Evolutionary trade-offs may interact with physiological constraints to maintain color variation

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Abstract. Animal coloration is a multifaceted trait with many ecological roles and related to a variety of developmental and physiological processes. Consequently, coloration is often subject to a variety of selective pressures, leading to the evolutionary maintenance of variation. In this study, we investigated hypotheses related to the maintenance of dorsal color variation in wood frogs (Rana sylvatica). First, we tested for multimodality, and whether color correlates with body size or condition or varies by sex or age class. We combined behavioral trials with visual modeling to test for sex recognition. We also considered visual models for predators and tested for an interaction between discriminability indexes (JND) of color channel (chromatic vs. achromatic) and predator type (birds vs. snakes), as well as for a within individual trade-off between the JND of chromatic and achromatic coloration. Finally, we tested for disruptive viability selection on color using predation trials, and for antagonistic directional selection between viability selection and reproductive investment of females. We found that wood frogs present continuous color variation that does not correlate with body size or condition, but that changes with age. Wood frogs present subtle sexual dichromatism, but we found no evidence for a role of color in sex recognition. Instead, we discuss the possibility that sex differences might, at least in part, have a demographic explanation. Predator visual models indicated that wood frogs cannot solely rely on dorsal coloration for camouflage. Moreover, different predators might present selective pressures in different color channels, while individuals’ achromatic and chromatic coloration trade-off in JND. Therefore, different selective pressures caused by different predators might interact with ontogenetic changes and developmental/physiological trade-offs to maintain color variation. We found no relationship between color and survival or reproductive investment, suggesting further work is required to fully understand selection on color. Our results highlight the importance of understanding evolutionary trade-offs and developmental/physiological constraints in combination with one another, and suggest the potential for an interaction between these proximate and ultimate mechanisms in the evolutionary maintenance of variation. These results likely extend beyond color expression in amphibians, and exemplify a more general process for such evolutionary outcomes.

Key words: camouflage; coloration; phenotypic variation; selection; trade-offs; variable selection.

INTRODUCTION

The numerous ecological functions of coloration have made the study of color a fruitful source of evolutionary insight. Animal coloration influences behavioral interactions within a species and predator–prey interactions among species. Within a species, color is important during inter- and intrasexual selection, as it can signal reproductive status, age, or individual quality (e.g., birds [Part and Qvarnström 1997, Keyser and Hill 1999, Badyaev and Duckworth 2003, Hanssen et al. 2006], mammals [Sethell et al. 2006, Bergman et al. 2009], amphibians [Brenes-Soto et al. 2017, Zamora-Camacho and Comas 2019], fish [Hipple 1999], reptiles [Cuadrado 2000, Weiss 2006], insects [Kemp 2007, Willink et al. 2019], reviewed in Cuthill et al. [2017]). Among species, color can honestly advertise unpalatability levels to predators (Boyden 1976, Schlee 1986, Maan and Cummins 2012) or, alternatively, deceive predators through background matching, masquerade, or Batesian mimicry (reviewed in Stoddard 2012, Merilaita et al. 2017, Rojas 2017). Due to these various ecological roles, animal coloration may be under natural and sexual selection, which can be either reinforcing or opposing, and potentially result in spatial diversification and/or the
maintenance of variation within a species (Jameson and Pequetgnat 1971, Endler 1980, 1992, Rudh et al. 2007, 2011, Nokelainen et al. 2012, 2014, Willink et al. 2014).

Color discrimination is both subjective and context dependent because it depends on species-specific anatomical and physiological mechanisms of visual processing (reviewed in Bennett et al. 1994, Endler and Mielke 2005, Kemp et al. 2015, Renoult et al. 2017), and varies with environmental conditions under which signals are transmitted (Endler 1990, 1992, Endler and Théry 1996). Color is also a composite trait, which can be parsed into chromatic (color per se or spectral composition) and achromatic (brightness or luminance) components. These two components represent separate visual channels in animals (Fleishman and Persons 2001, Hempel de Ibarra et al. 2001, Kelber 2005, Osorio and Vorobyev 2005) and are affected differently by variation in the environmental conditions (Endler 1993, Endler and Théry 1996). Therefore, different color components could be under different selective pressures depending on the combination of receivers and environmental conditions, as well as the relative reliance of each receiver on these different visual channels (Siddiqi et al. 2004, Stuart-Fox et al. 2008, Bybee et al. 2012, Maan and Cummings 2012, Barry et al. 2015). Moreover, developmental and physiological mechanisms of color production, whether genetically and/or environmentally induced, could potentially affect both components simultaneously, independently, or by different magnitudes (Flores et al. 2013, Tang et al. 2014, Brenes-Soto et al. 2017, see also Maan and Cummings 2009, Crothers and Cummings 2013), leading to complex interactions between proximate (physiological and developmental) and ultimate (evolutionary) mechanisms.

Such complexity suggests that understanding the evolutionary maintenance of color variation requires an integrative approach. Therefore, in this study, we investigated numerous hypotheses considering the role of proximate and ultimate mechanisms behind the maintenance of intraspecific color variation, in both intra- and interspecific ecological contexts. Using the wood frog (Rana sylvatica), a species that presents remarkable intraspecific color variation and sexual dimorphism (Banta 1914, Martof and Humphries 1959, King and King 1991, Lambert et al. 2017), we first describe color variation in our study populations. Variation provides clues about potential signaling functions. For example, in an intraspecific context, a signal providing information on individual quality is predicted to show unimodal and continuous variation, while a signal used for sexual recognition should have a discrete, bimodal distribution (Dale 2006). Variation in camouflage coloration should also be unimodal and continuous, unless environmental variation is high, in which case the variation is expected to be multimodal and discontinuous (Dale 2006). Moreover, the modality of color distribution allows us to test if color variation represents discrete color polymorphism. Color polymorphic systems are of interest in the study of evolutionary processes as the distinguishable phenotypes provide a means for characterization of individuals' genotypes, and color morphs often present associated differences in morphology, physiology, and/or behavior (Cuthill et al. 2017, see also Svensson 2017).

Second, we investigated the role of color in intraspecific communication. We tested whether color correlates to metrics of individual quality, and whether it varies ontogenetically (i.e., with age and sex). For example, in the natterjack toad (E. calamita) individuals become lighter in color as they age (Zamora-Camacho and Comas 2019). In other anuran species, color correlates with nutritional status (Brenes-Soto and Dierenfeld 2014, Brenes-Soto et al. 2017) and hormone levels (Brown 1976, Camargo et al. 1999, Tang et al. 2014, reviewed in Nilsson Sköld et al. 2012). Together, ontogenetic variation and correlations between color and body condition suggest a direct effect of physiology in color determination, leading to the possibility for such information to be exploited during intraspecific communication. We combined behavioral trials and visual modeling to test an implicit assumption of intraspecific communication studies: that conspecifics are able to visually discriminate color variation.

Next, we investigated the role of coloration in interspecific contexts. We modeled the chromatic and achromatic perceptual ability of two predators, snakes and birds, under a variety of natural backgrounds and lighting conditions. We tested whether discriminability values change with visual channel, predator, and background types. Although predator-driven natural selection should favor more cryptic coloration, variation in discriminability due to changes in background or variation in perceptual ability across predators might lead to antagonistic selection (Jameson and Pequetgnat 1971, Endler 1980, Nokelainen et al. 2014, Willink et al. 2014). For example, specialization in perceptual ability in one visual channel could trade-off with the perceptual ability in the other channel (Ghim and Hodos 2006). Moreover, different backgrounds could represent environments in which one or another channel is more easily discriminable. Therefore, if discriminability is higher in one channel for a particular predator, but higher in another channel for a different predator (Rößler et al. 2019), variation could be maintained in the population.

