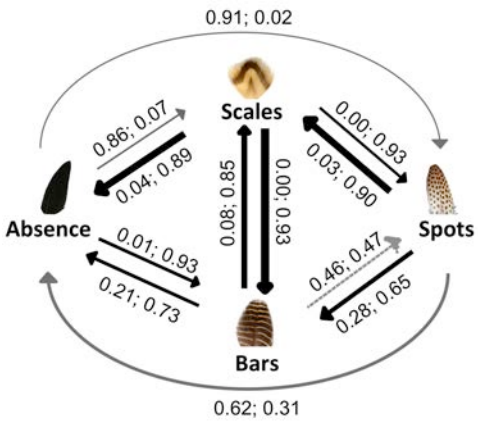


DORSOVENTRAL AXIS

Dorsal



Scales are derived



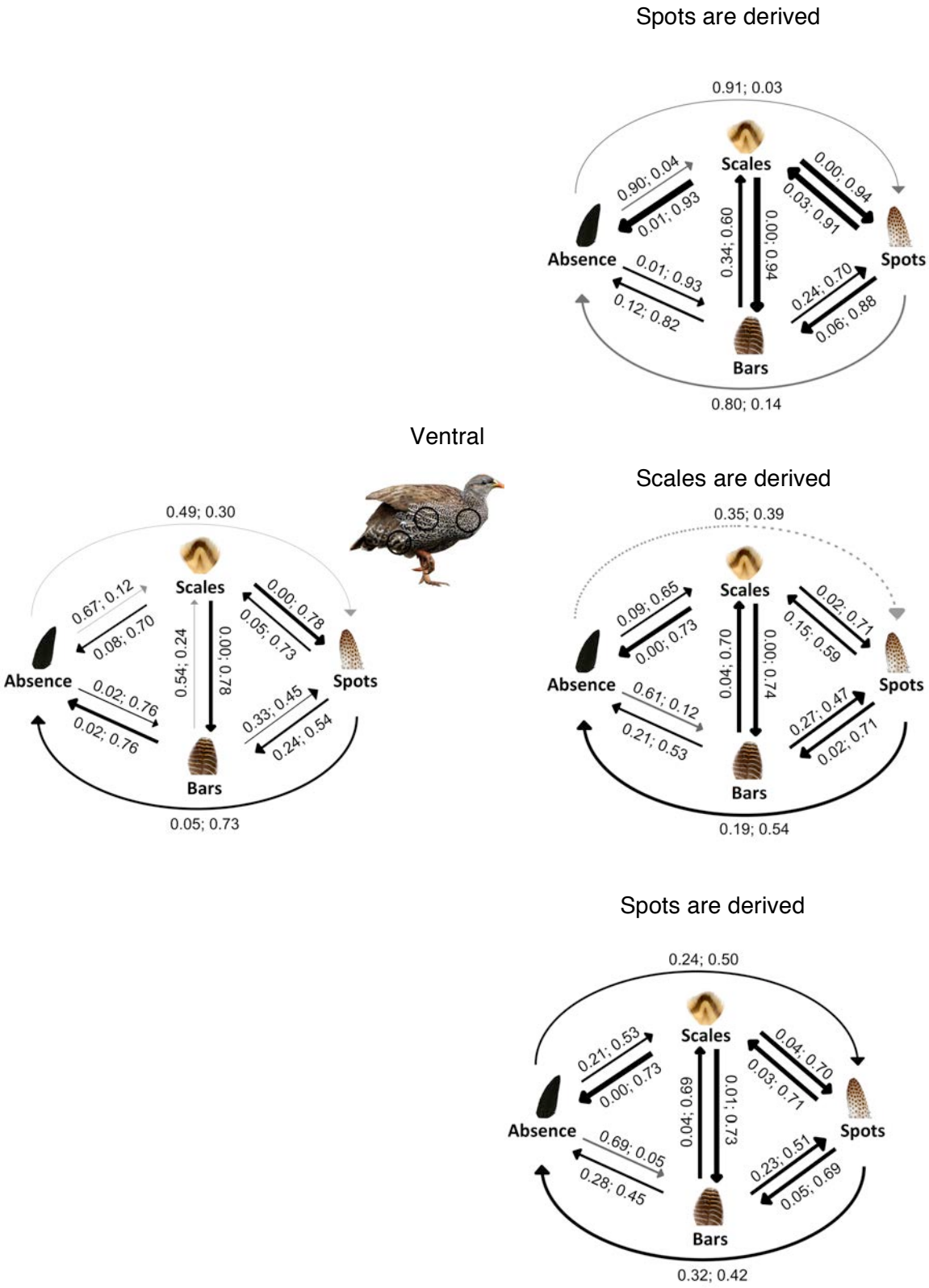


Fig. S3.3. Regional pattern evolution within regions in Anseriformes and Galliformes. To examine the effects of uncertainty in the order of plumage pattern evolution in Galliformes we modeled the effect of scales and spots being more derived. The width

of each evolutionary step is proportional to the average rate in the top model set. Beside each evolutionary step is the marginal probability of each transition not occurring, followed by the marginal probability of it occurring. Where the transition probably does not occur, the transition line is grey. Conversely, where the transition probably does occur, the transition line is black. Equivocal transitions, where the marginal probability is =< 0.05 difference between not occurring and occurring, are indicated by a grey dashed line.

