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Testing visual ecology hypotheses in avian brood parasite-host systems: the role of UV-light perception and egg-nest contrast in foreign egg rejection

by

Zachary Aidal

A dissertation submitted to the Graduate Faculty in Psychology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York Graduate Center

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Abstract

Testing visual ecology hypotheses in avian brood parasite-host systems: the role of UV-light perception and egg-nest contrast in foreign egg rejection

by

Zachary Aidala

Adviser: Dr. Mark E. Hauber

Color signals are highly important features of animal communication systems, particularly among birds, which possess exquisitely complex visual perception systems. Birds possess tetrachromatic vision, and some species are sensitive to ultraviolet (UV) wavelengths. Because human and avian visual systems dramatically differ (i.e. humans are not sensitive to UV wavelengths), biologically relevant sensory models are necessary to accurately assess the function of avian color signals. In this dissertation, I primarily use brood parasite-host interactions as a model for studying the behavioral function of avian-perceivable visual stimuli. In Chapter 1, I review the importance of employing biologically relevant sensory-perceptual visual models when testing visual ecology hypotheses. Most models of avian visual space require the input of physiological parameters, such as the relative densities of cone photoreceptors. I also review methodologies that can be employed to increase the accuracy of visual models themselves. One such method is DNA sequencing of the short-wavelength sensitive type 1 (SWS1) opsin to assess the degree of UV-light sensitivity. Avian species possess variable sensitivities to UV wavelengths based on the amino acids present at key ‘spectral tuning’ sites, and DNA sequencing of the SWS1 opsin gene allows for accurate assessment of the photoreceptor opsin’s maximal sensitivity. In Chapters 2 and 3, I report predicted sensitivities to
UV light signals based on DNA sequencing of the key ‘spectral tuning’ region of the SWS1 opsin in a number of species spanning four avian lineages, including passerine hosts of obligate brood parasitic North American brown-headed cowbird (*Molothrus ater*) and Australasian shining-bronze cuckoo (*Chrysococcyx lucidus*) and long-tailed cuckoo (*Eudynamis taitensis*). I specifically tested the UV-matching hypothesis, which suggests that seemingly non-mimetic parasitic eggs (based on human vision) may be accepted by hosts due to parasite eggshell mimicry at UV wavelengths. While the UV-matching hypothesis garnered some previous empirical support among African parasite-host systems, I did not find evidence of UV-matching as it relates to egg rejection behaviors by hosts of the brown-headed cowbird. In absence of support for the UV-matching hypothesis, in Chapter 4 I tested the long-standing but largely untested assumption of brood parasitism that visual comparisons between eggs *per se* drive egg rejection behavior. To do this, I examined whether egg-nest visual contrasts contribute to egg rejection decisions in the American robin, a robust rejecter of natural cowbird parasitism. I experimentally increased/decreased parasitic egg-nest contrast in an artificial brood parasitism experiment, and predicted that foreign eggs with low visual contrast against the nest lining (i.e. were more cryptic) would be rejected more often than foreign eggs with high visual contrast against the nest lining. I employed a perceptual modeling approach that compares reflectance spectra across the avian spectral sensitivity range to assess the degree of contrast between eggs and nests. I found that egg-nest contrast did not significantly affect artificial egg ejection rates, instead artificial eggs were rejected at rates similar to those observed in non-manipulated nests. In this host-parasite system, egg rejection behavior is most likely driven by differences between eggs themselves. In Chapter 5, I show novel phylogenetic relationships of the previously unresolved endemic New Zealand Passeriformes genus *Mohoua*, only one species of which is an
ejector host of artificial long-tailed cuckoo (*Eudynamis taitensis*) eggs. Because the predicted sensitivity to UV wavelengths now exists for only one *Mohoua* species, such well-resolved phylogenies are integral for comparative analyses that map life history traits with respect to the evolution of defenses against brood parasitism. Overall, the collection of manuscripts presented in this dissertation test specific sensory hypotheses related to the visual ecology of brood parasite hosts. Specifically, I found minimal empirical support for a major role of UV wavelengths and egg-nest visual contrasts in parasitic egg rejection among hosts of the brown-headed cowbird. Lastly, phylogenetic analysis of a largely under-studied New Zealand brood parasite-host system paves the way for novel tests of visual ecology hypothesis from a comparative perspective.
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Chapter 1

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Avian Egg Coloration and Visual Ecology

Zachary Aidala & Mark E. Hauber

Evolutionary processes have generated diverse color patterns of avian feathers, skin patches, and egg shells, which perform countless functions, including mimicry, crypsis, prey detection, predator avoidance, and signaling individual identity or mate quality (Hill & McGraw 2006). If color patterns function to communicate information, then do we need to understand the sensory and perceptual systems responsible for detecting these complex patterns? Recent technological and theoretical efforts have revolutionized the study of avian vision so that we now can use genetic sequencing of the opsin genes involved in avian color vision to reconstruct light-receptor sensitivity parameters, and this information can be used in perceptual models of birds' vision. Combined, these two approaches allow for a better understanding of the role that visual ecology plays in the evolution of avian communication and recognition systems, especially in the study of egg mimicry, ultraviolet (UV) light sensitivity, and their role in shaping the sensory ecology and behavioral patterns of diverse bird species (Hubbard et al. 2010).

The Evolution of Egg Color Patterns

Why do bird eggs range in color from uniformly white to brightly colored and/or densely speckled (maculated; see Banner photo)? Based on a comparison of eggshell patterns between different avian families, one of the most prevalent ecological factors responsible for the diversity of egg coloration is the interaction between brood parasites and their hosts (Kilner 2006). Obligate brood parasitic birds lay their eggs in nests of other species, thereby imposing a cost on
hosts to raise genetically unrelated young (Davies 2000). Egg coloration and maculation play important roles in whether hosts accept or reject the fitness costs imposed by parasitism. For example, the blackcap (*Sylvia atricapilla*) is a host of the parasitic common cuckoo (*Cuculus canorus*) in Europe and typically rejects all non-mimetic (dissimilar) eggs (Honza *et al.* 2004). By experimentally parasitizing blackcap nests with host-like mimetic eggs (using eggs of other blackcaps), egg rejection drops to 36% (Polacikova *et al.* 2007). Accurate rejection of foreign eggs even at seemingly low rates can still be an adaptive behavior because the host reduces its chances of spending time and energy raising overly needy and genetically unrelated offspring.

These and other cuckoo hosts appear to have evolved a simple rule of thumb to direct their behavior: "eject the egg unlike your own". But how does a bird know what its own eggs look like? Researchers have tackled this question by experimentally manipulating the appearance of the bird's own egg by dying one, more, or all eggs in the same brood (Figure 1.1). Such studies reveal that great reed warbler (*A. arundinaceus*) hosts rely on both color differences between eggs and learned memories of their own eggs to recognize and reject cuckoo eggs (Moskát *et al.* 2010).
Truly astounding, though, is that selection for visual cues of recognition has resulted in the evolution of extreme level of egg color mimicry of specific hosts by different parasitic
cuckoos (Figure 1.2). Through the process of coevolutionary arms race, egg mimicry also has likely influenced the perceptual sensitivities of hosts and their abilities to correctly identify and reject foreign eggs from the nest. The perceptual acuity necessary to make a correct rejection invites direct investigation; researchers can experimentally parasitize the nests of hosts with eggs of varying degrees of similarity, in order to determine the thresholds in color and maculation at which hosts make decisions to reject dissimilar, and likely foreign, eggs from their nest.

The cone photoreceptors of the vertebrate retina (Figure 1.3) are

**Figure 1.2**: Nests with both host and parasitic common cuckoo eggs, illustrating near-perfect mimicry to the human eye. Black arrows identify cuckoo egg.

**Avian Color Perception**

Before an experimenter can set out to manipulate egg colors, especially the ones hypothesized to be important for foreign egg rejection, it must be first established which colors that host species can see. The cone photoreceptors of the vertebrate retina (Figure 1.3) are
The genes for opsins encode specific photopigments expressed in these cones, generating different combinations of proteins with maximal sensitivities to a particular wavelength of light ($\lambda_{\text{max}}$). The cone cells of all color-sensitive vertebrates express opsins. Avian retinas differ from those of mammals in many ways, notably in the number of cone types that they possess. Unlike mammals, which typically have only two or three different cone types.
types (Figure 1.4), bird species possess 4 distinct single cone types in their retinas, making them tetrachromatic (Figure 1.5; Hunt et al. 2009). Tetrachromats are theoretically able to see twice as many colors as trichromats (e.g., humans). For example, two eggs might appear indistinguishable to us, but a bird might see them displaying two distinct colors. This has direct implications for scientific investigations of avian perception — how can we manipulate egg colors when birds themselves may be more sensitive than we are to subtle differences in color?

Some avian lineages, including many passerines, are able to see light in the ultraviolet (UV) range, which humans cannot see (Hart 2001). The hosts of brood-parasitic cuckoos in
Europe and brown-headed cowbirds (*Molothrus ater*) in North America are passerines, implying that they might be able to perceive cryptic (to human) UV differences between their own and parasitic eggs to discriminate and reject parasitic eggs. One of the types of opsins (SWS1) is sensitive to the shortest wavelengths of light, and is found in all vertebrate classes (Hazel *et al.* 2006). In humans and many bird species, the SWS1 opsin is expressed in cones that respond maximally to violet light (such species are termed violet-sensitive, VS). In some passerine species, however, the SWS1 opsin gene codes for a photoreceptor with a $\lambda_{\text{max}}$ that crosses into the UV portion of the light spectrum (Figure 1.5). Species with this type of SWS1 cone are UV-sensitive (UVS; Hart 2001; Ödeen & Håstad 2003; Hunt *et al.* 2009).
A UV-sensitive SWS1 is apparently ancestral among vertebrates, but was subsequently lost in primates and birds (Yokoyama 2000; Jacobs & Rowe 2004; this is a great review for those interested in the evolution of color vision in vertebrates). Among birds, however, UVS has re-evolved independently at least 4 times via a shift in SWS1 sensitivity (Hunt et al. 2009). UV-sensitivity in turn can serve a number of adaptive behavioral and ecological functions, including sexual displays, predator/prey detection, intraspecific communication to avoid detection by VS predators (Håstad et al. 2005), and defense mechanisms against egg mimicry in brood parasitism (Honza et al. 2007; Underwood & Sealy 2008). These two latter studies demonstrate that the UV-reflectance of eggs differs between host and parasite, suggesting that hosts can use UV-only visible patterns to discriminate between their own and foreign eggs. Whether UV-sensitivity evolved as a response to brood parasitism or was already available for hosts to utilize at the onset of their evolutionary history with brood parasitism, remains still unknown (Underwood & Sealy 2008).

Regarding other ecological contexts, eggs of cavity-nesting species tend to have higher UV-reflectance than eggs of open cup nesters (Aviles et al. 2006), providing further evidence that UV light can both be seen and be informative for parental birds' behavioral decisions. Accordingly, cavity-nesting spotless starlings (Sturnus unicolor) are more likely to accept experimental eggs placed just outside the nest cup within the cavity (by pulling them into the nest) with high UV-reflectance than eggs with low UV-reflectance.

But how can we know whether the SWS1 opsin of a particular bird species will be maximally sensitive to UV or violet wavelengths of light? Much of our knowledge of the avian sensory world now derives from physiological and molecular techniques which describe the sensitivities of opsins present in the eye. The traditional method of microspectrophotometry
allowed researchers to determine the \( \lambda \)-max of any photoreceptor by transmitting light through it and measuring which wavelengths are absorbed (Govardovskii et al. 2000). More recently, DNA sequencing of the SWS1 opsin gene has allowed researchers to assign VS/UVS states in a more cost-effective and non-lethal manner, relevant for large scale comparative studies (Ödeen & Håstad 2003), including work with bird species of conservation concern for which invasive studies cannot be done (Igic et al. 2010).

The molecular machinery of the SWS1 photoreceptor requires only one amino acid substitution in a select few sites of the protein's amino-acid chain to change a VS species or individual to a UVS species or individual (Yokoyama et al. 2000). Genetic sequencing of the SWS1 opsin gene is now regarded as an accurate, reliable and economical alternative to microspectrophotometry (but see Smith et al. 2002).

**Perceptual Modeling of the Avian Visual System**

Integrative research spanning the fields of molecular genetics, physical light reflectance measurements, and behavioral experiments, has allowed researchers to quantify color patterns as birds would see and use them (Vorobyev & Osorio 1998; Endler & Mielke 2005). To interpret physiological and genetic data, however, requires perceptual models which are mathematical representations of what a bird can see, based on a number of different parameters, including the amount of light that reaches the retina and the relative abundance and type of photoreceptors present in that particular species' eyes. Using physiological data generated from genetic sequencing of the opsin genes (Ödeen & Håstad 2003), researchers can now produce reasonably accurate models of avian visual perception and its behavioral implications in egg rejection decisions (Cassey et al. 2008). Typically, the light reflectance of surfaces of interest, such as
eggshells, is measured with a spectrophotometer and the resulting relative light reflectance data are then filtered through the perceptual model's equations to assess whether a species in question can see differences between particular light reflectance patterns, or colors.

**Color Vision Links Sensory Ecology with Behavioral Decisions**

Perceptual modeling has been adapted to study a wide range of phenomena; these include the perceived variability in eggshell colorations across many bird species (e.g. Cassey *et al.* 2009; Cassey *et al.* 2010), the adaptive use of human-made refuse as nesting material (Igic *et al.* 2009), as well as sexual dimorphism (Igic *et al.* 2010). Future studies of perceptual modeling should focus on the differences in egg colors between brood parasites and their hosts, and whether or not hosts are visually equipped to perceive these differences. Overall, molecular techniques and sensory modeling now allow researchers to begin to study the mechanisms underlying avian color vision, and do not require severely invasive methods. These integrative approaches make it possible for future researchers to accurately describe and manipulate salient color information in studies of mimicry, crypsis, mate quality, and other behavioral functions critical for survival and reproduction across diverse species of birds and other visually oriented animal lineages.
References and Recommended Reading


Chapter 2

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**Ultraviolet visual sensitivity in three avian lineages: paleognaths, parrots, and passerines**

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**Keywords**

Avian communication, sensory ecology, perching birds, SWS1 opsin, ultraviolet vision
Abstract

Ultraviolet (UV) light-transmitted signals play a major role in avian foraging and communication, subserving functional roles in feeding, mate choice, egg recognition, and nestling discrimination. Sequencing functionally relevant regions of the short wavelength sensitive type 1 (SWS1) opsin gene that is responsible for modulating the extent of SWS1 UV-sensitivity in birds allows predictions to be made about the visual system’s UV sensitivity in species where direct physiological or behavioral measures would be impractical or unethical. Here, we present SWS1 segment sequence data from representative species of three avian lineages for which visually based cues for foraging and communication have been investigated to varying extents. We also present a preliminary phylogenetic analysis and ancestral character state reconstructions of key spectral tuning sites along the SWS1 opsin based on our sequence data. The results suggest ubiquitous ultraviolet SWS1 sensitivity (UVS) in both paleognaths, including extinct moa (Emeidae), and parrots, including the nocturnal and flightless kakapo (Strigops habroptilus), and in most, but not all, songbird (oscine) lineages, and confirmed violet sensitivity (VS) in two suboscine families. Passerine hosts of avian brood parasites included both UVS and VS taxa, but sensitivity did not co-vary with egg rejection behaviors. The results should stimulate future research into the functional parallels between the roles of visual signals and the genetic basis of visual sensitivity in birds and other taxa.
2.1. Introduction

Color cues are ubiquitous features of avian orientation, foraging, and communication systems, and studies of (co)variation in visual signals and their associated sensory bases of vision in birds have provided a critical model system for the evolution of avian molecular, morphological, sensory, and behavioral diversity (Hill and McGraw 2006). In many bird species, color perception provides the behavioral basis for mate choice, predator avoidance, prey acquisition, and egg or nestling identification (Hubbard et al. 2010). Birds have highly complex visual systems, possessing five separate classes of cone photoreceptors, four of which directly contribute to color perception by absorbing maximally at wavelengths in the range of 360 – 570 nm of light, resulting in tetrachromacy (Hunt et al. 2009). In recent years the role of avian visual signals outside of the human perceptual range, specifically in the UV wavelengths (< 400 nm), has garnered increasing attention especially with respect to the evolution of private communication channels (Hauber et al. 2000; Hart 2001; Hauber et al. 2001; Hauber and Sherman 2001; Ödeen and Håstad 2003; Goth and Evans 2004; Cuthill 2006; Underwood and Sealy 2008). Accordingly, many UV-based visual signals are known to play important roles in both the interspecific and intraspecific communication behaviors of many bird species (e.g. Bennett and Cuthill 1994).

2.1.1 Molecular basis of UV-sensitivity

Sequencing a short ‘spectral tuning’ region of the avian SWS1 opsin gene allows for accurate prediction of the degree of UV-sensitivity in diverse avian taxa (Ödeen and Håstad 2003; Carvalho et al. 2011; Machovsky Capuska et al. 2011). Site-directed mutagenesis, combined with in vitro expression work, has implicated a number of “spectral tuning” sites along
the SWS1 photoreceptor, all of which are in the transmembrane (TM) II region of the protein (Hunt et al., 2009). For example, a single C90S substitution (following the bovine Bos taurus rhodopsin numbering) in the UV-sensitive SWS1 opsin of the budgerigar (Melopsittacus undulatus) produces a long-wave shift, consistently altering the SWS1 photoreceptor’s maximal sensitivity by approximately 35 nm to light from 363 nm to 398 nm (Wilkie et al. 2000). Similarly, the same substitution in the UV-sensitive SWS1 opsin of the zebra finch (Taeniopygia guttata) shifts the SWS1 opsins peak spectral sensitivity from 359 nm to 397 nm, from UV towards the violet portion of the light spectrum (Yokoyama et al. 2000). The converse S90C shift in both the violet-sensitive pigeon (Columba livia) and chicken (Gallus gallus) SWS1 opsins produces a short-wave shift in spectral sensitivity from 393 nm towards the UV range of 359 nm and from 415 nm to 369 nm, respectively (Yokoyama et al. 2000). Shi et al. (2001) demonstrated five separate residues as important spectral tuning sites among mammals, though residues 86, 90, 93, and 118 appear to be the most important spectral tuning sites among avian species. Specifically, A86S, T93V, and A118T substitutions alter the maximal sensitivity of the SWS1 opsin -1 nm and 3 nm, and 3 nm respectively in the budgerigar SWS1 opsin (Wilkie et al. 2000). This suggests that residue 90 is singularly important in mediating the spectral tuning of the SWS1 opsin.

By convention, avian SWS1 opsins with a maximal sensitivity < 400 nm are designated UV-sensitive (UVS) while those with a maximal sensitivity ≥ 400 nm are designated violet-sensitive (VS; Hart 2001; Ödeen and Håstad 2003; Hunt et al. 2009). Sequencing the short ‘spectral tuning’ region of the SWS1 photoreceptor may complement the need for terminal microspectrophotometry, intensive site-directed mutagenesis/in vitro protein expression, invasive physiological analyses and/or extensive behavioral experiments to assess the degree and function
of UV-sensitivity in different bird species (Ödeen and Håstad 2003; Hunt et al. 2009; Aidala and Hauber 2010).

2.1.2. Avian UV-sensitivity and visual ecology

Previously it was suggested that a VS SWS1 opsin is the ancestral state in birds (Yokoyama and Shi, 2000; Hunt et al. 2001; Shi et al. 2001; Hunt et al. 2009), with UVS SWS1 independently evolving at least four times among perching birds (Passeriformes), parrots (Psittaciformes), rheas (Struthioniformes), trogons (Trogoniformes), and gulls and terns (Ciconiiformes) (Mullen and Pohland 2008; Hunt et al. 2009; Ödeen et al. 2010; Machovsky Capuska et al. 2011).

2.1.2.1 Paleognaths

Paleognath birds are a diverse ancient lineage that includes both extant and extinct taxa with varied ecology and distribution (Davies 2002). This group encompasses ostriches (Struthionidae), rheas (Rheidae), cassowaries and emu (Casuariidae), kiwis (Apterygidae), tinamous (Tinamidae), and the extinct moa of New Zealand (Emeidae), with all except the tinamous being flightless. Being predominantly diurnal species (with the exception of the nocturnal kiwi), color vision is likely to play a major role in paleognath behavior, although the extent to which they are UV-spectra sensitive is not fully known. Interestingly, tinamou eggs are among the most colorful of all avian eggs with some reflecting in the UV range (Igic et al. 2010a). However, the specific functions of tinamou egg color remains unknown in this group (Brennan 2010). Even less is known about the visual ecology of the extinct New Zealand moa, with most available information, gleaned from the fossil record, suggesting vision was important as these birds foraged mainly on low hanging branches, shrubs, and herbs in and on the margins
of forests (Burrows 1989; Horrocks et al. 2004; Wood et al. 2008). The importance of visual sensory processing and perception in moa species has been confirmed in studies of cranial morphology (Ashwell and Scofield 2008; Corfield et al. 2008).

Microspectrophotometry studies of emu (Dromaius novaehollandiae), brushland tinamou (Nothoprocta cinerascens) and Chilean tinamou (Nothoprocta perdicaria) retinas did not detect any SWS1 cones (see Mullen and Pohland 2008), however earlier work by Wright and Bowmaker (2001) on ostrich (Struthio camelus) and common rhea (Rhea americana) isolated a SWS cone with maximal sensitivity around 400 nm, suggesting a VS state in both species. Sequencing the ‘spectral tuning’ region of the SWS1 opsin gene suggested that the ostrich and rhea are likely to possess VS SWS1 and UVS SWS1 opsins, respectively (Ödeen and Håstad 2003). Spectrophotometric measurements of rhea and ostrich plumage have failed to detect any UV-reflectance (Mullen and Pohland 2008), suggesting that variation in UV-sensitivity may not be driven by variation in UV-containing plumage color.

