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THE EVOLUTION OF OPSINS AND COLOR VISION: CONNECTING GENOTYPE TO A COMPLEX PHENOTYPE

Conectando el genotipo a un fenotipo complejo: evolución de las opsinas y la visión a color

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ABSTRACT

Dissecting the genetic basis of adaptive traits is key to our understanding of evolutionary processes. A major and essential step in the study of evolutionary genetics is drawing link between genotype and phenotype, which depends on the difficult process of defining the phenotype at different levels, from functional to organismal. Visual pigments are a key component of the visual system and their evolution could also provide important clues on the evolution of visual sensory system in response to sexual and natural selection. As a system in which genotype can be linked to phenotype, I will use visual pigments and color vision, particularly in birds, as a case of a complex phenotype. I aim to emphasize the difficulties in drawing the genotype-phenotype relationship for complex phenotypes and to highlight the challenges of doing so for color vision. The use of vision-based receiver models to quantify animal colors and patterns is increasingly important in many fields of evolutionary research, spanning studies of mate choice, predation, camouflage and sensory ecology. Given these models impact on evolution and ecology, it is important to provide other researchers with the opportunity to better understand animal vision and the corresponding advantages and limitations of these models.

Keywords: avian visual pigments, color vision, complex phenotypes, genotype-phenotype, opsins.

RESUMEN

Entender la base genética de los rasgos adaptativos es un paso crítico en el estudio de los procesos evolutivos. Para estudiar la conexión entre genotipo y fenotipo es importante definir el fenotipo a diferentes niveles: desde las proteínas que se construyen con base en un gen, hasta las características finales presentes en un organismo. Las opsinas y los fotopigmentos son elementos primordiales de la visión y entender cómo han evolucionado es fundamental en el estudio de la visión en los animales como un caracter derivado de selección natural o sexual. Este artículo se enfoca en este sistema, en el que se pueden conectar genotipo y fenotipo, como ejemplo de fenotipo complejo para ilustrar las dificultades de establecer una relación clara entre genotipo y fenotipo. Adicionalmente, este artículo tiene como objetivo discutir el funcionamiento del sistema de fotorrecepción, con énfasis particular en las aves, con el fin de enumerar varios factores que deben ser tenidos en cuenta para predecir cambios en la visión a partir del estudio de los fotopigmentos. Dado que los modelos basados en la visión de aves son cada vez más usados en diversas áreas de la biología evolutiva tales como: selección de pareja, depredación y camuflaje; se hace relevante entender los fundamentos y limitaciones de estos modelos. Por esta razón, en este artículo discuto los detalles y aspectos prácticos del uso de los modelos de visión existentes para aves, con el fin de facilitar su uso en futuras investigaciones en diversas áreas de evolución.

Palabras clave: fenotipos complejos, fotopigmentos, genotipo-fenotipo, opsinas, visión a color.

INTRODUCTION

The last decades have witnessed outstanding advancements in our understanding of the genetic basis of key adaptive traits. The genetic underpinnings of morphological adaptions like the change in beak shape of Darwin finches (Abzhanov *et al.*, 2004; Abzhanov *et al.*, 2006) or the pelvic girdles of sticklebacks (Shapiro *et al.*, 2004) have been uncovered, contributing critical knowledge to our understanding of the evolutionary processes like adaptation and speciation. The same technological advances that have allowed these discoveries have also made it possible to find and study the evolutionary dynamics of many genes for which we have little functional information or with a less clear relationship to an organisms phenotype and fitness (Bochner, 2003).

We sequence genes, study their molecular evolution using sophisticated statistical models to inquire about the selective pressures shaping their evolution, investigate how and why they duplicate, how they interact with other genes. But at the end our ultimate goal is to understand how changes in the genes we study affect an organism's phenotype and fitness, how they change over time, and how to predict and treat diseases: we want to understand that essential link between genotype and phenotype.

The phenotype controlled by any gene can be defined by changes in its sequence (mutations). In addition to coding sequence variation, regulatory changes altering the expression patterns of a gene can be a key mechanism of phenotypic evolution (Hoekstra and Coyne, 2007; Carroll, 2008). Gene expression levels, timing or tissue specificity can have an important impact on phenotype (Harrison *et al.*, 2012). However, the phenotype can be defined at different levels: from protein function to organismal effects. The relationship between genotype and phenotype becomes increasingly difficult to establish as one gets closer to the latter more complex level of the phenotype. Beyond the direct alterations in protein function that arise from mutations in a gene or changes in its expression, the epistatic and pleiotropic interactions with other genes (Wagner and Zhang, 2011) and the specifics of the pathway and physiological system that gene is part of, make it harder to link changes in the gene itself with the changes it ultimately causes in an organism.

How then, do we make the connection between genotype and phenotype necessary to study phenotypic evolution? In order to clearly score the phenotype associated with changes in a gene we need to compare organisms where only the interest gene changes. This can be accomplished in laboratory organisms, like yeast for example, that are easy to grow and have short generation times, and in which we can generate different lines that only differ in the gene of interest. Even in these circumstance it is a slow and laborious process. Beyond these model laboratory organisms, scoring the phenotype associated with differences in genes and their expression can be extremely difficult.

Here, I will focus on the evolution of opsin genes and color vision with a strong emphasis on birds, as an example of a system with enormous potential. We can directly measure how substitutions in opsin genes translate into changes in the corresponding protein function allowing us to study the genotype-phenotype connection in a key adaptive trait. As the fist step in the phototransduction cascade that mediates vision, opsin genes are also part of the deeply complex visual system. By elaborating on important aspects of the visual system beyond opsins, I will highlight on the difficulties of predicting how an organisms color vision will change in response to opsin variation, illustrating the complexity associated with understanding the genotype-phenotype relationship in complex systems.

WHAT ARE OPSINS?