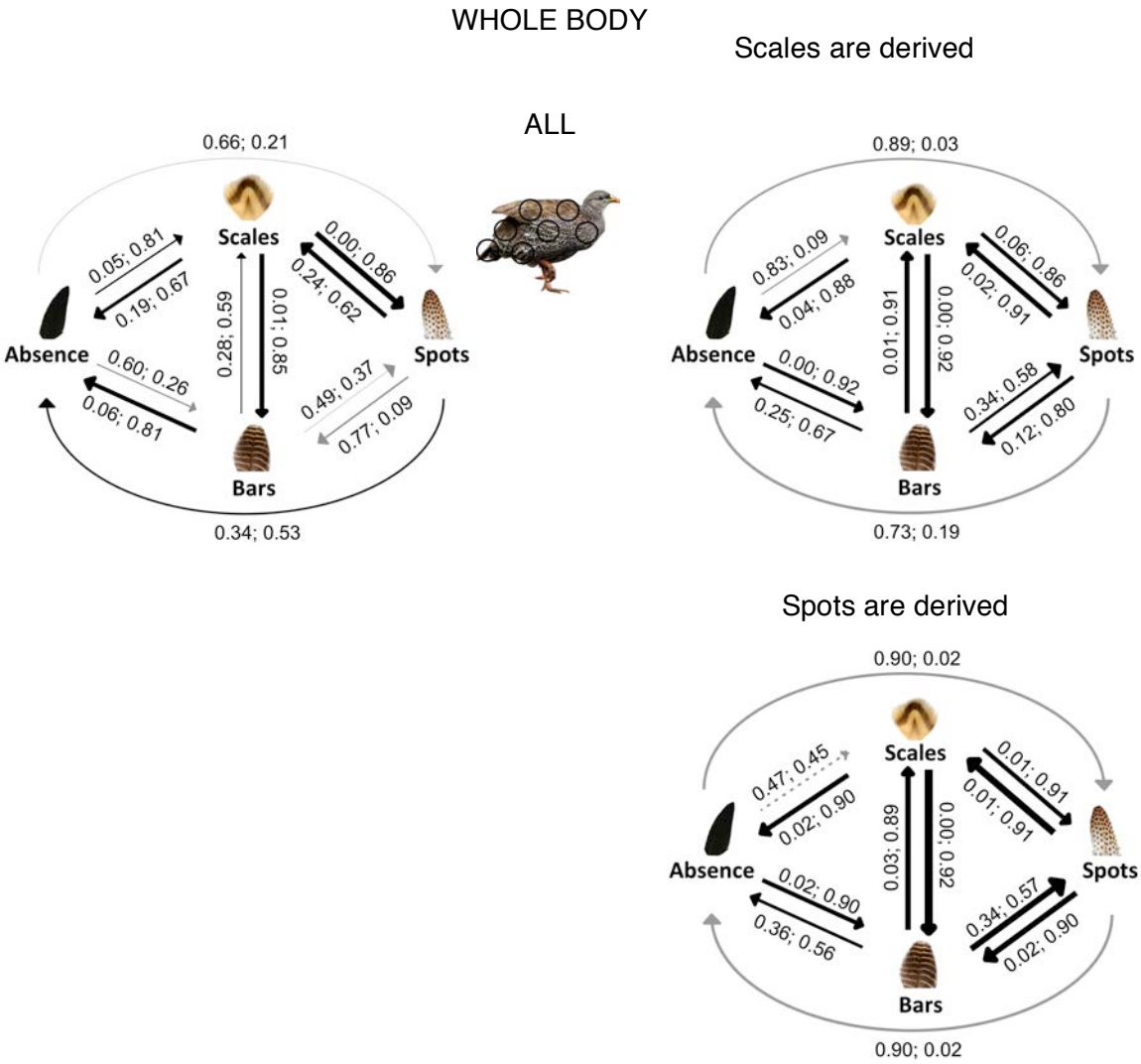


Fig. S3.4. Pattern evolution over the whole body, in Anseriformes and Galliformes. To examine the effects of uncertainty in the order of plumage pattern evolution in Galliformes we modeled the effect of scales or spots being more derived. The width of each evolutionary step is proportional to the average rate in the top model set. Beside each evolutionary step is the marginal probability of each transition not occurring, followed by the marginal probability of it occurring. Where the transition probably does not occur, the transition line is grey. Conversely, where the transition probably does occur, the transition line is black. Equivocal transitions, where the marginal probability is =< 0.05 difference between not occurring and occurring, are indicated by a grey dashed line.

Table S3.1. Prior probability of encountering models of <12 parameters. Calculations are comprised of binomial for Z (where Z = n parameters that are set to 0 i.e. do not occur) and Bell numbers for models with 12 possible transition rates.

No. Zeros (z)	Binomial for z	12 - z	Bell number for 12 - z	Binomial for z x bell number for 12 - z	Prior
0	1	12	4213597	4213597	0.1524211599
1	12	11	678570	8142840	0.0245463499
2	66	10	115975	7654350	0.0041952384
3	220	9	21147	4652340	0.0007649641
4	495	8	4140	2049300	0.0001497589
5	792	7	877	694584	0.0000317243
6	924	6	203	187572	0.0000073432
7	792	5	52	41184	0.0000018810
8	495	4	15	7425	0.0000005426
9	220	3	5	1100	0.0000001809
10	66	2	2	132	0.0000000723
11	12	1	1	12	0.0000000362

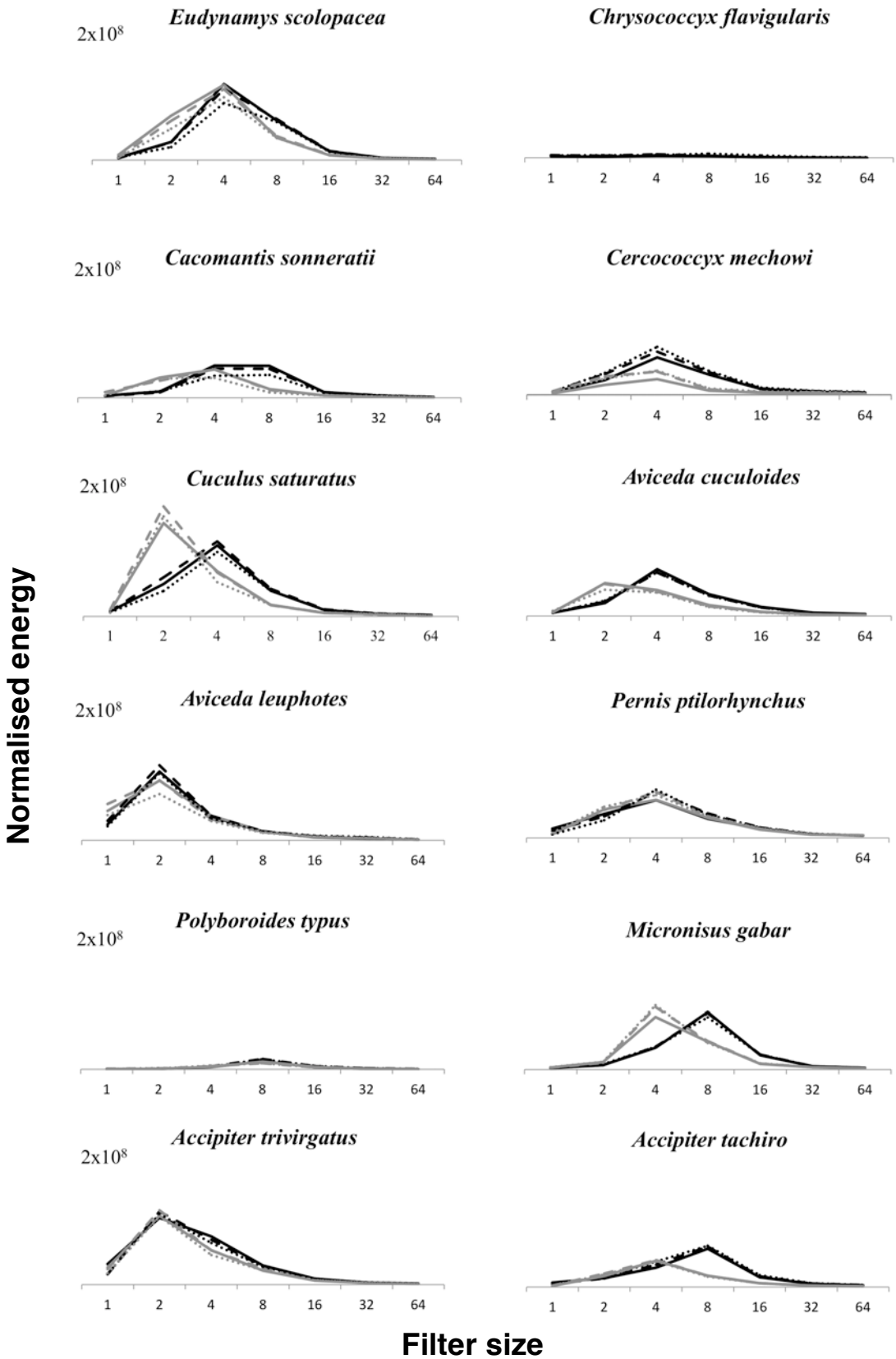
Table S3.2. Prior probability of encountering models of <6 parameters. Calculations were comprised of binomial for Z (where Z = n parameters that are set to 0 i.e. do not occur) and Bell numbers for models with three pattern states encompassing 6 possible transitions.

No of zeroes	Binomial for z	6-z	Bell number for 6-z	Binomial for z x bell number for 6-z	Prior
0	1	6	203	203	0.23173516
1	6	5	52	312	0.04577465
2	15	4	15	225	0.01381216
3	20	3	5	100	0.00514933
4	15	2	2	30	0.00221239
5	6	1	1	6	0.00113507

Table S3.3. The number of unique models as well as the ancestral state of patterns per body region in Anseriformes and Galliformes.

				Ancestral state							
	Unique models			Average probability				Marginal probability			
	Posterior sample	Top model set (BF>=2)	Full model	Absence of patterns	Scales	Bars	Spots	Absence of patterns	Scales	Bars	Spots
Anseriformes											
Whole body	142	88	98: BF = 0.34	0.3	0.24	0.27	0.2	0.11; 0.75	0.84; 0.02	0.79; 0.08	0.85; 0.01
Anterior	219	158	35: BF = 0.12	0.3	0.25	0.22	0.23	0.23; 0.56	0.79; 0.00	0.72; 0.07	0.64; 0.15
Middle	216	144	13: BF = 0.04	0.25	0.25	0.25	0.25	0.31; 0.59	0.89; 0.01	0.62; 0.28	0.87; 0.03
Posterior	199	140	2: BF = 0.00	0.25	0.25	0.25	0.25	0.34; 0.59	0.92; 0.00	0.64; 0.29	0.88; 0.05
Ventral	181	190	43: BF = 0.14	0.23	0.27	0.22	0.28	0.37; 0.41	0.71; 0.07	0.74; 0.04	0.52; 0.26
Dorsal	273	191	23: BF = 0.08	0.28	0.25	0.22	0.24	0.05; 0.79	0.82; 0.02	0.82; 0.02	0.82; 0.02
Galliformes											
Whole body (scales)	114	76	7: BF = 0.023	0.88	0.05	0.05	0.02	0.00; 0.92	0.92; 0.00	0.92; 0.00	0.92; 0.00
Whole body (spots)	95	63	6: BF = 0.020	0.84	0.09	0.06	0.01	0.00; 0.92	0.00; 0.00	0.00; 0.00	0.00; 0.00
Anterior (scales)	149	99	11: BF = 0.04	0.72	0.13	0.09	0.06	0.00; 0.92	0.00; 0.00	0.00; 0.00	0.00; 0.00
Anterior (spots)	139	89	12: BF = 0.04	0.71	0.14	0.1	0.05	0.00; 0.93	0.00; 0.00	0.00; 0.00	0.00; 0.00
Middle	129	82	14: BF = 0.05	0.84	0.06	0.07	0.03	0.00; 0.87	0.00; 0.00	0.00; 0.00	0.00; 0.00