We also tested whether within an individual frog, chromatic and achromatic discriminability values are negatively correlated with one another, as reported in other amphibians (e.g., Dendrobates pumilio; Crothers and Cummings 2013). Amphibian coloration is the product of three skin-layers (the dermal chromatophore unit; Bagnara et al. 1968, reviewed in Bagnara et al. 1978, Bell and Zamudio 2012, Nilsson Sköld et al. 2012), which house xanthophores containing pteridines (synthesized by chromatophores) or carotenoids (obtained from diet), iridophores containing platelets of purine crystals, and melanophores containing eumelanin. Skin color variation is caused by changes in the
composition, quantity, or aggregation of pigment cells (Nielsen 1979, Tang et al. 2014, reviewed in Nilsson Sköld et al. 2012) as a result of genetic (Baker 1951, Merrell 1972, Tang et al. 2014) and/or hormonal control (Brown 1976, Bagnara et al. 1978, Camargo et al. 1999, Tang et al. 2014). For example, in the Buergeria robusta frog, changes in dispersal of xanthophores due to testosterone injections causes concomitant changes in hue, saturation and brightness of the dorsal coloration (Tang et al. 2014). Moreover, different hormones can interact synergistically or agonistically (Bagnara et al. 1978, Camargo et al. 1999, Tang et al. 2014), simultaneously affecting aggregation and dispersal in more than one pigment cell type (Brown 1976, Tang et al. 2014), and the differentiation of xanthophore pigments and the purine in the iridophores seem to be linked through a common biochemical pathway (reviewed in Bagnara et al. 1978). Therefore, changes in color pigments should affect discriminability in both channels simultaneously, either reinforcing or opposing each other. For instance, if pigments with lower discriminability values in one channel have higher discriminability in the other against a particular background, an individual would not be able to be camouflaged in both channels at the same time. In this case, a trade-off would be reflected by individuals with high values of discriminability in one channel having low values in the other, and vice-versa.

Finally, we tested for the association between color and two components of fitness: survival and reproductive investment. Specifically, disruptive selection can lead to the maintenance of, or even an increase in, phenotypic variance (Scharloo 1964, Skibinski and Thoday 1979, Spichtig and Kawecki 2004, Pelabon et al. 2010), and the relationship between fitness components and color could be opposite when considering survival and reproduction, such that the resulting antagonistic selection may contribute to the maintenance of variation (Jameson and Pequegnat 1971, Endler 1980, 1992, Rudh et al. 2007, 2011, Nokelainen et al. 2012, 2014, Willink et al. 2014).

**METHODS**

**Study species**

Wood frogs (*Rana sylvatica*) are diurnal (Bellis 1962), small to medium-sized frogs (35–60 mm snout–vent length in our populations). They have a large geographical range throughout much of North America (Martof and Humphries 1959). Wood frogs arrive at breeding ponds in early spring, soon after the onset of above-freezing temperatures, when snow melts and the ground thaws. Males stay in the breeding ponds, chorusing, throughout the duration of the explosive breeding period (ranging between 4–7 d), typically mid to late April at our study sites. Gravid females entering the pond are quickly pursued by males, and we often observe multiple males grasping a single female. Oviposition does not seem to occur until a single male is in the correct amplexus position (Howard and Kluge 1985; D. Goedert, personal observation). After breeding, wood frogs leave the ponds but are commonly found nearby during the day. Adult wood frogs experience predation by a variety of sources including garter snakes (*Thamnophis sirtalis*; Gregory and Stewart 1975, Rittenhouse et al. 2009), and birds (we have found evidence of American Kestrels, *Falco sparverius*, feeding on frogs near our breeding ponds on at least one occasion).

Across their geographic range, wood frogs have been described as having regional morphotypes that differ not only in morphology (i.e., relative leg length), but also in body coloration and patterning, with background colors ranging from a tan or chestnut brown to a dark brown (Martof and Humphries 1959). They also exhibit considerable color variation within populations (King and King 1991, Lambert et al. 2017; D. Goedert, personal observation; Appendix S1: Fig. S1) that has been partially explained by sexual dimorphism, with males expressing darker and yellower coloration, and females being lighter and redder than males (Banta 1914, King and King 1991, Lambert et al. 2017). Sexual dichromatism in wood frogs has been proposed as a mechanism for sexual recognition, with anecdotal evidence that lighter-colored, redder females are more readily recognized by males than are darker colored females when entering breeding aggregations (Banta 1914). Previous studies have also suggested that sex recognition should be particularly favored in explosive breeders, since the male biased sex ratio at breeding aggregations results in high intrasexual competition (Sztatecsny et al. 2010, 2012), such as in the case for wood frogs (Howard 1980, Berven 1981). Therefore, sexual dichromatism could evolve and be maintained because reproductive fitness should be higher for males that readily distinguish the sex of individuals entering ponds, and that are able to avoid claspers’ attempts from other males (Sztatecsny et al. 2012).

**Data collection**

**Animal collection.**—Our study ponds are located near the towns of Hanover, New Hampshire and Norwich, Vermont, USA (43.7153° N, 72.3079° W). Adult wood frogs were collected from 10 breeding ponds in the spring of 2016 (n = 225) and 2017 (n = 303) using pitfall traps or minnow traps. Pitfall traps were set at ~2 m from the margin of breeding ponds, prior to the arrival of breeding frogs. Minnow traps were deployed after individuals were observed in the ponds, always near the margin of the ponds where water was shallow enough that captured frogs could swim up to the surface. Both types of traps were checked daily. Captured individuals were taken to the Life Sciences Center at Dartmouth College, where they were kept in a temperature-controlled room at 4°C, in a 14:10 light:dark cycle with full spectrum fluorescent lights, and in individual 1-L plastic
containers containing dechlorinated tap water and leaves of Acer spp. and Quercus spp. until experiments were finalized. At the end of the study, all animals were returned to the ponds where captured.

All animals were sexed based on the presence of nuptial pads in males, measured for snout–vent length with calipers, and weighed with a digital scale, before photographs and spectrophotometry data were collected. In 2016, while collecting eggs for a different study (Goedert and Calospeek 2019), we counted number of eggs for a subset of females ($n = 39$), and marked all animals with a toe clip before releasing. Since our field procedure required trips to multiple populations within a day, some animals were housed in the temperature controlled room for many hours before processing. Therefore, all individuals ($n = 525$) were allowed to acclimate, undisturbed, within their housing containers to a water temperature of 20–23˚C before processing. Full processing took on average less than 5 minutes, and is unlikely to have caused color changes as a consequence of stress from processing. Since we did not obtain photographs of the animals under natural conditions and immediately after capture, however, it is unclear how the stress related with transportation, time in captivity, or the artificial conditions (e.g., housing conditions, temperature, lighting) affected the color of the animals.