Vision starts as photons hit the photosensitive cells in the retina, the rod and cone photoreceptors, the first step in a complex biochemical cascade that ultimately leads to vision. Rods and cones are morphologically distinct photoreceptors (Ebrey and Koutalos, 2001) that serve different systems in vision. Rods contribute to scotopic vision, or vision at low light levels, a highly sensitive process that lacks color information or has low acuity. Cones on the other hand, are only active in bright light contributing to photopic vision (vision in bright light), and are at the basis of color vision. Because color vision relies on comparing the output from different cone classes (Kelber *et al.*, 2003; Shevell, 2003; Solomon and Lennie, 2007), it requires a minimum of two cone types and an opponent mechanism wired into the nervous system that can compare the outputs of the different cone types (Bowmaker, 2008).

The spectral absorption properties of rod and cone photoreceptors are determined in large part by the type of visual pigment they express. Visual pigments are trans-membrane molecules contained in the outer segment of photoreceptors. They consist of an opsin protein moiety covalently bound to a light-sensitive chromophore. Opsin proteins belong to the superfamily of G-protein coupled receptor proteins (GPCR) involved in multiple cellular signaling processes (Arshavsky *et al.*, 2002). They consist of seven α-helical trans-membrane regions enclosing the chromophore-binding pocket. The chromophore gives opsins their unique light-sensitive properties and are found in two forms: most vertebrate opsins bind a A1 chromophore, 11-*cis*-retinaldehyde (retinal) and some bind a A2 chromophore, 3,4-dehydroretinaldehyde (3,4-dehydroretinal) (Bowmaker, 2008). The spectral sensitivity of a visual pigment depends on the type of chromophore it binds (A2 chromophore cause pigments to be slightly long-wavelength shifted), and on the identity of specific amino acids throughout the opsin protein that interact with the chromophore (Applebury and Hargrave, 1986; Yokoyama, 2000; Bowmaker, 2008). When a photon hits a visual pigment, the chromophore changes

from a 11-*cis*-retinal conformation to an all-*trans*-retinal, initiating the phototransduction cascade that leads to vision (Lamb *et al.*, 2007).

Rod and cone visual pigments differ in many aspects of their structure and function that cause them to be active at different light levels and serve different purposes in the visual system. In addition to the rod pigment or Rhodopsin (RH1), there are also several types of cone visual pigments that differ in their wavelength of maximum absorbance (λmax, Figs. 1 and 2) and belong to four distinct spectral classes (Lamb *et al.*, 2007; Bowmaker, 2008). The LWS-MWS family is maximally sensitive in the mid to long-wavelengths region of the spectrum ("reds" and "greens" between 490-570nm), the RH2 family is sensitive to middle-wavelengths (480-535nm), the short-wavelength sensitive SWS2 family ("blue-violet" between 410-490nm) and the SWS1 family also sensitive to short wavelengths generally including ultraviolet (355-440nm) (Yokoyama, 2000).

The ancestral opsin gene duplicated early during vertebrate evolution to give rise to the four (4) opsin families. Different vertebrate groups have independently retained, lost and/or duplicated genes of the various opsin families (Fig. 1) (Bowmaker, 2008). For example mammals have lost SWS2 and RH2 and fish have maintained multiple duplications within each opsin gene family causing them to have as many as ten opsins like the Guppy, *Poecilia reticulata* (Hoffmann *et al.*, 2007; Ward *et al.*, 2008; Watson *et al.*, 2011). Birds have retained all the five opsins types inferred to be present in the vertebrate ancestor. In addition to the rhodopsin (RH1), birds posses four spectrally distinct cone visual pigments believed to mediate color vision (Figs. 1 and 2): long-wavelength sensitive (LWS), medium-wavelength sensitive

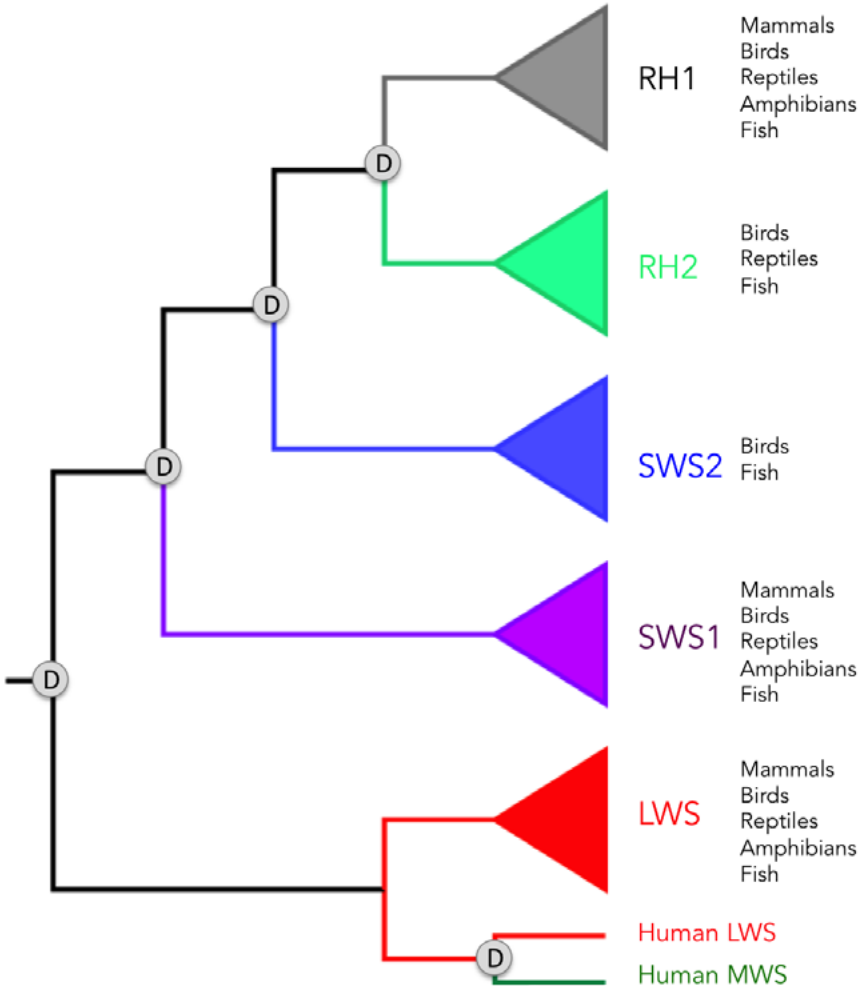

Figure 1. Relationships between opsin spectral classes.
Duplications are indicated by circles at relevant nodes. Except for the duplication that resulted in human LWS and MWS pigments, all duplications are believed to have occurred very early in vertebrate evolution. Clades listed refer to the ones where each opsin class is represented (Bowmaker, 2008).