2.1.2.2 Parrots

Parrots (Psittaciformes) are widely known for their extravagant plumage and integument (scale and skin) coloration (Berg and Bennett 2010), and many parrot species possess highly UV-reflective/-fluorescent plumages (Hausman et al. 2003). The functional role(s) that coloration plays in parrot species remains unclear, although mate-choice studies have implicated UV-reflectance as a major factor. For example, female budgerigars prefer males with UV-reflecting plumage over those where UV-reflectance was removed (Pearn et al. 2001). Further, both male and female budgerigars prefer conspecifics with UV-fluorescent plumage over experimentally removed fluorescence (Arnold et al. 2002). Recently reported SWS1 opsin gene
partial sequences from fourteen parrot species spanning three families, support UVS SWS1 among all member families of the parrot order (Carvalho et al. 2011).

2.1.2.3 Passerines

Among perching birds (Passeriformes), UV signals play a major functional role for many species, aiding in foraging (Honkavaara et al. 2002), mate choice (e.g. Bennett et al. 1996), nest/nestling and egg discrimination (Jourdie et al. 2004; Avilés et al. 2006). For example, perceptual modeling work showed that frugivorous UVS birds possess an enhanced ability to detect fruits against their background color versus VS species (Schaefer et al. 2007). The mouth gapes of nestling passerines reflect UV light, especially in contrast to dark, non-UV-reflective nests, which is thought to facilitate nestling recognition (Hunt et al. 2003). Female European starlings (Sturnus vulgaris) may rank prospective mates based on the degree to which male plumage reflects UV spectra, apparently preferring males with higher UV-reflectance (Bennett et al. 1997). Male blue tits (Cyanistes caeruleus) possess sexually dichromatic ornamental crown patches which highly reflect UV-spectra and are likely informative in mate choice and acquisition (Andersson et al. 1998).

2.1.3 Egg coloration, recognition, rejection and host-parasite interactions

The UV-reflectance of the eggs of cavity-nesting species such as the spotless starling (Sturnus unicolor) is thought to aid in egg detection (Avilés et al. 2006). Blackcaps (Sylvia atricapilla) rely on UV signals to discriminate between their own and foreign parasitic common cuckoo (Cuculus canorus) eggs in their nest (Polačíková et al. 2007). Similarly, there is evidence that the eggs of some rejecter hosts of parasitic brown-headed cowbirds (Molothrus ater) differ in their degree of UV-reflectance from parasitic eggs, so that hosts may be able to attend to these
differences in order to discriminate their own from parasitic eggs (Underwood and Sealy 2008). Further, the UV-matching hypothesis (Cherry and Bennett 2001), specifically predicts that the larger the difference in UV-reflectance between host and brood parasitic eggs, the better hosts can discriminate foreign vs. own eggs.

2.1.4 Hypothesis

Here we sequenced the SWS1 opsin gene ‘spectral tuning’ region, targeting the critical residue 90 (Wilkie et al. 2000) of representative members of the songbirds, parrots, and both extant and extinct paleognaths to test for UV-sensitivity. Using our generated sequences, we then conducted phylogenetic analysis followed by ancestral character state reconstructions for known spectral tuning sites in order to ensure sequence quality as well as to better assess the history of critical amino acid substitutions throughout evolutionary time. Among passerines, we focus on North American hosts of brood parasitic brown-headed cowbirds and endemic New Zealand hosts of brood parasitic cuckoos. We replicate and expand on previously published UVS states of parrot and passerine species (Ödeen and Håstad 2003; Carvalho et al. 2011), and include the New Zealand endemic nocturnal kakapo (Strigops habroptilus) (Gil 2010). We also broaden our current knowledge of UV-sensitivity among extant and extinct paleognath species, with an emphasis on endemic New Zealand taxa. We expect a co-variation of UV-sensitivity in relation to the use of UV signals in species’ ecology. We predict a VS state in nocturnal species such as the kakapo and in non-ejector passerine hosts of brood parasitic cuckoos and cowbirds, in keeping with predictions of the UV-matching hypothesis (Cherry and Bennett 2001).

2.2. Materials and Methods

2.2.1 Taxon Sampling
Our paleognath samples included 16 individuals representing 15 species over 5 families, including the extinct moa family Emeidae of New Zealand (Table 2.1). DNA or tissue samples were also obtained from 14 parrot species with representatives of each of the three recognized Psittaciformes families (Table 2.2). Finally, we sampled 17 individuals representing 16 Passeriformes species over 7 North American families (Emberizidae, Icteridae, Mimidae, Parulidae, Passeridae, Turdidae, and Tyrannidae), 3 Australasian families (Acanthizidae, Pachycephalidae, and Petroicidae), and 1 South American family (Pipridae) (Table 2.3).
Table 2.1.
Predicted VS/UVS visual sensitivity among sampled paleognath species based on DNA sequencing of the SWS1 photoreceptor.
Target amino acid sites 86, 90, and 93 are in bold. Previously predicted sensitivities were compiled from SWS1 sequencing (s) and/or λ-max (m) values reported in Ödeen and Håstad (2003). Intraordinal amino acid variations are shaded.
† denotes an extinct taxon.

<table>
<thead>
<tr>
<th>Scientific Name</th>
<th>Common Name</th>
<th>Amino Acid Sequence</th>
<th>Predicted Sensitivity</th>
<th>Previously Predicted Sensitivity</th>
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<td></td>
</tr>
<tr>
<td>Casuariidae</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>SLGGFI[^90]CVLCVF^F</td>
<td>UVS</td>
<td></td>
</tr>
<tr>
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<td>UVS</td>
<td></td>
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</tr>
<tr>
<td>Emeidae</td>
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</tr>
<tr>
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<th>Previously Predicted Sensitivity</th>
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<td>Rhynchotus rufescens</td>
<td>Red-legged Tinamou</td>
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<td>UV5</td>
</tr>
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<td>Great Tinamou</td>
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<td>UV5</td>
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Table 2.2.
Predicted VS/UVS visual sensitivity among sampled parrot species based on DNA sequencing of the SWS1 photoreceptor. Target amino acid sites 86, 90, and 93 are in **bold**. Previously predicted sensitivities were compiled from SWS1 sequencing and/or \( \lambda \)-max (\( \text{m} \)) values reported by Carvalho et al. (2011). Intraordinal amino acid variations are shaded.

<table>
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<th>Scientific Name</th>
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<th>Accession Number</th>
<th>Amino Acid Sequence</th>
<th>Predicted Sensitivity</th>
<th>Previously Predicted</th>
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<td>UVS</td>
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<td>( \text{UVS}^{s.m} )</td>
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Table 2.3.
Predicted VS/UVS visual sensitivity among sampled Passeriformes species based on DNA sequencing of the SWS1 photoreceptor. Target amino acid sites 86, 90, and 93 are in **bold**. Parasitic species (BHCO = brown-headed cowbird, LTCU = long-tailed cuckoo, SBTU = shining-bronze cuckoo, NP = not parasitized) and rejecter status (R = rejecter, I = intermediate rejecter, A = accepter, NP = not parasitized) are also shown. Intraordinal amino acid variations are shaded.

<table>
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<tr>
<th>Scientific Name</th>
<th>Common Name</th>
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<th>Amino Acid Sequence</th>
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<td>Eastern Phoebe</td>
<td>BHCO</td>
<td>A</td>
<td></td>
<td><strong>SVSGEMCCIPSVPT</strong></td>
<td>VS</td>
</tr>
<tr>
<td>Tyrannus tyrannus</td>
<td>Eastern Kingbird</td>
<td>BHCO</td>
<td>R</td>
<td></td>
<td><strong>SVSGEMCCIPSVPT</strong></td>
<td>VS</td>
</tr>
</tbody>
</table>
2.2.2 SWS1 Sequencing

2.2.2.1 Paleognaths

DNA from extinct moa species (Table 2.1) was extracted from moa bone following the procedures required for ancient material. We incubated approximately 20 mg of bone shavings with rotation overnight at 56 °C in 300 µl of 0.25M EDTA and ~50 µg of proteinase K. The mix was extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by one volume of chloroform. DNA was precipitated from the mix with 0.5 volumes of 7.5 M ammonium acetate, 10 µl of 0.25% linear polyacrylamide (LPA), and 2.5 volumes of ethanol. The mix was incubated at -20 °C for 20 minutes and then centrifuged in a benchtop microfuge at full speed for 15 minutes. The resulting pellet was resuspended in 25 µl of Milli-Q H2O and then desalted by passage through 300 µl of dry Sephacryl S200HR (GE Healthcare, Buckinghamshire, UK). Extant ostrich, rhea, emu, and cassowary (*Casuarius casuarius* 1: see Table 2.1) tissue samples were extracted using DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions.

The SWS1 gene was then amplified using the forward primer 5’-agtgcagcttctagttTACATCCTGGTGAAACATCT-3’ and reverse primer 5’-catgctacctgctactgtTATCCCTGAGCTGmGAT-3’. Lower case letters at the 5’ ends of each primer are generic sequences that allow direct of short PCR products. Amplification reactions were conducted in 10 µl reaction volumes consisting of 50 mM Tris-Cl pH 8.8, 20 mM (NH4)2SO4, 2.5 mM MgCl2, 1 mg/ml BSA, 200 µM of each dNTP, 0.5 µM of each primer, and approximately 1.5 µl extracted DNA. Thermal cycling was conducted in an ABI GeneAmp 9700 (Applied Biosystems, Foster City, CA, U.S.A.) with an initial denaturation step of 94 °C for 2
minutes, then 40 cycles of: 94 °C for 20 s and 54 °C for 1 min. Amplified products were then checked by gel electrophoresis in 2% agarose in 0.5 xTBE, and purified by centrifugation through dry Sephacryl S200HR (GE Healthcare, Buckinghamshire, UK). PCR products were sequenced using ABI BigDye Terminator v3.1 chemistry, then edited and aligned in Sequencher v4.10.1 (Genecodes, Ann Arbor, MI, USA). Predicted protein sequences were derived using Geneious v. 5.1 (Drummond et al. 2010).

We extracted total DNA from six species of tinamou using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany). For all but one of these, DNA was extracted from frozen tissue following the standard protocol for tissue. For *Casuarius casuarius* (*Casuarius casuarius* 2: see Table 2.1), preserved tissue was unavailable, so DNA was extracted from a sliver of toepad collected from the hallux of a museum skin at the Yale Peabody Museum (YPM 86855). For this extraction we modified the standard tissue protocol by adding 19 µl of 1 M dithiothreitol to the initial lysis reaction and reducing the final elution to 50 µl to ensure adequate DNA concentration.

We amplified a 119 base pair fragment of the SWS1 opsin gene using the degenerate primer pair SU149a/SU306b (Ödeen and Håstad 2003) and GoTaq Hot Start Polymerase (Promega, Fitchburg, WI, USA) following the manufacturer’s instructions. We used a thermocycler touchdown protocol similar to Groth and Barrowclough (1999), but with an initial annealing temperature of 58˚ C. Correct amplicon size was verified on agarose gels and the PCR product was prepared for sequencing by enzymatic digestion with Exonuclease 1 and Shrimp Alkaline Phosphatase (Werle et al. 1994). Sequencing was performed on an ABI 3730 Sequencer v4.10.1 and alignment was conducted by eye in Sequencher (Genecodes, Ann Arbor, MI, USA).
2.2.2.2 Parrots

All parrot samples were supplied as either purified DNA or blood samples stored in Queen’s Lysis buffer (Seutin et al. 1991). DNA was extracted from pure blood samples using a DNeasy® (Qiagen, Hilden, Germany) following the manufacturer’s instructions. We amplified the SWS1 opsin gene using previously published forward primers SU149a or SU193 paired with the reverse primer SU306b (Ödeen and Håstad 2003), modified to include M13-tails. We conducted PCR amplifications in 25 µl reaction volumes containing 60 mM Tris-HCl ph 8.5, 15 mM \((NH_4)_2SO_4\), 2.5 mM MgCl\(_2\), 0.3 mM of each dNTP, 0.2 µM of each primer and 0.5U of Platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA). Thermal cycling reactions were performed using an ABI GeneAmp 9700 thermocycler following the protocol published by Ödeen and Håstad (2003): an initial denaturation at 94 °C for 2 minutes, followed by 5 cycles at 94 °C for 30 seconds, 54 °C for 30 seconds and 72 °C for 1 second, then 38 cycles where the extension time was lengthened to 5 seconds and a final 10 minute extension at 72 °C.

PCR products were then purified using Exo/SAP treatment. We added 5 µl PCR product to 0.2 µl Exo I (GE Healthcare, Buckinghamshire, UK), 0.1 µl Shrimp Alkaline Phosphatase (GE Healthcare, Buckinghamshire, UK) and 1.7 µl UltraPure water (Invitrogen, Carlsbad, CA, USA). Mixtures were incubated for 30 minutes at 37 °C, then for 15 minutes at 80 °C in order to inactivate the enzymes. We sequenced samples in both directions using BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA) with M13 forward and reverse primers. Each sequencing reaction contained 1 µl BigDye Terminator Mix, 3.5 µl 5X Sequencing Buffer, 0.2 µM primer, 1 µl DMSO and 2 µl PCR product. Sequencing reactions were purified using Agencourt CleanSeq (Beckman Coulter, Brea, CA, USA) according to manufacturer’s instructions and analyzed using an ABI 3100 automated sequencer. Sequences
were edited using Chromas Pro (Technelysium Pty. Ltd.) and exported to BioEdit (Hall 1999), where they were aligned, translated and compared to other avian opsin sequences downloaded from GenBank. Amino acid sequences were then aligned with our paleognath sequences (see below) using Geneious v. 5.1 (Drummond et al. 2010).

2.2.2.3 Passerines

All songbird samples were provided either as frozen tissue samples (North American species) or blood samples stored in Queen’s Lysis buffer (Australasian species) (Seutin et al. 1991). DNA was extracted from samples using DNeasy kits (Qiagen, Hilden, Germany) following standard protocols. Initial attempts to amplify the whitehead (Mohoua albicilla) SWS1 gene sequence with the primers of Ödeen and Håstad (2003) were unsuccessful. We then designed a new set of primers based on alignments of SWS1 sequences from the zebra finch and the chicken. We designed two forward primers (SWS1_F1: 5’- CSCCCACGTGGGCTTCTACC - 3’; SWS1_F2: 5’- GTACCACATCGCSCCCATGTG - 3’) and two reverse primers (SWS1_R1: 5’ – GTGCCACCCTGTACCAGTC – 3’; SWS1_R2: 5’ – CASGTGGCCRCSCGCACCAGC – 3’).

Amplification reactions contained a total volume of 10 µl and consisted of 1 µl undiluted genomic DNA (10-50 ng/µl concentration), 10 µM Tris-HCl (pH 8), 50 µM KCl, 4 µM MgCl₂, 0.25 mM of each nucleotide, 0.25 mM of each primer, and 0.025 U Jumpstart Taq polymerase (Sigma, St. Louis, MO, USA). Thermal cycling reactions were conducted in PTC-220 Dyad Thermal Cyclers (MJ Research, Waltham, MA, USA). Cycling profiles followed an initial denaturing at 95 °C for 4 minutes 30 seconds; 30-35 cycles of denaturing at 95 °C for 45 seconds,
annealing at 54º C for 1 minute, an extension at 72 ºC for 1 – 2 minutes 20 seconds, and a final extension at 72 ºC for 5 minutes.

Amplification and fragment size confirmation was performed on PCR products via electrophoresis in 1.5% agarose TAE gels. We then added 0.5 U each of Shrimp Alkaline Phosphatase (USB) and Exonuclease (USB) to each remaining 7 µl PCR product and incubated for 30 minutes at 37 ºC followed by 10 minutes at 90 ºC in order to digest unincorporated nucleotides and primers. Cycle sequencing reactions were conducted using the amplification primers as well as the previously published forward primer SU193a and reverse primer SU306b (Ödeen and Håstad 2003) which sit internal to our designed amplification primers. Cycle sequencing reactions were performed using a BigDye 3.1 (Applied Biosystems, Foster City, CA, USA) sequencing kit using recommended cycling conditions. Sequences were then read using Applied Biosystems model 3100 or 3730 automated Genetic Analyzers. Both strands were sequenced in order to verify fragments, and sequences were checked and assembled used Sequencher 4.5 (Genecodes, Ann Arbor, MI, USA). The North American brown-headed cowbird, house sparrow (Passer domesticus), eastern phoebe (Sayornis phoebe), American robin (Turdus migratorius), red-winged blackbird (Agelaius phoeniceus) and the Australasian robin (Petroica sp.) sample were successfully sequenced using these primers. However, the whitehead sample still failed to sequence successfully using these new primers.

We then designed a “passerine-specific” primer using these sequences as well as previously published SWS1 sequence from the zebra finch (Ödeen and Håstad 2003) in order to target the Mohoua genus. We designed one forward primer, SWS1_F4: 5’ - CTACCTGCAGACCATCTTCATGG – 3’, which we successfully combined with previously published reverse primer SU306b (Ödeen and Håstad 2003). We used this combination of
primers to sequence song sparrow (*Melospiza melodia*), gray catbird (*Dumetella carolinensis*), common grackle (*Quiscalus quiscula*), wood thrush (*Hylocichla mustelina*), northern mockingbird (*Mimus polyglottos*), yellow warbler (*Setophaga petechia*), and eastern kingbird (*Tyrannus tyrannus*) species. The same protocol was followed for all amplification, purification, and sequencing reactions described in this section. Finally, the white-bearded manakin (*Manacus manacus*) and grey warbler (*Gerygone igata*) samples were sequenced following protocols described for tinamou SWS1 sequencing in section 2.2.1 and parrot SWS1 sequencing in section 2.2.2.2 respectively.

### 2.2.3 SWS1 phylogenetic analysis and ancestral character state reconstruction

We aligned all SWS1 DNA sequences, including a SWS1 DNA sequence from *Gallus gallus* for use as the outgroup (Accession NM205438) using ClustalW (Thompson et al. 1994) implemented in Geneious v. 5.1 (Drummond et al. 2010). The alignment was then inspected visually and edited to match the maximum length of our sample sequence alignment (a gapless 226 bp sequence) for use in phylogenetic analysis. We used jModeltest v. 0.1.1 (Guindon and Gascuel 2003; Posada 2008) to determine the appropriate model of DNA evolution using Akaike Information Criteria (AIC) calculations to select the best model of evolution (Posada and Buckley, 2004). Bayesian inference analysis was conducted using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), estimating all model parameters during the analysis. The analysis was run for 10 million generations sampled every 100 generations employing Markov Chain Monte Carlo (MCMC) tree searches comprised of 2 runs of 3 heated chains at a temperature of 0.5 and 1 cold chain each. The first 25% of samples were discarded as burn-in (Nyári et al. 2003). By this point all average standard deviations of split frequencies were ≤ 0.1 (0.004) sand all log likelihood value fluctuated within a stable range,
suggesting that convergence had been reached. The majority rules consensus tree was edited using TreeGraph2 (Stöver and Müller 2010) and Adobe Creative Suite 5.0 (Adobe Systems Inc. 2010). Ancestral character state reconstructions of amino acids were conducted using the majority rules consensus tree in Mesquite v. 2.75 (Maddison and Maddison 2011) under a parsimony model with unordered character states.

2.3. Results

2.3.1 Paleognath SWS1 opsin sequences

In families where more than one species was represented (Casuariidae, Emeidae, and Tinamidae), only the Tinamidae contain intrafamily variation in the SWS1 amino acid sequence (Table 2.1; complete DNA sequences and amino acid translations for all species available in Supplementary Tables S1 and S2, respectively). While our common rhea sample was identical to a previously published SWS1 amino acid sequence of the same species, our ostrich sample contained numerous substitutions differing from a previously published sequence (Ödeen and Hästad 2003). Most importantly, our ostrich sample contained C90 while Ödeen and Hästad (2003) reported S90. Our ostrich C90 result has been replicated using a different sample than the one included here and was conducted in a separate laboratory (A Fidler, unpublished data; Tables S1 and S2; all supplementary materials available through published online article). All paleognath samples in our study, both extant and extinct, possess C90, strongly suggesting a ubiquitous UVS SWS1 opsin among paleognaths.

2.3.2 Parrot SWS1 opsin sequences

SWS1 sequencing of our 16 parrot individuals resulted in a 74 base-pair (bp) sequence including the codon for target amino acid site 90. The amino acid translations among the parrots
were highly conserved across all three families studied. We observed little variability in the SWS1 amino acid sequences for all parrot samples (Table 2.2; complete DNA sequences and amino acid translations available in Tables S1 and S2, respectively). The presence of C at site 90 for all parrot species studied here suggests a UVS SWS1 opsin (Table 2.2; Ödeen and Håstad 2003) for all members of the Psittaciformes Order.

2.3.3 Passerine SWS1 opsin sequences

The predicted SWS1 spectral tuning sequences (residues 80 – 92) showed intra-ordinal variation at residues 84, 85, and 88 – 92 (Table 2.3; complete DNA sequences and amino acid translations available in Tables S1 and S2, respectively). Unlike either the parrots or paleognaths, we observed intrafamily variability within the target amino acid sequence of perching birds, although only among the suboscine family Tyrannidae. No intrafamily variation was detected at residue 90 (Table 2.3). Among our samples, only the two suboscine tyrant flycatchers (Tyrannidae) and the white-bearded manakin (Pipridae), and the New Zealand oscine grey warbler (Acanthizidae) are predicted to possess VS SWS1 opsins. All other species sampled are predicted to possess UVS SWS1 opsins (Table 2.3). Our white-bearded manakin sample possessed 3 amino acid differences relative to a previously published SWS1 sequence (Ödeen and Håstad 2003); our sample possessed V instead of F at position 81, M instead of I at position 85, and C instead of S at position 86 (See Table 2.3).

Among our passerine brood parasite hosts (Table 2.3), we did not observe a clear relationship between acceptance/rejection of parasitic eggs and predicted SWS1 opsin sensitivity. Of the eight accepter species, six were predicted to possess UVS SWS1 opsins. Of the three rejecter species, two were predicted to possess UVS SWS1 opsins. The one
intermediate rejecter (northern mockingbird) was predicted to have a UVS SWS1 opsin. There was also no obvious relationship between parasitic egg acceptance/rejection and predicted SWS1 sensitivity by geographical location. The two Australasian host species, the accepter whitehead and the accepter grey warbler, were assigned UVS and VS SWS1 opsins, respectively. Similarly, among the North American brown-headed cowbird hosts, accepter and rejecter hosts varied in their predicted SWS1 opsin sensitivities (Table 2.3).