Color measurements—photographs.—Animals were photographed with a Nikon D7100 camera (Nikon, Melville, New York, USA) and a 50 mm Zeiss Makro-Planar ZF.2 macro lens (Zeiss, Oberkochen, Germany) attached to a tripod. We used two led lamps as illuminant, placed behind a white box built out of white heavyweight poster paper. The white balance was set manually (see Stevens et al. 2007, Troscianko and Stevens 2015). Since this information was not available for the camera used, 301 individuals captured in 2017 were measured using a handheld spectrometer (Ocean Optics Jaz) with a built-in light source (PX2, range 220–750 nm; Ocean Optics, Dunedin, Florida, USA). The fiber optic probe was fitted into a custom probe holder to exclude ambient light. Measurements were taken relative to a thick layer of barium sulfate as a 99% reflectance standard (Grum and Luckey 1968), using a black surface as a dark reference. The spectrometer was configured (“scans to average” option) as to record the average of 10 sequential spectral acquisitions. Individuals were patted dry before measures. We recorded two spectra per individual: one from the head, and one from the dorsum, avoiding regions with black dots, dorsal stripes or other color alterations. This procedure was repeated for the following background materials found around breeding ponds: exposed soil, grass leaves, and leaves from the leaf litter, including birch (Betula sp.), oak (Quercus sp.), and maple (Acer sp.) leaves, as well as pine needles (Pinus sp.). Because birch and oak leaves can vary in shade, “light” and “dark” leaves were measured. We also measured wet birch and maple leaves. For the background materials, three measures were taken per object.

We used the package pavo (v. 2.0; Maia et al. 2013, 2019) in R (v. 3.4.1; R Core Team 2017) to import and process the reflectance spectra, and to model visual perception using the receptor-noise model implemented in the package. For all frogs and background spectra, we averaged the repeated spectra per sample, applied a LOESS-smoothing ($\alpha = 0.15$) and rescaled the spectra to minimum values of zero, to remove minor electrical noise and to correct for spurious negative values.

The receptor-noise model does not assume specific color opponent mechanisms for the chromatic discrimination thresholds (Vorobyev and Osorio 1998, Vorobyev et al. 1998, 2001) and, therefore, is not species specific (Renoult et al. 2017, Olsson et al. 2018). This model disregards the achromatic signal for the chromatic
discrimination, justified by evidence that color is processed independently from brightness in many animals, and that this model predicts color discrimination in a variety of animals, with the exception of crepuscular species, or under conditions of low light (Vorobyev and Osorio 1998, Vorobyev et al. 2001, reviewed in Endler and Mielke 2005, Kemp et al. 2015). This visual model allows for the estimation of chromatic and achromatic contrast in units of “just noticeable difference” (JND), which are noise-corrected contrasts (Vorobyev and Osorio 1998, Vorobyev et al. 2001). In this scale, contrasts of JND < 1 are considered as indistinguishable by the viewer (Vorobyev et al. 2001). The parameter choices for the models are detailed below. Since not all parameters are known for the species considered, we conducted sensitivity analyses and reran analyses considering different parameter values for the visual models (see Bitton et al. 2017).

Visual models: parameters, models and sensitivity analyses

Photoreceptor sensitivities.—We estimated the photoreceptor sensitivities of kestrels, snakes, and frogs, using the function sensmodel in pavo (Maia et al. 2013, 2019). Since peak wavelengths for photoreceptors, oil droplets, and the ocular transmission were not available for all the species used at the time of this study, we used information for the closest related species available. This was justified based on sensitivity analyses indicating that shifts in the spectra within 20 nm do not considerably affect color discrimination abilities (Lind and Kelber 2009, reviewed in Olsson et al. 2018). Moreover, the spectral sensitivity of cones is mostly conserved across species of birds (Hart 2001, Hart and Vorobyev 2005, Hart and Hunt 2007), as it seems to be for species of Rana sp. (see Liebman and Entine 1968, Harosi 1982, Koskelainen et al. 1994, Govardovskii et al. 2000).

Birds are tetrachromats, having a long-wavelength (LWS), a medium-wavelength (MWS), and two short-wavelength sensitive cones (SWS1 and SWS2). The SWS1 sensitive cone can be of the violet (VS) or ultraviolet (UVS) type (reviewed in Hart 2001, Endler and Mielke 2005). Birds in the Falconidae family, including kestrels, have the VS-type of SWS1 cone, with maximum absorbance (λ<sub>max</sub>) at 405 nm (Odeen and Hart 1992, Odeen and Hästad 2003). For the other photoreceptors, we considered the photoreceptor absorbance of the American Kestrel to be equal to the ones of the tawny owl (λ<sub>max</sub> at SWS2: 463 nm; MWS: 503 nm; LWS: 555 nm; reviewed in Hart and Hunt 2007; Bowmaker and Martin 1978), as recent studies have identified similar amino-acid substitutions in the LWS and SWS2 genes of Falconidae and Stringigidae (Wu et al. 2016). We generalized the spectral transmittance of the oil droplets from species with similar photoreceptor spectra (λ<sub>cut</sub> of T, 300 nm; C, 445 nm; Y, 506 nm; R, 562 nm; Hart and Vorobyev 2005). We used ocular transmittance empirically determined for the congener species Falco tinnunculus (Lind et al. 2013).

Garter snakes (Thamnophis sirtalis), as other diurnal snakes, are trichromats, with three single cones that lack oil droplets (proportions 1:1.6:7.3; λ<sub>max</sub> at SWS1, 360 nm; MWS, 482 nm; LWS, 554 nm; Sillman et al. 1997). Since the eyes of garter snakes are covered by a spectacle, which can also filter light, we combined the transmission values from the lens and spectacle estimated by Simões et al. (2016) by choosing the lowest value among the two transmission values for each wavelength.

Although no information on photoreceptors was available for wood frogs at the time of this study, congeneric species (R. pipens, R. temporaria, and R. catesbeiana) have been described as having a single RS cone (λ<sub>max</sub> at 562–575 nm), a BS cone (λ<sub>max</sub> at 431 nm), and a green-red double cone (DC; λ<sub>max</sub> at 502 and 575 nm; Liebman and Entine 1968, Harosi 1982, Koskelainen et al. 1994). While double cones (DC) do not play a role in chromatic discrimination in birds, there is evidence that they could play such a role in the reef fish Rhinecanthus aculeatus (Pignatelli et al. 2010). Moreover, DC have been shown to respond in a blue-yellow color opponent manner in frogs (reviewed in Donner and Reuter 1976). In this case, the spectral sensitivity of each member of the DC is combined, resulting in a peak sensitivity of 560 nm for the green-red DC of the frog (Reuter and Virtanen 1972, Donner and Reuter 1976). Therefore, these studies suggest that wood frogs are dichromats, with photoreceptor sensitivities at λ<sub>max</sub> of 433 and ~562 nm. We disregarded the presence of oil droplets because oil droplets are rare in frogs and presumed to be of no functional significance (Liebman and Entine 1968, Siddiqi et al. 2004). We used the ocular transmissions values described for R. temporaria (λ<sub>max</sub> at 500 nm; Govardovskii and Zueva 1974), obtained by digitizing the figure with the package metaDigitise (Pick et al. 2018).

Chromatic perception.—Although the von Kries transformation for chromatic adaptation or color constancy (von Kries 1905 as cited in Kemp et al. 2015, also reviewed in Endler and Mielke 2005, Renoult et al. 2017) is commonly used in visual models, it is unclear to which conditions and to what extent this chromatic adaptation applies (reviewed in Endler and Mielke 2005, Kemp et al. 2015, Renoult et al. 2017). At the least, chromatic adaptation is known to be imperfect (reviewed in Renoult et al. 2017), explaining why an organism would time their displays to take advantage of certain ambient light conditions (Endler and Théry 1996, Sicé et al. 2013). Therefore, for this study we followed recommendations from Renoult et al. (2017) and compared results of models with and without the von Kries transformation. To account for the ambient light conditions without the color constancy transformation, we considered the light spectra of “open areas” and
“forest shade” as defined by Endler (1991, 1993) and provided in the R package pavo (Maia et al. 2013, 2019). We considered only conditions of bright daylight for the visual perception of frogs and predators, under which a fixed Weber fraction to the absorbed quanta represents a good approximation of the receptor noise (Vorobyev et al. 1998, Olsson et al. 2015, 2018).