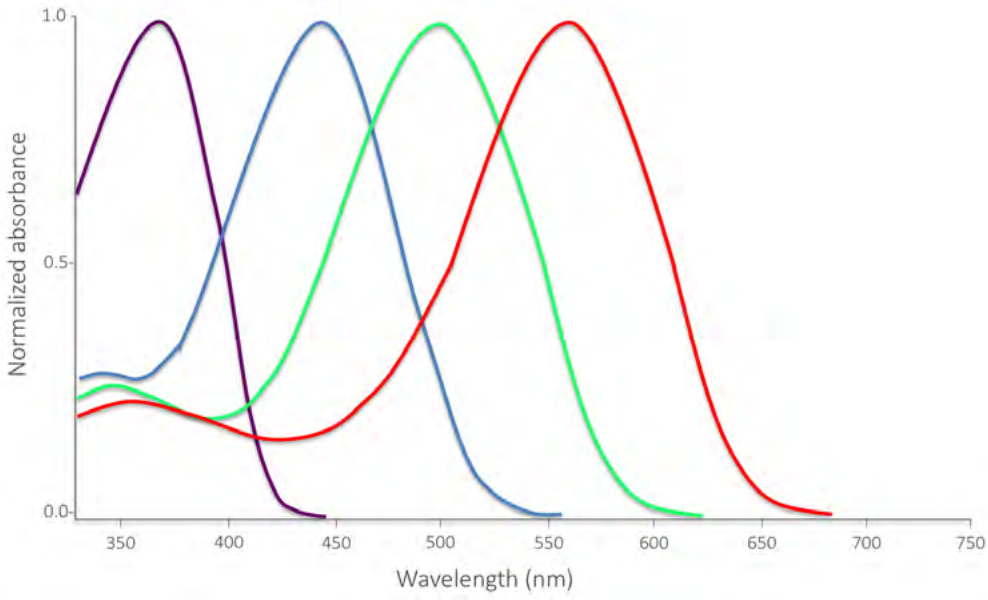

Figure 2. Spectral sensitivities of vertebrate visual pigments.
LWS in red, MWS or RH2 in green, SWS2 in blue and, purple for SWS1 are represented. These are just examples of the spectral sensitivities of visual pigments for each family. The actual maximum sensitivity values and number of visual pigments in each family varies in different species (see text). In birds SWS1 can have a peak of maximum absorbance in the ultraviolet (UV) region or in the violet region.

(RH2), short-wavelength sensitive (SWS2) and very-short wavelength sensitive visual pigments (SWS1).

WHY STUDY OPSINS?

Visual pigments are a key component of the visual system and their evolution could also provide important clues on the evolution of visual sensory systems. As the first step of the visual transduction cascade, photoreceptors and their associated visual pigments, are the only contributors to color vision in direct contact with the environment (Baylor, 1996) and are thus good candidates to look for signatures of selection.

The visual signals and displays of animals have been extensively studied, particularly in an attempt to evaluate the information content of signals and the role of courtship displays in species formation (Endler *et al.*, 2005; Price, 2008; Seehausen *et al.*, 2008; Maan and Seehausen, 2011). While we know a fair amount about how signal evolution responds to both sexual and natural selection, little is known about the role of the underlying visual sensory system. Multiple hypotheses to explain color diversity have been developed (Lande, 1981; Ryan, 1990; Andersson, 1994; Endler and Basolo, 1998; Zahavi *et al.*, 1999; Boughman, 2002; Bradbury and Vehrencamp, 2011). All these hypotheses implicate a fundamental role of the visual system in color diversification but lead to different predictions regarding the extent to which the visual system and color co-evolve, and on the role the environment plays in the evolutionary process. Unlike color and ecology, visual systems have not been studied in many systems and thus most of these predictions remain untested.

We have evidence, mainly accumulated from fish, that visual pigments respond to pressures imposed by the light environment, helping organism adapt to their habitat. For example fish living in turbid waters, which are rich in longer wavelength, have generally lost their short-sensitive opsin genes, do not express them or have visual pigments shifted to match their light environments (Yokoyama *et al.*, 1999; Terai *et al.*, 2002; Sugawara *et al.*, 2005; Seehausen *et al.*, 2008; Hofmann *et al.*, 2009). More recent research indicates selection from the light environment could also be driving the evolution of opsin expression in birds (Bloch, 2015).

In addition to their crucial and poorly understood role in mate choice, visual pigments are a system in which genotype can be clearly linked to the resulting visual pigment spectral tuning (i.e. function). Visual pigments and opsin genes provide an opportunity to link genotype to protein function providing a synthetic approach to examine evolutionary processes. The availability of an *in vitro* assay (Chang, 2003; Yokoyama, 2008) that allows for visual pigments to be expressed in cultured cells, in order to measure their absorbance spectra, makes this a system where we can take a holistic approach identifying the precise molecular basis for functional changes. In these assays cultured primate or human cells are used to express opsin proteins that are then reconstituted with their corresponding chromophore, either A1 or A2 (Fig 3). This process produces functional, purified visual pigments in enough quantity to measure

their spectral sensitivity (Chang, 2003; Bloch *et al.*, 2015a; Bloch *et al.*, 2015b) and more recently other less understood functions (Bickelmann *et al.*, 2012; Morrow and Chang, 2015). In addition to studying the function of extant visual pigments, these assays can be combined with site-directed mutagenesis in order to recreate and express ancestral visual pigments, thus providing a way to trace the evolution of genes and function all the way from a clade's ancestor (Chang, 2003; Bloch *et al.*, 2015a) and to study to role of specific substitutions on spectral tuning (Yokoyama and Radlwimmer, 2001; Yokoyama *et al.*, 2008a).