2.3.4 SWS1 phylogenetic analysis and ancestral character state reconstruction

Model selection analysis in jModelTest proposed a general time-reversible evolutionary model following a gamma rate distribution (GTR+Γ). Here we show the majority rules consensus tree produced by Bayesian inference (Fig. 2.1). Despite the relatively short DNA sequence, our analysis produced three distinct, basally well-resolved clades of Passeriformes, Pstittaciformes, and paleognath species. The only samples falling outside of the three clades were, incidentally, the Passeriformes species predicted to possess VS opsins (Table 2.1, Fig. 2.1). However, these samples showed strong intra-species (G. igata) and moderate intra-familial posterior probability support (Tyrannidae).

Within the paleognath clade, members of both the Casuariidae and Tinamidae grouped strongly while the rhea, ostrich, and the extinct moas were not as well-resolved. The lack of resolution among these species is most likely due to their being the shortest of the SWS1 sequences in this study. Nonetheless, the analysis firmly placed them within the paleognath clade with a posterior probability of 0.92. The parrot clade, despite including all parrot species in this study with a posterior probability of 1.00, was less well-resolved at the family and genus levels than the paleognath clade. However, three members of the Psittacidae (E. roratus, P. derbiana,
*P. krameri manillensis* grouped together as did the kea and New Zealand kaka samples (Nestoridae).

**Fig. 2.1.** Phylogram based on SWS1 nucleotide sequences produced by Bayesian inference in MrBayes (avg. std. dev. of split frequencies = 0.003) with posterior probabilities shown. * denotes posterior probability of 1.00.
The majority of the Passeriformes also formed a distinct basal clade in which the two Turdidae species had moderate support for each other, as did our Emberizidae and Parulidae species. The Australasian whitehead and *Petroica* sp. samples also grouped strongly with each other. The Icteridae all grouped into the same clade with a high (0.94) posterior probability but did not definitively resolve with one another, instead forming a polytomy with the house sparrow. Outside of the larger Passeriformes clade, the Tyrannidae grouped together as did the two Australasian warbler samples, all of which were predicted to be VS taxa.

All samples in this study possessed either C90 or S90. Ancestral character state reconstruction for this site under the produced phylogeny suggests C90 as the ancestral state for both Psittaciformes and paleognaths. Assuming that S is the ancestral state of all birds (Yokoyama et al., 2000) it appears that C evolved at site 90 some time later. The Passeriformes lineage is divided seemingly on the basis of the amino acid present at site 90 in which the larger clade possesses C90 and the less resolved species possess S90 (Fig. 2.2). Interestingly, site 90 is the only amino acid that is definitively distributed in that manner among our sequences. Amino acid residue 86, varied across, but not within all orders. All Passeriformes possessed C86, all Psittaciformes possessed A86, all Paleognaths possessed F86, while the outgroup Galliformes species possessed S86 (Fig. 2.2).

At amino acid residue 93, all Passeriformes and Psittaciformes possessed T93. The paleognaths possessed M93, though this is predicted by the character state reconstruction as the most parsimonious state for the extinct moas, rhea, emu, and ostrich because the sequence did not extend to site 93 (Table 2.2). Given that all the tinamous and the cassowary possessed M93, the character state reconstruction at this site (Fig. 2.2) is likely accurate. The Galliformes outgroup possessed V93.
Fig. 2.2. Ancestral character state reconstruction of key SWS1 spectral tuning sites 86 (A), 90 (B), and 93 (C) produced in Mesquite using the phylogram from the Bayesian analysis. Each amino acid is color coded and labeled (S = blue, C = purple, A = orange, F = green, V = yellow, T = red, M = black). Grey indicates an equivocal state.
Based on the phylogenetic and ancestral character state reconstruction analyses of amino acid residues 86, 90, and 93 (Fig. 2.2), both the paleognath and Psittaciformes clades appear to retain their phylogenetic history while the Passeriformes clades are additionally split depending on which amino acid (C or S) is present at site 90.

2.4. Discussion

2.4.1 Predicted SWS1 opsin sensitivity

In this study we expanded on previous work in which the ‘spectral tuning’ region of the SWS1 opsin gene was used to predict the degree of avian SWS1 sensitivity to UV spectra (< 400 nm), and therefore infer the degree of several bird species’ UV visual sensitivity (e.g. Ödeen and Håstad 2003). To date, all avian species examined prior to this study using direct SWS1 DNA sequencing (e.g. Ödeen and Håstad 2003; Carvalho et al. 2011) and/or site-directed mutagenesis followed by in vitro protein expression possess C90 or S90 (e.g. Yokoyama et al. 2000; Wilkie et al. 2000). All of our samples possessed either C90 or S90, allowing the predicted assignment of UVS or VS SWS1 opsins to be fairly straightforward in the current dataset. Our results suggest ubiquitous UVS SWS1 opsins in both the Psittaciformes and paleognaths, while the Passeriformes contain species with both VS and UVS SWS1 opsins (Tables 2.1 – 2.3).

In addition to showing both intra- and extra-ordinal variation at spectral tuning site 90, we also report extra-ordinal variation at two additional well-studied spectral tuning sites along the avian SWS1 opsin – residues 86 and 93 (e.g. Yokoyama et al. 2000; Wilkie et al. 2000). Our Passeriformes species invariably possessed C86 and T93, both of which have been reported elsewhere (Table 2.3; Ödeen and Håstad 2003). Similarly, other Psittaciformes species have also been shown to possess A86 and T93 (Table 2.2; Ödeen and Håstad 2003; Carvalho et al. 2011).
The majority of paleognaths definitively possessed F86 and M93 (exceptions being the emu, rhea, ostrich, one cassowary, and the extinct moas, where the sequence did not encompass residue 93. Character state reconstruction analyses predicted M93 for our paleognath samples; (see Fig. 2.2), both of which have been previously documented in paleognath species (Ödeen and Håstad 2003). Variation at amino acid residues 86 and 93 does not covary well with the assignment of VS/UVS SWS1 opsins among avian species (e.g. Ödeen and Håstad 2003; Hunt et al. 2009; Tables 2.1 – 2.3; Fig. 2.2), although S86 never accompanies a UVS SWS1 opsin (Carvalho et al. 2007; Hunt et al. 2009). This is likely because the resultant changes in spectral sensitivity due to amino acid substitutions at sites 86 and 93 are much lower than that at residue 90 (Yokoyama et al. 2000; Wilkie et al. 2000).

Our findings largely coincide with and expand on the assigned VS/UVS avian SWS1 opsin states of earlier studies. Our Passeriformes samples demonstrated extra-familial variation at site 90, supporting recent findings that UVS/VS opsins have been acquired and lost at least eight times within the Passeriformes lineage (Ödeen et al. 2011). However, our white-bearded manakin SWS1 sequence differed from a previously published sample, possessing M85 and C86 instead of the previously reported I85 and S86 (Ödeen and Håstad 2003). Though the reasons for this discrepancy are not clear, our sample possessed a sequence identical to the brown-crested flycatcher (Myiarchus tyrannulus) reported in the same study (Ödeen and Håstad 2003) as well as to our own eastern phoebe and eastern kingbird Tyrannidae samples. In turn, our galah (Eolophus roseicapillus), sulphur-crested cockatoo (Cacatua galerita), crimson rosella (Platycercus elegans), and kea (Nestor notabilis) samples are identical to those reported by Carvalho et al. (2011), supporting their conclusion that UV-sensitivity is ubiquitous among all parrots.
We found F86, V88, and C90 in the ostrich while an earlier study reported S86, I88, and S90 in the same species (Ödeen and Håstad 2003), leading us to predict a UVS instead of the previously predicted VS SWS1 opsin. Our phylogenetic analysis places the ostrich firmly within the paleognath clade (Fig. 2.1). It is also a unique sequence in our data set (Table 2.1), making the discrepancy due to mislabeling of samples unlikely. However, earlier microspectrophotometry studies have reported a maximal absorbance of the ostrich SWS1 photoreceptor at 405 nm, which makes up 1.5% of the total cones present in the ostrich retina, also suggesting violet sensitivity (Wright and Bowmaker 2001). Our sequence data suggests that the ostrich possesses F86 and C90, identical to our and an earlier published SWS1 sequence of the common rhea (Ödeen and Håstad 2003). Although maximal absorbance of the common rhea’s SWS1 photoreceptor is not available, the SWS1 photoreceptor comprises 2.5% of all the cone-types found its retina (Wright and Bowmaker 2001). This is comparable to the low-end of the SWS1 cone distribution in the UVS European starling, in which the SWS1 cone comprises 3-7% of all cone-types (Hart et al. 1998). Given that UV plumage and integument (skin) signals play major roles in parrot behavior (Berg and Bennett 2010), even though in their retinas the SWS1 cone is again the least represented of the cone-types (9% in the budgerigar; Wilkie et al. 1998), it is not unreasonable to suggest that UV signals likely possess behavioral relevance in paleognath species as well, though the reason for the discrepancy in the reported DNA sequences between our and Ödeen and Håstad’s (2003) remains unknown.

Additional analyses such as microspectrophotometry, site-directed mutagenesis/in vitro expression, and/or behavioral experiments to determine the presence and/or functional relevance of UV-sensitivity would be ideal components to include in any study addressing UV-sensitivity at the molecular and/or behavioral levels. However, they are not available or practical for all
avian species such as extinct or endangered taxa. UV-sensitivity can further be mediated by the level of ocular media transparency to UV wavelengths as well as by higher-level neural processes (see Machovsky Capuska et al. 2011), as well as the relative abundance/density of SWS1 cones in the retina, making behavioral studies a primary goal for assessing the degree of UV-sensitivity in any species.

2.4.2 Phylogenetics and character state reconstruction

SWS1 opsin DNA sequence has been used to produce a reliable phylogeny among most vertebrate Classes consistent with both morphological and molecular phylogenies most likely due to its relatively homogeneous substitution rates (van Hazel et al. 2006). Our phylogenetic analysis of the SWS1 nucleotide sequences showed three distinct, basally resolved clades of Passeriformes, Psittaciformes, and paleognaths (Fig. 2.1). The lack of high resolution at the apices is likely due to the relatively short lengths of our sequences as well as the inclusion of only one gene, yet the overall intra-Order affinities of our samples ensures the quality/validity of our sequence data. It is indeed compelling that the Passeriformes clade appears to be split functionally as VS SWS1-assigned (S90) taxa resolved outside the UVS SWS1-assigned (C90) taxa whereas the ubiquitously UVS SWS1 Psittaciformes and paleognaths appear to better retain their phylogenetic history within their respective clades (Fig. 2.1).

The close phylogenetic relationship between Passeriformes and Psittaciformes is increasingly well established (Hackett et al. 2008; Suh et al. 2011), our phylogenetic analysis also places Psittaciformes with Passeriformes. Future work including multiple gene sequences and/or a longer SWS1 sequence is needed both to produce a phylogeny in better consensus with the established literature, and increase resolution within the phylogeny. Nonetheless, the
phylogeny reported here allowed for informative ancestral character state reconstruction of key spectral tuning sites along the SWS1 opsin within each separate clade (Fig. 2.2).

The ancestral state of the vertebrate SWS1 opsin is believed to be UVS (Yokoyama and Shi 2000; Shi et al. 2001), with the avian lineage having a VS SWS1 ancestral state, evolving an UVS SWS1 opsin multiple times (Ödeen and Håstad 2003; Carvalho et al. 2007; Hunt et al. 2009; Ödeen et al. 2011). Here we show that an ancient lineage of birds, the paleognaths, likely possess UVS SWS1 opsins due to the presence of C90 and F86 (Fig. 2.2), the latter of which is highly conserved in UV-sensitive mammals (Hunt et al. 2009). A F86S substitution has been shown to be the integral substitution involved in the evolution of a VS SWS1 opsin in avian ancestors (Carvalho et al. 2007). The ubiquitous presence of F86 in the ancient paleognath lineage is particularly exciting because it was present in the ancestral UVS SWS1 opsin of all vertebrates (Yokoyama and Shi 2000; Shi et al. 2001; Hunt et al. 2009), especially because the paleognaths were the earliest clade to diverge within the avian lineage (e.g. Hackett et al. 2008).

If UV-sensitivity was lost among avian ancestors and subsequently reacquired in the avian lineage, our results suggest this may have occurred earlier than has been previously suggested. Recent phylogenetic work placed the extinct moa as closely related to the cassowary, emu, kiwi, and tinamous with the rhea and ostrich having diverged earlier (Phillips et al. 2010). If all paleognath species have UVS SWS1 pigments as our results suggest, a UVS state is likely to have evolved in a species ancestral to all the paleognaths rather than only among the rheas, as has been previously suggested (e.g. Ödeen and Håstad 2003).

A single amino acid substitution (C90S) was responsible for the evolution of UV SWS1 sensitivity in avian species (Yokoyama et al. 2000), though no paleognath species were included
in that study. To date, however, no avian species have been identified that do not possess either S90 or C90, suggesting site 90 is a highly conserved, functionally relevant site among all avian lineages. Our reconstruction analyses did not support F86 or C90 as the root ancestral states (Fig. 2.2), though this is probably due to the choice of outgroup (Galliformes), the relatively short sequence used in the phylogenetic analysis, and the lack of diverse taxon sampling throughout the avian (and vertebrate) lineage.

2.4.3 Functional relevance of UV-sensitivity among paleognaths

Our results suggest that all paleognath birds have the molecular pigments for perceiving UV-light, though the function of this perception is not clear. There is no evidence that paleognath plumage reflects UV light, so it is not clear whether UV signals play a major role in mate choice (Mullen and Pohland 2008). However, moa species with predominantly paternal care (Huynen et al. 2010) may have used UV-reflectance spectra in egg identification, which is potentially important in numerous other bird species (e.g. Cherry and Bennett 2001; Polačiková et al. 2007; Honza et al. 2007), particularly those that nest in enclosed, dark cavities (Avilés et al. 2006). Further, extant tinamous lay conspicuously colored eggs despite being ground nesting species which typically lay camouflaged eggs (Kilner 2006). These non-cryptic eggs may serve as an intraspecific signal of female quality to incubating males and/or a signal of nest location for laying females (Brennan 2010).

For example, males may attend to UV-reflectance of eggs as a predictor of female quality, as has been suggested in spotless starlings and garnering moderate, if correlative, empirical support (López-Rull et al. 2007). Eggshells of extinct moa species are often found in caves, suggesting that UV egg reflectance may have facilitated egg location and recognition in
these species. Ostrich, emu, and extinct stout-legged moa eggshells are known to reflect UV light (Igic et al. 2010a) which may play a functional role in signaling quality of both mother and chicks in other species (Moreno et al. 2005; López-Rull et al. 2007; López-Rull et al. 2008).

Fadzly et al. (2009) has examined the spectral reflectance curves of lancewood tree (Pseudopanax crassifolius) leaves once exploited by moa species as a food source. The leaves showed some, albeit minimal, reflectance in the UV portion of the light spectrum and it is possible that moa could have used this UV-reflectance to locate suitable saplings. However, the relative densities of UV-sensitive cones within their retinas are unknown, making this claim purely speculative.

2.4.4 Functional relevance of UV-sensitivity among parrots

All parrot species sampled in this study possess a C at amino acid site 90, with very little variation along the entire length of the sequence and are identical in all parrot lineages sampled (Table 2.2). Although the behavioral relevance of UV-spectra among parrot species is fairly well-documented (see Berg and Bennett 2010), little is known about its role in the kakapo, an endangered, nocturnal parrot of New Zealand. Evolving nocturnality tends to favor either an increased or decreased reliance on visual stimuli; nocturnal species relying heavily on visual stimuli tend to possess large eyes with poor acuity (Hall and Ross 2007), whereas those that rely on senses other than vision often possess smaller eyes (e.g. Martin et al. 2007). A recent study by Corfield et al. (2011) showed the kakapo has evolved a visual system not typically observed in nocturnal animals as it possesses visual system traits characteristic of both nocturnal and diurnal birds. This could be because the kakapo evolved from a diurnal parrot ancestor (Corfield et al. 2011). Given that our data suggest that the kakapo has pigments sensitive to UV-spectra, future
studies are necessary to establish its role in the kakapo’s behavioral repertoire and communication behavior.

2.4.5 Functional relevance of UV-sensitivity among passerines

Each of the North American species from the present study has been reported as a host of the brown-headed cowbird, allowing us to assess whether or not UV-sensitivity is related to parasitic egg rejection rates. According to Peer and Sealy (2004), rejecter hosts (those that typically remove parasitic cowbird eggs) include the eastern kingbird, American robin, and gray catbird. The northern mockingbird is classified as an intermediate rejecter (with some foreign eggs rejected from its nests), and the eastern phoebe, wood thrush, yellow warbler, song sparrow, red-winged blackbird, and common grackle are classified as accepter species. Our results suggest that the tyrant flycatchers (eastern kingbird and eastern phoebe) possess a VS visual system, as has been previously shown among the Tyrannidae (Ödeen and Håstad 2003). In keeping with Underwood and Sealy’s (2008) conclusions, we found no obvious relationship between hosts’ predicted UV-sensitivity and their rejecter status and thereby do not find support for the UV-matching hypothesis (Cherry and Bennett 2001) in North American hosts of the brown-headed cowbird.

The New Zealand endemic whitehead is a non-ejector host of the long-tailed cuckoo (Eudynamis taitensis) (McLean and Waas 1987, Briskie 2003), so it is unlikely that UV-sensitivity plays a functional role in egg discrimination. However, it may be involved in detecting the sexual dichromatism of whitehead feathers (Igic et al. 2010b). The grey warbler is a non-ejector host of the shining bronze-cuckoo (Chrysococcyx lucidus) (Briskie 2003) but unlike the whitehead, it is predicted to be a VS species. However, because these were the only two
Australasian brood parasite hosts sampled, support for the UV-matching hypothesis in this system remains equivocal.

Although we did not find overwhelming support for the UV-matching hypothesis between egg accepters and rejecters (Cherry and Bennett 2001; Underwood and Sealy 2008) in any of our sampled songbirds, alternative roles of UV-reflectance in behavioral repertoires in songbirds remains unclear. Our results support ubiquitous UVS pigments in the true sparrows and a VS state in suboscines (Tyrannidae), as has been previously shown (Ödeen and Håstad 2003). By including previously unsequenced representative songbird (oscine) Passeriformes from the New World sparrows, New World blackbirds, mimids, thrushes, whistlers, Australasian robins, and Australasian warblers, we show that all appear to be UVS, except the New Zealand G. igata which is predicted to be VS.

2.4.6 Summary

Our results suggest a ubiquitous UVS SWS1 opsin for Psittaciformes and paleognath species based on the presence of SWS1 C90 in all samples tested, and is the first study to investigate SWS1 pigments in extinct paleognaths. We also predict variable SWS1 opsin sensitivity among the Passeriformes based on the presence of S90 or C90. Although further confirmation (microspectrophotometry, site directed mutagenesis, ocular media transparency, behavioral studies) would be ideal, genetic sequencing of the SWS1 photoreceptor gene is the only method currently available for studying extinct taxa. Given that paleognath eggshells reflect a large proportion of UV light suggests that UV-sensitivity may be behaviorally relevant for these species. Our results also expand and support previous suggestions of ubiquitous UVS pigments for the entire parrot order (Psittaciformes). Our passerine samples yielded the only
taxonomic group to demonstrate any variability in predicted UV-sensitivity throughout the order, though we did not observe any intra-family variability. Where possible, behavioral, microspectrophotometric, and ocular media transparency studies should follow up on the results presented here in order to better describe the function of UV signals in these species.

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Manacus manacus YPM 137115 and second Casuarius casuarius (YPM 86855) samples. Finally, extinct moa samples Pachyornis elephantopus (CM SB301), Emeus crassus (CM Av13775), Euryapteryx gravis (OM Av9821), Euryapteryx curtus (AIM B6595ii), and Pachyornis geranoides (W336) were kindly provided by Canterbury Museum, Otago Museum, Auckland Museum, and Whanganui Museum, respectively. We would also like to thank two anonymous reviewers for their helpful comments on an earlier version of the manuscript.
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Chapter 3
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Predicted visual sensitivity for short-wavelength light in the brood parasitic cuckoos of New Zealand

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Abstract

Different lineages of birds show varying sensitivity to light in the ultraviolet (UV) wavelengths. In several avian brood parasite-host systems, UV-reflectance of the parasite eggs is important in discriminating own from foreign eggs by the hosts. In turn, for parasitic females it may be beneficial to lay eggs into host clutches where eggs more closely match the parasite’s own eggs. While the visual sensitivities of numerous cuckoo- and cowbird-host species have been described, less is known about those of their respective parasites. Such sensory characterization is important for understanding the mechanisms underlying potential perceptual coevolutionary processes between hosts and parasites, as well as for better understanding each species’ respective visual sensory ecology. We sequenced the short wavelength-sensitive type 1 (SWS1) opsin gene to predict the degree of UV-sensitivity in both of New Zealand’s obligate parasitic cuckoo species, the Shining Cuckoo (*Chalcites* [*Chrysococcyx*] *lucidus*) and the Long-tailed Cuckoo (*Urodynamis* [*Eudynamis*] *taitensis*). We show that both species are predicted to possess SWS1 opsins with maximal sensitivity in the human-visible violet portion of the short-wavelength light spectrum, and not in the UV. Future studies should focus on the (mis)matching in host-parasite visual sensitivities with respect to host-parasite egg similarity as perceived by the avian visual system and the behavioral outcomes of foreign egg rejection.