**Achromatic perception.**—Birds are generally thought to use double cones in achromatic discrimination, and we model the achromatic perception of the Kestrel using the absorbance values of double cones of Blue-tits, as implemented in pavo (Maia et al. 2013, 2019). For frogs and snakes, we considered the LWS cone to be involved in the achromatic perception (Fleishman and Persons 2001, Siddiqi et al. 2004, Stuart-Fox et al. 2008, Kemp et al. 2015).

**Receptor-noise model.**—We obtained the chromatic and achromatic contrast in the “just noticeable difference” (JND) unit, which are noise-corrected contrasts (Vorobyev and Osorio 1998, Vorobyev et al. 2001), using the coldest function (Maia et al. 2013, 2019). In this scale, contrasts of JND < 1 are considered as indistinguishable by the viewer (Vorobyev et al. 2001).

For the chromatic channel of the kestrel, we considered the photoreceptors proportion to be 1:2:3:3 (SWS1: SWS2:MWS:LWS), with a Weber fraction of 0.1, which are commonly encountered values for birds (reviewed in Hart 2001, Bitton et al. 2017, Olsson et al. 2018). We considered the Weber fraction for the achromatic channel to be 0.18 (reviewed in Olsson et al. 2018), following from the derivation that this fraction equals the noise in the receptor mechanism for the log-linear visual models (Vorobyev et al. 2001).

Since RS cones are dominant in the retina of *Rana* sp. (Liebman and Entine 1968, Hárosi 1982, Koskelainen et al. 1994), and DC possibly present similar wavelength sensitivity to the RS cone, we used a photoreceptor relative abundance of 1:8 (BS:RS) for the frog. Note that, since the noise in the other photoreceptors are calculated as the square-root of the ratio of the abundance of RS cone to the abundance of the cone of interest (reviewed in Olsson et al. 2018), a biased ratio of photoreceptor toward a higher proportion of long-wavelength cones results in a conservative estimate of chromatic discrimination in comparison to a more even ratio.

For both snakes and frogs, we used a Weber fraction of 0.05 for the chromatic and achromatic channels (following Siddiqi et al. 2004, Stuart-Fox et al. 2008).

**Sensitivity analyses.**—We reran analyses with different parameter values for some of the variables. For the kestrel visual model, we considered the possibility that the oil droplets were absent or transparent ($\lambda_{\text{cut}}$ of 300 nm). We also estimated achromatic perception using the LWS cone, as raptors including kestrels have been reported to not present double cones in their fovea (Mitkus et al. 2017, see also Campenhausen and Kirschfeld 1998). For both channels in the frog vision, we considered the ocular transmissions values described for *R. pipens* ($\lambda_{\text{max}}$ at 503 nm; Kennedy and Milkman 1956). We also considered a range of photoreceptor proportions for the frog, from 1:1 to 1:7. Finally, we considered a Weber fraction of 0.1 for the chromatic and achromatic channels of frogs and snakes in the estimation of noise-corrected JNDs.

**Experiments**

**Sex discrimination experiments.**—To investigate whether color variation is used in mate recognition by wood frogs, we conducted a traditional dichotomous choice experiment (Bisazza et al. 1989, Rios-Cardenas et al. 2007). We conducted these experiments between 20 and 22 April 2017, within 1–3 d of capturing the animals, and between 09:00 and 21:00 hours. We simultaneously presented each male frog with two gravid females of different coloration, a light-colored and dark-colored female, classified according to human perception. In a total of 22 trials conducted, we used 22 males and 37 females. Two light-colored females and two dark-colored females were reused but paired with a different female, one female was used as a dark-colored female in one trial and as a light-colored female in another trial, and one pair of females was used twice. Otherwise, different individuals were used in each trial (see Kroodsma et al. 2001).

The pair of females was introduced simultaneously at opposite ends of a tank (60 × 136 cm) filled with water, separated from a central arena by a transparent plastic wall. Males were placed in the central arena and allowed to freely move toward each of the females. This central arena (60 cm in width by 80 cm in length) was divided lengthwise into three zones of equal size: a central-most zone that we considered a “neutral zone,” and the two remaining thirds of the central arena nearest to the females that we considered “choice zones” for each of the females. To replicate the visual environment of local breeding ponds we covered the exterior sides of the tank with artificial turf and placed the tank over a bed of leaves collected from around the breeding ponds. To prevent females from hiding, we did not place any leaves inside the tanks.

At the start of the experiment, males were placed in the neutral zone of the central arena and prevented from moving by a transparent plastic divider for a total of two minutes. The divider was attached to a string such that after this initial acclimatization period we were able to gently remove the divider, freeing the male without approaching the arena. We ran the experiment for 8 min, and counted the total time, in seconds, that a male spent in each of the choice zones. To account for potential side preference biases, we then placed the male back in the neutral zone under the plastic divider, switched the position of the females, and repeated the
procedure. Because in such an experiment, males are not able to reach the females, we considered the total time a male spent in the third of the tank closest to each female as indicative of preferential association with that female.

Selection experiments.—Survival data were reanalyzed from selection experiments described in Goedert and Cališek (2019). Briefly, 196 individuals were placed in semi-natural enclosures near one of the breeding ponds in four replicates, in the spring of 2016 and 2017. In 2016, replicates consisted of 50 and 46 frogs, with approximately even sex ratio (27:23 and 26:20, males:females). However, due to the low number of females captured in 2017, only males were used for the other two replicates (50 males each replicate). In these enclosures, animals were exposed to natural predation from small mammals and birds of prey.

Data analyses

All analyses were conducted in R (v. 3.4.1, 3.6 or 3.6.2; R Core Team 2017, 2019). Whenever relevant, we assessed normality through visual inspection of quantile plots and used an F test to check for equality of variance. Initial inspection of the data using quantile plots and regressions between variables estimated from the spectrophotometry and photography procedures indicated that 16 out of 301 individuals consistently appeared as extreme values, suggesting the possibility of errors during the data collection (e.g., spectrophotometer probe was not properly placed). Therefore, except for the most computationally intensive analyses, we ran all analyses with and without these individuals. The results were qualitatively identical, so we present the results with the full data set (see data repository for other results). For the ANOVA with permutation and multi-response Bayesian models, we only conducted analyses without these individuals.

Color variation.—We reduced the dimensionality of the RGB color values with a principal component analysis and used the first principal component (PC1) for the analyses. To help interpretation and comparison with previous studies, we additionally transformed color data from RGB to HSV scale using the function rgb2hsv in R (R Core Team 2017, 2019), and reanalyzed the data for the relevant analyses. In the HSV scale, hue (H) represents what is commonly referred as “color”; the saturation (or chroma, S) is a representation of the purity of the color, where a saturation of 0 represents gray, and a saturation of 1 represents fully saturated hue; and the brightness (or value, V) represents the intensity of light reflected, in a scale from 0 (black) to 1 (white).

To assess modality of distribution for color variables, we estimated Hartigan’s dip statistics (HDS, Hartigan and Hartigan 1985), and used a permutation procedure to test the null hypothesis of unimodality. The HDS and permutation test were conducted using the package diptest (Maechler 2016) in R (R Core Team 2019).