HOW COLOR VISION WORKS

It is important to note that the spectral sensitivity curves illustrated in Figure 2 describe the probability of a visual pigment of capturing a photon of a certain wavelength. In other words, it means the probability that a visual pigment will capture a photon is highest for light of wavelengths that match its peak of maximum absorbance (λ_{max}) and decreases for other wavelengths. Even if the probability that a photon is captured by a visual pigment changes with wavelength, the phototransduction cascade started by this process remains the same independent of the wavelength of the absorbed photon. The fact that the response depends on the number of photons absorbed, but not their wavelength, is called the *Principle of Univariance* (Mitchell and Rushton, 1971). An important consequence of this principle is that a visual pigment, and its corresponding cone, on its own is color-blind. Color vision can only be achieved by comparing the output of the different visual pigment/cone types and *how* these outputs are compared to each other.

The notion of color opponency was first raised by Ewald Hering, by concentrating on the fact that in humans there are two pairs of opponent colors, green-red and yellow-blue. What this means is that no color can simultaneously be defined as red and green or blue and yellow (i.e. a "bluish-yellow" is unthinkable). Color opponency is a consequence of how the information from the cone photoreceptors is processed by the rest of the neurons in the retina and the brain. The physiological basis and mechanisms of color

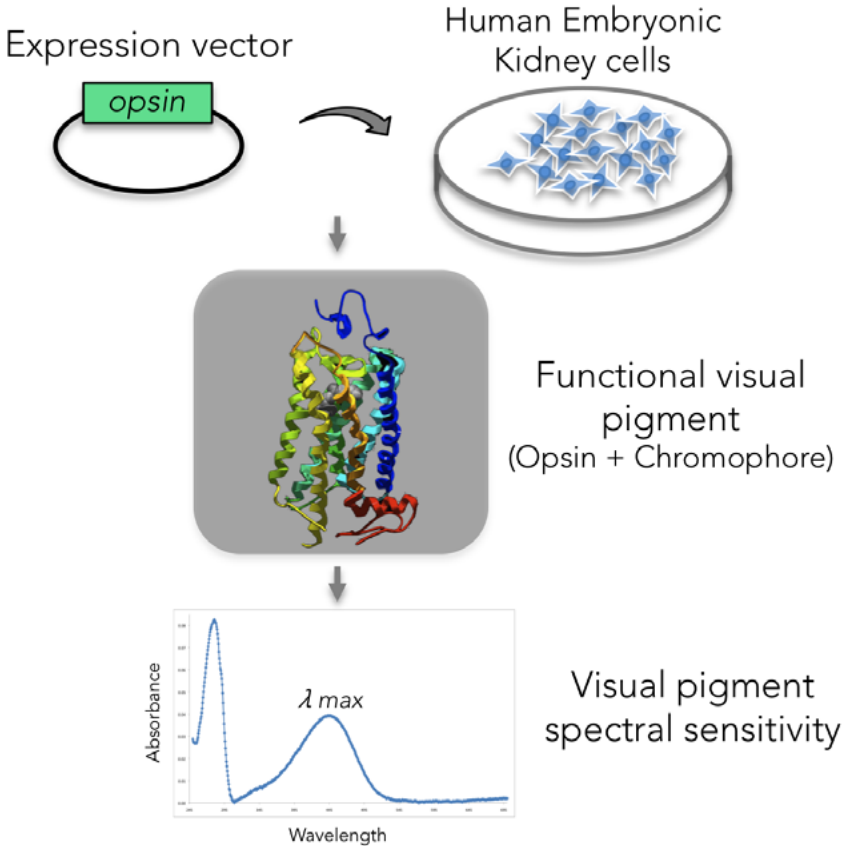

Figure 3. Opsin *in vitro* expression and visual pigment regeneration.
In order to express visual pigments in vitro the opsin gene of interest initially inserted into the appropriate expression vector – p1D4-hrGFP II (Morrow and Chang, 2010), which is the used for transient transfection of human or primate cells. The resulting opsin protein is then incubated with the 11-cis-retinal in order to reconstitute a functional visual pigment. After purification by immunoafinity with 1D4 monoclonal antibody, the absorbance of visual pigment is measured with a double-beam spectrophotometer to obtain accurate values of λmax (Chang, 2003; Morrow and Chang, 2010).

opponency have been extensively researcher in primates, but remains poorly understood in other taxa. Interestingly for evolutionary biologists, although the way that photoreceptors transmit information (phototransduction cascade) seems to be extremely conserved across animals, the photoreceptor types and how the information they output is processed by the rest of the visual system seem to vary from species to species, most likely allowing them to adapt to their environment and needs (Kelber *et al.*, 2003; Neumeyer, 2012).

Research in primates has shown that the integration of the information from cones required to have color vision, and thus the basis of color opponency, are two types of cells in the next layer of the retina: bipolar and ganglion cells. It is beyond the scope of this article to go in detail about the neuronal basis of visual information processing so my description of these process will be greatly summarized. Information from the cones is transmitted to the bipolar cells which then pass it to ganglion cells of two mayor classes, the magnocellular and the parvocellular cell layers (Shevell, 2003). The way these cells integrate information and which signals they compare determines the number and nature of the opponent channels. Humans, for which opponent channels have been well described, have three opponent channels, two of which carry color information (Fig. 4). The first "red-green" opponent channel compares the output of LWS and MWS cones (L-M) and the second, the "Blue-Yellow" channel compares the output of the three human cones as S-(L+M). Visual information then travels outside of the retina via the axons of ganglion cells, which form the optic nerve, to the Lateral Geniculate Nucleus (LGN) of the thalamus and from there to the visual cortex. These post-retinal or "higher centers" is where more complex of visual information processing occurs resulting in perception and recognition among others.