Posterior (scales)	145	112	1: BF = 0.003	0.82	0.09	0.07	0.02	0.01; 0.97	0.97; 0.01	0.97; 0.01	0.97; 0.01
Posterior (spots)	129	92	0	0.81	0.1	0.07	0.02	0.01; 0.96	0.96; 0.01	0.96; 0.01	0.96; 0.01
Ventral (scales)	186	113	112: BF = 0.38	0.76	0.11	0.07	0.06	0.00; 0.74	0.00; 0.00	0.00; 0.00	0.00; 0.00
Ventral (spots)	203	122	86: BF = 0.28	0.74	0.13	0.07	0.06	0.00; 0.74	0.00; 0.00	0.00; 0.00	0.00; 0.00
Dorsal (scales)	129	84	0	0.79	0.1	0.08	0.03	0.00; 0.93	0.93; 0.00	0.93; 0.00	0.93; 0.00
Dorsal (spots)	106	73	5: BF = 0.02	0.78	0.11	0.09	0.02	0.00; 0.94	0.94; 0.00	0.94; 0.00	0.94; 0.00


Eudynamys scolopacea
Chrysococcyx flavigularis
Cacomantis sonneratii
Cercococcyx mechowi
Cuculus saturatus
Aviceda cuculoides
Aviceda leuphotes
Pernis ptilorhynchus
Polyboroides typus
Micronisus gabar
Accipiter trivirgatus
Accipiter tachiro
2x10^8
1 2 4 8 16 32 64
Normalised energy
Filter size

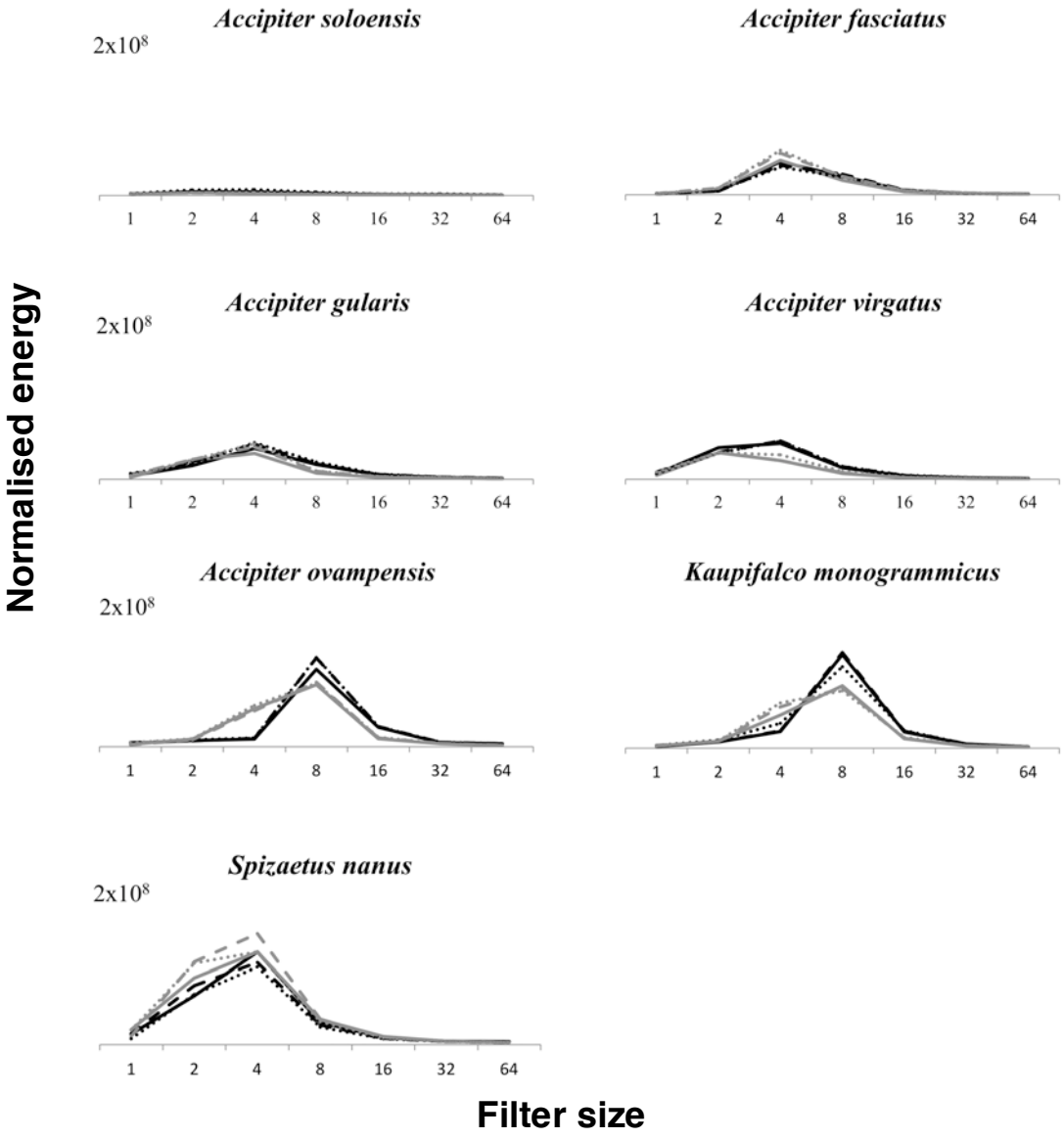


Fig. S4.1. Granularity spectra of the ventral and flank area of each species sampled. Black lines indicate ventral patterns, and grey lines indicate the flank. To assess the variability of patterning within each patch of patterning sampled, we compared the top, middle and bottom section for the ventral and flank area respectively. A continuous line represents the top section, a dashed line represents the middle, and the dotted line represents the bottom section.

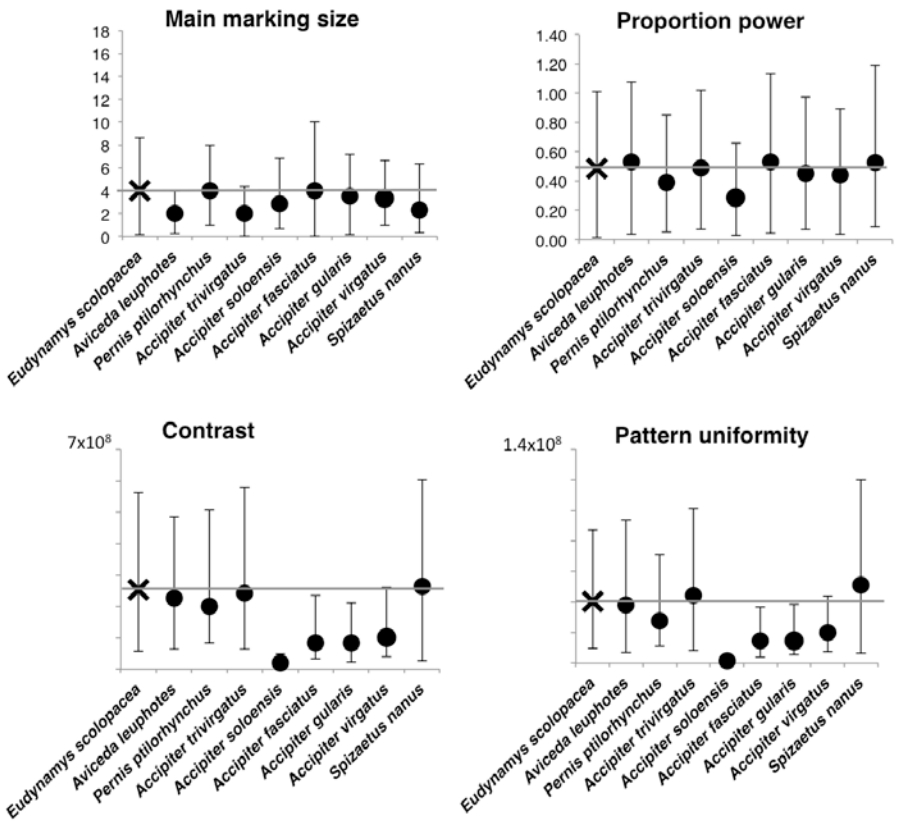


Fig. S4.2a. Median pattern attributes for *Eudynamys scolopacea.*

Confidence intervals are bootstrapped values and the grey line indicates the median of *Eudynamys scolopacea.*