Intraspecific contexts.—To test for sexual dichromatism with data in the HSV scale, we used a PERMANOVA implemented in the function adonis of the R package vegan (v. 2.5-6; Oksanen et al. 2019). Hue, saturation and brightness measured from pictures were response variables. Sex, year, breeding pond, the three-way interaction term, and the pairwise interaction terms between variables were initially added as independent variables. Subsequently, we conducted two post-hoc PERMANOVA analyses. First, we added sex, year, and the interaction between the two variables as the independent variables, while adding breeding pond as a grouping variable. For the second analysis, we added sex, breeding pond, and the interaction between the two variables as the independent variables, while adding year as a grouping variable. We removed three breeding ponds from these analyses, because they had fewer than 20 individuals captured in each year. Finally, we conducted post-hoc univariate Wilcoxon tests to investigate sex differences in each of the response variables.

To test for a relationship between individual quality or nutritional status on coloration, we used the R package lmerTest (v.3.1-1; Kuznetsova et al. 2017; using the package lme4 v. 1.1-21; Bates et al. 2015) to fit a mixed effects model between PC1 and body size and body condition (taken as body weight). We added breeding pond as a random effect, and included sex, year, and the two-way interactions between sex and body size, and sex and body condition as fixed effects. The interactions were subsequently removed if not significant, as forced correlations between variables can influence the estimates per variable.

We tested whether individuals recaptured in 2017 differed in PC1 values between the two years using a paired t-test for samples with equal variance. To facilitate interpretation, we compared hue, saturation and brightness between years using a PERMANOVA adding individuals as a grouping factor and conducted post-hoc paired Wilcoxon-tests for each of the response variables.

To test whether color differences are perceptually discriminable when accounting for the visual system of the frogs, we estimated the distance between the geometric means of male and female coloration in the JND scale (Vorobyev et al. 2001, Maia and White 2018). We then used a bootstrap procedure (n = 1,000) to estimate a 95% confidence interval. This analysis was conducted using the function bootcoldist from the package pavo (v. 2.3; Maia et al. 2019), as described in Maia and White (2018).

To test whether males behaviorally discriminate between light and dark-colored females, we used a paired t-test on the total time spent with each female in the first and second rounds of the experiment. We transformed the recorded total time values into period objects
using the R package lubridate (Grolemund and Wickham 2011).

Inter specific contexts.— We tested whether males and females differ when considering the noise-corrected discriminability values of predators using the function bootcoldist from the package pavo (v. 2.3; Maia et al. 2019).

To test for changes in discriminability values per channel, predator and background type we conducted an ANOVA on the three-way interaction between these categorical variables, adding individual as an error term. Since the assumptions of the analyses could not be met even with transformation of the response variable, we estimated statistical significance through a permutation procedure. We estimated the $F$ statistic for the interaction term of 4,999 permutations of the response variable, and estimated significance using the correction $P = (r + 1)/(k + 1)$, where $r$ is the number of permutations resulting in an equal or higher value of $F$, and $k$ is the total number of permutations (Davison and Hinkley 1997). We also performed the permutation procedure constrained within the individual level. As post-hoc analyses, we repeated the permutation procedures, fitting the ANOVA with the interaction between predator and channel type for each of the backgrounds considered.

We fitted a mixed effect model to test for a within individual trade-off between the contrast indices of the chromatic and achromatic channels using the package MCMCglmm (v 2.29; Hadfield 2010). The response variable was a vector of all contrast indices, and the random term included the interaction between channel type and individual identity (us variance structure). Therefore, indices across predators and backgrounds were statistically treated as repeated measures, such that we were able to estimate variance per channel and covariance between channels. The correlation between channels was calculated as the covariance divided by the square-root of the product of the variances. We included background, predator and channel types as fixed effects, and removed the intercept. The residual covariance structure was set to be heterogeneous in variance for each channel type (idh variance structure). As priors for fixed effects, we used diffuse normal distributions with mean of zero, and for the residual variance, we used an inverse Wishart. For the random term, we choose a prior that is expected to be uniform in the range of $-1$ to $1$ ($V = \text{diag}(n) \times 0.02$, nu = $n + 1$; following Hadfield 2019:71).

We tested for the relationship between discriminability values in JND units and the PC1 scores of the RGB values for each of the backgrounds, predator and channel type combinations. Since we did not have reflectance data for animals captured in 2016, this analysis allows us to infer how ontogenetic changes in color affect discriminability values for each of the predators. We fitted a multi-response linear mixed-effect model for each of the predator and channel combinations (i.e., four models: snake-chromatic, snake-achromatic, kestrel-chromatic, and kestrel-achromatic), with breeding pond of origin as a random term. The response variables were the JND values of each of the ten backgrounds. To fit such a multi-response model, we used a Bayesian framework (package MCMCglmm; Hadfield 2010) and fitted the models so as to estimate a regression coefficient between discriminability indices and PC1, and an intercept for each of the 10 background types (i.e., fixed effects were fitted as PC1:trait + trait - 1; where “trait” represents each of the backgrounds). The priors for the fixed effects were the default Gaussian distribution, and for the random term we used parameter extended priors.

For all models fitted using the package MCMCglmm, we visually inspected the MCMC chains for mixing, and checked that the autocorrelation between stored iterations was below 0.1 (Hadfield 2019:22). We additionally conducted a Heidelberger and Welch’s convergence diagnostic as implemented in the package coda (Plummer et al. 2006). All models were run a second time with different starting values, and rerun twice with inverse Wishart priors for the random terms. Results for all models were robust across runs and priors. All statistical models were refitted considering alternative parameter values, as explained in the section “sensitivity analyses.”

Fitness components.— To test for the relationship between coloration and survival, we fit a generalized linear model with a binomial distribution to account for variation in survival (live vs. dead), and the following fixed effects: replicate, body size, PC1, the interaction between body size and PC1, and a quadratic term for PC1. We refit the model twice, first removing the non-significant interaction between PC1 and body size, and then additionally removing the nonsignificant quadratic term for PC1.

To test for a relationship between coloration and reproductive investment in females, we fit a linear model where the square-root of the number of eggs was the response variable. Because the variance of the response variable was a hundred times the mean, we did not use a generalized linear model with a Poisson distribution for the number of eggs and, instead, we assumed a Gaussian distribution for the square-root transformation of egg number, which presented a distribution that reasonably approximated normality. The model was initially fit with body size, PC1, and the interaction between the two variables. Subsequently, the model was refit without the interaction term, as this term was not significant.

Results

Color variation

The first principal component based on color metrics obtained from digital images was positively correlated with all of the variables (PC1 loadings: red = 0.837, green = 0.451, and blue = 0.310), and explained 96.6% of the total variance (Fig. 1B). Hue ranged from 0.06 to
0.33, saturation ranged from 0.11 to 0.49, and brightness ranged from 0.2 to 0.57 (Fig. 1, Appendix S1: Fig. S2). Color variation in PC1 indicated that wood frogs exhibit unimodal variation in color (Hartigan’s dip statistics, HDS: 0.012, \( P = 0.935 \)). Reflectance spectra revealed discontinuous variation in the wavelength of maximum reflectance, with 40% of the frogs exhibiting peak reflectance between 534 and 538 nm, and the remaining 60% of frogs exhibiting peak reflectance above 605 nm (HDS: 0.172, \( P < 0.001 \)). However, the reflectance spectrum of individuals often included multiple peaks (Appendix S1: Fig. S3). By contrast, the distribution of JND in the chromatic and achromatic channels had a unimodal distribution both when considering the visual system of frogs (HDS range: 0.01 to 0.025, \( P > 0.24 \)) and predators (HDS range: 0.01 to 0.024, \( P > 0.24 \)).