Ultimately, the perception and discrimination of color will be a result of how all the components of the visual system work. Importantly, we need to keep in mind that how color information is integrated beyond photoreceptors is a crucial component of the visual system and color processing. Thus, our inferences about color vision in any organism need to consider aspects beyond opsins and photoreceptor number, and we need to be particularly cautious when we do not have knowledge of how these work, as it is the case for the vast majority of animals.

When it comes to defining the phenotype associated with opsin genotype, we can take into consideration two levels of complexity: The functional phenotype, or the function of the corresponding visual pigment, and the organismal phenotype, referring to the way an animal will perceive color.

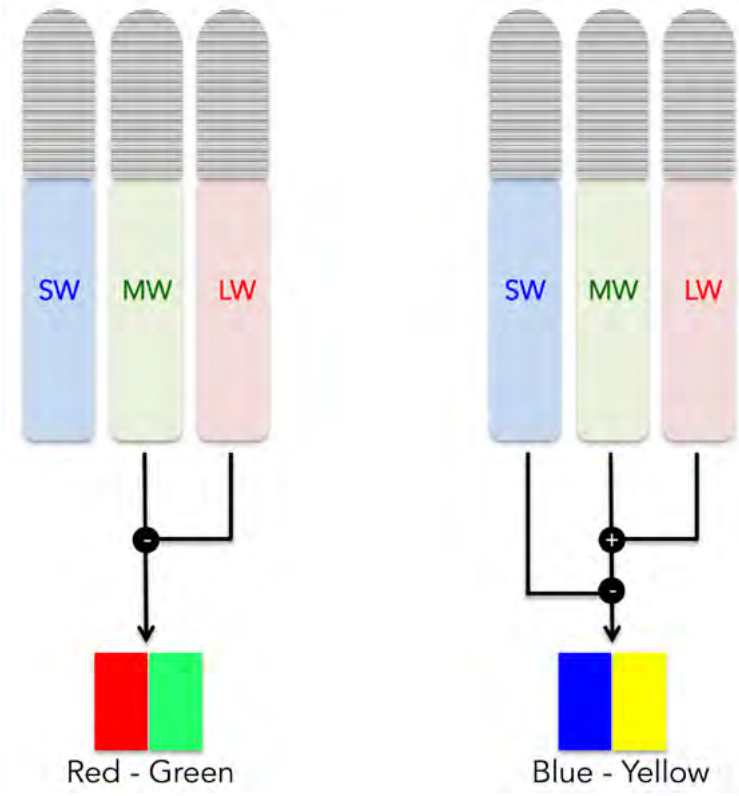

Figure 4. Human chromatic opponent channels.

Left: "Red-Green" opponent channel (L-M). Right: "Blue-Yellow" opponent channel S-(L+M).

FROM OPSIN GENES TO SPECTRAL TUNING

The most immediate and direct phenotype associated with opsin genes is the spectral sensitivity of the resulting visual pigment. As explained here, the spectral tuning of a visual pigment can change in two ways: with changes in some of the opsins amino acid that will affect the way the opsin interacts with the chromophore, or by changing the type of chromophore within the visual pigment from an 11-*cis*-retinal (A1) to an 3-dehydroretinal (A2). A2 chromophores are not present in birds and mammals. Generally speaking, species of marine fish and terrestrial animals posses A1 chromophores while fresh water fish, amphibians and reptiles have A2 chromophores (Bowmaker, 2008). It is well understood how changes in the type of chromophore affect the spectral tuning of a visual pigment. We know that for short-wavelength sensitive pigments, two visual pigments based on the same opsins sequence but different chomophores will only differ by a few nanometers, but for long wavelength sensitive pigments the chromophore switch causes a large difference in spectral sensitivity of up to 50nm (Parry and Bowmaker, 2000). How substitutions in the opsin protein affect spectral tuning, on the other hand, can be a more complex matter. The effects of many substitutions on the spectral tuning of the different visual pigment types has been investigated. This has been accomplished by comparing the visual pigments of different species (Yokoyama and Yokoyama, 1996; Yokoyama *et al.*, 2008a; Bloch *et al.*, 2015a; Bloch *et al.*, 2015b) and conducting sophisticated site-directed mutagenesis experiments (Yokoyama, 2000; van Hazel *et al.*, 2013). The combination of phylogenetic methods to identify key substitution that occur multiple times throughout vertebrate evolution, site-directed mutagenesis to introduce and reverse those substitutions in visual pigments and *in vitro* expression has contributed immensely to our understanding of spectral tuning. For example, we know that nine amino acid substitutions that have occurred throughout the evolution of rhodopsin in vertebrates explain the evolution of this pigment's spectral tuning (Yokoyama, 2008; Yokoyama *et al.*, 2008a). Also, a so-called "five-sites rule" has been established for long-wavelength visual pigments. According to this rule, the identity of the amino acid at positions 180, 197, 277, 285 and 308 allows to predict the λmax of and LWS/MWS visual pigment (Yokoyama and Radlwimmer, 1998).

However, expressing visual pigments in the laboratory and going through the step-wise process of introducing mutations one at a time to get at the basis of spectral tuning is a difficult, time consuming or sometimes impossible task in the case of some visual pigments. The only alternative to *in vitro* expression is doing MSP (microspectrophotometry) directly on the retinas of sacrificed animals, which is a highly difficult technique that requires custom made equipment and unfortunately produces measurements with large experimental error compared to *in vitro* expression. It thus becomes a natural and logical step to infer changes in spectral tuning when the substitutions studied by the above mentioned functional studies are identified in the opsin genes of other organism. This is particularly true for evolutionary ecology studies that do not focus on the functional aspects of the opsin protein but rather on visual adaptations to different environments or mating systems (for an example see Hofmann *et al.*, 2009). As this becomes a more and more frequent practice in evolutionary studies we are faced with the unavoidable question of how good our models are. Do we have enough data to predict spectral tuning from the sequence of an opsin? How predictable is the relationship between opsin genotype and phenotype?