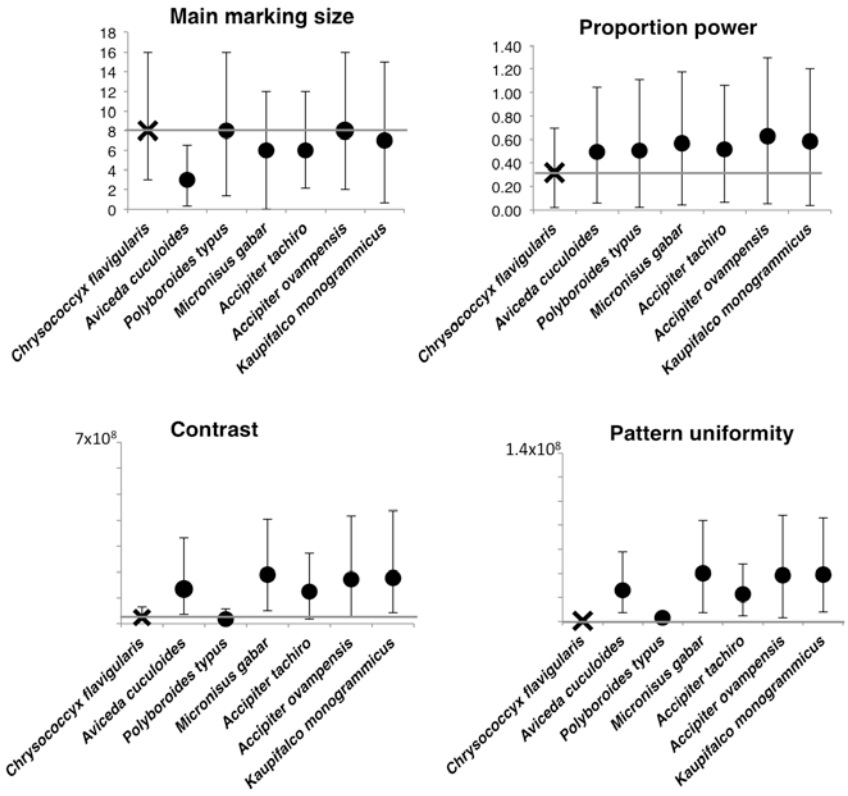


Fig. S4.2b. Median plumage pattern attributes for *Chrysococcyx flavigularis.* Confidence intervals are bootstrapped values and the grey line indicates the median of *Chrysococcyx flavigularis.*

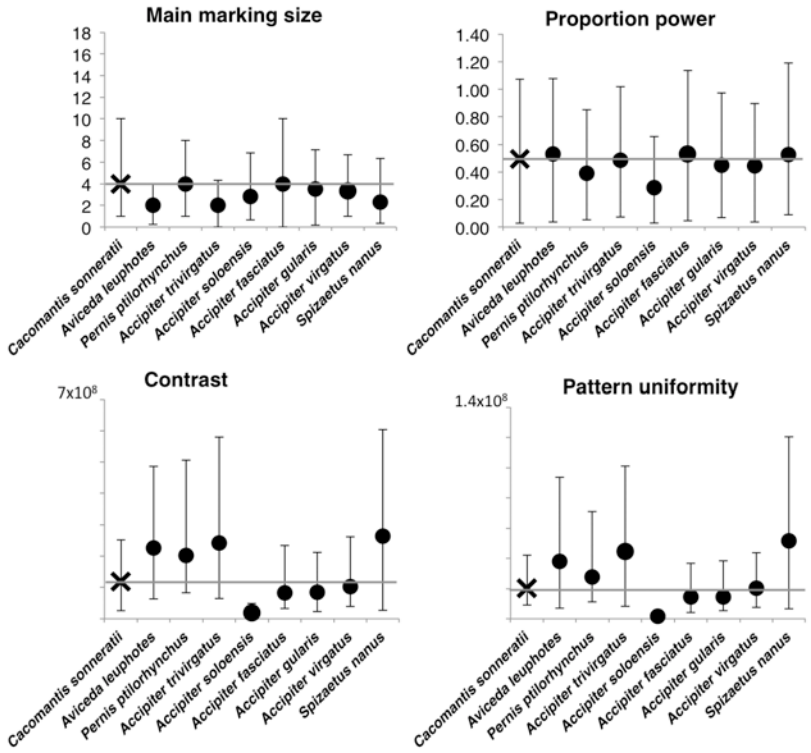


Fig. S4.2c. Median plumage pattern attributes for *Cacomantis sonneratii.* Confidence intervals are bootstrapped values and the grey line indicates the median of *Cacomantis sonneratii.*

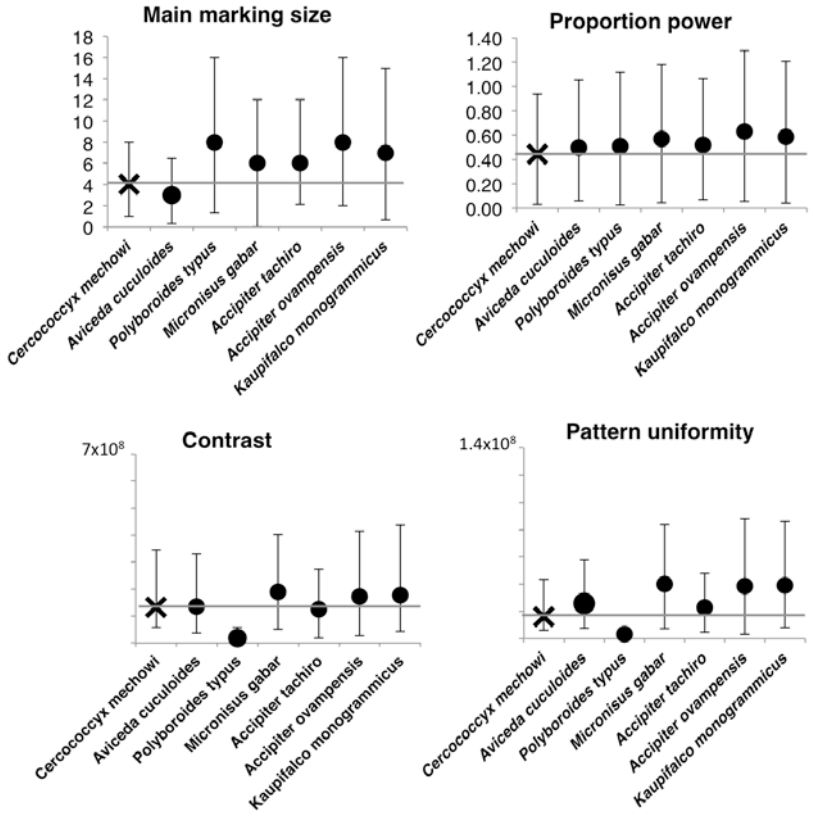


Fig. S4.2d. Median plumage pattern attributes for *Cercococcyx mechowi.* Confidence intervals are bootstrapped values and the grey line indicates the median of *Cercococcyx mechowi.*