**Intraspecific context**

Color data in the HSV scale corroborated previous findings of sexual dichromatism in wood frogs (PERMANOVA \( R^2 = 0.046, P = 0.001 \); post-hoc Wilcoxon-tests \( P < 0.00001 \) for all variables). Specifically, females in our study are redder in coloration compared with males, with lower hue (females, 0.098 ± 0.038; males,

![Fig. 1](image-url)

**Fig. 1.** Wood frogs present subtle sexual dimorphism in coloration, as seen by (a) the distribution of hues for males (continuous line, not shaded) and females (dashed lines, shaded); the mean (open symbols) and the median (closed) hue of males (M, circles) is further to the right than those of females (F, triangles). Therefore, while the distribution of females is centered on a red-orange hue, males have a mean hue in the yellow color region. Similar results can be seen when considering (b) the first principal component of a principal components analysis (PCA) on the RGB values, with (c) males presenting lower PC1 scores than females and (d) lower PC1 scores corresponding to higher values of hue (see also Appendix S1: Fig. S2). For panels b–d, open circles represent males and solid triangles represent females.
0.134 ± 0.065; mean ± SD; Fig. 1A), and higher saturation (females, 0.31 ± 0.094; males, 0.257 ± 0.084). Additionally, males are darker in coloration (lower brightness) than females (males: 0.317 ± 0.066, females: 0.355 ± 0.083). Notably, wood frog coloration seems to be comprised of two axes of variation, such that individuals with hue values near 0.1 (red-orange) show variation in brightness and saturation, while individuals with larger hue values, in the yellow-green range, seem to show a narrow range of low values of both brightness and saturation (Appendix S1: Fig. S2). Such low values of both saturation and brightness result in individuals appearing mostly greyish and dark in human perception, regardless of their hue.

All of the interaction terms in the PERMANOVA were significant (P < 0.05), indicating differences in the degree of sexual dichromatism across breeding ponds and years. However, the sum of squares for each interaction term represented a very small proportion of the total color variation (R² ranged from 0.007 to 0.021). Similarly, coloration differed between years (R² = 0.171, P = 0.001) and among breeding ponds (R² = 0.02, P = 0.038). Results were identical when considering year or breeding pond as grouping factors. For these analyses, we removed individuals from three populations due to the low number of individuals captured in both years (2016: n₁ = 8, n₂ = 7, and n₃ = 3; 2017: n₁ = 14, n₂ = 3 and n₃ = 0), making the interpretation of interaction terms difficult. Including these populations in the analyses did not affect the significance of the interaction between sex and year in the model with population as a grouping factor. However, the three-way interaction between year, population, and sex in the model without a grouping factor, as well as the interaction between population and sex in the model with year as a grouping factor became marginally nonsignificant (P < 0.1). Since the R² values for these terms were extremely small, even when terms were statistically significant, we consider these differences to be of minor consequence.

There was no evidence for a relationship between color and proxies for individual quality and nutritional status. We found no interaction between sex and body size or sex and body condition (Appendix S1: Table S1). After removing the interaction terms, we found no relationship between coloration measured as PC1 and body size or body condition (Table 1). The random term (breeding pond) represented 3.1% of the total variance. We detected two outlying data points (based on large residual values). Removing these points from the analyses did not qualitatively influence the results.

Eleven individuals (4 males, 1 female) were recaptured in 2017, and differed in coloration between years (RGB scale, PC1: t₁₀ = 3.783, P = 0.004; HSV scale, PERMANOVA R² = 0.163, P = 0.007; Fig. 2). In the second year, recaptured individuals showed higher values of PC1 (first year, −19.815 ± 20.854 vs. second year, −3.721 ± 17.638; mean ± SD), saturation (0.213 ± 0.079 vs. 0.264 ± 0.082; Wilcoxon V = 11, P = 0.054), and brightness (0.267 ± 0.062 vs. 0.312 ± 0.055; Wilcoxon V = 7, P = 0.019), and lower values of hue (0.184 ± 0.071 vs. 0.12 ± 0.041; Wilcoxon V = 64, P = 0.003).

Although sexes differed statistically in coloration metrics, mean pairwise JND was below the discriminability threshold for both the chromatic (mean = 0.047, 95% CI 0.003–0.255) and achromatic channel (mean = 0.20, 95% CI 0.032–2.11; Fig. 3), when considering the visual perception of conspecífics. Results were consistent when considering alternative parameter values.

In the dichotomous choice experiments, there was no difference in the amount of time a male spent with the light or the dark-colored female (paired t test, t₂₀ = 0.421, P = 0.678; Fig. 4). Moreover, the proportion of males that spent more time with the lighter females was equal to that of males that spent more time with the darker females (i.e., 1:1).

Table 1. Results of the mixed-effects model, with breeding pond as the random effect, indicating no relationship between coloration (PC1) and body size or body condition.

| Factor         | Estimate | SE   | df  | t    | P    |
|----------------|----------|------|-----|------|------|
| Intercept      | 2.068    | 4.201| 218.2| 0.492| 0.623|
| Sex (M)        | −1.049   | 1.749| 451.9| −0.600| 0.549|
| Body condition | −1.428   | 1.074| 336.7| −1.329| 0.185|
| Year (2017)    | 16.045   | 2.277| 450.3| 7.046| 0.000|

Note: Statistically significant values are highlighted in bold-face type.

Interspecific contexts

Visual models for the potential predators across the set of environmental conditions considered indicated...
JND was frequently above and much higher than the discriminability threshold of 1 (mean JND per channel, predator, and background type ranged from 0 to 56.2; median 0.89–23.22; Appendix S1: Fig. S4). Males and females did not differ in mean pairwise JND when considering the perceptual ability of snakes (chromatic mean = 0.444, 95% CI 0.055–1.029; achromatic mean = 0.763, 95% CI 0.045–2.293) or kestrels (chromatic mean = 0.69, 95% CI 0.283–1.313; achromatic mean = 0.28, 95% CI 0.015–0.692).

The mean JND changed across the combinations of channel, predator, and background types, as indicated by a three-way interaction ($F_{9, 11,076} = 134.98$; constrained permutation, $P = 0.038$; Fig. 5, thick lines). Sensitivity analyses indicated that this result was robust to variation in parameter values of the visual models (Appendix S1: Table S3). Within each background type, the interaction between channel and predator types was significant for all but a few parameter combinations within the dark birch leaves and dark oak leaves backgrounds (Appendix S1: Table S4).

We found a negative individual-level correlation between discriminability values in the chromatic and achromatic channels (posterior mode $r = -0.308$; 95% credible interval $-0.438$ to $-0.135$; Fig. 5, thin lines). Sensitivity analyses returned negative correlations for all parameters considered (Appendix S1: Table S5).

For the chromatic channel of both predators, in all of the backgrounds considered, individuals with higher PC1 values presented better camouflage, as indicated by the negative slope (PMCMC $< 0.05$; Fig. 6) between the discriminability indices and PC1 (coefficients ranged from $-0.012$ to $-0.023$ for the kestrel, and from $-0.017$ to $-0.038$ for the snake). In the achromatic channel, this was also true for both predators in six of the leaf litter backgrounds (coefficients were $-0.03$ or $-0.031$ for the kestrel, and ranged from $-0.1$ to $-0.095$ for the snake; Appendix S1: Tables S8–9). The relationship between discriminability indices and PC1, however, was reversed in three backgrounds for both predators (kestrel: exposed soil = 0.021, birch leaves, wet = 0.024, and maple leaves, dry = 0.007; snake: exposed soil = 0.076, birch leaves, wet = 0.082, and maple leaves, dry = 0.053). No relationship was found for either predator in the grass background (Appendix S1: Tables S8, S9). Results for the models with alternative parameter values yielded qualitatively similar results (Appendix S1: Tables S6–S9, Appendix S1: Fig. S5).