Keywords

Brood parasitism, coevolution, Cuculiformes, SWS1 opsin, ultraviolet vision, visual ecology
Introduction

Interactions between avian obligate brood parasites and their hosts remain one of the most robust examples of coevolutionary arms races (Davies, 2000; Stoddard & Stevens, 2010; Kilner & Langmore, 2011). The best studied and historically most prominent example of such interactions is the evolved mimicry of host eggs by parasites (Moksnes & Røskaft 1995; Cherry et al., 2007a; Moskát et al., 2008; Moskát et al., 2010; Spottiswoode & Stevens, 2010; Soler et al., 2012). Despite the extensive similarities in the appearance of host and parasitic eggs (Grim, 2005), many host species possess the ability to discriminate between own and foreign eggs (Stoddard & Stevens, 2011). Much attention has recently been given to the functional roles of light wavelengths beyond the human perceptual range in avian egg discrimination, including the role of the shorter, ultraviolet (UV) wavelengths (λ < 400 nm) (e.g. Honza et al., 2007), to which different species of birds within distantly related lineages are varyingly sensitive (e.g. Ödeen & Håstad, 2003; Machovsky Capuska et al., 2011; Aidala et al., 2012). For example, UV-reflectance is important in recognizing and rejecting foreign eggs in the Blackcap (Sylvia atricapilla) (Honza & Polačíková, 2008) and the Song Thrush (Turdus philomelos) (Honza et al., 2007). However, comparatively less emphasis has been given to describing the visual sensitivities, UV or otherwise, of avian obligate brood parasites themselves.

Describing the visual sensitivities of specific bird species is vital, especially because the avian visual world differs substantially from that of humans. For example, unlike trichromatic humans, who possess only three classes of cone photoreceptor, birds possess five classes, four of which are directly responsible for color perception (Hunt et al., 2009). The short wavelength-
sensitive type 1 (SWS1) photoreceptor, which is responsible for short-wavelength light detection, differs in its maximal sensitivity depending on the amino acids present at key ‘spectral tuning’ sites 86, 90, and 93 (following the bovine *Bos taurus* rhodopsin numbering) (Wilkie et al., 2000; Yokoyama et al., 2000; Shi et al., 2001). Of these, amino acid residue 90 is particularly important for mediating the degree of UV-sensitivity in avian species (Wilkie et al., 2000; Hunt et al., 2009). Those species possessing serine at site 90 (S90) are designated as having violet-sensitive (VS) pigments with a maximal sensitivity > 400 nm, and those possessing cysteine (C)90 are designated as having UV-sensitive (UVS) pigments with a maximal sensitivity < 400 nm (Hart, 2001). Site 90 is also highly conserved, with S90 proposed to be the ancestral state in all birds (Yokoyama & Shi, 2000; Hunt et al., 2009), though recent analyses of basal paleognaths (which were not included in these earlier analyses) including extinct moa from New Zealand, predicted a uniform UVS SWS1 for all ratites and tinamou allies (Aidala et al., 2012). Therefore, it is likely that C90 has (re-)evolved independently several times among avian lineages (Hunt et al., 2009; Ödeen et al., 2010; Machovsky Capuska et al., 2011; Ödeen et al., 2011, Aidala et al. 2012). Because microspectrophotometric and genetic data are in accord with one another in avian taxa for which both types of data are available (i.e. those possessing S90 have VS SWS1 opsins and those possessing C90 have UVS SWS1 opsins), DNA sequencing of the SWS1 opsin gene therefore permits accurate assessment of the degree of UV-sensitivity in any given avian species (Ödeen & Hästad, 2003) before the need for invasive and terminal physiological experimentation to confirm the sequence-based predictions (Aidala & Hauber, 2010).

Much of the work on the functional role of UV-reflectance and sensitivity in brood parasitic birds has focused on explaining the lack of eggshell color-based egg rejection to
seemingly non-mimetic parasitic eggs. Cherry and Bennett’s (2001) UV-matching hypothesis suggests that matching host/parasitic egg reflectance along a UV-green opponency (which humans cannot see) may explain the lack of rejection in acceptor host species. Empirical support for this hypothesis, however, is equivocal. For example, blocking the UV-reflectance of Great-spotted Cuckoo (Clamator glandarius) eggs does not affect rejection in Common Magpies (Pica pica) (Avilés et al., 2006). However, the UVS/VS SWS1 sensitivity in this parasite-impacted host species has not been described, although other Corvidae species are predicted to be VS based on SWS1 DNA sequencing (Ödeen & Håstad, 2003). More critically, no apparent relationship between accepter/rejecter status and UVS/VS SWS1 sensitivity appears to exist among hosts of the North American generalist brood parasite, the Brown-headed Cowbird (Molothrus ater) and its many hosts (Underwood & Sealy, 2008; Aidala et al., 2012).

The degree of UV egg color-matching/UV light sensitivity in New Zealand obligate brood parasite-host systems is not yet described using reflectance spectrophotometric or avian perceptual modeling data. The endemic Grey Warbler (Gerygone igata) is an accepter host of the local subspecies of the native Shining Cuckoo (in Australia, called the Shining-bronze Cuckoo; Chalcites [Chrysococcyx] lucidus) (McLean & Waas, 1987; also reviewed in Grim, 2006). In turn, the Whitehead (Mohoua albicilla), Yellowhead (M. ochrocephala), and Brown Creeper (M. novaeseelandiae) are endemic hosts of the also endemic Long-tailed Cuckoo (Urodoynamis [Eudynamis] taitensis) (Payne 2005). The Whitehead and Yellowhead are both considered accepter hosts (McLean & Waas, 1987; Briskie, 2003), while the Brown Creeper ejects artificial Long-tailed Cuckoo eggs at a rate of 67% (Briskie, 2003). DNA sequencing of the SWS1 photoreceptor in the Grey Warbler and the Whitehead predicted a VS and a UVS SWS1 maximal sensitivity, respectively (Aidala et al., 2012), whereas the predicted sensitivities
of their respective parasites are not well known.

Compared to the large amount of effort spent characterizing the visual sensitivities of host species, those of brood parasites themselves, especially to UV-wavelengths, have received considerably less attention. To date, the SWS1 sensitivities have not been described in any Cuculiformes species, although a study measuring UV-reflectance in feather patches of 24 of 143 (17%) total cuckoo species showed that 5 of the species (21% of those measured) showed peaks in UV-reflectance (Mullen & Pohland, 2008). As there are increasingly more known inter-and intra-order variations in avian UV-sensitivity (Ödeen & Håstad, 2003; Machovsky Capuska et al. 2011, Aidala et al., 2012; Ödeen et al., 2012), and because visual systems among closely related species may vary widely, and are likely to reflect species-specific sensory ecologies (Machovsky Capuska et al. 2012), reliance on species for which SWS1 sensitivity data are available even within a lineage to approximate the degree of UV-sensitivity may be inaccurate.

Characterization of the UV-sensitivities of brood parasitic species is important for several reasons. First, it will allow for stronger analysis of comparative perceptual coevolution between hosts and parasites (Anderson et al. 2009). For example, recent egg color work using spectrophotometric measurements across the entire avian visible range have provided new insights into the direction of coevolutionary processes between hosts and parasites. Great Reed Warblers (Acrocephalus arundinaceus) are more likely to reject mimetic Common Cuckoo (Cuculus canorus) eggs when this hosts’ own eggs exhibit higher intraclutch variation, a finding not in line with traditional predictions of coevolutionary theory, but validated by spectrophotometric measurements of host eggs (Cherry et al., 2007a; Antonov et al., 2012). Similarly, Common Cuckoos may preferentially parasitize host nests with eggs more closely resembling their own, also out of line with the theoretical assumption that female cuckoos
randomly choose local nests to parasitize (Cherry et al., 2007b). Second, describing the visual sensivities of brood parasitic cuckoo species will better inform studies examining cuckoo-cuckoo competition (Brooker et al., 1990) over host nesting sites using visual modeling analyses. Third, it will allow for more accurate analysis of VS/UVS SWS1 opsin ancestral states among avian species (Hunt et al., 2009, Aidala et al. 2012). Here, we report the predicted maximal sensivities of the SWS1 opsins in two New Zealand native brood parasitic cuckoos based on DNA sequencing of the SWS1 ‘spectral tuning’ region. In keeping with the general theoretical framework that host egg rejection selects for egg color matching, and in turn, favors UV-sensitivity in hosts, which in turn selects for UV-sensitivity in parasites, we expect the Shining Cuckoo that parasitizes the VS-predicted Grey Warbler to possess VS SWS1 opsins and the Long-tailed Cuckoo that parasitizes the UVS-predicted Whitehead to possess UVS SWS1 opsins.

Methods

We collected ~100 μl blood samples that were stored in Queen’s lysis buffer from live Shining Cuckoos captured in mistnets during our field studies on avian host-parasite interactions (Anderson et al. 2009). We also obtained tissue samples from frozen Long-tailed Cuckoos that died from migration-related window-collisions and were stored in the Auckland Museum collection (Gill & Hauber 2012). Our collecting protocols were approved by governmental and institutional animal research committees. Total genomic DNA was extracted from tissue samples stored in ethanol using the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer’s instructions. DNA concentration (ng/μl) was estimated using Nanodrop spectrophotometer.

Forward primers SU149a (Shining Cuckoo) or SU193 (Long-tailed Cuckoo) and reverse
primer SU306b (Ödeen and Håstad, 2003), modified to include M13-tails, were used to sequence the SWS1 opsin gene. PCR amplifications were carried out in 25 μl reaction volumes of 60 mM Tris-HCl ph 8.5, 15 mM (NH₄)2SO₄, 2.5 mM MgCl₂, 0.3 mM of each dNTP, 0.2 μM of each primer and 0.5 U of Platinum Taq polymerase (Invitrogen). Thermal cycling followed conditions outlined in Ödeen and Håstad (2003) and was conducted in an ABI GeneAmp 9700 thermocycler.

An Exo/SAP treatment was used to purify PCR products: 5 μl PCR product was added to 0.2 μl of Exo I (GE Healthcare), 0.1 μl Shrimp Alkaline Phosphatase (GE Healthcare) and 1.7 μl UltraPure water (Invitrogen). We incubated mixtures for 30 min at 37 °C, then for 15 min at 80 °C to ensure enzyme inactivation. A BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) was used to sequence samples in both directions with M13 forward and reverse primers. Each sequencing reaction consisted of 1 μl BigDye Terminator Mix, 3.5 μl 5X sequencing buffer, 0.2 μM primer, 1 μl DMSO and 2 μl PCR product. Agencourt CleanSeq (Beckman Coulter) was used according to manufacturer’s instructions to purify sequencing reactions and analyzed using an ABI 3100 automated sequencer. Chromas Pro (Technelysium Pty. Ltd.) was used to edit sequences following which they were exported to BioEdit (Hall, 1999) for alignment and translation.

**Results**

The two Shining Cuckoo samples generated a sequence length of 119 base pairs (bp) each. The two Long-tailed Cuckoo samples generated a sequence length of 74 bp each. All sequences have been made available on GenBank (Accession numbers HM159121 – HM159124). We detected no intraspecific or intrafamilial variation in either the gene or amino
acid sequences, except for the codons at residue 95; however, both of these code for the amino acid phenylalanine (Table 3.1). We found only two ambiguities in one Long-tailed Cuckoo sample, whereas the other Long-tailed Cuckoo possessed the same codons and amino acid residues as the two Shining Cuckoo samples (Table 3.1). After alignment, all samples possessed S86, S90, and T93, which predict VS for both of these cuckoo species’ SWS1 opsin photoreceptors.

Discussion

This is the first study to report on the sequence of SWS1 receptors and to predict short-wavelength visual sensitivities of New Zealand’s brood parasitic native Shining Cuckoos and endemic Long-tailed Cuckoos. Substituting S for A at amino acid residue 86 (A86S substitution) produces a short-wave shift of 1 nm, a T93V substitution produces a long-wave shift of 3 nm, and a C90S substitution produces a 35 nm long-wave shift in the UVS SWS1 opsin of the Budgerigar (*Melopsittacus undulatus*) (Wilkie et al. 2000). The same C90S substitution in the Zebra Finch (*Taeniopygia guttata*) produces a similar-magnitude long-wave shift of SWS1 maximal sensitivity from 359 to 397 nm (Yokoyama et al., 2000). Thus, despite possessing S86 and T93 in both species, the presence of S90 predicts that the SWS1 maximal sensitivities of our cuckoo samples should be well within the visible-violet portion of the light spectrum, or VS (Table 3.1).

This finding is contradictory to our original prediction that only the Long-tailed Cuckoo should possess UVS SWS1 opsins due to the predicted UVS SWS1 of its Whitehead host (in contrast with the VS SWS1 of the Shining Cuckoo’s Grey Warbler host; Table 3.1).
Accordingly, we did not observe a distinct pattern between predicted SWS1 sensitivities of our cuckoo samples and those of their hosts. Both the Grey Warbler and Whitehead are non-ejector hosts of the Shining and Long-tailed Cuckoos respectively, yet these host species differ in their predicted SWS1 maximal sensitivities; DNA sequencing of the SWS1 photoreceptor gene predicted a VS SWS1 in the Grey Warbler but a UVS SWS1 in the Whitehead (Aidala et al. 2012). Predicted sensitivities of the other two Long-tailed Cuckoo hosts, the non-ejector Yellowhead, and the artificial egg-ejecting Brown Creeper are not yet described from molecular sequencing data. Also undocumented is the degree of physical or perceptual host-parasite egg color matching, in the UV-portion specifically, and in the avian-visible spectrum overall, in these two host-parasite systems. Nonetheless, human-visible assessment suggests some level of mimicry between Long-tailed Cuckoos and their hosts (Briskie, 2003), whereas the dark Shining Cuckoo’s eggs may be cryptic, and not mimetic, in the enclosed nests of the Grey Warbler hosts (see Langmore et al., 2009).

An alternative to perceptual coevolutionary processes mediating the detection of parasitic eggs in New Zealand hosts is that the cost of accepting parasitic eggs might be offset by recognizing and rejecting parasitic cuckoo chicks (Davies, 2000). Despite a lack of direct behavioral or sensory data in our focal systems, there is evidence of parasitic chick detection and ejection based on visual appearance in the closely related Australian Large-billed Gerygone.
(Gerygone manirostris)/Little Bronze-cuckoo (Chalcites [Chrysococcyx] minutiliss) (Sato et al., 2010;)
and Superb Fairy-wren (Malurus cyaneus)/Shining Cuckoo host-parasite systems
(Langmore et al., 2003; see also Langmore et al., 2011). Further, there is evidence of evolved
call-matching of the begging calls of Grey Warblers by Shining Cuckoo chicks based on both
sound recordings (McLean & Waas, 1987) and comparative phylogenetic inference (Anderson et
al., 2009). Similarly, McLean and Waas (1987) noted and Ranjard et al. (2010) provided
bioacoustic evidence for the evolved similarity between the begging calls of the Long-tailed
Cuckoo and its Mohoua spp. hosts. Other parasitic cuckoo-host systems, including the
Horsefield’s Bronze-cuckoo (Chalcites [Chrysococcyx] basalis) and its Superb Fairy-wren
(Malurus cyaneus) hosts (Langmore et al., 2003; Langmore et al., 2008; Colombelli-Negrel et
al., 2012), the Diederick Cuckoo (Chrysococcyx caprius) and its hosts, and the Koel (Eudynamis
scolopacea) and its House Crow (Corvus splendens) hosts have also been shown to have similar
begging calls (reviewed in Grim, 2006).

Characterizing the visual sensitivities of diverse avian lineages, including parasitic
cuckoo species, is an important step in understanding the coevolution of visual
perception/parasitic egg rejection behaviors in host-parasite interactions and sensory ecology.
These studies form the basis for future visual modeling and sensory-physiological studies for
more accurate description of the perceptual systems of focal cuckoo species. Future studies
should investigate the behavioral significance of egg color matching in driving sensory
coevolution using appropriate visual perceptual modeling analyses of both host and parasitic
species (Aidala & Hauber, 2010). Additional Cuculiformes species should also be included in
future analyses in order to better describe the degree of V/UV-matching in host-parasite egg
color mimicry and its perception and the ecological variables that may drive or hinder the
evolution of UV-sensitivity amongst parasitic and non-parasitic cuckoos (Krüger et al., 2009).
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Chapter 4

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The role of egg-nest contrast in the rejection of brood parasitic eggs

Aidala Z, Croston R, Schwartz J, Tong L, Hauber ME

SUMMARY

Hosts of avian brood parasites can avoid the reproductive costs of raising genetically unrelated offspring by rejecting parasitic eggs. The perceptual cues and controls mediating parasitic egg discrimination and ejection are well studied: hosts are thought to use differences in egg color, brightness, maculation, size, and shape to discriminate between own and foreign eggs. Most models of brood parasitism implicitly assume that the primary criteria to which hosts attend when discriminating eggs are differences between the eggs themselves. However, this assumption is confounded by the degree to which chromatic and achromatic characteristics of the nest lining co-vary with egg coloration, in that egg-nest contrast per se might be the recognition cue driving parasitic egg detection. Here we systematically tested whether and how egg-nest contrast itself contributes to foreign egg discrimination. In an artificial parasitism experiment, we independently manipulated egg color and nest lining color of the egg-ejector American robin (Turdus migratorius), a host of the obligate brood parasitic brown-headed cowbird (Molothrus ater). We hypothesized that the degree of contrast between foreign eggs and the nest background would affect host egg rejection behavior. We predicted that experimentally decreasing egg-nest chromatic and achromatic contrast (i.e. rendering parasitic eggs more cryptic against the nest lining) would decrease rejection rates, while increasing egg-nest contrast would increase rejection rates. In contrast to our predictions, egg-nest contrast was not a significant predictor of
egg ejection patterns. Instead, egg color significantly predicted responses to parasitism. We conclude that egg-egg differences are the primary drivers of egg rejection in this system. Future studies should test for the effects of egg-nest contrast *per se* in predicting parasitic egg recognition in other host-parasite systems, including those hosts building enclosed nests and parasites laying cryptic eggs, as an alternative to hypothesized effects of egg-egg contrasts.

**Key words: Brood parasitism, visual modeling, visual ecology, egg rejection**

**INTRODUCTION**

Obligate brood parasites circumvent the costs of parental care and lay their eggs in the nests of other species (Davies, 2000). By accepting the burden of raising genetically unrelated offspring, brood parasite hosts suffer major fitness costs (Øien et al., 1998; Lorenzana and Sealy, 2001; Hauber, 2003a; Hauber, 2003b; Hoover, 2003). The rejection of foreign eggs in the nest is a potent defense against brood parasitism (Rothstein, 1975; Grim et al., 2011; Kilner and Langmore, 2011), which places reciprocal selective pressure on parasites to evolve egg coloration and/or maculation to match that of its host. This then selects for increasingly fine-tuned discrimination by hosts (Davies and Brooke, 1989; Stoddard and Stevens, 2010; Davies, 2011; Stoddard and Stevens, 2011). Such an arms-race is a canonical example of coevolutionary processes driving both perceptual and signaling mechanisms (Davies and Brooke, 1989; Davies, 2011, Igic et al., 2012; Stoddard et al., 2014).

The proximate, perceptual controls underlying egg rejection behavior have been intensively studied in various brood parasite-host systems (Kilner and Langmore, 2011). Generally, an egg should be perceived as foreign if it differs beyond a given threshold from the variation present within a host female’s natural clutch (Reeve, 1989; Rodriguez-Gironés, 1999).
Such recognition is dependent on a number of factors, including the population parasitism rate (Davies et al., 1996), the number of host eggs present, and the timing of egg parasitism (e.g. Moskát and Hauber, 2007). Hosts’ acceptance thresholds also vary according to experience, even within a single clutch (Hauber et al., 2006). Hosts can respond to differences in eggshell background color (Avilés et al., 2005; Honza et al., 2007; Honza and Polačiková, 2008; Moskát et al., 2008; Avilés et al., 2010; Bán et al., 2013; Croston and Hauber, 2014a), maculation pattern (Lawes and Kirkman, 1996; Lahti and Lahti, 2002; López-de-Hierro and Moreno-Rueda, 2010, Spottiswoode and Stevens, 2010), egg brightness (Lahti, 2006; Gloag et al., 2014), egg size (Rothstein, 1982; Marchetti, 2000), and egg shape (Guigueno and Sealy, 2012) when discriminating own from foreign eggs.

While above-threshold visual contrast is increasingly known to induce egg rejection among brood parasite hosts, it is not firmly established whether comparing own vs. foreign eggs is a more reliable cue than other visual comparisons available in the host’s nest environment (Endler and Mielke, 2005; Thorogood and Davies, 2013). For example, relatively few studies have examined whether and how nest lining color influences behavior (but see Bailey et al., 2014). Regarding parasitic egg rejection, the role of egg-nest contrast has similarly not been well-established (Siefferman, 2006), and only a handful of studies have experimentally tested the hypothesis that visual contrasts between eggs and their background (i.e. the nest lining) affect egg rejection decisions (Gloag et al., 2014; Honza et al., 2014). Growing evidence suggests that there is selective pressure for brood parasites to evolve dark, cryptic eggs among Australasian cuckoo-host systems, making egg detection by hosts or competing parasites difficult by blending in with the nest background (Langmore, 2005; Langmore et al., 2009; Gloag et al. 2014). While similar arguments have also been made for other host-parasite systems (Mason and Rothstein,
1987; Honza et al., 2011; Honza et al. 2014), experimental tests of whether egg-nest contrast affects parasitic egg discrimination in the context of both natural and experimental egg color variation are lacking.

We focused on the North American brown-headed cowbird (Molothrus ater; hereafter, cowbird) - American robin (Turdus migratorius; hereafter, robin) parasite-host system. Robins are a suitable study host in that they are one of fewer than 30 documented cowbird host species to eject cowbird eggs at rates above 75% (Briskie et al., 1992; Peer and Sealy, 2004), allowing for the testing of specific sensory hypotheses mediating egg rejection in this system. Previous work on this species-pair showed that natural cowbird eggs are perceptually distinct from natural (conspecific) robin eggs: they are rejected from 100% of experimental nests, whereas conspecific robin eggs are not rejected (Briskie et al., 1992; Croston and Hauber, 2014a; Fig. 4.1A). Because egg color variability within robin clutches is significantly lower than egg color variability between clutches, robins may compare foreign eggs against the relatively low color variability present within the entire clutch in their egg rejection decisions (Abernathy and Peer 2014; see also Fig. 4.1B).