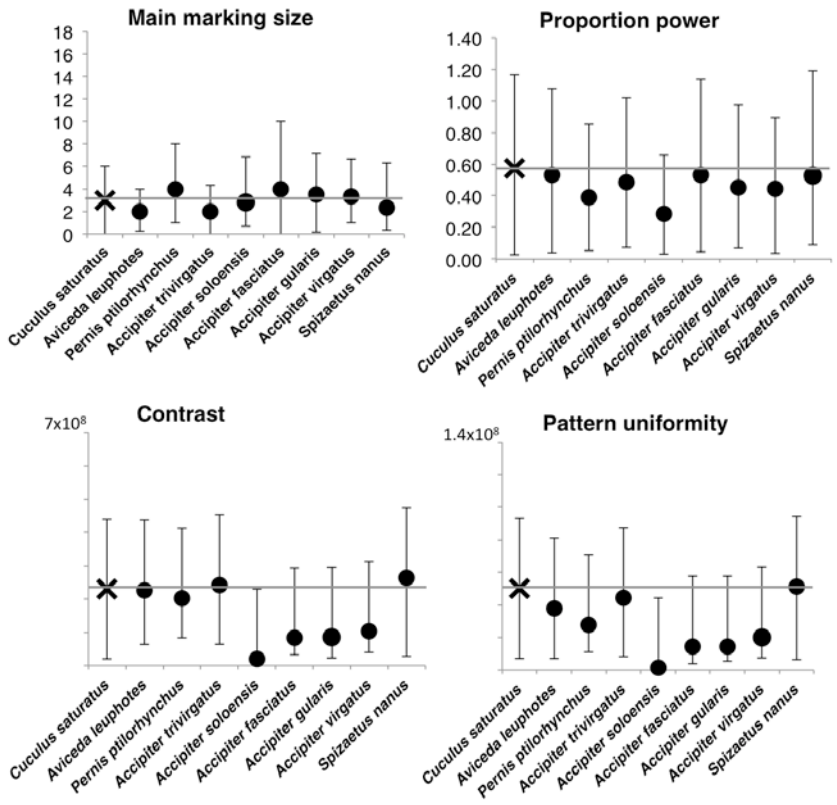


Fig. S4.2e. Median plumage pattern attributes for *Cuculus saturates.*
Confidence intervals represent bootstrapped values and the grey line indicates the median of *Cuculus saturatus.*

Table S4.1. Comparison of distribution overlap from a taxonomic source (Payne and Sorensen 2005; Christie and Ferguson-Lees 2010) with a regional field guide (Sinclair and Ryan 2010).

	Chrysococcyx flavigularis		*Cercococcyx mechowi*	
	Taxonomic source	Field guide	Taxonomic source	Field guide
Aviceda cuculoides	<1	<1	<1	<1
Polyboroides typus	<1	<1	<1	<1
Micronisus gabar	<0.25	<0.25	<0.50	<0.25
Accipiter tachiro	<1	<0.25	<0.75	<0.25
Accipiter ovampensis	<0.25	<0.25	<0.25	<0.25
Kaupifalco monogrammicus	<1	<1	<1	<1

Table S4.2. Cuckoo and raptor host prey species
(Brown et al. 1982; Del Hoyo et al. 1994; Payne 2005).

CUCKOOS	Species	Host/prey
	Eudynamys scolopacea	Crows (*Corvus splendens, C. macrorhynchos, C. enca, C. florensis, Urocissa erythrorhyncha*)
		Drongo (*Drongo macrocercus*)
		Common starlings
		Mynas (*Acridotheres tristis, A. grandis, Gracula religious*)
	Chrysococcyx flavigularis	Tit-flycatcher (*Myioparus griseigularis*)
	Cacomantis sonneratii	Loras (*Aegithina tiphia, A. viridissima*)
		Bulbuls (*Pycnonotus jocose*)
		Yuhinia (*Yuhinia zantholeuca*)
		Cuckoo-shrike (*Pericrococtus flames*)
		Babblers (*Stachyris*)
	Cercococcyx mechowi	Illadopsis (*Trichastoma fulvescens*)
		Forest robin (*Stiphrornis erythrothorax*)
		Monarch-flycatcher (*Trochocercus nitens*)
	Cuculus saturatus	Warblers (genus *Seicercus, Phylloscopus occipitals, P. reguloides*)
RAPTORS	*Aviceda cuculoides*	Small birds
	Aviceda leuphotes	Asian palm swift (*Cypsiurus batasiensis*)
	Pernis ptilorhyncus	Small birds (Passerines)
	Polyboroides typus	Weavers
		Swifts
		Herons
		Barbet
		Roller
		Kingfisher
		Birds eggs - sparrow to darter (*Anhinga melanogaster*).
	Micronisus gabar	Pipits
		Weaver birds
		Starlings

	Barbets
	Thrush
	Coucals
	Francolins
Accipiter trivirgatus	Small birds
	Pigeon (*Treron*)
Accipiter tachiro	Doves (*Streptopelia semitorquata*)
	Cuckoo (*Chrysococcyx klaas*)
	Mousebirds
	Trogon
	Hornbills
Accipiter soloensis	Pigeon (*Columba livia*)
Accipiter fasciatus	Oriole (*Oriolus chinensis*)
	Pigeons
	Ducks
	Herons
	Rails
	Poultry
Accipiter gularis	Fruit doves (*Ptilinopus*)
	Sparrows (*Passer montanus*)
	Buntings (*Emberiza*)
	Tits (*Parus*)
	Warblers
	Nuthatches (*Sitta*)
	Magpie (*Cyanopica cyanea*)
Accipiter virgatus	Gamebirds (nudifigous young *Gallus*)
	Warblers
	Thrushes
	Barbets
Accipiter ovampensis	Doves
	Bee-eaters
	Hoopoes
	Woodpeckers
	Pipits
	Weaver birds

	Prinia
Kaupifalco monogrammicus	Rarely small birds
Spizaetus nanus	Young or injured birds including Blackbirds (*Turdus merula*)
	Some birds

Table S4.3a. The individual types of habitat inhabited by cuckoos and sympatric raptors of Africa (Payne and Sorensen 2005; Christie and Ferguson-Lees 2010).

Habitat	*Chrysococcyx flavigularis*	*Cercococcyx mechowi*	*Aviceda cuculoides*	*Polyboroides typus*	*Micronisus gabar*	*Accipiter tachiro*	*Accipiter ovampensis*	*Kaupifalco monogrammicus*
Primary forest	Yes	-	Yes	-	-	Yes	-	Yes
Secondary and gallery forest	Yes	-	-	Yes	-	Yes	-	-
Lowlands	Yes	-	-	-	-	Yes	-	-
Lowland mature forest	-	Yes	-	-	-	Yes	-	-
Montane forest	-	Yes	-	-	-	-	-	-
Forest edge	-	Yes	-	Yes	-	-	-	Yes
Riverside gallery	-	-	Yes	Yes	Yes	Yes	-	Yes
Savannah	-	-	Yes	Yes	Yes	Yes	Yes	Yes
Eucalyptus and pine plantations	-	-	Yes	Yes	-	Yes	Yes	-
Suburban gardens	-	-	Yes	-	Yes	Yes	-	Yes
Dry woodland	-	-	-	-	-	Yes	Yes	Yes
Mountain woodland	-	-	-	-	-	Yes	-	-

Tropical rainforest	-	-	-	-	-	Yes	-	-
Mangroves	-	-	-	-	-	Yes	-	-
Broadleaf woodland	-	-	-	-	-	-	-	Yes
Open woodland	-	-	-	-	Yes	-	-	-
Moist woodland	-	-	-	Yes	-	-	-	-
Hill country	-	-	-	Yes	-	-	-	-

Table S4.3b. The individual types of habitat inhabited by cuckoos and sympatric raptors of Oceania (Ferguson-Lees and Christie 2001; Payne 2005).

Habitat	*Eudynamys scolopacea*	*Cacomantis sonneratii*	*Cuculus saturatus*	*Aviceda leuphotes*	*Pernis ptilorhyncus*	*Accipiter trivirgatus*	*Accipiter soloensis*	*Accipiter fasciatus*	*Accipiter gularis*	*Accipiter virgatus*	*Spizaetus nanus*
Primary forest	Yes	Yes	Yes	Yes	-	Yes	-	Yes	-	-	Yes
Lowlands	Yes	Yes	-	-	Yes	Yes	Yes	-	Yes	-	Yes
Forest edge	Yes	Yes	-	Yes	-	-	-	Yes	-	-	-
Secondary forest	Yes	Yes	-	Yes	-	Yes	-	Yes	Yes	Yes	Yes
Remnant woodlands with large trees	Yes	-	-	-	-	-	Yes	-	-	-	-
Monsoon forest	Yes	-	-	-	-	-		-	-	-	-
Riverine scrub	Yes	-	-	-	-	-	Yes	-	-	Yes	-
Cultivated/plantations	Yes	Yes	-	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-
Heath forest	Yes	-	-	-	-	-	-	Yes	-	-	-
Gardens	Yes	Yes	-	Yes	-	-	Yes	Yes	-	-	-
Mangrove/swamp	Yes	-	-	Yes	-	-	Yes	-	Yes	-	-
Coniferous-deciduous forest	-	-	Yes	Yes	-	Yes	-	-	Yes	Yes	-
Larch taiga	-	-	Yes	-	-	-	-	-	-	-	-
Broad-leafed forest	-	Yes	Yes	-	Yes	-	-	-	-	-	-