Despite their important implications for study of the genotype-phenotype relationship, well beyond opsins and color vision, these questions might not have a simple answers. In some cases models have been shown to successfully predict a visual pigment's λ_{max}. Some examples include experimental verifications of the "five-sites rule" (Yokoyama and Radlwimmer, 1998; Yokoyama *et al.* , 2008b) and the effect of SWS1 substitutions of large effect that cause this pigment to shift from the violet to the UV region in birds (Ödeen *et al.*, 2009; Hauser *et al.*, 2014). However, predicting λ_{max} from opsin sequence can be far from simple and highly dependent on genetic background. My own research has identified concrete examples in which this is not the case (Bloch *et al.*, 2015a). Two residues shown to vary in the SWS2 opsin genes across warblers (residues 49 and 269) have been previously studied using site-directed mutagenesis in other organisms. In warblers these residues do not cause any significant shift in λ_{max} however, in SWS2 in the green anole and goldfish (Yokoyama, 2003), as well as LWS in human (Asenjo *et al.*, 1994) and bovine rhodopsin (Chan *et al.*, 1992), they were shown to cause shifts between 5 and 12 nm. Other work has reached similar conclusions for SWS1 pigments in mammals (Hauser *et al.,* 2014). It has now become quite common to predict whether visual pigments will differ in spectral tuning based on observed substitutions at sites known to cause spectral shifts in other organisms. Despite the common nature of this practice, one should be very careful when doing this if comparing opsins from different species that differ at many amino acid sites or that do not carry the same substitution (Ward *et al.*, 2008; Hofmann *et al.*, 2009).

In addition to variation in opsin gene sequence, opsin expression has been shown to vary significantly across species in various systems (Laver and Taylor, 2011; Sandkam *et al.*, 2015), in many cases in a way consistent with variation in the light environment (Fuller *et al.*, 2004; Hofmann *et al.*, 2009; Fuller *et al.*, 2010; Bloch, 2015) or even measures of sexual selection (Bloch, 2015). The immediate consequences of changes in gene expression remain poorly understood. In many systems it has been shown that opsin expression levels match the relative abundance of the different cone

types leading to the conclusion that opsin expression reflects changes in relative cone abundances (Hagstrom *et al.*, 1998). However, preliminary data suggest this might not be the case in birds (Bloch, 2015) and data is still not available for enough species, particularly beyond fish, to know whether opsin expression is generally a reflection of relative photoreceptor abundances (Fuller *et al.*, 2004; Fuller and Claricoates, 2011).

The relationship between opsin genotype and phenotype, defined at the molecular and most direct level is thus not simple. This relationship only becomes more difficult to predict as one considers the consequences of opsin gene mutations at the organismic level, which comes down to connecting opsin genes to color vision.

BEYOND OPSINS AND INTO COLOR VISION

Opsins are a critical component of the visual system in direct contact with the external environment, which makes them an obvious target of selection. However, as explained above, they are only one piece of the visual system and ultimately the perception of color is dependent on many other visual components beyond opsins that determine how visual information is integrated.

Starting with the first step of information integration after photoreceptors, how color is perceived is determined in part by the nature and number of opponent channels. To better understand the importance of color opponent channels it is helpful use an example drawn from our own perceptual experience. As mentioned in previous sections: due to the wiring of human opponent channels (Fig. 4) it is impossible for us to perceive or imagine a yellowish-blue. This is due to the way human ganglion cells integrate information. How the visual information captured by cones is integrated into opponent channel depends on the properties of ganglion cells, which potentially, and most likely vary widely across the animal world. Color opponency has mainly been studied and modeled in a trichromatic framework due to the nature of our own visual system. Beyond this, for animals that are tetrachromatic, we are faced with a much larger universe of possible opponent channels and a dearth of data to help us reduce it. One of the rare animals with four visual pigments for which we have physiological and behavioral data to describe opponent channels is the turtle, where as many as 12 opponent cells have been described, of which 5 are involved in color vision (Rocha *et al.*, 2008). The existence of opponent channels and our knowledge of psychophysics (the study of the relationship between physical stimuli and how they are perceived), provide important cautionary notes when trying to infer vision phenotypes from changes in visual pigments or opsin expression.

First, it is important to keep present that the number of photoreceptors an organism posses does not necessarily match the dimensionality of its color vision. In order to elaborate on this idea I will again rely on the human visual system and the knowledge we have gained on vision from the study of psychophysics in humans. As stated before in this paper and now widely known, humans with normal color vision are trichromats. This not only means we have three independent receptors to process color information. In strict terms the definition of trichromacy is derived from psychophysical color matching experiments: trichromacy means that to match any color, the mixing of three colors are *necessary* and *sufficient*. This means that in order to match the appearance of a color stimulus perfectly, a person with normal vision needs a mix of the appropriate amounts of three colors, also referred to as primaries (monochromatic rays of light). Two will not be enough and four would be superfluous (Shevell, 2003). Even if humans are trichromatic while having three types of photoreceptors dedicated to color vision it does not mean this correspondence is obligatory. It is possible for an animal to posses three cone photoreceptors and be a dichromat if only two of those receptors are compared to each other, or to have four cone photoreceptors and not be a tetrachromat. Equating the number of cones with the dimensionality of color is a misconception that occurs often when studying visual pigments in diverse organisms, including birds. Birds have four cone photoreceptors and are more often than not assumed to be tetrachromats. This however, has never been demonstrated. There are rare and valuable behavioral studies aimed at understanding bird color vision (Osorio *et al.*, 1999; Ham and Osorio, 2007; Lind and Kelber, 2009; Lind *et al.*, 2014; Olsson *et al.*, 2015) among others. Some have contributed some initial insight into opponency in birds (Wright, 1972; Goldsmith and Butler, 2005). But the fact remains, we still do not know what are the opponent channels birds' color vision is based on, and how photoreceptor information is integrated in birds. Thus, based on the human definition for tetrachromacy, it can be argued we still lack a demonstration that birds are tetrachromats.

Obtaining information on opponent channels requires complex electrophysiological manipulations or equally intricate behavioral experiments that are not only prohibitive but also beyond the scope of many studies on visual pigment evolution. However, as these studies come to light we should be aware of the complexity involved in color vision and thus the relationship between genotype and phenotype in this case.