In this host-parasite system, artificially colored and natural eggs also exhibit strongly and positively correlated chromatic contrast against both natural robin eggs and natural robin nest linings, as measured by avian visual modeling (Fig. 4.1B). Similar to robins’ intra-clutch color variability, natural robin nest linings show low spectral variability across the avian visible range (SFig. 4.1) as well as low avian-perceived chromatic and achromatic contrasts when compared against each other (SFig. 4.2). Further, avian-perceived visual chromatic and achromatic contrasts between robin eggs and natural nest linings are generally low (see below). Thus, egg-nest contrast potentially confounds the degree to which we understand egg-egg contrasts to serve
as the necessary and/or sufficient cues for parasitic egg discrimination in this and other host-parasite systems.

Here, we hypothesize that artificial eggs that more closely resemble the nest background (i.e. are cryptic) are more likely to be accepted. We experimentally tested the degree to which egg-nest contrast affects egg rejection, independent of egg-egg contrast, predicting that in/decreasing egg-nest contrast (thereby rendering eggs less/more cryptic), would in/decrease parasitic egg ejection rates. Alternatively, egg-nest contrast may not itself affect hosts’ rejection decisions, which would support the role of foreign vs. own egg differences themselves as the primary cue for parasitic egg discrimination. To establish the degree to which egg-nest contrast *per se* influences parasitic egg discrimination, we manipulated the nest-lining color of robin nests (Fig. 4.2) in an artificial brood parasitism experiment.

We parasitized robin nests with plaster-of-Paris eggs painted the same colors as our nest lining manipulations (cowbird-ground color mimetic – hereafter, beige; blue-green – hereafter, robin-mimetic; and red), and whose rejection rates in non-manipulated nests are known from our published work (Table 4.1; Fig. 4.2; these egg colors and their rejection rates in natural nests were sourced from Croston and Hauber, 2014a). To determine the extent to which we successfully manipulated artificial egg-nest lining contrast, we conducted avian visual modeling analyses on egg and nest-lining reflectance spectra (Fig. 4.3), as well as analyzed raw reflectance spectra themselves (see Supplementary Materials) as a methodological check. We specifically predicted that artificially increasing the visual contrasts (measured as just-noticeable-differences, or JNDs, from visual modeling analyses) between experimental parasitic eggs and the nest background would result in increased rejection rates, while artificially decreasing contrast would
decrease rejection rates (Table 4.1). We then tested our predictions by assessing the extent to which artificial egg-nest lining achromatic and chromatic contrasts predicted egg rejection rates.

**RESULTS**

**Covariation of egg-egg vs. egg-nest contrasts with published egg rejection rates**

Natural robin eggs (which elicit no ejection; Briskie et al., 1992), possessed significantly lower chromatic contrasts than natural cowbird eggs (which are always ejected) when compared to natural robin eggs sourced from different conspecific nests ($U_{(1)} = 21.77$, $p < 0.0001$; Fig. 4.1A). In parallel, natural robin eggs possessed significantly lower chromatic contrasts against natural robin nest linings relative to natural cowbird eggs ($U_{(1)} = 6.00$, $p = 0.01$, Fig. 4.1A). Further supporting our claim that there is a quantitative confound between egg-egg chromatic contrast and egg-nest chromatic contrast, we found a strong and significant positive relationship between artificial egg-natural robin egg and artificial egg-natural nest chromatic JNDs ($F_{(1,4)} = 30.24$, $p = 0.0053$; Fig. 4.1B) by including natural and artificial egg stimuli analyzed in Croston and Hauber (2014a). We also show in the Supplementary Materials that color variation among natural robin nest linings is low (SFig. 4.1; SFig. 4.2), suggesting that the nest lining itself presents a reliable cue to be used by robins to perceptually discriminate own from foreign eggs.

**Perceptual outcomes of egg/nest lining color manipulations**

We found that natural robin egg-egg chromatic contrasts ($M = 1.91$, S.E.M. = 0.30) were significantly lower than natural egg-natural nest lining contrasts ($M = 3.98$, S.E.M. = 0.19; $U_{(1)} = 9.60$, $p < 0.001$; Fig. 4.1). In contrast, achromatic natural egg-egg contrasts (2.84, S.E.M. = 0.65) were not significantly different from egg-natural nest lining contrasts ($M = 3.70$, S.E.M. = 1.53; $U_{(1)} = 0.154$, $p = 0.69$) when compared to natural nest lining. Together, these results suggest that
it is chromatic contrast against the natural nest lining that may provide a strong cue against which to compare foreign eggs.

We compared avian-perceived chromatic differences between all eggs and nest-lining colors to test our predictions outlined in Table 4.1. We found a significant effect of nest-lining color ($n_{\text{beige nest}} = 15$, $n_{\text{red nest}} = 15$, $n_{\text{robin-mimetic nest}} = 15$, $n_{\text{natural nest}} = 5$) on chromatic contrast among beige eggs ($H(3) = 44.63$, $p < 0.0001$). All pairwise comparisons were significant ($p < 0.05$; Fig. 4.4A): beige eggs had the highest chromatic contrast in red nests, followed by robin-mimetic nests, natural nests, then beige nests. We also found a significant effect of nest-lining color on chromatic contrast among robin-mimetic eggs ($H(3) = 42.67$, $p < 0.0001$). Red nests had the highest chromatic contrast with robin-mimetic eggs, followed by beige nests, natural nests, and robin-mimetic nests. The amount of chromatic contrast between robin-mimetic eggs and nests differed significantly among all pairs ($p < 0.05$), except between beige and natural nests (Fig. 4.4C). Last, we found a significant effect of nest-lining color on chromatic contrast among red eggs ($H(3) = 45.00$, $p < 0.0001$). All pairwise comparisons were significant ($p < 0.05$; Fig. 4.4E); red eggs in robin-mimetic nests had the highest chromatic contrast, followed by beige nests, natural nests, and red nests.

The analyses above were conducted using an ultra-violet sensitive (UVS) perceptual model for robin vision (based on Aidala et al. 2012), and we carried out a separate set of visual model analyses using violet-sensitive (VS) visual model parameters (see Supplementary Materials). The results followed the same chromatic contrast patterns as above, although JND values were generally much larger using this model than in our UVS visual model (Fig. 4.4; SFig. 4.3A, 4.3C, 4.3E). Similarly, chromatic distance analyses of chromatic principal components (PC) 2 and 3 scores (see Supplementary Materials; STable 4.1; SFig. 4.4) of raw
reflectance spectra (as a measure of chromatic distance) between eggs and nest linings corroborated the patterns seen in both of our visual modeling analyses (See Supplementary Materials; Fig. 4.4A, 4.4C, 4.4E; SFig. 4.5) and followed the same pattern when compared against rejection rates as chromatic JNDs (Fig. 4.5A, SFig. 4.6A SFig. 4.7A).

We also compared avian-perceived achromatic differences between all eggs and nest-lining colors (Fig 4.4 B, 4.4D, 4.4F). We found a significant effect of nest-lining color on achromatic contrast among beige eggs ($H(3) = 43.54, p < 0.0001$; Fig. 4.4B). There was also a significant effect of nest-lining color on achromatic contrast among robin-mimetic eggs ($H(3) = 38.03, p < 0.0001$; Fig. 4.4D) and red eggs ($H(3) = 41.38, p < 0.0001$; Fig. 4.4F). Neither our VS visual modeling analysis (SFig. 4.3) nor our PC 1 distances (as a measure of achromatic distance – see Supplementary Materials; SFig. 4.5B, 4.5D, 4.5F) paralleled the visual contrasts in our achromatic UVS visual model. However, because neither PC 1 distances nor achromatic JNDs (from either visual modeling analysis) between artificial eggs and nest linings were significantly related to rejection rate (see below; Fig. 4.5B, SFig. 4.6B, SFig. 4.7B), we only included achromatic JNDs in further behavioral analyses so as to be consistent with our analysis of chromatic JNDs. Due to the similarities in chromatic contrasts for both the VS visual model and analysis of chromatic principal components, we focus on our primary UVS visual modeling data (JNDs) for our behavioral analyses (see below).

**Behavioral experiments**

We conducted a total of 94 artificial parasitism experiments with model eggs ($n_{beige\ egg} = 34$, $n_{robin-mimetic\ egg} = 29$, $n_{red\ egg} = 31$), in nests with beige ($n = 17$), red ($n = 19$), and robin-mimetic ($n = 12$) linings. When combined with egg rejection rate data of artificial egg colors in
natural nests from Croston and Hauber (2014a), the mean chromatic contrasts in egg-nest treatments were not significantly related to the rejection rate in our egg-nest manipulations, and the regression slope was slightly negative and thus in the opposite direction of our predictions ($F_{(1, 10)} = 0.49, p = 0.50, R^2 = 0.05; \text{Fig. 4.5A}$). Similarly, when natural nest data were removed from this analysis, the relationship trended in the same direction but remained non-significant ($F_{(1, 7)} = 0.25, p = 0.63, R^2 = 0.03$). Achromatic contrasts were also not significantly related to rejection rate in our nest manipulations both with natural nest rejection data from Croston and Hauber (2014a) included ($F_{(1, 10)} = 0.43, p = 0.53, R^2 = 0.04; \text{Fig. 4.5B}$) and removed ($F_{(1, 7)} = 0.0004, p = 0.98, R^2 < 0.01$).

A Friedman ANOVA revealed a consistent effect of egg colors on robins’ egg rejection behaviors: relative egg rejection rates were consistently ordered as beige > red > mimetic eggs across, and irrespective of, the three colors of experimental and one natural nest lining types ($\chi^2_{(2)} = 8.00, p = 0.018; \text{Fig. 4.5C}$). To confirm these results, we fit GLMM binomial logistic models to further describe predictors of egg rejection. In order to be conservative in the analysis and interpretation of our data, we first controlled for individual females’ known propensity to consistently reject or accept foreign eggs irrespective of egg coloration (Croston and Hauber 2014b; See Supplementary Materials). We then combined our dataset with published egg rejection data of artificial eggs in natural, non-manipulated robin nests (Croston and Hauber, 2014a). The full model significantly predicted artificial egg rejection/acceptance outcome ($\chi^2_{(8)} = 41.19, p < 0.0001$). The only significant predictor of egg rejection in this model was egg color ($\chi^2_{(2)} = 39.77, p < 0.0001; \text{Table 4.2A}$). Last, we fit a GLMM including all above predictors, as well as chromatic and achromatic contrast between egg and nest-lining colors. Again, the whole model significantly predicted egg acceptance/rejection behavior ($\chi^2_{(10)} = 42.82, p < 0.0001$), but
neither chromatic nor achromatic egg-nest JNDs were a significant predictor of egg rejection. As in our above models, the only significant predictor of egg rejection was egg color ($\chi^2_{(2)} = 34.05$, $p < 0.0001$; Table 4.2B).

To further confirm these results, we ran post-hoc tests on the single significant predictor (egg color) in the final GLMM model (Table 4.2D). The post-hoc $\chi^2$ test of egg color against a reject/accept outcome variable showed a significant difference in egg rejection behavior by egg color ($\chi^2_{(2)} = 40.39$, $p < 0.0001$; Fig. 4.5C). Irrespective of nest type, beige eggs were rejected in 33 out of 39 trials, robin-mimetic eggs were rejected in 5 out of 35 trials, and red eggs were rejected in 23 out of 43 trials. When split by nest type (Fig. 4.5C; Table 4.1), there was a significant difference in rejection rate of each egg type in beige nests ($\chi^2_{(2)} = 24.69$, $p < 0.0001$). In beige nests, beige eggs were rejected in 12 of 13 trials, robin-mimetic eggs were rejected in 0 of 9 trials, and red eggs were rejected in 4 of 12 trials. Further analysis showed that beige eggs were rejected significantly more often than both robin-mimetic eggs ($\chi^2_{(1)} = 23.27$, $p < 0.001$) and red eggs ($\chi^2_{(1)} = 10.34$, $p = 0.0013$; Table 4.1). Red eggs were similarly rejected more often than robin-mimetic eggs ($\chi^2_{(1)} = 5.17$, $p = 0.02$; Table 4.1). There was no significant difference in egg rejection by egg color in robin-mimetic nests ($\chi^2_{(2)} = 4.68$, $p = 0.10$; Table 4.1). In robin-mimetic nests, beige eggs were rejected in 6 out of 8 trials, robin-mimetic eggs were rejected in 2 of 8 trials, and red eggs were rejected in 4 out of 6 trials. There was also no significant difference in egg rejection by egg color in red nests ($\chi^2_{(2)} = 3.97$, $p = 0.14$; Table 4.1). In red nests, beige eggs were rejected in 8 of 11 trials, robin-mimetic eggs were rejected in 3 of 10 trials, and red eggs were rejected in 6 of 11 trials (Table 4.1).

**DISCUSSION**
Natural nest linings represent a reliable cue against which robins could compare own vs. foreign eggs; natural robin nests have low variation in raw reflectance spectra (SFig. 4.1) and avian-perceived chromatic and achromatic visual contrasts across different nests (SFig. 4.2). Furthermore, egg-egg contrasts between natural and artificial egg colors are positively related to egg-nest contrasts in robin nests, thus potentially confounding the interpretation of host-parasite egg rejection studies focusing on egg-egg contrasts only. Yet, our experimental manipulations of nest lining did not reliably alter egg rejection rates. Although we successfully altered the degree of egg-nest visual contrast both above and below natural levels (Table 4.1; Fig 4.4; SFig. 4.3, SFig. 4.5), we show here that the degree of perceivable color difference between foreign eggs and the nest background does not induce a predictable change in rejection rates of foreign eggs in the American robin. We minimally predicted egg-nest contrast would affect rejection rate of red eggs, which are rejected at intermediate rates in natural nests (Fig. 4.5C; Croston and Hauber, 2014a). Here, red eggs were rejected at intermediate rates irrespective of nest-lining color. Similarly, ejection rates of beige eggs and robin-mimetic eggs remained high and low, respectively, in all experimental nest-lining color conditions (Fig. 4.5C).

All same color egg-nest combinations produced the lowest chromatic contrast (i.e. were the most cryptic) when compared with other nest types (e.g. beige egg-beige nest), while different egg-nest combinations consistently yielded high chromatic contrasts (Fig. 4.4A, 4.4C, 4.4E). However, the degree of egg-nest chromatic contrast did not have a significant effect on rejection rates in our linear regression analysis (Fig. 4.5A), and remained non-significant in our GLMM analysis (Table 4.2B). There was similarly no discernible pattern, nor significant predictive effect, of achromatic contrast on egg rejection (Fig. 4.4B, 4.4D, 4.4F, Fig. 4.5B-C, Table 4.2). Because only four natural robin eggs went missing throughout the course of this
study, excluding the predation of the entire nest (See Materials and Methods), we conclude that rejection responses by robins were specifically directed at experimental egg colors, and that manipulation of the nest lining did not induce rejection of the robins’ own eggs.

Based on the consistent patterns of relative egg rejection rates between different artificial colors, irrespective of nest type (Fig. 4.5C; Table 4.2), we are therefore confident to reject the hypothesis that altering egg-nest contrast affects egg rejection in American robins. Unfortunately, robin identity, breeding age, prior experience with natural cowbird parasitism, and/or prior experience with our own experimentation was unknown in this study. Though age and experience may influence egg rejection decisions in other brood-parasite host systems, with more experienced individuals typically more likely to correctly identify and reject parasitism (e.g. Moskát et al., 2014a), it is not clear to what extent experience influences rejection decisions in American robins in our study population.

Evidence for parasitic egg crypsis via egg-nest color matching in other brood parasite systems is increasingly well documented in enclosed-nesting species. For example, some bronze-cuckoos (Chalcites spp.) have evolved dark egg pigmentation, which is cryptic in the domed nests of their hosts (Langmore et al., 2009). Manipulations could next establish whether host species and/or competing parasites respond differentially to parasitic eggs (Gloag et al., 2014) when experimentally illuminating the nest interior (Cassey, 2009; Honza et al., 2014) or when altering egg-nest contrasts independent of egg-egg contrast (this study). Whether cowbird eggs have a cryptic function in host nests has also not been studied in detail across different Molothrus cowbird-host systems (but see Mason and Rothstein, 1987; Siefferman, 2006). For example, cowbird eggs may be crypic or difficult to see in the open cup nests of the eastern phoebe (Sayornis phoebe), which are often built under eaves/bridges or in caves and may be less
illuminated than the open cups of robin nests; in turn, phoebes always accept cowbird parasitism (Hauber, 2003a; Peer and Sealy, 2004). Conversely, cowbird eggs have a greater avian-perceivable chromatic contrast against natural robin nest linings than do robin eggs themselves (Fig. 4.1A), making it unlikely that cowbird eggs are at all cryptic in robin nests.

That foreign eggs’ rejection does not depend on the degree of contrast between eggs and the nest lining (this study), provides support for earlier findings in hosts of egg-mimetic brood parasites that egg rejection is driven mechanistically by differences between foreign and own host eggs (Cassey et al., 2008; Stevens et al., 2013; Moskat et al., 2014b). In contrast, cowbird eggs in robin nests are exceptional to this pattern: Croston and Hauber (2014a) showed that while robins’ responses to artificial egg colors are generally predicted by chromatic JNDs differentiating foreign vs. host eggs, artificial cowbird ground color-mimetic (beige) eggs are rejected in 100% of trials, despite their relatively low avian-perceivable chromatic difference from robin eggs (Fig. 4.1B). In keeping with this pattern, our experimental manipulations showed that neither chromatic nor achromatic contrasts differentiating foreign eggs from nest linings were significant predictors of egg rejection – thus, cowbird egg rejection is likely the result of comparison between host and foreign eggs in robins (Croston and Hauber, 2014a). Future work should investigate the role of egg-nest contrast in egg rejection using ordinarily non-ejecting hosts.

We should note that higher chromatic contrasts do not necessarily correspond to more robust behavioral responses (Ham and Osorio, 2007). For example, chromatic JNDs differentiating artificial parasitic eggs and natural robin eggs do seem to drive rejection in robins. However, cowbird-mimetic model eggs are rejected at the highest rates despite having relatively low chromatic contrast from robin eggs (Croston and Hauber 2014a). In the present study, both
visual models showed similar patterns of chromatic contrasts between eggs and nest linings, and our supplementary analyses of physical distance using chromatic PCs largely confirm the outputs of both of our visual models. Despite the corroboration of our visual contrast analyses, we cannot not assume that higher JND values in the supra-threshold range necessarily correlate with stronger behavioral responses.

Another caveat in this, and other studies based on the analysis of avian visual modeling data, is that the magnitude of chromatic difference (whether between eggs or between eggs and nests) is not always a linear means of predicting egg rejection (or any vision-dependent) behavior. Chromatic distance is but one component of broader sensory/perceptual (de la Colina et al., 2012) and cognition-dependent (Hauber and Sherman, 2001; Moskát and Hauber, 2007) processes that ultimately result in the complex behavioral decision to accept or reject a parasitic egg. For example, there are a growing number of studies showing that perceptual difference alone does not fully explain patterns of egg rejection behavior (Moskát and Hauber, 2007; Moskát et al. 2010, Cassey et al., 2008; Stoddard and Stevens 2011, Bán et al. 2013; Stevens et al., 2013, Croston and Hauber, 2014a).

Aside from specific perceptual/cognitive processes mediating egg rejection behavior, variation in the predictive power of avian visual models may be partly due to the physiological assumptions made within visual sensory models themselves. For example, visual models are based on a limited subset of bird species, including a handful of UVS oscines, none of which are common hosts of brood parasites (Grim et al., 2011, Aidala et al., 2012). For example, for this study we used parameters for the robin’s visual system from the congeneric European blackbird (T. merula). This potentially confounds the degree to which we can model and understand host-parasite coevolution to shape hosts’ perceptual sensitivities. It is possible, then, that the visual
models used in this and in previous studies do not accurately represent the sensory physiology of the American robin. Likewise, inter-individual differences in sensory physiology could confound our results, such that egg rejection reflects unaccounted-for differences in individual sensory physiology rather than at the level of decision-making. Accordingly, within-species differences in sensory physiology have recently been described in the brown-headed cowbird (Fernández-Juricic et al., 2013). Future studies should endeavor not only to obtain and incorporate species-specific models of avian sensory physiology, but also describe the degree of inter-individual variation at both the behavioral and physiological levels.

We have shown here that egg-nest contrast is not a significant predictor of egg rejection by the American robin. Instead, egg rejection in robins is statistically explained, and likely perceptually driven, by differences between their hosts’ own eggs and foreign egg colors. Future work should focus on improving visual models by incorporating physiologically-appropriate, individual specific cone densities/absorbance spectra, as well as nest-site specific egg, nest lining, and ambient-light availability data.

**MATERIALS AND METHODS**

**Behavioral experiments**

All behavioral experiments were conducted in the vicinity of Ithaca, Tompkins County, New York, USA from May – July of the 2013 breeding season. We located active robin nests (n = 48), as defined by dry nest content, warm eggs, and/or defense or attendance by adult robins, through focusing on suitable nest sites near human-built structures, as this species is highly commensal (Sallabanks and James, 1999). Nest sites were also located with the help of local citizens via advertising in community listserves and businesses, and returning to locations with
known robin nests from previous years (Croston and Hauber, 2014a; Croston and Hauber 2014b).