Thickets	-	-	Yes	-	-	-	-	-	-	-	-
Oak rhododendron forests	-	-	Yes	-	-	-	-	-	-	-	-
Subtropical woodlands	-	-	Yes	-	-	-	Yes	-	-	-	-
Mountain forest	-	-	-	-	-	Yes	-	-	Yes	Yes	-
Savannah	-	-	-	-	-	-	-	Yes	-	-	-
Rainforest	-	-	-	-	Yes	-	-	-	-	-	-
Coastal plains	Yes	-	-	-	-	-	-	-	-	-	-
Wooded hills	Yes	-	-	-	-	-	-	-	-	-	-

Table. S5.1. A representative sample of literature of pattern type and pattern function in different contexts (stationary or moving) in animals. Literature cited covers birds, mammals, fish, reptiles, insects, cephalopods, crustaceans, and amphibians. Literature is categorized as 1) Hypotheses (theoretical hypotheses), 2) Experimental (direct empirical evidence), 3) Correlational/observational, 4) Comparative phylogenetic analyses, and 5) Methodological (proposing and/or testing new methods). Camouflage mechanisms cover the following: Stationary camouflage (Background matching [BM], disruptive camouflage [DC]) and motion camouflage (motion-dazzle [MD]), flicker-fusion [F-F]).

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
Irregular	Stationary	X		BM	N/A	N/A	(Poulton, 1890)	Hypotheses
	Stationary	X		BM and DC	N/A	N/A	(Thayer, 1909)	Hypotheses
	Stationary	X		DC	N/A	N/A	(Cott, 1940)	Hypotheses
	Stationary	X		BM and DC	Juvenile cuttlefish (*Sepia officinalis*)	N/A	(Hanlon & Messenger, 1988)	Experimental
	Stationary	X		DC	Frogs (*Limnodynastes tasmaniensis*)	Garter snake (*Thamnophis sirtalis*)	(Osorio & Srinivasan, 1991)	Experimental
	Stationary	X		BM	*Aythya* and *Somateria* ducks	N/A	(Hohman et al., 1992)	Hypotheses

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
	Stationary	X		DC	Marine isopod *Idotea baltica* (white-spotted phenotype *albafusca*)	N/A	(Merilaita, 1998)	Experimental
	Stationary	X		BM	Artificial paper moths	Captive trained birds	(Merilaita et al., 2001)	Experimental
	Stationary					Fish predators	E.g. (Chiao & Hanlon, 2001; Barbosa et al., 2004; Chiao et al., 2005; Barbosa et al., 2007; Chiao et al., 2007; Mäthger et al., 2007; Shohet et al., 2007; Barbosa et al., 2008; Mäthger	
Irregular	Stationary	X		BM and DC	Cuttlefish (*Sepia officinalis*)	Fish predators (Akkaynak et al., 2013; Chiao et al., 2013; Hanlon et al., 2013)		Experimental
		X		BM and DC	Cuttlefish (*Sepia officinalis*)			Experimental

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
							et al., 2008; Chiao et al., 2010; Chiao et al., 2011; Akkaynak et al., 2013; Chiao et al., 2013; Hanlon et al., 2013)	
	Stationary	X		BM	Artificial paper moths	Wild birds	(Cuthill et al., 2005)	Experimental
	Stationary	X		BM and DC	Artificial paper moths	Captive trained birds	(Merilaita & Lind, 2005)	Experimental
	Stationary	X		DC	Artificial paper moths	Wild birds	(Stevens & Cuthill, 2006)	Experimental
	Stationary	X		DC	Artificial paper moths	Wild birds	(Stevens et al., 2006)	Experimental
Irregular	Stationary	X		BM and DC	Artificial paper	Wild birds	(Cuthill et al., 2006)	Experimental

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
					moths			
	Stationary	X		BM and DC	Artificial paper moths (modelled on *Thyatira batis*)	Wild birds	(Schaefer & Stobbe, 2006)	Experimental
	Stationary	X		BM	Fiddler crabs (*Uca vomeris*)	Wild and dummy birds	(Hemmi et al., 2006)	Experimental
	Stationary	X		DC	Computer-generated moth images	Humans	(Fraser et al., 2007)	Experimental
	Stationary	X		DC (surface disruption)	Artificial paper moths	Wild birds	(Stevens et al., 2009)	Experimental
	Moving and stationary	X		DC/MD	Cuttlefish (*Sepia officinalis*)	N/A	(Zylinski et al., 2009)	Experimental
	Stationary	X		BM and DC	Artificial paper moths	Captive trained birds	(Dimitrova & Merilaita, 2010)	Experimental
	Stationary	X		BM	Octopuses (*Octopus cyanea* & *O.vulgaris*)	N/A	(Josef et al., 2012)	Experimental

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
Irregular	Stationary	X		DC	Computer-generated moth images	Humans	(Troscianko et al., 2013)	Experimental
	Stationary	X		BM and DC	Computer-generated moth images	Humans	Webster et al., 2013)	Experimental
	Moving and stationary	X		BM and DC	Computer-generated stimuli	Humans	(Hall et al., 2013)	Experimental
	Stationary	X		BM	Japanese quail (*Coturnix japonica*)	N/A	(Lovell et al., 2013)	Experimental
	Stationary	X		BM and DC	Moths (*Jankowskia fuscaria*)	N/A	(Kang et al., 2012, 2013a, 2013b; Kang et al., 2014)	Experimental
	N/A		X	N/A	Red-legged partridge (*Alectoris rufa*)	Red-legged partridge (*Alectoris rufa*)	(Pérez-Rodríguez et al., 2013)	Experimental
	Stationary	X		BM	Artificial paper	Captive trained	(DImitrova &	Experimental

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
					moths	birds	Merilaita, 2014)	
	Stationary	X		BM	Artificial paper moths	Captive trained birds	(Merilaita & DImitrova, 2014)	Experimental
Irregular TOTAL	**Stationary = 42 Moving & stationary = 2**	**44**	**1**	**BM = 35 DC = 33 MD = 1**	**9 species: birds, insects, cephalopods, crustaceans, amphibians**	**4 groups: birds, fish, snakes, humans**	**Ca. 45+**	**Experimental = 41/45 (91%) Hypotheses = 4/45 (8%)**

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
Regular								
	Moving	X		F-F	Brightly-coloured snakes, e.g. coral snakes	N/A	(Pough, 1976)	Hypotheses
	Moving	X		F-F	North American	N/A	(Jackson et al.,	Correlational/

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
					snakes (132 spp.)		1976)	observational
Regular	Moving	X		F-F	Guppies (*Poecilia reticulata*)	N/A	(Endler, 1980)	Experimental
	Moving	X		MD and F-F	Garter snakes (*Thamnophis ordinoides*)	N/A	(Brodie, 1989, 1992, 1993) *but see* Allen et al. 2013)	Correlational/ observational
	N/A	X		BM	Tiger (*Panthera tigris*)	N/A	(Godfrey et al., 1987)	Experimental
	N/A		X	N/A	Peafowl (*Pavo cristatus*)	Peafowl (*Pavo cristatus*)	(Petrie et al., 1991)	Correlational/ observational
	N/A	X		BM	Zebra, tigers	N/A	(Kiltie et al., 1994)	Methodological
	Moving	X		F-F	Vipera snakes	N/A	(Shine & Madsen, 1994) *but see* Allen et al. (2013)	Hypotheses

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
Regular	N/A		X	N/A	Zebra finches (*Taeniopygia guttata*)	Zebra finches (*Taeniopygia guttata*)	(Swaddle & Cuthill, 1994)	Experimental
	Moving	X		F-F	Adders (*Vipera berus*)	N/A	(Lindell & Forsman, 1996)	Correlational/ observational
	N/A		X	N/A	Mallard ducks (*Anas platyrhynchos*)	Mallard ducks (*Anas platyrhynchos*)	(Omland, 1996)	Correlational/ observational
	N/A	X		BM	Mammalian carnivores	N/A	(Ortolani, 1999)	Comparative phylogenetic
	N/A		X	N/A	Barn owls (*Tyto alba*)	Barn owls (*Tyto alba*)	(Roulin, 1999)	Experimental
	N/A	X		N/A	Artiodactyls	N/A	(Stoner et al., 2003)	Comparative phylogenetic
	N/A		X	N/A	N/A	N/A	(Kenward et al., 2004)	Comparative phylogenetic
	Moving and	X	X	Private UV	Damselfish	N/A	(Siebeck, 2004)	Experimental