**Fitness components**

Combined survival of males and females was 28.1% (ranging from 18% to 38% across replicates). Neither the quadratic relationship between survival and coloration, nor the interaction between PC1 and body size were statistically significant ($P > 0.05$), so these terms were removed from the model. Variation in coloration was not correlated with differences in survival (Table 2). Adding an interaction term between sex and PC1 to the model indicated the slope of the relationship between survival and coloration differed between males and females (interaction term 0.007 ± 0.003, estimate ± SE, $P = 0.043$, Appendix S1: Table S10). However, the slope of this relationship did not differ from zero for either of the sexes (female 0.006 ± 0.003, $P = 0.062$; male 0.001 ± 0.002, $P = 0.394$; Appendix S1: Table S10).

There was no relationship between coloration and reproductive investment of females, estimated from individual fecundity, nor was this relationship influenced by the size of the female (Table 3). The removal of the
interaction between coloration and female body size did not change the results.

**DISCUSSION**

We investigated a variety of possible explanations for the maintenance of color variation in wood frogs, considering both intra- and interspecific ecological contexts. We first described aspects of the color variation, ruling out the possibility of discrete color polymorphism. We tested whether color variation is explained by body size, body condition, sex, and age of individuals. Using dichotomous choice experiments and considering visual models for color vision in frogs, we then tested a century-old hypothesis that sexual dichromatism is maintained because male wood frogs use color for sex-recognition during breeding. In addition, we investigated the role of coloration in camouflage, asking if antagonistic selection during predation contexts could explain the maintenance of color variation. Within such context, we considered whether discriminability indices obtained from predator visual models vary across natural backgrounds, between different predators, or between different visual channels of a predator, and whether there is a negative correlation between chromatic and achromatic discriminability values. Finally, we tested for disruptive selection on survival, and for antagonistic selection between reproductive investment and survival.

We have shown that wood frog coloration exhibits gradual and unimodal variation for most of the variables considered, including the first axis of variation for color measured from digital images. Though peak reflectance was bimodal at wavelengths corresponding to either green or orange-red, the reflectance spectrum of individual frogs often included multiple peaks in this range of wavelengths, with near maximal reflectance values. Moreover, we found no evidence for bimodality in discriminability values (noise-corrected JND), which
consider perceptual abilities of conspecifics and predators. We suggest that these results are not consistent with the presence of discrete color morphs in our study breeding ponds. Instead, the continuous color variation may indicate individual quality or camouflage (see Dale 2006).

The role of coloration in mating interactions has been increasingly highlighted in amphibians, even among nocturnal frogs (Gomez et al. 2009, 2010). However, wood frogs are explosive breeders, and as females enter the breeding ponds, males compete to rapidly pursue and amplex them. As with congeneric species with similar natural histories (R. temporaria [Sztatecsny et al. 2010], Rana arvalis [Sztatecsny et al. 2012]), female choice is not a key aspect of the mating system in wood frogs. Instead, intrasexual competition among wood frog males appears to be the main avenue for sexual selection, with little to no opportunity for female choice (Berven 1981, Howard and Kluge 1985). The absence of female choice could explain why we found no evidence for correlations between color and body size or body condition. Yet, such correlations could still be expected in males, as the ability to assess the size of potential opponents before engaging in competitive interactions over females is evolutionarily favored (Maynard Smith and Parker 1976, Enquist and Leimar 1983). In wood frogs, such an evolutionary outcome via discrimination of color differences is likely hindered by constraints of color perception mechanisms.

We found evidence for ontogenetic color changes in wood frogs, as recaptured individuals were redder and lighter in coloration in the second year. Additionally, we found color differences between males and females, with females being redder (i.e., lower hue and higher saturation) and males being yellower and darker (i.e., lower brightness), similar to what has been previously reported in the literature (Banta 1914, King and King 1991, Lambert et al. 2017). In wood frogs, such an evolutionary outcome via discrimination of color differences is likely hindered by constraints of color perception mechanisms.

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**Table 2.** Results of the generalized linear model with binomial distribution for survival.

| Factor                  | Estimate | SE   | t    | P     |
|-------------------------|----------|------|------|-------|
| Intercept               | −0.009   | 0.440| −0.21| 0.984 |
| Body size               | 0.008    | 0.008| 0.900| 0.369 |
| Coloration (PC1)        | 0.000    | 0.001| 0.267| 0.790 |
| Replica2                | −0.005   | 0.091| −0.057| 0.955 |
| Replica3                | −0.158   | 0.104| −1.526| 0.129 |
| Replica4                | −0.136   | 0.104| −1.310| 0.192 |

**Table 3.** Results of the linear model for female reproductive investment (square-root of the number of eggs).

| Factor                  | Estimate | SE   | t    | P     |
|-------------------------|----------|------|------|-------|
| Intercept               | 12.849   | 13.633| 0.942| 0.352 |
| Body size               | 0.181    | 0.255| 0.711| 0.482 |
| Coloration (PC1)        | −0.235   | 0.466| −0.504| 0.618 |
| Body size × PC1         | 0.004    | 0.009| 0.477| 0.636 |

**Figure 6.** Slope of the regression and respective credible intervals between discriminability indices in JND units and PC1 scores, when considering the chromatic and achromatic visual channels of two different predators, across a variety of natural backgrounds. The slope of the regression is negative for all of the backgrounds in the chromatic channel and for the majority of backgrounds in the achromatic channel of both predators, indicating a decrease in conspicuousness against the background for individuals with higher color PC1 scores (see also Appendix S1: Fig. S5).
in age structure of reproductive individuals in our breeding ponds (Brady et al. 2019). This suggests that the commonly reported sexual dichromatism in adult wood frogs could be explained, at least in parts, by demographic factors (i.e., age differences between breeding males and females) when sampling reproductive individuals. Yet, demography cannot be the sole explanation, as male and female wood frogs show color differences within months after metamorphosis, and sex differences are found even when accounting for variation in larval developmental time (Lambert et al. 2017). Thus, additional studies should explore the role of physiological and developmental mechanisms (e.g., hormones) in sexual dichromatism.

Sex differences in coloration were statistically significant but very small, and the unimodal and continuous color variation is inconsistent with a possible role of coloration in sex recognition (see Dale 2006). If males used sex differences in coloration as a sex-recognition mechanism, we would expect males to have preferentially associated with lighter colored females in our dichotomous choice experiment. Instead, we found that males associated with both types of females indiscriminately. Our interpretation is supported by the visual perception models, which suggest that wood frogs are unable to distinguish the sex of conspecifics based on either chromatic or achromatic components of coloration. It is important to note, however, that natural lighting conditions are likely to be highly variable between sites and times of day, and water temperatures during breeding are substantially colder than lab conditions. Such conditions could affect both color perception and individual coloration. Moreover, future studies using color data collected from individuals immediately after capture are necessary to investigate potential changes in coloration caused by stress related to transportation and housing of the animals.