After an active nest containing eggs was located, it was assigned in a balanced random procedure to an experimental nest type (1 treatment per nest) and sequential egg treatments (1-3 artificial eggs per nest). Robin nests were assigned one of three artificially colored nest linings, and paired with an artificial egg of one of the same three colors (see below for artificial egg and nest details; Fig. 4.2). Painted felt nest-linings (see below for details) were inserted and affixed to the inner bottom lining of robin nests using fast-drying, non-toxic glue (Liquid Fusion ®). An experimental egg was then added to the clutch without replacement (removal of one host egg), following methods used by Briskie et al. (1992) for American robins. Although egg replacement by cowbirds has been documented in one third of parasitized yellow warbler (Setophaga petechia) nests (Sealy, 1992) and in most parasitized eastern phoebe (Sayornis phoebe) nests (Hauber 2003a), the addition of an experimental egg does not affect rejection rates in related, European Turdus thrushes (Davies and Brooke, 1989; Grim et al., 2011) and allowed us to compare our new data to previous studies on robins (Rothstein, 1982; Briskie et al., 1992, Hauber and Croston, 2014a). Following the initiation of an experiment, we remained within sight of the nest to ensure that the new nest lining was not removed by adults upon their return to the nest. Nest lining removal occurred in only 3% of trials, and we returned and replaced the lining. If the experimental nest lining was removed by an adult robin three consecutive times, the experiment was abandoned at that nest. This occurred at only one nest site throughout the entire study.

All nests were checked daily after each experiment was initiated. Eggs were considered rejected if they were missing from a nest upon the return visit, unless the entire clutch was
missing (presumed predation) or nestlings had begun to hatch (to avoid conflating egg rejection with eggshell removal, as in nest sanitation: Hauber, 2003c). If an artificial egg remained in the nest on the 5th day after addition, it was considered accepted (Rothstein, 1975; Briskie et al., 1992). In a previous study using the same focal robin population, all ejected model eggs were rejected within 1 - 4 days of being parasitized (mean = 1.69 days; Croston and Hauber, 2014a), justifying a 5-day acceptance threshold. If a model egg remained in the nest through hatching, we continued monitoring for up to three days post-hatching due to well-documented asynchronous hatching in robin broods (Sallabanks and James, 1999; Z.A. personal observations). Following the acceptance or rejection of a first experimental egg, a second egg of a different color was introduced. Up to three different eggs were introduced into robin nests in this way during the laying and incubation periods. The same egg color was not introduced repeatedly into the same nest. The experimental protocols followed in this study were approved by the Hunter College Institutional Animal Care and Use Committee, and all experiments conducted on private properties were done so with the express permission and mostly enthusiastic support from the landowners (Hauber, 2003a; Wagner et al., 2013).

**Experimental eggs and nest linings**

We constructed model cowbird eggs within the natural variation of natural brown-headed cowbird eggs’ shape, size (21 mm x 16 mm), and weight (2.6 g – 3.4 g) as documented near our field site in upstate New York, USA (Lowther, 1993; Croston and Hauber, 2014a; Z.A. personal observation). Model eggs were made from plaster-of-Paris, using the silicone molds that were used for Croston and Hauber (2014a). Experimental nest-lining inserts were circular discs cut from white felt to fit the bottom of the robin’s nest cup dimensions (mean disc diameter = 94.14 mm) at our study site (Fig. 4.2). Eggs and felt were then painted red, natural cowbird ground
color-mimetic (beige), or blue-green (robin-mimetic), using the same latex or acrylic paint as used in Croston and Hauber (2014b). We utilized the three egg and nest lining colors by considering the general shape and peak of their reflectance curves and by the relative photon catches of each avian cone photoreceptor (Endler, 1990; Endler and Mielke, 2005; Fig. 4.3), predicted to induce sharply different sensory responses of the UV-sensitive visual range of American robins (Aidala et al. 2012). We also chose these three egg/nest colors because they represented known behavioral variation in egg ejection responses in natural nests within the same population of robins: beige (100% rejected), red (64% rejected), and robin-mimetic (0% rejected; Croston and Hauber, 2014a). These extreme and intermediate egg color rejection rates allowed us to design a two-tailed experiment, whereby both increased, decreased, and unchanged rejection rates would be predicted as a result of our experimental manipulations (Table 4.1).

As an internal experimental control for our invasive manipulations, we monitored the fate of naturally laid robin eggs in each clutch: a total of four robin eggs (at N= 48 nests monitored, mean natural clutch size per nest = 3.3 eggs) went missing during our study in 2013 (outside of complete nest predation events), implying that egg rejection responses were limited to experimental model eggs, and that own-egg rejection was not related to experimental manipulation of the nest lining. It was unclear in these instances whether these eggs were missing due to depredation events or failed rejections. In turn, as experimental controls for the nest lining manipulation, we contrasted the data from all of our experiments (single and multiple-presentation nests, barring those from the two significant sites in the GLMM model described above and in Table 4.2, in 2013) with the published behavioral egg rejection data in natural, unmanipulated robin nests in response to our three experimental egg colors from Croston and Hauber (2014a). We acknowledge the limitation that using the published egg rejection data from
natural nests is at best a partial methodological control for our nest lining manipulations, and full experimental control should conceivably include adding a see-through felt, or felt dyed with a natural nest-reflectance matching color. Furthermore, those data were derived mostly during the 3 years prior to our experiments; however, egg rejection rates did not vary between years in our study population: Croston and Hauber, 2014a; Croston and Hauber, 2014b).

**Spectral measurements and visual modeling**

We obtained spectral measurements of natural robin (n = 76) and cowbird (n = 15) eggs by combining our dataset from 2013 with that of Croston and Hauber (2014a). In 2013, we also collected reflectance spectra from natural robin nest linings (n = 19), as well as from our artificial eggs and nest backgrounds. Spectral measurements were taken with an Ocean Optics USB2000 Miniature Fiber Optic Spectrometer, connected to a laptop computer running OOIBase32 software, and using a UV-Vis DT mini-lamp light source (Ocean Optics, Inc. Dunedin, FL) or an Ocean Optics Jaz spectrometer with UV-VIS light source (Ocean Optics, Inc. Dunedin, FL). All measurements were taken at a 90° angle to the egg or nest-lining surface. We took nine measurements each from individual nests, linings, and eggs: three measurements each from the nests’ upper inner cup, lower inner cup, and bottom, and three measures each from the blunt pole, middle portion, and narrow pole of natural and artificial eggs. The spectrometer was re-calibrated frequently, using the Ocean Optics WS-1 white reflectance standard, and a dark reference made from a cardboard box, lined with black felt, and pierced to create a small hole for the probe (blocking any incident light; Igic et al., 2009; Igic et al., 2010; Croston and Hauber, 2014a). We averaged the 9 spectra per egg/nest to generate a composite spectra profile for each egg and nest included in our visual modeling analyses. As a methodological check, we compared
the mean achromatic and chromatic spectra of each nest lining area prior to compiling composite natural nest lining spectra.

Visual modeling analyses were conducted using AVICOL v.6 (Gomez, 2006). We applied a 15 nm triangular correction to raw spectra, available as a function within AVICOL, to attenuate and minimize the effect of spectrometer noise on the visual model. We ran a tetrachromatic receptor noise-limited color opponency model (Vorobyev and Osorio, 1998), assuming noise independent of the neural signal, and set the Weber fraction to 0.1 (Vorobyev et al., 1998; Igic et al., 2010; Croston and Hauber, 2014a; Croston and Hauber, 2014b). This type of opponency contrast model is preferable over avian visual models only accounting for properties of the photoreceptors themselves because such models do not agree with behavioral psychophysics data (see Vorobyev & Osorio, 1998). The model incorporates maximal absorbance and relative densities of each cone type as well as other physiological variables such as oil droplet and ocular media transmittance, allowing for analysis of both chromatic and achromatic contrasts (Vorobyev and Osorio, 1998; Vorobyev et al., 1998).

Because no photoreceptor absorbance or relative cone density data are currently available for robins, we approximated photoreceptor abundances and relative cone densities based on published data of the closely related UVS European blackbird (Hart et al., 2000). The use of a congener Turdus may be suitable as the American robin is predicted to also possess a UVS SWS1 photo-pigment, based on the results of our molecular genetic analyses of the SWS1 opsin gene of the robin (Aidala et al., 2012). In this model, we set the relative cone densities (UVS: 1, SWS: 1.78, MWS: 2.21, LWS: 1.96) based on cone density data measured by Hart et al. (2000). Ambient light level irradiance data of a generic ‘open-cup’ nesting species were extracted from Avilés et al. (2008) and were kindly provided by B. Igic (Igic et a. 2012), as ambient light levels
can affect both the risk of parasitism and parasitic egg detection (Langmore et al., 2005; Muñoz et al., 2007; Avilés, 2008; Honza et al., 2011).

Achromatic contrasts were calculated by summing MWS and LWS cone spectra (Osorio and Vorobyev, 2005; Gomez, 2006; Osorio and Vorobyev, 2008), as their combined sensitivities are thought to be comparable to those of the non-color sensitive rod and double cone (Osorio et al., 1999) photoreceptors across avian taxa (Hart et al., 1998; Hart et al., 2000; Igic et al., 2009). Using the model parameters described above, AVICOL generated separate chromatic and achromatic perceptual distances between two objects as JNDs; a calculated JND value greater than 1.0 suggests that two stimuli are discriminable from one another, while a JND less than that suggests that they are not (Gomez, 2006).

Although our visual modeling is based on the known retinal physiology of a closely related UVS *Turdus* species, the European blackbird, we augmented our visual modeling analyses by also computing a VS visual model (See Supplementary Materials). We also analyzed raw reflectance spectra of nests and eggs (see Supplementary Materials), as reliance on avian visual modeling alone introduces untested assumptions about a focal species’ physiology (Stoddard and Stevens, 2011), and can be avoided by analyzing raw spectra instead (Cherry and Bennett, 2001; Starling et al. 2006; Cherry et al., 2007; but see Endler and Mielke, 2005). These additional analyses allowed for increased explanatory power of our behavioral results as they relate to visual contrast. The results of these analyses complemented the statistical and qualitative conclusions drawn from our JND analyses (see Supplementary Materials).

**Data analysis**
In order to confirm that our natural nest lining composite spectra were representative of all three nest areas measured, and not biased towards one nest area over the others, we compared avian-perceived (a)chromatic differences between natural nest lining areas (upper inner cup, lower inner cup, and bottom). No achromatic or chromatic within nest area comparison was higher than 1.65 JNDs. Because the visual contrasts between nest areas were so low, we used the composite natural nest lining spectra including the nine measurements from the three nest areas in all analyses. In order to show that there exists a methodological confound between egg-egg chromatic contrast and egg-nest chromatic contrast, we conducted nonparametric Mann-Whitney U tests between natural robin and cowbird eggs against conspecific natural robin eggs and natural robin nest linings, respectively. We also conducted a linear regression analysis to test the relationship between egg-natural robin egg and egg-natural robin nest lining chromatic contrasts using artificial egg stimuli sourced from Croston and Hauber (2014a). We next confirmed that our nest lining manipulations resulted in experimental alteration of chromatic and achromatic contrasts between eggs and nests, using nonparametric Kruskal-Wallis rank sums tests and post-hoc pairwise comparisons following the Wilcoxon method. Prior to analysis, we randomized our comparisons such that only one egg-nest combination was used in each type of egg-nest contrast comparisons.

We examined the statistical relationship between (a)chromatic egg-nest and egg-egg contrasts and rejection rates for both natural and artificial eggs using linear regression analyses. A non-parametric 2-way Friedman ANOVA was run to test whether nest color affected egg rejection behavior across the different egg color stimuli. To further examine the role of egg-egg and egg-nest contrasts in parasitic egg rejections, we fit binomial Generalized Linear Mixed Models (GLMMs, with accept/reject as the outcome variables) using Firth-adjusted bias
estimates to determine the degree to which nest color influenced egg rejection behavior. In these models, we included egg color, nest color, nest site, experimental date, presentation order, and natural clutch size as predictor variables (Table 4.2; STable 4.2). After controlling for individual females’ tendencies to accept or reject experimental eggs irrespective of egg/nest treatments (See Supplemental Materials), we included rejection rates in natural nests (Croston and Hauber 2014a) for the three egg colors used in this study. We included the same predictors listed above except nest site. Last, we included chromatic and achromatic JNDs between eggs and nests in the GLMM model to explicitly test the role of avian-perceived contrasts in egg rejection frequencies. Post-hoc analyses of significant predictors in this final GLMM were run using chi-square tests. All analyses were run using JMP v. 10 (SAS Institute, Inc., Cary, NC), Statview 5.1 (SAS Institute, Inc., Cary, NC), and GraphPad Prism v. 6 (GraphPad Software, Inc., La Jolla, CA). Figures were compiled and edited using Adobe Creative Suite 5 (Adobe Systems, Inc., San Jose, CA).

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COMPETING INTERESTS

The authors declare no competing interests for this work.

AUTHOR CONTRIBUTIONS
Z.A. and M.E.H. designed this study. Z.A., R.C., J.S. and L.T. conducted the experiments and collected data. Z.A. and M.E.H. analyzed the data and Z.A. wrote the first draft of the manuscript, with all authors contributing to critical interpretation of data and results, writing, and editing of subsequent drafts of the manuscript.

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**REFERENCES**


### Table 4.1

Experimental nest/egg color manipulations showing the predicted and observed effects on rejection rates in robins. Predicted Change in Chromatic Contrast and Observed Change in Chromatic Contrast refers to the predicted change in avian perceivable color contrast between experimental eggs-natural nests relative to experimental eggs-experimental nests. Rejection rates in natural nests (unmanipulated nest lining) were sourced from Croston and Hauber (2014a). Observed rejection rates and 95% Confidence Intervals of experimental egg colors in each experimental nest lining also shown. P-values from χ² analyses of experimental egg colors are split by nest lining color and shown as significant or not significant at α = 0.05.

<table>
<thead>
<tr>
<th>Nest Color</th>
<th>Egg Color</th>
<th>Rejection Rate in Natural Nests</th>
<th>Observed Change in Chromatic Contrast</th>
<th>Predicted Change in Rejection Rate</th>
<th>Observed Percent of Experimental Eggs Rejected</th>
<th>Rejection Rate 95% Confidence Interval</th>
<th>χ² Test Comparing Rejection by Experimental Nest Lining Color</th>
</tr>
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<tbody>
<tr>
<td>Beige</td>
<td>Beige</td>
<td>100%</td>
<td>Decrease</td>
<td>Decrease</td>
<td>92%</td>
<td>0.67 - 0.99</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Mimetic</td>
<td>0%</td>
<td>No Change</td>
<td>Increase</td>
<td>0%</td>
<td>0.00 - 0.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>64%</td>
<td>Increase</td>
<td>Increase</td>
<td>33%</td>
<td>0.14 - 0.61</td>
<td></td>
</tr>
<tr>
<td>Mimetic</td>
<td>Beige</td>
<td>100%</td>
<td>Increase</td>
<td>No change</td>
<td>75%</td>
<td>0.41 - 0.93</td>
<td>p = 0.10</td>
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<td></td>
<td>Mimetic</td>
<td>0%</td>
<td>Decrease</td>
<td>No change</td>
<td>25%</td>
<td>0.07 - 0.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>64%</td>
<td>Increase</td>
<td>Increase</td>
<td>67%</td>
<td>0.30 - 0.90</td>
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</tr>
<tr>
<td>Red</td>
<td>Beige</td>
<td>100%</td>
<td>Increase</td>
<td>No change</td>
<td>73%</td>
<td>0.43 - 0.90</td>
<td>p = 0.14</td>
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<tr>
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<td>Increase</td>
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<td>0.11 - 0.60</td>
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<tr>
<td></td>
<td>Red</td>
<td>64%</td>
<td>Decrease</td>
<td>Decrease</td>
<td>55%</td>
<td>0.28 - 0.79</td>
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Table 4.2. Generalized Linear Mixed Model (GLMM) fits with binomial distribution (outcome variable: accept/reject) of parameters used to assess variables predicting egg rejection behavior. Egg rejection data in natural nests sourced from Croston and Hauber (2014a). In (A), experimental nests from (STable 4.2B) and sites at which only one trial was conducted were included. In (B), all trials included in (A) were re-run adding achromatic and chromatic egg-nest lining contrasts as predictor variables. Significant models and predictor variables are denoted by an asterisk (*).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>df</th>
<th>$\chi^2$</th>
<th>p-value</th>
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<td>Whole model</td>
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<td>41.19</td>
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<tr>
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<td>39.77</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Clutch size</td>
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<td>0.35</td>
<td>0.55</td>
</tr>
<tr>
<td>Presentation order</td>
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<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Experiment date</td>
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<td>0.03</td>
<td>0.87</td>
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<tr>
<td>Egg color</td>
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<td>34.05</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Nest color</td>
<td>3</td>
<td>1.84</td>
<td>0.61</td>
</tr>
<tr>
<td>Chromatic JND</td>
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<td>0.31</td>
<td>0.58</td>
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<tr>
<td>Achromatic JND</td>
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<td>1.51</td>
<td>0.22</td>
</tr>
<tr>
<td>Clutch Size</td>
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<td>0.67</td>
</tr>
<tr>
<td>Presentation order</td>
<td>1</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Experiment date</td>
<td>1</td>
<td>0.13</td>
<td>0.72</td>
</tr>
</tbody>
</table>
Fig. 4.1. (A) Comparison of chromatic contrasts of robin eggs, which elicit no ejection (Briskie et al., 1992), and cowbird eggs, which elicit 100% ejection by robins against robin eggs and natural robin nest linings. Conspecific robin eggs had significantly lower chromatic contrasts than cowbird eggs against both robin eggs ($U_{(1)} = 21.77, p < 0.0001$) and natural nest linings ($U_{(1)} = 6.00, p = 0.01$). (B) Linear regression between mean egg-egg and egg-nest chromatic JNDs for artificial egg types ($F_{(1,4)} = 30.24, p = 0.0053$) using combined data from Croston and Hauber (2014a). For comparison, natural robin and cowbird egg chromatic contrasts are also shown.
Fig. 4.2. Artificial eggs in natural (top row), beige (second row), robin-mimetic (third row), and red (bottom row) nests. Artificial eggs were constructed of painted plaster-of-Paris (measuring 21 mm x 16 mm). Experimental nests were lined with a felt pad (mean disc diameter = 94.14 mm) that was painted with the same paint colors as artificial eggs, which was affixed to the bottom of American robin nests using fast-drying, non-toxic glue.
Fig. 4.3. Mean interpolated spectra of natural (A) and artificial (B) eggs and nests used in visual modeling analyses. Spectral measurements were taken across the avian-visible range (300 nm – 700 nm; see Materials and Methods).
Fig. 4.4. (A-F) Mean (S.E.M) chromatic (A, C, E) and achromatic (B, D, F) contrasts between experimental eggs and all nest linings. Images below each column indicate the experimental egg-nest lining pair measured. All comparisons made using Kruskal-Wallis rank sums tests followed by Wilcoxon pairwise comparisons. Significant pairwise comparisons indicated by letters in/above each column – columns bearing the same letter are not significantly different. In (A)
and (B), beige egg-nest lining chromatic ($H(3) = 44.63$, $p < 0.0001$) and achromatic ($H(3) = 43.54$, $p < 0.0001$) contrasts were significant. In (C) and (D), robin-mimetic egg-nest lining chromatic ($H(3) = 42.67$, $p < 0.0001$) and achromatic ($H(3) = 38.03$, $p < 0.0001$) contrasts were significant. In (E) and (F), red egg-nest lining chromatic ($H(3) = 45.00$, $p < 0.0001$) and achromatic ($H(3) = 41.38$, $p < 0.0001$) contrasts were significant.
Fig. 4.5. The effect of model egg and nest lining color manipulations on egg rejection rates by American robins. In (A) and (B), data points refer to egg colors (tan = beige, blue = robin-mimetic, and red = red) and text refers to nest linings (B_N = beige nest, M_N = robin-mimetic nest, and R_N = red nest). (A) The relationship between chromatic JND of eggs-nest linings and rejection rate was not significant (F_{(1, 10)} = 0.49, p = 0.50). (B) The relationship between achromatic JND of eggs-nest linings and rejection rate was also not significant F_{(1, 10)} = 0.43, p = 0.53. (C) Egg ejection rates plotted by nest and egg types, which showed a significant effect of egg type on rejection rate, irrespective of nest treatment (Friedman ANOVA χ^2_{(2)} = 8.00, p = 0.018). Egg color was also the only significant predictor of egg rejection in our GLMM analysis (χ^2_{(2)} = 34.05, p < 0.0001), while nest color was not (χ^2_{(3)} = 1.84, p = 0.61). Post-hoc chi-square tests showed that rejection behavior was consistently ordered by egg colors (χ^2_{(2)} = 40.39, p < 0.0001).
SUPPLEMENTARY MATERIALS

Supplementary Methods

VS visual modeling analysis

We supplemented our primary visual modeling analyses by using a VS-based avian visual model, as differences in retinal physiology between the European blackbird and the American robin are unknown. In this second visual model, we used the cone absorbance spectra from the VS pigeon (*Columba livia*; Bowmaker et al., 1997; Vorobyev and Osorio, 1998) and the relative cone densities (UVS: 1, SWS: 1.9, MWS: 2.2, LWS: 2.1) of the peafowl (*Pavo cristatus*) as measured by Hart (2002). All other visual modeling parameters remained the same as in our UVS visual model (See Materials and Methods).

Analyzing raw reflectance spectra

We conducted principal components analysis (PCA) on covariances of interpolated egg and nest reflectance spectra over 1 nm intervals from 300 nm to 700 nm as a supplementary analysis to our avian visual models. In such analyses of eggshell and other avian coloration, principal component 1 (PC 1) typically explains the vast majority of the variance among spectral data and is typically a measure of achromatic/brightness variation (Cherry and Bennett, 2001; Endler and Mielke, 2005). When plotting eigenvectors as a function of wavelength, PC 1 is represented as a relatively straight horizontal line (SFig. 4.4A). Subsequent PCs are typically a measure of chromatic variation. In our analysis, the first three PCs explained 98.6% of the variance in the data (STable 4.1; PC 1 = 67.14%, PC 2 = 28.53%, and PC 3 = 2.93%). Subsequent PCs explained less than 1% of the variance and were eliminated from further analysis. We calculated the absolute value distance between PC 1 scores for achromatic egg-nest
contrasts (following Igic et al., 2012). As a measure of chromatic distance, we calculated the Euclidean distance between principal components scores for PCs 2 (described by $x$ and $y$ coordinate values of $p_1$ and $p_2$) and 3 (described by $x$ and $y$ coordinate values of $q_1$ and $q_2$) (SFig. 4.4B) using the standard distance formula:

$$d = \sqrt{(q_1 - p_1)^2 + (q_2 - p_2)^2}$$

Using these distance scores (PC 1 distance scores as a measure of achromatic contrast and PC 2 – PC 3 Euclidean distance scores as a measure of chromatic contrast), we examined the relationship between distance and rejection rates between experimental eggs and nest linings using linear regression analyses.