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
	stationary			signals	(*Pomacentrus amboinensis*)			
	Stationary	X		DC	Clay models of adders (*Vipera latastei gaditana*)	Wild birds	(Niskanen & Mappes, 2005)	Experimental
	N/A		X	N/A	Red-legged partridge (*Alectoris rufa*)	Red-legged partridge (*Alectoris rufa*)	(Bortolotti et al., 2006)	Correlational/ observational
	Moving	X		MD	Cuttlefish (*Sepia officinalis*)	N/A	(Shohet et al., 2006)	Experimental
	Moving	X		MD and F-F	Computer-generated moving stimuli	Humans	(Stevens et al., 2008)	Experimental
	Stationary	X		DC	Artificial paper butterflies (modelled on *Limenitis camilla*)	Wild birds	(Stobbe & Schaefer, 2008)	Experimental

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
Regular	Stationary	X		Coincident disruptive camouflage	Artificial paper moths/computer-generated stimuli	Wild birds/humans	(Cuthill & Székely, 2009)	Experimental
	N/A		X	N/A	N/A	N/A	(Gluckman & Cardoso, 2009)	Methodological and Experimental
	N/A		X	N/A	Barn owls (*Tyto alba*)	Barn owls (*Tyto alba*)	(Roulin et al., 2010)	Correlational/ observational, Experimental and Comparative phylogenetic
	N/A	X		BM	Felidae	N/A	(Allen et al., 2011)	Comparative phylogenetic
	N/A		X	N/A	Barred buttonquails (*Turnix suscitator*)	Barred buttonquails (*Turnix suscitator*)	(Muck & Goymann, 2011)	Correlational/ observational

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
	Moving	X		MD and F-F	Computer-generated moving stimuli	Humans	(Scott-Samuel et al., 2011)	Experimental
	Stationary	X		BM	The least killfish (*Heterandria formosa*)	Predatory fish	(Kjernsmo & Merilaita, 2012)	Experimental
	Moving	X		MD	Computer-generated moving stimuli	Humans	(von Helversen et al., 2013)	Experimental
	Moving	X		MD	Computer-generated moving stimuli	Locusts (*Schistocerca gregaria*)	(Santer, 2013)	Experimental
	Moving	X		MD	Computer-generated moving stimuli	Motion detection algorithm	(How & Zanker, 2013)	Experimental
Regular TOTAL	**Stationary = 4 Moving = 14**	**24**	**10**	**F-F = 8 MD = 7 BM = 5**	**Ca. 13 species: birds, reptiles, cephalopods, fish,**	**4 groups: fish, insects, birds, humans**	**Ca. 33+**	**Experimental = 17/33 (52%) Correlational**

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
	Moving & stationary = 1			**DC = 3 Private signals = 1**	**mammals**			**comparative = 14/33 (42%) Hypotheses = 6%**
Irregular and regular	N/A	X		BM	Juvenile plaice (*Pleuronectes platessa*)	N/A	(Kelman et al., 2006)	Experimental
	Moving and stationary	X		MD, F-F, BM, DC	Computer-generated moving and static stimuli	Humans	(Stevens et al., 2011)	Experimental
	Stationary	X		BM	Artificial paper moths	Captive trained birds	(Dimitrova & Merilaita, 2012)	Experimental
	N/A	X		BM and DC	*Galaxias nebula* fish (Galaxiidae)	N/A	(Magellan & Swartz, 2013)	Experimental
	Moving	X		MD, F-F, BM, distractive	Computer-generated moving stimuli	Humans	(Hughes et al., 2014)	Experimental

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
				markings				
Irregular and regular TOTAL	**Stationary = 2 Moving & stationary = 1**	**5**	**0**	**BM = 5 DC = 2 MD, F-F = 3**	**2 species (fish)**	**2 groups (birds and humans)**	**Ca. 5+**	**Experimental = 5/5 (100%)**

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
Bimodal	Moving and stationary	X	X	Camouflage vs. signal efficacy	Guppies (*Poecilia reticulata*)	Various aquatic predators	(Endler, 1978)	Hypotheses and Experimental
	Moving and stationary	X	X	Predator avoidance vs. signal	N/A	N/A	(Endler, 1987)	Experimental

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
				efficacy				
	Moving and stationary	X	X	Distance-dependence	Reef fish (e.g. *Pygoplites diacanthus*)	Predatory fish	(Marshall, 2000)	Experimental
Bimodal	Stationary	X	X	Signal partitioning	Australian agamid lizards (*Agamidae*)	N/A	(Stuart-Fox & Ord, 2004)	Comparative phylogenetic
	N/A	X	X	Signal partitioning	*Bicyclus* butterflies	N/A	(Oliver et al., 2009)	Comparative phylogenetic
	N/A	X	X	Signal partitioning	Birds	N/A	(Gluckman & Cardoso, 2010)	Comparative phylogenetic
	Stationary	X	X	Background-matching vs. conspicuous signalling	Giant cuttlefish (*Sepia apama*)	N/A	(Zylinski et al., 2011)	Experimental
	N/A	X	X	Predator avoidance vs. sexual	Australian dragon lizards (*Agamidae*)	N/A	(Chen et al., 2012)	Comparative phylogenetic

Pattern type	Behavioural context	Camouflage	Communication	Camouflage mechanism	Prey or signaller (where tested)	Predator or signal receiver (where tested)	References	Type of literature/ evidence
				dichromatism				
	Stationary	X	X	Signal partitioning	Australian mallee dragon lizards (*Ctenophorus fordi*)	Avian predators	(Garcia et al., 2013)	Experimental
Bimodal TOTAL	**Stationary = 3 Moving & stationary = 3**	**9**	**9**	**Multiple function = 5 Signal partitioning =4**	**Ca. 4+ species: reptiles, cuttlefish, birds, insects, fish**	**2 groups (fish, birds)**	**Ca. 9+**	**Experimental = 5/9 (55%) Comparative = 4/9 (45%)**

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Table S5.2. The number of bird species with plumage patterns in the 16 habitat types of the world across the class Aves.
Plumage patterns represent breeding and non-breeding plumage of males and females, as well as juveniles, on the ventral and dorsal surface.
Species numbers are averaged across eco-regions for each habitat. T = Temperate; and T/S - Tropical and Subtropical.

Sex/ age	Season	Location	Pattern	Boreal forest /taiga	Desert and xeric shrubland	Flooded grassland and savanna	Inland water	Mangrove	Mediterranean forest, woodland and scrub	Montane grassland and shrubland	Rock and Ice	T broadleaf and mixed forest	T conifer forest	T grassland savanna and shrubland	T/S coniferous forest	T/S dry broadleaf forest	T/S grassland savanna and shrubland	T/S moist broadleaf forests	Tundra
Male	Breeding	Ventral	All	23.4	33.5	39.7	96.7	66.4	27.3	49.9	32.0	27.4	32.4	30.3	49.4	49.7	72.8	54.2	12.1
			Mottled	11.9	16.6	19.1	40.3	32.2	13.1	23.0	15.7	14.0	16.5	15.1	23.6	22.3	34.1	25.3	5.4
			Scaled	1.0	3.8	5.2	12.7	9.0	3.6	5.7	2.0	3.2	3.3	3.0	7.2	7.6	9.0	7.6	0.5
			Barred	11.4	12.0	13.7	41.0	22.4	9.8	19.1	14.7	10.4	12.7	11.6	16.9	17.0	26.1	18.9	6.8
			Spotted	1.6	3.3	4.0	8.3	6.1	3.3	4.7	2.3	1.9	2.9	2.4	5.4	5.6	6.9	5.1	0.8
		Dorsal	All	26.5	44.8	51.7	110.7	83.4	37.1	65.3	39.3	35.3	41.7	36.9	60.2	62.0	90.8	67.5	13.5
			Mottled	14.5	24.9	29.6	60.0	47.8	22.2	37.4	23.0	21.0	23.6	21.2	31.1	34.3	52.8	38.0	7.6
			Scaled	2.2	4.4	4.2	11.7	8.5	3.6	6.7	4.0	4.2	4.3	3.8	6.1	6.1	7.3	6.7	0.7
			Barred	8.1	10.7	13.2	36.0	21.5	9.1	18.0	13.7	9.5	11.4	8.7	16.7	16.5	26.4	19.0	5.3
			Spotted	6.5	10.4	10.8	18.3	15.5	8.1	12.3	7.3	6.6	8.6	7.8	13.9	12.1	15.4	12.0	3.0
	Non-breeding	Ventral	All	23.7	33.7	39.9	96.7	66.9	26.9	49.8	32.0	27.5	32.8	30.3	50.1	50.0	72.2	54.4	12.3
			Mottled	12.3	16.7	19.6	40.7	33.4	13.1	23.3	16.0	14.5	16.9	15.3	24.4	23.1	34.6	26.1	5.6
			Scaled	0.9	3.5	4.9	12.3	8.2	3.3	5.3	1.3	2.9	3.1	2.8	7.2	7.0	8.6	7.0	0.5
			Barred	11.2	12.5	14.0	41.3	23.2	9.8	19.3	15.7	10.5	13.2	11.6	18.1	18.0	25.7	19.4	6.7
			Spotted	1.6	3.1	3.6	8.0	5.4	3.2	4.4	1.7	1.8	2.5	2.3	4.3	4.7	6.7	4.5	0.8
		Dorsal	All	27.1	45.8	52.5	112.7	86.5	37.2	66.2	40.0	35.9	42.6	37.3	64.2	64.9	91.5	69.4	13.8
			Mottled	15.7	25.5	30.6	62.3	49.7	22.4	38.0	23.0	21.5	24.0	21.5	33.3	36.2	53.7	39.2	8.3