THE PARTICULAR CASE OF BIRDS AND COLOR VISION MODELING

Visual pigments in birds

Opsin genes and visual pigments in birds are generally assumed to be rather invariant (Hart and Hunt, 2007). The only well documented differences have been found in SWS1, which can be of two types in birds: some species have a VS or violet-sensitivite type SWS1 pigment and other species have a UVS or ultraviolet-sensitive SWS1, with λ_{max} values

shifted to shorter wavelengths into the UV. This difference has been well studied and we know it can be explained by a few substitutions of large effect (Hauser *et al.,* 2014). The ancestor of birds was inferred to have a VS type SWS1 pigment and SWS1 is believed to have shifted to UVS three or four times independently across birds (Odeen *et al.,* 2012).

Recent comparative studies have shown that there is more variation at the molecular level within visual pigment types of closely related species than previously acknowledged (Bloch *et al.*, 2015a; Bloch *et al.*, 2015b). However, differences in the sequence of opsin genes across birds does not always translate into spectral shifts (Coyle *et al*., 2012), and the spectral tuning of visual pigments has been shown to evolve very slowly in passerines (Bloch *et al.*, 2015a; Bloch *et al.*, 2015b).

Hart and Hunt (2007) present a comprehensive review of all the available data on the spectral sensitivities of avian visual pigments until 2007. Since then a few studies have measured more species (Coyle *et al*., 2012; Bloch *et al.*, 2015a; Bloch *et al.*, 2015b) adding to this body of data. Looking at these data reveals that there is some variation in the visual pigments of birds beyond the VS-UV shift of SWS1 pigments (Table 2–Hart and Hunt, 2007). However, it remains unknown to what extent this variation impacts the color vision of different bird species.

Avian color vision models

Color spaces are a commonly used method to predict the changes in color vision phenotype that might arise from spectral shifts in visual pigments (Vorobyev and Osorio, 1998; Endler and Mielke, 2005; Stoddard and Prum, 2008) (Fig. 5). This type of chromaticity diagrams are most commonly used for trichromats and tetrachromats, like birds.

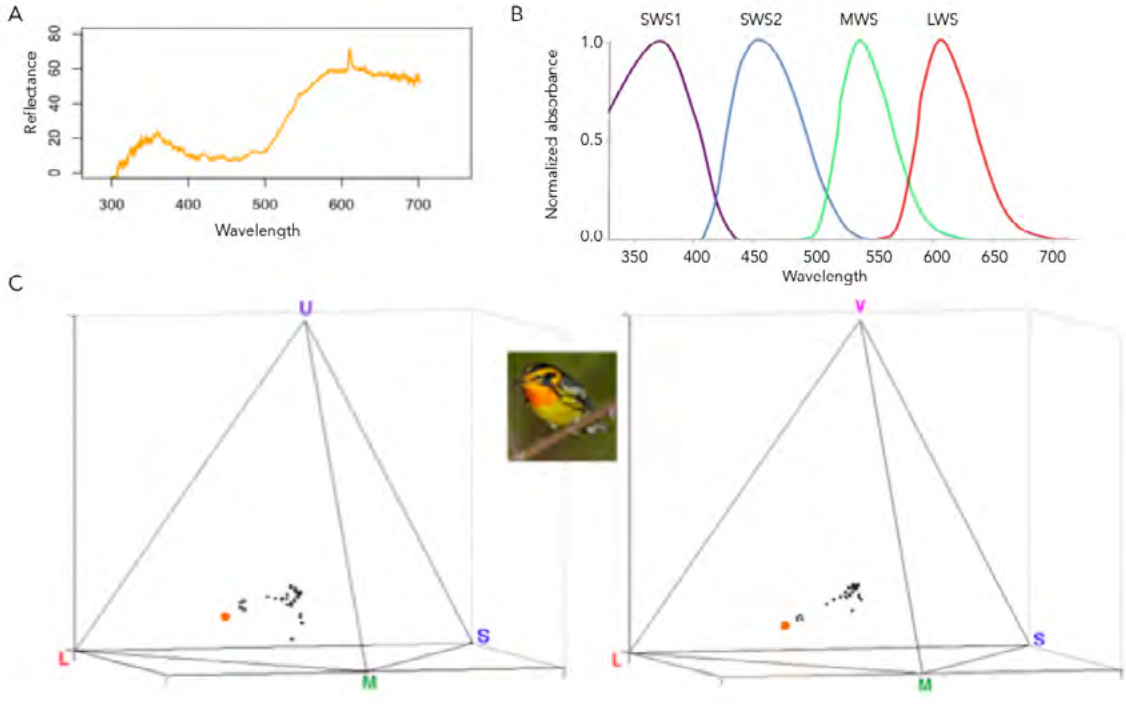

Figure 5. Projection of colors into avian color space.
(A) Example of a reflectance absorption spectrum for a patch of a bird's plumage. Here reflectance is represented in function of wavelength for the orange throat patch of a Blackburnian warbler, *Setophaga fusca* (illustrated below). This is the average of 5 separate spectrophotometric measurements. **(B)** Spectral sensitivities of four avian cones, LWS, RH2, SWS2 and SWS1. These curves represent predicted photon catches after filtering by the oil droplets is taken into account (adapted from Hart and Hunt, 2007). SWS1 can be of two types in birds: in some species (most passerines) SWS1 peaks in the ultraviolet region and is therefore labeled UVS. In other species this pigment peaks in the violet region instead and is thus labeled VS. Here, cone sentivity curves have been **(C)** Plumage patches for *Setophaga fusca*, projected on to color space for a typical 'UV' bird *(*left; based on *Parus caeruleus* – blue tit) and for a typical bird with a 'VS' visual pigment (right; based on *Columba livia*–pigeon). These projection were obtain using the program Tetrahedral Color Space provided by M.C. Stoddard (Stoddard and Prum, 2008). Each point represents the color of 1 of 29 different locations of a bird measured using a spectrophotometer at the Chicago Field Museum, with locations chosen to thoroughly cover variation across all species; the orange (leftmost) point represents the reflectance spectrum illustrated in Figure 5A. Color volume (the minimum spanning volume covering all the points) is more than 2x larger under UV ($4x10^{-4}$) than under V ($1.7 x10^{-4}$). This implies that species with a UVS cone, which include *S. fusca*, have greater discrimination among patches than species with a VS cone.