**Analyzing individual females’ responses to multiple parasitism experiments**

We first examined the known effects of the same individual female robins’ tendencies to consistently accept or reject the differently colored eggs across the different nest treatments (Croston and Hauber, 2014b). We conducted our logistic regression analyses (See Materials and Methods) with site nested within nest color to test for the known effects of the same individual female robins’ tendencies to consistently accept or reject in each nest treatment (Croston and Hauber, 2014b; STable 4.2). In these models, we only included nest sites where more than one egg had been presented. We then re-ran the analysis to include all experiments save those where nest site was a significant predictor of rejection (See Results; Table 4.2).

**Supplementary Results and Discussion**

VS visual modeling analysis
We found a significant effect of experimental nest treatment on chromatic JNDs among beige eggs \((H(3) = 45.00, p < 0.0001)\). All pairwise comparisons were significant \((p < 0.05; \text{SFig. 4.3A})\): beige eggs against red nests had the highest chromatic contrast, followed by robin-mimetic nests, then natural nests, then beige nests. We also found a significant effect of nest treatment on chromatic JNDs among robin-mimetic eggs \((H(3) = 43.86, p < 0.0001)\). Again, all pairwise comparisons were significant \((p < 0.05; \text{SFig. 4.3C})\): robin-mimetic eggs possessed the highest chromatic contrast against red nests, followed by natural nests, then beige nests, then robin-mimetic nests. Lastly, we found a significant effect of nest treatment on chromatic JNDs among red eggs \((H(3) = 44.56, p < 0.0001)\). All pairwise comparisons were significant \((p < 0.05; \text{SFig. 4.3E})\): red eggs against robin-mimetic nests had the highest chromatic contrast, followed by beige nests, then natural nests, then red nests.

We found a significant effect of experimental nest treatment on achromatic JNDs among beige eggs \((H(3) = 42.29, p < 0.0001)\). All pairwise comparisons were significant, except for those between natural and red nests (SFig. 4.3B). Beige eggs had the lowest achromatic contrasts against beige nests, and the largest against robin-mimetic nests. We also found a significant effect of experimental nest treatment on achromatic JNDs among robin-mimetic eggs \((H(3) = 38.65, p < 0.0001)\). Robin-mimetic eggs had significantly higher achromatic contrasts against beige nests than against natural, red, or robin-mimetic nests (SFig. 4.3D). Robin-mimetic eggs also had significantly higher achromatic contrasts against robin-mimetic nests than red nests. No other pairwise comparisons were significant in this analysis. Lastly, we found a significant effect of experimental nest treatment on achromatic JNDs among red eggs \((H(3) = 36.83, p < 0.0001)\). All pairwise comparisons were significant, except for those between natural and red nests (SFig. 4.3E).
Red eggs had the highest achromatic contrasts against beige nests, followed by robin-mimetic nests, then red nests, then natural nests.

Our VS visual modeling analysis suggested that even using model parameters that differ from those of the more closely related European blackbird, the general patterns of experimentally induced egg-nest chromatic contrasts remained consistent. Overall chromatic JND values were inflated in this model but it is not clear how these higher JND values would translate into different behavioral responses, given that increasing JND values do not always correspond to more robust behaviors (Stevens et al. 2013). In this analysis, however, neither egg-nest chromatic JNDs ($F_{(1, 10)} = 0.32, p = 0.58$) nor egg-nest achromatic JNDs ($F_{(1, 10)} = 0.75, p = 0.41$) were significantly related to egg rejection rate (SFig. 4.7).

**Analysis of raw reflectance spectra**

We found a significant effect of experimental nest treatment on chromatic (Euclidean) distance of among beige eggs ($H_{(3)} = 45.00, p < 0.0001$). All pairwise comparisons were significant ($p < 0.05$; SFig. 4.5A); beige eggs in red nests had the highest Euclidean distance followed by natural nests, beige nests, then robin-mimetic nests. We also found a significant effect of nest type on Euclidean distance among robin-mimetic eggs ($H_{(3)} = 43.43, p < 0.0001$). All pairwise comparisons were again significant ($p < 0.05$; SFig. 4.5C); robin-mimetic eggs had the highest Euclidean distances in red nests, followed by natural nests, beige nests, then robin-mimetic nests. Last, we found a significant effect of nest type on Euclidean distance among red eggs ($H_{(3)} = 45.00, p < 0.0001$). All pairwise comparisons were also significant ($p < 0.05$, SFig. 4.5E); red eggs had the highest Euclidean distances in robin-mimetic nests, followed by beige nests, natural nests, then red nests. Euclidean distances had a significant, positive correlation
with chromatic JNDs (R = 0.95, p < 0.0001), and followed the same general pattern among egg and nest combinations (SFig. 4.5A, 4.5C, 4.5E; Fig. 4.4A, 4.4C, 4.4E). Because these analyses corroborate the data from our visual modeling analyses, we focused on our avian visual model’s chromatic JND data for our behavioral analyses as response measures predicted by experimental variation of nest-egg chromatic contrasts.

We also found a significant effect of nest type on PC 1 (achromatic) distance scores for beige eggs (H(3) = 35.84, p < 0.0001). All pairwise comparisons were significant (p < 0.05) except between red nests-natural nests and natural nests-robin-mimetic nests. Beige eggs had the largest PC 1 distance in robin-mimetic nests, followed by natural nests, red nests, then beige nests (SFig. 4.5B). We also found a significant effect of nest type on PC 1 distance scores for robin-mimetic eggs (H(3) = 40.33, p < 0.0001). Again, all pairwise comparisons were significant (p < 0.05) except between natural nests and robin-mimetic nests. Robin-mimetic eggs had the largest PC 1 distance in beige nests, followed by mimetic nests, natural nests, then red nests (SFig. 4.5D). Last, we found a significant effect of nest type on PC 1 distance scores for red eggs (H(3) = 41.24, p < 0.0001). All pairwise comparisons were significant (p < 0.05) except between red nests-natural nests and natural nests-robin-mimetic nests. Red eggs had the highest contrast in beige nests, followed by robin-mimetic nests, then natural nests, then red nests (SFig. 4.5F).

The PC 1 distance analysis (SFig. 4.5B, 4.5D, 4.5F) did not parallel the achromatic JND analyses (Fig. 4.3B, 4.3D, 4.3F) as strongly as the Euclidean distance analysis (SFig. 4.5A, 4.5C, 4.5E) paralleled the chromatic JND analysis (Fig. 4.4A, 4.4C, 4.4E), although there was a significant positive correlation between PC 1 distances and achromatic JNDs (R = 0.50, p < 0.0001). This is possibly because the PC 1 eigenvectors did not form a completely straight horizontal line when plotted as a function of wavelength (SFig. 4.4A), particularly at short
wavelengths. A number of earlier studies employing PCA to analyze reflectance spectra analyzed mimetic parasite and host eggs (e.g. Cherry and Bennett, 2001; Cherry et al., 2007), which would explain why most of the variation in PCA is due to achromatic variation. Because we included reflectance spectra from a broad range of colors, it is possible that a degree of chromatic variation leached into the PC 1 scores, which would be sufficient to cause the eigenvectors to correlate at varying strengths and directions across different wavelengths.

However, linear regression analysis showing Euclidean distance between PCs 2 and 3 as a function of rejection rate was not significant ($F_{(1, 10)} = 0.37, p = 0.56, R^2 = 0.04$; SFig. 4.6A), nor was the linear regression analysis showing PC 1 distance as a function of rejection rate ($F_{(1, 10)} = 1.48, p = 0.25, R^2 = 0.13$; SFig. 4.5B). We also did not find a significant relationship between PC distance scores and rejection rate (SFig. 4.5A-B), paralleling the non-significant relationship between (a)chromatic JND and rejection rate (Fig. 4.5A-B). Nonetheless, our chromatic JND (Fig. 4.4A, 4.4C, 4.4E) and chromatic PC (SFig. 4.5A, 4.5C, 4.5E) analyses complemented each other with respect to egg-nest lining contrasts. We therefore focused on chromatic and achromatic JNDs for subsequent behavioral analysis.

*Individual females’ responses to multiple parasitism experiments*

In the first model testing for the effects of individual females’ responses to accept or reject model eggs at each nest, we nested site as a predictor variable within nest lining color (site[nest color]). The overall model was a significant predictor of acceptance or rejection of artificial eggs ($\chi^2_{(33)} = 70.25, p = 0.0002$). Only egg color ($\chi^2_{(2)} = 27.86, p < 0.0001$) and site[nest color] ($\chi^2_{(33)} = 44.23, p = 0.03$) were significant predictors of egg rejection in the model (Table 4.2A). Although only one site was significant (red nest, all egg colors accepted; $p = 0.04$) in the
model, we also removed a second site that approached significance (robin-mimetic nest, all egg colors rejected; p = 0.06) to be conservative, thereby excluding two sites at which female robins responded to neither egg color nor nest lining treatments. We therefore excluded a total of six experiments at these two nests from further behavioral analysis. We re-ran this model excluding these two sites, leaving n = 88 experiments analyzed in subsequent models. The model significantly predicted egg rejection/acceptance behavior ($\chi^2_{(31)} = 63.89, p = 0.0005$; Table 4.2B). The only significant predictor of egg rejection in this model was egg color $\chi^2_{(2)} = 27.03, p < 0.0001$; Table 4.2B), providing experimental support for the independence of egg-egg and egg-nest color contrasts on females’ behavioral responses to accept or reject artificial eggs.
SFig. 4.1. Mean (S.E.M.) reflectance spectra across the avian visible spectrum of all natural nests. Data are batched over 10 nm intervals.
SFig. 4.2. Mean (S.E.M.) chromatic (A) and achromatic (B) JNDs between randomly-paired natural robin nest linings.
SFig. 4.3. (A-F) Mean (S.E.M.) chromatic (A, C, E) and achromatic (B, D, F) contrasts between experimental eggs and all nest linings using a VS visual perceptual model. Images below each column indicate the experimental egg-nest lining pair measured. All comparisons are made using Kruskal-Wallis rank sums tests followed by Wilcoxon pairwise comparisons. Significant pairwise comparisons are indicated by letters in/above each column – columns bearing the same letter are not significantly different. In (A) and (B), beige egg-nest lining chromatic ($H_{(3)} = 45.00$, $p < 0.0001$) and achromatic ($H_{(3)} = 42.29$, $p < 0.0001$) contrasts were significant. In (C) and (D), robin-mimetic egg-nest lining chromatic ($H_{(3)} = 43.86$, $p < 0.0001$) and achromatic ($H_{(3)} = 38.65$, $p < 0.0001$) contrasts were significant. In (E) and (F), red egg-nest lining chromatic ($H_{(3)} = 44.56$, $p < 0.0001$) and achromatic ($H_{(3)} = 36.83$, $p < 0.0001$) contrasts were significant.
SFig. 4.4. (A) Eigenvectors as a function of wavelength for the first three PCs from PCA on eggs and nests. PC 1, PC 2, and PC 3 refer to principal components 1, 2, and 3, respectively. (B) PC score plot for PC 2 and PC 3 following principal components analysis of interpolated reflectance spectra of eggs and nests. The first three principal components explained over 98% of the variance in the model. PC 1 is a positive correlate of achromatic variation (Cherry and Bennett, 2001; Endler and Mielke, 2005), and explained 67.14% of the variance in our data. PC 2 (28.53% of the variance) and PC 3 (2.93% of the variance) were used as descriptors of chromatic variation. Distances in (a)chromatic metrics between eggs and nests were calculated using both PC 1 (for achromatic distances) and PC 2 and PC 3 scores (for chromatic distances, by calculating Euclidian distances).
SFig. 4.5. (A-F) Mean (S.E.M) Euclidean distance between PC 2 and PC 3 scores (A, C, E) and PC 1 distance scores (B, D, F) from PCA on interpolated spectra between experimental eggs and all nest linings. Images below each column indicate the experimental egg-nest lining pair measured. All comparisons made using Kruskal-Wallis rank sums tests followed by Wilcoxon pairwise comparisons. Significant pairwise comparisons indicated by letters in/above each column – columns bearing the same letter are not significantly different. In (A) and (B), beige egg-nest PC 2 – PC 3 Euclidean distances ($H_{(3)} = 45.00, p < 0.0001$) and PC 1 distances $H_{(3)} = 35.84, p < 0.0001$ were significant. In (C) and (D), robin-mimetic egg-nest PC 2 – PC 3 Euclidean distances ($H_{(3)} = 43.43, p < 0.0001$) and PC 1 distances ($H_{(3)} = 40.33, p < 0.0001$) were significant. In (E) and (F), red egg-nest PC 2 – PC 3 Euclidean distances ($H_{(3)} = 45.00, p < 0.0001$) and PC 1 distances ($H_{(3)} = 41.24, p < 0.0001$) were significant.
SFig. 4.6. The effect of model egg and nest lining color manipulations on egg rejection rates by American robins. In (A) and (B), data points refer to egg colors (tan = beige, blue = robin-mimetic, and red = red) and text refers to nest linings (B_N = beige nest, M_N = robin-mimetic nest, and R_N = red nest). (A) The relationship between PC 2 and PC 3 Euclidean distances between eggs and nest linings and rejection rate was not significant (F_{(1, 10)} = 0.37, p = 0.56, R^2 = 0.04). (B) The relationship between PC 1 distances between eggs and nest linings and rejection rate was also not significant (F_{(1, 10)} = 1.48, p = 0.25, R^2 = 0.13).
The effect of model egg and nest lining color manipulations on egg rejection rates by American robins following a VS visual model. In (A) and (B), data points refer to egg colors (tan = beige, blue = robin-mimetic, and red = red) and text refers to nest linings (B_N = beige nest, M_N = robin-mimetic nest, and R_N = red nest). (A) The relationship between chromatic JND of eggs-nest linings and rejection rate was not significant ($F_{(1, 10)} = 0.32$, $p = 0.58; R^2 = 0.03$). (B) The relationship between achromatic JND of eggs-nest linings and rejection rate was also not significant ($F_{(1, 10)} = 0.75$, $p = 0.41; R^2 = 0.07$).
**Table 4.1.** The three principal components (PCs) from principal components analysis of interpolated egg and nest spectra that explain over 98% of the variance in spectral data.

<table>
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<tr>
<th>PC</th>
<th>Eigenvalue</th>
<th>Percent Variance Explained</th>
<th>Cumulative Percent</th>
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<th>Df</th>
<th>P-value</th>
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<td>67.14</td>
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<td>80600</td>
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<td>2</td>
<td>24958.96</td>
<td>28.53</td>
<td>95.67</td>
<td>1416452</td>
<td>80199</td>
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<tr>
<td>3</td>
<td>2563.19</td>
<td>2.93</td>
<td>98.60</td>
<td>1256024</td>
<td>79799</td>
<td>&lt; 0.0001*</td>
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Table 4.2. Generalized Linear Mixed Model (GLMM) fits with binomial distribution (outcome variable: accept/reject) of parameters used to assess individual robins’ acceptance/rejection of parasitic eggs irrespective of egg/nest treatments. In (A), nest sites at which more than one parasitism trial was conducted were included as a nested predictor within nest lining color to test for individuals’ reactions to parasitism, irrespective of nest and egg type. In (B), the analysis from (A) was re-run with two sites removed (one significant site and one site approaching significance).

<table>
<thead>
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<th>df</th>
<th>$\chi^2$</th>
<th>p-value</th>
</tr>
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<td>27.86</td>
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<tr>
<td>Site [Nest color]</td>
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(B) Multiple experiments, excluding significant sites from (A)

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<th>Predictor</th>
<th>df</th>
<th>$\chi^2$</th>
<th>p-value</th>
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<td>Egg color</td>
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Chapter 5

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Phylogenetic relationships of the genus *Mohoua*, endemic hosts of New Zealand’s obligate brood parasitic Long-tailed Cuckoo (*Eudynamis taitensis*)

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\textsuperscript{b} Department of Psychology, Hunter College of City University of New York, 695 Park Ave, New York, NY 10065, USA
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\textsuperscript{f} Allan Wilson Centre of Molecular Ecology and Evolution, Department of Zoology, University of Otago, Dunedin, New Zealand,
\textsuperscript{g} School of Biological Sciences, University of Canterbury, Christchurch, New Zealand
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\textsuperscript{i} Auckland War memorial Museum, Auckland, New Zealand
Abstract

The three species of New Zealand’s endemic *Mohoua* genus are sole hosts of the obligate brood parasitic Long-tailed Cuckoo (*Eudynamis taitensis*), making their intrageneric phylogenetic relationships particularly important for coevolutionary studies. Recent molecular phylogenetic analyses have also not identified the family-level placement of this genus. To resolve both intrageneric and family relationships, we generated new nuclear and mitochondrial sequence data and conducted phylogenetic analyses using Bayesian inference among representatives of endemic New Zealand passerines and Australasian ‘core Corvoidea’ lineages. The results establish strong intrageneric relationships of all three *Mohoua* species, confirm the monophyly of the genus, and suggest its placement in a re-erected monotypic family: Mohouidae.

Keywords

Core Corvoidea; Pachycephalidae; Phylogenetics
Introduction

Taxonomic classification of the most diverse and speciose avian order, the Passeriformes, has attracted ongoing close scrutiny (e.g. Barker et al. 2004), especially because well-resolved phylogenies are necessary for comparative studies of sensory and behavioral evolution (Anderson et al. 2009). The Corvoid assemblage of oscine Passeriformes, or ‘core Corvoidea,’ comprises the largest oscine radiation in the Australo-Papuan region (Norman et al. 2009). Although extensive new molecular data have recently become available for the analysis of the phylogenetic relationships within the Corvoid assemblage (Norman et al. 2009), many systematic details remain unresolved, especially with respect to several endemic lineages of New Zealand oscine species (Ewen et al. 2006, Driskell et al. 2007, Lanfear and Bromham 2011). The oscine genus *Mohoua* is endemic to New Zealand and includes the Whitehead (*M. albicilla*) of the North Island, and the Yellowhead (*M. ochrocephala*) and Brown Creeper (*M. novaeseelandiae*) of the South and Stewart Islands. All three species are hosts to the endemic brood parasitic Long-tailed Cuckoo (*Eudynamis taitensis*). The phylogenetic placement and affinities of the species within the genus *Mohoua* are required to understand the sensory, morphological, and behavioral coevolution within this group with their specialist brood parasite (Ranjard et al. 2010). For example, Brown Creepers reject some artificial Long-tailed Cuckoo eggs, whereas Whiteheads and Yellowheads accept foreign eggs (Briskie 2003). In turn, Long-tailed Cuckoo chicks produce begging calls that resemble those of all three *Mohoua* hosts (McLean and Waas 1987, Ranjard et al. 2010).

*Mohoua* is one of the most enigmatic and elusive genera within the New Zealand oscine radiation, and is currently considered as a ‘core Corvoidea’ oscine within the family Pachycephalidae, based on both DNA-DNA hybridization (Sibley and Ahlquist 1990) and,
earlier, morphological taxonomy (Keast 1977). However, recent molecular phylogenetic analyses suggest that the placement of *Mohoua* within the paraphyletic Pachycephalidae was ambiguous and poorly resolved (Norman et al. 2009; Jønsson et al. 2011), although only one species, *M. albicilla*, was included in those genetic analyses. Regarding intrageneric relationships, DNA-DNA hybridization work suggested *M. albicilla* was a congener of the Brown Creeper (then *Finschia novaeseelandiae*) (Sibley and Ahlquist 1987); this was later supported by morphological evidence (Olson 1990a), and is reflected in the current taxonomy of the Brown Creeper as *Mohoua novaeseelandiae* (Gill 2010). Here, we generated the first molecular sequence data for the phylogenetic analysis of all intrageneric relationships of *Mohoua*. We also compared the affinities of *Mohoua* amongst extant New Zealand species (Lanfear and Bromham 2011; Jetz et al. 2012), as well as explored the broader placement of the genus among Australasian families in the ‘core Corvoidea’ passerine lineages (Norman et al. 2009).

**Materials and Methods**

We analyzed the phylogenetic relationships among 13 extant native New Zealand oscine species using a sample of 25 individuals from 8 families, as well as one Shining Cuckoo (or Shining Bronze-cuckoo: *Chalcites lucidus*) specimen. We generated 2 mitochondrial gene (mtDNA) sequences for 19 of the 26 specimens which were uploaded by us to Genbank prior to 2011 (Table 5.1; Lanfear and Bromham 2011, Jetz et al. 2012). The remaining sequences were sourced from Genbank (Table 1). We also investigated the family-level phylogenetic relationships of *Mohoua* spp. by conducting a multi-gene analysis comprised of representative species of 14 Australasian passerines using nuclear DNA obtained during our study (accession numbers JQ065727-JQ065732) and mtDNA sequences (above), and additional data from GenBank (Table 5.1).
### Table 5.1
Species and samples included in the endemic New Zealand (NZ), and/or the broader Australasian (AU) phylogenetic analyses, organized by standing family classification

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<th>Genbank accession numbers</th>
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Mohoua spp. have traditionally been placed in families considered to be part of ‘core Corvoidea’, including Acanthizidae (Mayr et al. 1986; Gill and Wright 2006) and Pachycephalidae (Higgins and Peter 2002; del Hoyo and Elliott 2007). Indeed, despite placing Mohoua in Pachycephalidae, del Hoyo and Elliott (2007) considered that placement to be unlikely. Therefore, we selected taxa thought to be closely related to Pachycephalidae and other ‘core Corvoidea’ species for our broader, family-level analyses.