In wood frogs, reproductive success is highly skewed among males within a breeding season, while the probability of returning for a subsequent breeding event seems to be low (Berven 1981, Howard and Kluge 1985). We expect the high intrasexual competition among males in the breeding aggregations to favor the evolution of sex recognition. Yet, non-discriminating individuals should be favored if discrimination is imperfect (Trimmer and Houston 2014), as in the case of subtle sexual dichromatism, due to the risk of males failing to pursue a female at all. Moreover, sex recognition might have negative consequences for females, due to the high fitness cost that male mating harassment imposes on females (Howard 1980, Howard and Kluge 1985; Sztatecsny et al. 2012). For instance, we have found female wood frogs in amplexus with a ruptured abdomen, likely the result of overly enthusiastic male claspers (Appendix S1: Fig. S6). Sexual conflict, therefore, might impede the evolution of sex recognition by males, and even favor the evolutionary maintenance of color variation through balancing selection (Cordero et al. 1998, Svensson et al. 2005, Rivera and Sánchez-Guillén 2007, Le Rouzic et al. 2015).

Our tests of predator perception of the different wood frog dorsal colors revealed, surprisingly, that wood frog coloration is not cryptic. Instead, all variants are discriminable (i.e., JND > 1) by both snakes and kestrels against at least a few of the backgrounds, and for at least one of the predator’s visual channels. These results suggest that wood frogs cannot solely rely on dorsal coloration for camouflage. Still, we cannot rule out a function of coloration in predator avoidance. For instance, detection can be affected by the interaction between animal coloration and background complexity (Dimitrova and Merilaita 2010), or by surface disruption resultant of the contrast between dorsal and abdominal coloration, (Stevens et al. 2009; see also Merilaita et al. 2017). It is also possible that color discrimination depends on the visual acuity of the predator, such that over longer distances frogs may not be distinguishable by predators (Rodriguez-Morales et al. 2018). Additionally, coloration could be under frequency dependent selection arising from a predator’s search image based on most frequently found colorations (i.e., apostatic selection; Allen and Clarke 1968, Endler 1980, Bond 2007). Wood frogs match all of the criteria for apostatic selection to occur. They are abundant amphibians without toxic chemical defenses and are preyed upon by visually oriented predators capable of learning (see Milstead et al. 1974).

Discriminability indices differed across combinations of background, predator, and channel type. The channel in which contrast values were higher was frequently opposite between the two predators, although the direction and magnitude of this difference changed across backgrounds. In general, contrast values were higher in the achromatic channel for males, but in the chromatic channel for kestrels. Moreover, we found that chromatic and achromatic contrast values traded-off at the intra-individual level, such that, for each predator and background combination, individuals with larger contrast values in the chromatic channel had lower contrast values in the achromatic channel and vice versa. If developmental or physiological trade-offs in color production prevent frogs from being cryptic in both channels simultaneously, while different visual perception abilities of predators result in opposing patterns of selection for each channel, balancing selection might occur as a consequence of response to selection for crypsis in one channel resulting in a correlated increased conspicuousness in the other channel.

Specialization trade-offs of the visual system could prevent predators from enhancing visual perception in both channels simultaneously (Ghim and Hodos 2006, Potier et al. 2018). It is unclear, however, how much the variation in perceptual ability across channels corresponds to a behavioral reliance on different visual channels for foraging. Kestrels are thought to rely on
chromatic discrimination for foraging, and Harris’ hawks are able to discriminate isoluminant objects based on color differences over a long distance (Potier et al. 2018). Therefore, behavioral responses might correspond to perceptual abilities. On the other hand, predators might be able to switch the channel they rely upon depending on whether the chromatic or the achromatic variation is the most reliable cue (Kelber 2005, see also Giurfa et al. 1997).

We found several negative relationships between discriminability values and PC1 scores across the various visual channels and backgrounds, with a few positive relationships found only in the achromatic channel. Therefore, older individuals and females should experience an overall reduced predation risk because they present higher PC1 scores. Moreover, these relationships may reflect variable selective pressures imposed by changes in the main predator across ontogeny. For instance, assuming garter snakes rely mostly on achromatic perception, and kestrels on chromatic perception, then younger individuals would be better camouflaged against snakes and older individuals against kestrels. Finally, variation in habitat composition could lead to spatially varying selection on coloration. For example, leaf litter backgrounds dominated by oak and pine needles should favor the evolution of lighter and redder individuals as adults. In birch-dominated forests or degraded environments where exposed soil and grass are predominant, however, chromatic and achromatic channels should impose opposing selective pressures, and potentially favor the maintenance of variation. These possibilities suggest interesting avenues for future studies, which could investigate the adaptive role of ontogenetic color changes in light of variation in predator-specific selective pressures, as well as the role of habitat degradation in the maintenance of color variation.

Finally, we found no evidence for disruptive viability selection or for antagonistic selection between survival and female reproductive investment. We assumed contrast variation should correlate with survival as studies have demonstrated preferential predation of birds upon frogs with dorsal coloration contrasting with the background (Tordoff 1980). Moreover, contrast in JND units has been shown to correlate to behavioral responses in insects (e.g., honeybees, Apis mellifera; Vorobyev et al. 2001), birds (e.g., Budgerigar, Melopsittacus undulatus; Goldsmith and Butler 2003), amphibians (e.g., strawberry poison frog, Dendrobates pumilio; Crothers et al. 2010), and lizards (e.g., brown anoles, Anolis sagrei; Fleishman et al. 2016). However, we found no relationship between color and survival. Because our survival experiments were not designed for measuring selection on color, we may have lacked sufficient variation to rule out more complex forms of selection, including apostatic or balancing selection. Furthermore, the enclosures may not have captured the full range of background variation found in natural habitats, potentially affecting the ability of some animals to match the color of their surroundings. Additional studies will be required to test for these effects (e.g., frequency, predator, background, or age-dependent effects; e.g., Allen and Clarke 1968, Jameson and Pequegnat 1971, Nokelainen et al. 2014, Willink et al. 2014).

CONCLUSION

Our results emphasize both the species-specific aspects of color perception and composite nature of color as a trait and, therefore, the importance of accounting for differing visual systems when studying color-based communication. For instance, although conspecifics have been commonly assumed to be able to visually discriminate between sexes based on human-observed sexual dichromatism, we found no evidence that color plays a role in sex recognition in the wood frog. Moreover, different types of predators, as well as different visual channels within a predator, vary in their ability to perceive the prey, potentially leading to opposing selective pressures on prey camouflage coloration. Additionally, we found intra-individual trade-offs between chromatic and achromatic contrasts, which potentially reinforce variable selective pressures to maintain intraspecific variation in coloration in wood frogs.

Color and pattern polymorphisms have been described in over 200 species of frogs (Hoffman and Blouin 2000), with over 100 showing sexual dichromatism (Bell and Zamudio 2012). Still, the processes that influence the evolution and maintenance of color variation in amphibians remain poorly understood (reviewed in Hoffman and Blouin 2000, Bell and Zamudio 2012, Merilaia et al. 2017, Rojas 2017). Our study takes new steps toward understanding these patterns, emphasizing the need for research that simultaneously addresses eco-physiological mechanisms as well as multiple hypotheses within the gamut of selective pressures influencing the evolution of coloration. We reiterate previous assertions (Kemp et al. 2015) that color research will benefit from an understanding of these proximate and ultimate mechanisms in combination with one another, and suggest that this interaction is likely general beyond the study of color or amphibians.

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Supporting Information

Additional supporting information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecm.1430/full

Data Availability

All data and R codes are available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.37pvmcvg2