For the latter, tetrahedral color spaces were first proposed by Burkhardt (1989) and initially used by Goldsmith (1990). These were later modified by others, including Endler and Mielke (2005) and Stoddard and Prum (2008), to incorporate different color discrimination models (such as the receptor noise model proposed by Vorobyev and Osorio (1998)). By making programs available for evolutionary biologist to use color spaces in common platforms like R (Maia *et al.*, 2013) and Matlab (Stoddard and Prum, 2008), the use of receiver models to quantify animal colors and patterns is becoming increasingly important in many fields of evolutionary research, spanning studies of mate choice, predation, camouflage and sensory ecology. Color spaces and color vision models are thus having a rapidly increasing impact on evolution and ecology, and it is important to understand animal vision and the corresponding advantages and limitations of this approach.

Like other color spaces, in avian tetrahedral color space any color is described as a point, which coordinates in the tetrahedron are determined by its relative stimulation of the four avian cone visual pigments (Fig. 5C). In tetrahedral color space each of the four vertices corresponds to a visual pigment, SWS1 (ultraviolet or violet sensitive type), SWS2, RH2 and LWS. Because the coordinates of each perceived color are calculated by its unique stimulation of each visual pigment it will have a unique set of relative stimulation values and thus a unique position in color space. Color spaces are an appropriate diagram to incorporate color vision models, aimed at estimating how colors are perceived and discriminated by animal receivers, to facilitate the presentation and interpretation of results.

A commonly used color vision model was proposed by Vorobyev and Osorio (1998). With this influential model Vorobyev and Osorio (1998) postulate that the main source of noise in color vision systems comes from photoreceptor noise, and explain this is proportional to the relative amounts of cones each color channel possesses. The noise in each channel is inversely proportional to the relative abundance of cones in that channel (Vorobyev and Osorio, 1998). This model fits available physiological data for several vertebrates, including birds, very well making this model a powerful tool for the study of bird vision and color. The receptor-noise model incorporates the relative abundance of the different cone types to calculate Just Discriminable Differences or JNDs. Here the distance between two colors is quantified in JNDs. When the receptor-noise model is incorporated into color space, the distance between two color points is proportional to how different they will appear to the modeled observer, and is thus a measure of its ability for color discrimination. Two different oranges for example, that are 2 JNDs apart will appear more similar to each other than two colors that are 5 JNDs apart. As a way to connect shifts in visual pigment spectral sensitivities to how colors are ultimately perceived by a bird, incorporating color vision models into color spaces is a useful way to connect vision phenotype to visual pigment function and even opsin genotype. This approach can help us model how changes in the visual pigments of different species will affect how a color is perceived, and also which colors will be perceived as different. This could be particularly important if one is interested in knowing whether small differences in plumage color among individuals, that could reflect differences in their condition or quality as mates, can be discriminated by conspecifics or whether the visual system of that species is adapted to discriminate among those differences.

One initial and important consideration is that, in the case of avian color spaces, JNDs are just an indicative calculation to estimate how different colors could appear when seen through the eyes of a bird. It does not mean however, that any colors that differ by ≥ 1 JND will be discriminable. In most species data to match JNDs to actual discrimination thresholds is not available and we need to be cautious when drawing conclusions based on very low JND values. As shown in recent behavioral experiment aimed at matching discrimination thresholds to JNDs needed for pairs of colors in different parts of the spectrum to appear different to chicks. This study showed that indeed one JND is not discriminable to chicks (Olsson *et al.*, 2015). Theoretically, we can only be certain that small JND values are discriminable in species like chicken in which JNDs have been adjusted to measured discrimination thresholds. This becomes particularly important when drawing conclusion on the evolution of color based on small differences in discrimination obtained in color space.

It is also important to understand the underlying assumptions of avian color vision models when using them to make inferences about vision phenotypes. As mentioned above, JND calculations find their basis on the relative abundance of the different cone types. Actually, the little data we have for vertebrates comes from humans, in which radically different ratios of M to L cones were proven not to affect performance in many color vision tasks, particularly color discrimination measures (Miyahara *et al.*, 1998). Moreover, like all models, color vision models rely on assumptions due to our current lack of knowledge of avian vision. In our current avian color models it is assumed that the output of every cone is compared to the output of all the other cones (see Vorobyev and Osorio (1998) for details on the equations). Even if this approximation is the best we can do given our lack of data on avian opponent channels, and it was demonstrated to be a good model of avian color vision, we know considering pairwise comparisons among all cone types is not physiologically accurate or realistic. Only running this model with multiple possible combinations of comparison could tell whether this has an important impact on threshold calculations or not. None of this takes from the

value or validity of avian color vision models, it just means that, like any other model, it relies on assumptions that need to be taken into consideration when those models are used.

Concluding remarks and implication for genotype-phenotype relationships

In conclusion, opsin genes and color vision are a complex system with the potential to offer important insight into the relationship between color and color perception to understand the evolution of color in nature. Additionally, it is a perfect system to make that elusive link between genotype and phenotype due to the availability of an *in vitro* assay to regenerate visual pigments in the laboratory and the inference we can make about an organism's color vision. However, phenotype in this case is complex and can be defined at different levels, from visual pigment function to color vision. As we attempt to connect opsin gene changes to the organism color vision phenotype the complexity of this connection also increases and requires considering both how color vision works and everything we still do not know about the color vision in many animals. As we continue to learn more about different components of their vision, increasing the number of species that have been studied and improving the models available to study their color vision, birds are becoming a system with enormous potential to help us understand how evolution of opsins and visual pigments impacts vision and ultimately how this influences the evolution of color perception.

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