Sex/ age	Season	Location	Pattern	Boreal forest /taiga	Desert and xeric shrubland	Flooded grassland and savanna	Inland water	Mangrove	Mediterranean forest, woodland and scrub	Montane grassland and shrubland	Rock and Ice	T broadleaf and mixed forest	T conifer forest	T grassland savanna and shrubland	T/S coniferous forest	T/S dry broadleaf forest	T/S grassland savanna and shrubland	T/S moist broadleaf forests	Tundra
			Scaled	2.0	4.6	4.0	10.7	8.1	3.7	6.3	4.0	4.1	4.8	3.9	7.0	6.1	6.5	6.5	0.6
			Barred	8.0	11.1	13.5	36.3	22.8	9.0	18.8	14.3	9.6	11.3	8.5	17.8	17.6	27.1	20.1	5.1
			Spotted	6.5	10.4	11.1	18.7	16.1	8.2	12.6	7.3	6.7	8.7	7.9	14.0	12.2	16.0	12.1	3.0
Female	Breeding	Ventral	All	32.4	43.3	49.5	117.0	81.3	37.7	61.9	43.3	36.6	43.8	40.0	61.1	59.5	89.4	66.9	17.3
			Mottled	16.2	21.3	24.4	51.3	41.4	18.5	30.2	21.7	18.4	21.9	19.0	29.9	27.5	45.1	32.2	7.8
			Scaled	4.2	5.4	7.0	15.7	10.4	5.1	7.1	3.3	4.9	4.9	5.1	8.6	8.8	10.8	9.1	2.2
			Barred	13.2	14.7	15.8	45.3	26.2	12.5	21.5	18.0	12.9	16.6	14.4	20.4	20.4	29.1	23.0	8.2
			Spotted	2.3	4.4	4.7	11.0	6.7	4.7	5.8	3.3	3.3	3.9	3.9	5.6	5.9	7.9	5.8	0.9
		Dorsal	All	29.2	49.9	56.8	121.7	94.5	40.6	71.8	45.0	39.1	46.5	39.6	71.4	70.1	99.1	75.7	15.2
			Mottled	17.5	28.9	34.5	69.3	56.8	23.8	43.7	27.3	23.4	27.0	23.8	39.2	40.3	61.4	44.6	9.3
			Scaled	1.6	4.4	4.3	11.0	8.1	4.1	5.9	3.7	3.7	4.1	3.1	6.9	6.4	7.1	6.7	0.4
			Barred	10.5	12.7	14.3	36.7	23.7	11.1	18.2	15.3	12.5	14.2	10.7	19.8	18.9	26.1	20.9	6.2
			Spotted	5.8	9.8	10.3	19.0	15.4	7.6	12.7	7.0	6.1	7.7	6.9	13.4	11.9	16.2	11.8	3.1
	Non-breeding	Ventral	All	32.1	43.2	49.4	116.7	81.5	37.4	61.7	43.3	36.2	43.5	39.5	61.2	59.7	89.3	67.1	17.1
			Mottled	15.9	21.2	24.2	51.3	41.5	18.4	30.2	21.7	18.2	21.7	18.8	30.1	27.8	45.0	32.3	7.6
			Scaled	4.2	5.4	7.0	15.7	10.3	5.1	7.0	3.3	4.9	4.9	5.1	8.6	8.8	10.8	9.1	2.2
			Barred	13.2	14.6	15.8	45.0	26.3	12.4	21.4	18.0	12.6	16.5	14.0	20.4	20.3	29.1	22.9	8.2
			Spotted	2.3	4.4	4.7	11.0	6.7	4.7	5.8	3.3	3.3	3.9	3.9	5.6	5.9	7.9	5.8	0.9
		Dorsal	All	28.9	49.9	56.8	121.3	94.5	40.4	71.7	45.0	38.7	46.2	39.2	71.4	70.0	99.1	75.6	15.0
			Mottled	17.2	28.9	34.6	69.3	56.8	23.7	43.7	27.3	23.2	26.8	23.6	39.2	40.3	61.4	44.6	9.1
			Scaled	1.6	4.4	4.3	11.0	8.0	4.1	5.9	3.7	3.7	4.1	3.1	6.9	6.4	7.1	6.7	0.4

Sex/ age	Season	Location	Pattern	Boreal forest /taiga	Desert and xeric shrubland	Flooded grassland and savanna	Inland water	Mangrove	Mediterranean forest, woodland and scrub	Montane grassland and shrubland	Rock and Ice	T broadleaf and mixed forest	T conifer forest	T grassland savanna and shrubland	T/S coniferous forest	T/S dry broadleaf forest	T/S grassland savanna and shrubland	T/S moist broadleaf forests	Tundra
			Barred	10.5	12.6	14.2	36.3	23.8	10.9	18.1	15.3	12.3	14.1	10.3	19.8	18.8	25.9	20.9	6.2
			Spotted	5.8	9.8	10.3	19.0	15.4	7.6	12.7	7.0	6.1	7.7	6.9	13.4	11.9	16.2	11.8	3.1
Juvenile	N/A	Ventral	All	24.7	38.7	44.7	102.0	74.1	32.6	54.3	33.3	30.2	36.0	31.8	57.8	55.6	76.1	59.7	12.3
			Mottled	15.6	23.6	27.2	59.3	45.1	17.7	31.7	19.3	19.0	22.8	19.8	36.0	31.8	43.7	33.7	7.3
			Scaled	2.4	3.4	4.8	9.0	6.3	3.1	4.4	1.7	2.7	2.8	3.2	4.8	5.1	8.2	5.4	1.3
			Barred	7.7	11.2	11.7	32.7	20.7	11.5	17.1	13.7	10.1	12.4	9.9	16.1	16.0	22.2	18.2	4.3
			Spotted	1.6	2.7	3.2	7.0	5.0	2.8	3.5	1.0	1.5	1.9	1.8	4.2	4.7	5.1	4.5	0.9
		Dorsal	All	23.1	39.8	46.7	96.7	75.9	36.1	57.9	35.0	31.3	36.0	31.2	53.9	54.1	84.0	61.6	12.0
			Mottled	14.8	25.4	31.6	62.7	49.4	21.2	38.1	22.0	20.2	23.4	20.9	36.7	35.7	53.0	38.3	7.7
			Scaled	0.9	4.3	4.9	9.3	8.6	4.2	4.9	2.7	3.0	3.3	2.4	5.2	6.5	8.6	7.4	0.4
			Barred	6.7	7.1	7.0	19.0	12.7	7.0	10.4	10.0	7.3	7.9	5.6	8.9	7.9	16.9	11.2	3.5
			Spotted	3.6	6.0	6.3	12.7	9.4	6.9	8.3	4.7	4.2	4.6	5.0	6.6	6.9	10.9	8.0	1.7

Dearest Africa, thank you for being my inspiration for change. Sorry I did not come back permanently, I ended up getting a PhD instead.

Ngorongoro crater, 2002.