Our general DNA sampling, extraction, and sequencing protocols are detailed elsewhere (Aidala et al. 2012). We amplified NADH dehydrogenase subunit 2 (ND2) and 12S ribosomal RNA (12S) using the published ND2 primer pairs L5216/H5766 and L5216/H6313 and the published 12S primer pair L1754/H2294 and reverse primer H1859 (Sorenson et al. 1999). Due to amplification difficulties for some samples, we designed a forward 12S primer, L1366 (5’-ACATGCAAGTATCCGCRCYCCCAG-3’), which was matched with the H1859 reverse primer. Amplification reactions contained a total volume of 25 μl comprised of 60mM Tris-HCl ph 8.5, 15 mM (NH₄)₂SO₄, 2.5mM MgCl₂, 0.4 mM of each dNTP, 0.2uM each primer, and 0.5U Platinum Taq polymerase. Cycling reactions were conducted using a touch-down protocol. The initial denaturation step was conducted for 3 minutes at 94° C, then 30 cycles of 25 seconds at 94°, 30 seconds at 60° C (temperature decreasing 0.5° C each cycle), and 90 seconds at 72° C, then 5 cycles with an annealing temperature at 45° C with a final 10 minute extension at 72° C. We followed an Exo/SAP treatment for PCR product purification; we added 5 μl PCR product to 0.2 μl Exo I, 0.1 μl Shrimp Alkaline Phosphatase and 1.7 μl UltraPure water. Samples were incubated at 37° C for 30 minutes, followed by 80° for 15 minutes to inactivate enzymes. A BigDye Terminator Cycle Sequencing kit v3.1 with PCR primers, and internal ND2 primer L5758 (Sorenson et al. 1999) was used to sequence purified samples in both directions. We
combined 2 µl BigDye Terminator Mix, 3.5 µl 5X Sequencing Buffer, 0.2-0.4 µM primer, 1 µl DMSO and 3-5 µl PCR product in each sequencing reaction. We edited sequences using Chromas Pro.

To augment GenBank data for nuclear genes already available for the Whitehead (Table 1), we amplified the recombination activating-protein 1 (RAG-1) and myoglobin (Myo) genes in one Yellowhead and two Brown Creeper individuals. Amplification and sequencing protocols followed those outlined in Norman et al. (2009). Sequences were aligned using ClustalW implemented in Geneious v. 5.1 after which they were inspected visually.

We determined the appropriate model of DNA evolution using jModelTest v. 0.1.1, with the best model selected from Akaike Information Criteria (AIC) calculations. We conducted Bayesian inference analyses in MrBayes v. 3.1.2. Analyses were partitioned with each gene analyzed using the best available model. Gaps were treated as missing data. All model parameters were estimated during the run. The New Zealand ND2 12S and the broader Australasian ND2 RAG-1 Myo analyses were run for 15 million and 25 million generations, respectively. All single gene analyses were run for 10 million generations each. The analyses employed Markov Chain Monte Carlo (MCMC) tree searches comprised of 2 runs of 4 chains each: 3 heated chains at a temperature of 0.500 and 1 cold chain. Trees were sampled every 100 generations, with the first 25% of samples discarded as burn-in. The ND2 12S and ND2 RAG-1 Myo analyses were rooted with the Shining Cuckoo and the suboscine Noisy Pitta (Pitta versicolor) for the New Zealand and Australasian analyses, respectively. Trees were compiled using TreeGraph2, and edited using Adobe Creative Suite 5.0.

Results
When combined with GenBank sequences, the *ND2* gene possessed a gapless maximum sequence length of 1041 bp and the *12S* gene possessed a maximum sequence length of 1003 bp. The broader Australasian *ND2* alignment possessed a maximum sequence length of 1059 bp due to short insertions (5 bp max) and the inclusion of flanking tRNA sequences at the beginning of one sequence (*Oreoica gutturalis*) and at the end of another (*Pitta versicolor*). The *RAG-1* sequences, after alignment and editing with the sequences obtained from GenBank, possessed a gapless 930 bp maximum length. After alignment with GenBank sequences, the *Myo* gene possessed a maximum sequence length of 734 bp.

The phylogenetic analysis of multiple genes from endemic New Zealand taxa generated very strong intrageneric support for a monophyletic genus *Mohoua*, with *M. albicilla* and *M. ochrocephala* placed as sister taxa (Fig. 5.1). The analysis of multiple genes from the broader, Australasian taxa also demonstrated strong intrageneric support for *Mohoua* spp., whereas there was no strong affinity of this genus with other Pachycephalidae genera (Fig. 5.2). All single gene trees (not shown) supported a monophyletic *Mohoua* clade with *M. albicilla* and *M. ochrocephala* as sister taxa, except the *RAG-1* analysis, which grouped *M. albicilla* and *M. novaeseelandiae* as sister taxa. Supporting our multi-gene analyses (Fig. 5.1), *Mohoua* demonstrated affinity for Rhipiduridae in the New Zealand-only *ND2* gene analysis, but did not group strongly with this, or any other clades in the broader Australasian single gene (*ND2, RAG-1, or Myo*, not shown) or the multi-gene analysis (Fig. 5.2).
Fig. 5.1. *ND2 12S* cladogram of representative endemic New Zealand taxa showing the majority rule consensus tree following Bayesian analysis in MrBayes (avg. std. dev. of split frequencies < 0.01) with posterior probabilities shown. Stars denote posterior probabilities of 1.00.
Fig. 5.2. ND2 RAG-1 Myo cladogram of the broader Australasian passerine taxonomic samples showing the majority rule consensus tree following Bayesian analysis in MrBayes (avg. std. dev. of split frequencies = 0.01) with posterior probabilities shown. Stars denote posterior probabilities of 1.00.
Discussion

We have generated the first nuclear and mtDNA sequence data for the phylogenetic analyses of intrageneric relationships for the entire New Zealand endemic genus *Mohoua* and conclude that all three species are congeneric, strongly supporting the previous synonymization of the genera *Mohoua* (Whitehead and Yellowhead) and *Finschia* (Brown Creeper; Sibley and Ahlquist 1987). These new data on within-*Mohoua* phylogenetic affinities reveal that the North Island endemic Whitehead and the South Island endemic Yellowhead are closer relatives than the Yellowhead is with the sympatric South Island endemic Brown Creeper. This parallels the findings of recent phylogenetic analyses (Lanfear and Bromham 2011, Jentz et al. 2012), which also used the publicly available mtDNA data generated and described here. In addition to confirming the monophyly of *Mohoua*, our analyses present a strongly supported phylogeny of native and endemic New Zealand oscines, which will be useful, amongst many other reasons, for coevolutionary studies of host-parasite adaptations (Anderson et al. 2009, Ranjard et al. 2010).

Although in our analyses the allopatric Whitehead and Yellowhead are aligned with each other as sister taxa, there are compelling lines of evidence to continue treating them as separate species. Not only are Whiteheads and Yellowheads distinct in their coloration, they differ in a variety of life history traits such as foraging and nesting behaviors (Gibb 1961; Read 1988a; Read 1988b; Stiller 2001). For example, the Yellowhead is considerably larger than the Whitehead (Gill and McLean 1986) and possesses skeletal specializations for foraging, clearly differentiating the two species (Olson 1990a; see also Olson 1990b). Indeed, Olson (1990) expressed skepticism that the two species would hybridize should they become sympatric. In the absence of direct evidence to the contrary, we maintain that the Whitehead and Yellowhead are heterospecific, in keeping with historical evidence and standing classification. Nonetheless,
future work should be directed to examine the extent of genetic divergence within each species-pair of *Mohoua* spp. in greater detail.

The *ND2 RAG-1 Myo* analysis placed the well-resolved *Mohoua* genus among the Campephagidae, Artamidae, and Oreicidae, but each of these affiliations were supported too weakly to draw any new conclusions (Fig. 5.2). Our analysis placed the *Mohoua* genus as a basal group within this clade, and because these families are well-nested within ‘core Corvoidea’ (Jønsson et al. 2011), we support the continued position of *Mohoua* spp. within the ‘core Corvoidea’ lineage. In parallel to Norman et al.’s (2009) results, we also confirmed that *Mohoua* was unlikely to reside within the paraphyletic Pachycephalidae (see also Jønsson et al. 2011). Given the lack of molecular evidence for a strong link to any other Australasian passerine family, and the presumed long isolation of these distinctive birds in New Zealand (Baker et al. 2004), we suggest that an appropriate taxonomic solution is to place *Mohoua* into its own monotypic family (Mohouidae), following Mathews (1946).

**Acknowledgements**

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Mathews GM (1946) A working list of Australian birds including the Australian quadrant and New Zealand. Shepherd and Newman, Sydney


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Chapter 6

Prospectus

Evolutionary processes have produced an astounding array of animal sensory/perceptual systems that mediate complex behaviors, including mate choice, predator/prey detection, dominance interactions, and mimicry detection. Given the diversity of sensory sensitivity ranges across all sensory-perceptual systems, a largely unresolved question in neuroethology and sensory ecology is the how the species we study process sensory information to make behavioral decisions. Addressing this question presents two major hurdles, the first being to describe the sensitivity parameters of the sensory system in question. The second is to identify and characterize the functional and behavioral relevance of sensory stimuli themselves. In order to better describe how organisms interact with and process sensory information to produce complex behavior, it is critical to characterize the nature of these sensory and perceptual processes. The collection of manuscripts in this dissertation characterizes previously unknown visual sensitivities to short-wavelength light across numerous avian lineages, with a specific focus on avian brood parasites and their hosts. It endeavors to further characterize specific visual stimuli that contribute to parasitic egg identification, as viewed by the avian eye. Specifically, a novel approach was to focus on the role of egg-nest visual differences, as opposed to differences between eggs themselves (Avilés et al. 2005; Mostkát et al. 2008; Spottiswoode and Stevens 2010; Gigueno and Sealy 2012; Croston and Hauber 2014; Gloag et al. 2014). The final chapter paves the way for more detailed comparative study of (co)evolution of antiparasite defenses in the generally less-well studied Mohoua spp. hosts of the long-tailed cuckoo.
In Chapters 2 and 3, I described the predicted UV-sensitivity of avian species spanning four avian lineages (Paleognathae, Psittaciformes, Passeriformes, and Cuculiformes) by sequencing the SWS1 opsin gene. These manuscripts have expanded the known degree of variation in SWS1 sensitivity across the avian lineage, and sets up future studies of the behavioral importance of UV signals in these species’ life histories. Although the pigmentation and/or structures responsible for producing UV-reflectance in eggs/plumage are largely unknown, UV-wavelengths provide important behavioral signals in avian mate choice (Bennett et al. 1996; Pearn et al. 2001), prey detection (see Stevens and Cuthill 2007), and parasitic egg identification (Avilés et al. 2006; Honza et al. 2007; Polačíková et al. 2007). A recent study has even indicated that UV-light may play a role in orientation during migration (Wiltschko et al. 2014).

In these manuscripts, I tested an assumption of the UV-matching hypothesis among Passeriformes hosts of the brown-headed cowbird as it relates to parasitic egg ejection. While previous work had shown that eggshell-reflectance at UV-wavelengths did not strongly correlate with brown-headed cowbird egg rejection behavior at the species level (Underwood and Sealy 2008), a potential caveat in this, and other studies (e.g. Cherry and Bennett 2001), is that it assumes that UV-wavelengths are maximally detectable by the SWS1 opsin, and therefore perceivable. I found that UV-sensitivity did not correlate with egg-rejecter status by species, and so supported the findings of Underwood and Sealy (2008) that UV-wavelengths are likely not functionally relevant for parasitic cowbird detection in hosts of the brown-headed cowbird.

While numerous studies have examined variation in predicted UV-sensitivity at higher taxonomic levels to describe the variation across avian lineages (e.g. Ödeen and Hästad 2003; Carvalho et al. 2006), increasingly more attention is being given to variation within lineages as it
relates to behavioral sensory ecology. For example, Ödeen et al. (2012) showed that SWS1 sensitivity to UV-wavelengths among the fairy-wrens (*Malurus spp.*) was strongly associated with plumage coloration where *Malurus spp.* with short-wavelength-reflecting plumage were shown to possess UVS SWS1 opsins. Their results also support at least two independent gains of a UV-sensitive SWS1 within Maluridae, as this clade’s ancestral state is believed to be a VS SWS1 opsin (Ödeen et al. 2012). These findings suggest that the evolution of UV-sensitivity in the avian lineage is far more complex than has been previously appreciated. To this end, it will be important to a) characterize the degree of variation in UV-sensitivity both within and across all avian lineages, and b) relate this variation to the behavioral sensory ecology of focal species by testing specific sensory hypotheses.

For example, I also showed that the SWS1 sensitivities of basal paleognaths, specifically the ostrich, directly conflicted with previous work at both the genetic and physiological levels, calling into question the currently proposed spectral sensitivity of the ancestral avian SWS1 photopigment. Our data indicated that the ostrich possesses a UVS SWS1, while previous genetic (Ödeen and Håstad 2003) and physiological (Wright and Bowmaker 2001) studies have shown that the ostrich possesses a VS SWS1. Interestingly, a more recent study published a correction to their original work, and now maintain that the ostrich possesses a UVS SWS1, based on direct DNA sequencing of the SWS1 opsin gene (Ödeen and Håstad 2013), and paralleling our findings. The genetic data, though now congruent, is contradicted by microspectrophotometric measurement of \( \lambda_{\text{max}} \) of SWS1 photopigments in the ostrich (Wright and Bowmaker 2001). The reasons for these conflicting results are not known, but such a resolution is will be necessary to most accurately predict the ancestral state of the SWS1 opsin among avian taxa. It would be helpful to test for UV-sensitivity from multiple levels in extant
paleognaths, including genetic, physiological, and behavioral methodologies, followed by
detailed ancestral character state mapping of SWS1 sensitivity across well-resolved avian
phylogenies (e.g. McCormack et al. 2013; Jetz et al. 2014) to better characterize the ancestral
state of the SWS1 opsin photopigment.

It will be enormously useful to identify and characterize spectral tuning sites (sensu
SWS1 opsin) for other avian opsins. At this time, spectral tuning sites for the avian SWS2,
MWS, and LWS photopigments are not well-described. Doing so may be particularly useful;
among trichromatic primates, for example, substitutions at three spectral turning sites have been
shown to be responsible for the shift in peak sensitivities between MWS and LWS cones, which
have $\lambda_{\text{max}}$ of 530 nm and 560 nm, respectively (see Osorio and Vorobyev 2008). However,
trichromacy in Old World monkeys and great apes emerged from a duplication of the LWS opsin
gene, resulting in distinct long-wave and medium-wave sensitive cone photopigments (Davies et

Despite the growing number of studies supporting the role of UV-wavelength perception
in avian behavior, a number of caveats exist concerning the functional role that UV-wavelengths
play in avian communication systems. First, the SWS1 opsin photoreceptor is generally the least
abundant photoreceptor in all birds studied to date (5 – 10 %; see Hunt et al. 2009). Second,
some researchers have also questioned the utility of UV-wavelengths as a long-distance
communication signal because UV-wavelengths are prone to scattering (see Stevens and Cuthill
2007). That is, UV-wavelengths may be maximally salient over short distances, i.e. examining
nest contents for parasitic eggs. Therefore, determining the extent to which a species/individual
is sensitive to UV-wavelengths (through SWS1 sequencing and/or microspectrophotometry)
would be immensely useful. It is especially important to determine the functional relevance of
UV-signals in concert with life history traits to better understand the sensory ecology of focal species. For example, the work presented in Chapters 2 and 3 did not demonstrate a significant relationship between UV-sensitivity and egg-rejection, suggesting that UV-wavelengths alone are not primary drivers of egg-rejection in brown-headed cowbird hosts (see also Croston and Hauber 2014). However, more direct experimental manipulations of parasitic egg UV-reflectance would more firmly establish this conclusion.

In the absence of support for the UV-matching hypothesis among hosts of the brown-headed cowbird, I opted to behaviorally examine the previously untested assumption of brood parasitism research that egg-egg comparisons are the primary criteria hosts make when discriminating own from foreign eggs (Chapter 4). In this set of experiments, I tested an alternative hypothesis that visual contrast between the nest lining and the parasitic egg contributes to the detection of foreign parasitic eggs. I experimentally altered the nest lining color of American robins (which reject natural brown-headed cowbird parasitism at a rate of 100%) using felt inserts glued directly to the natural nest lining. I painted the felt inserts one of three colors and artificially parasitized the robins with painted artificial plaster-of-Paris eggs painted one of the same colors as the artificial nest lining and whose rejection rates were already known in this population of robins (Croston and Hauber 2014). I hypothesized that same egg-nest color combinations would decrease egg rejection rates, while non-matching egg-nest color combinations would increase egg rejection rates. Using avian visual modeling to calculate the degree of (a)chromatic contrast between eggs and nests as processed by the visual system, I found that although I experimentally in/decreased egg-nest contrast in the hypothesized direction (same egg-nest combinations possessed lower visual contrasts than differing egg-nest combinations), egg rejection rates closely paralleled those reported those in natural nests
(Croston and Hauber 2014). I concluded that egg-nest contrast was not a significant predictor of egg-nest contrast in this host-parasite system.

One unexplored factor affecting the degree to which egg-nest contrast in open-cup nesting species such as robins and eastern phoebes impacts parasitic egg discrimination is the level of ambient light available at the nest. Ambient light availability is important in foreign egg discrimination in some brood parasite-host systems (Langmore 2005; Avilés et al. 2008; Honza et al. 2011), and might affect the degree to which both egg-egg and egg-nest contrast affects foreign egg discrimination. Both robins and eastern phoebes build open-cup nests, which are found in areas with highly variable ambient light conditions (Z.A. personal observation). Whether variation in robin egg rejection behavior is correlated with or caused by variation in ambient light levels at the nest site should be investigated from an experimental and comparative perspective by testing behavioral responses to parasitism under varying light conditions in a suite of accepter and rejecter host species. For example, same egg-nest color combinations may serve to reduce foreign egg discrimination and rejection as a function of ambient light availability at the nest site.

The increasingly widespread use of avian visual modeling in behavioral ecology research has allowed investigators to describe visual stimuli as they would be processed by the avian visual system, potentially removing the existing confound of characterizing visual stimuli based on the human visual system (Endler and Mielke 2005). Characterizing the spectral sensitivities of brood parasite hosts is therefore important for several reasons. First, physiology data used in avian visual models are only available for a select few number of species, none of which are common hosts of brood parasites (Grim et al. 2011). Second, visual ecologies vary widely, even among closely related species (Ödeen et al. 2012), and so even the use of congeners in such
models may inaccurately characterize the spectral sensitivities of focal species. Either (or both) of these caveats may result in inaccurate calculations of perceived visual contrasts in brood parasitism studies, leading to spurious conclusions regarding the eggshell reflectance parameters underlying foreign egg identification.

Future brood parasitism studies should employ microspectrophotometry (MSP) to improve the accuracy of visual models as well as characterize inter-individual variation in rejection behavior and spectral sensitivity. For example, inter-individual variation in egg rejection behavior may be directly related spectral sensitivity, thus explaining a lack of foreign egg ejection by certain individuals. For example, male brown-headed cowbirds possess higher color resolution than females based on cone density measurements from MSP (Fernández-Juricic et al. 2013), highlighting the importance of describing sex and individual differences in visual sensory physiology. A comparative approach to test for evolved coevolution of visual sensitivities as it relates to important visual stimuli of eggs used in the egg recognition process to datasets looking at retinal physiology differences between closely related host and non-host species could. For example, the extent to which host/parasite visual sensitivity has (or has not) coevolved with host parasitic egg discrimination has not been well studied.

Although MSP studies would greatly increase the accuracy and validity of visual models used in brood parasite research, MSP and the visual models relying on such sensory physiological data only produce a measure of the sensory apparatus. Future experiments should look to describe, behaviorally and physiologically, higher-order neural processes mediating rejection behavior. This will be especially important in systems where perceptual distance measured by visual models do not always predict behavioral outcomes (e.g. Croston and Hauber 2014). That the vast majority of cowbird hosts accept the costs of cowbird parasitism, despite a
lack of overt egg mimicry by the cowbird, reinforces the notion that, at least at the sensory level, cowbird eggs are in some way discriminable from a host’s own (Croston and Hauber, 2014). This is especially important for non-ejecting and intermediately ejecting species, as in some cases it appears that the host should be able to distinguish the parasitic egg from their own, based on avian visual modeling (ZA, unpublished data).

In Chapter 5, I addressed long-standing disagreement about the phylogenetic placement of *Mohoua* spp., which serve as hosts of New Zealand’s endemic long-tailed cuckoos (*Eudynamis taitensis*). Well-resolved phylogenies are critical to conduct accurate and valid comparative studies, particularly in largely understudied host-parasite systems such as those of New Zealand. Such characterization is necessary to better understand the evolution of anti-parasite behavior in this system. For example, long-tailed cuckoos do not demonstrate strong host-egg mimicry at the egg stage, but rather long-tailed cuckoo chicks are vocal mimics of their *Mohoua* hosts (Anderson et al. 2009; Ranjard et al. 2010). While the family-level placement still remains unresolved, this species-level resolution of the *Mohoua* genus has paved the way for robust comparative studies of anti-parasite behavior at both the egg and nestling stages. For example, the brown creeper (*M. novaeseelandiae*) is a reported foreign egg ejector, while the whitehead (*M. albicilla*) and yellowhead (*M. ochrocephala*) are not (Briskie 2003). It remains unclear what, if any, sensory-level differences explain these behaviors. To date, the degree of UV-sensitivity has only been described in the whitehead, which is predicted to possess a UVS SWS1 opsin (Aidala et al. 2012). Future studies would benefit from addressing anti-parasite behaviors from a sensory ecology and comparative perspective in this and other host-parasite systems.
In sum, the work presented in this dissertation provides a broad basis for more accurate tests of sensory-perceptual and visual ecology hypotheses among extant avian species by describing the SWS1 opsin photoreceptor’s UV light sensitivity. More specifically, it uses this data to test specific visual ecology hypotheses of brood parasitism in American robins, namely whether the nest lining itself presents a salient visual stimulus for identifying foreign parasitic eggs. Future studies should look to physiological assessment of individual spectral sensitivities through MSP to improve avian visual modeling analyses to test a broad range of visual ecology hypotheses.

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