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METHODS

Quantifying iridescent coloration in animals: a method for improving repeatability

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Abstract Quantification of animal colors is important to a variety of fields of research, especially those dealing with visual communication and sexual selection. Most animal colors are easily measured using well-established spectrophotometric techniques. However, the unique characteristics of iridescent colors present particular challenges and opportunities to quantify novel color metrics. Due to the fine-scale angle dependence of iridescent coloration, color metrics, such as hue and brightness, must be measured using methods that allow for repeatable comparison across individuals (e.g., by carefully controlling and measuring viewing geometry). Here, we explain how the optical characteristics of iridescent colors should be considered when developing measurement techniques, describe the pitfalls of some commonly used techniques, and recommend improved methods and metrics (angular degree of color change and breadth of reflectance) for quantifying iridescent color. In particular, most studies of iridescent birds to date have used less than ideal procedures and have not provided repeatability estimates for their methods. For example, we demonstrate here that measuring coloration from overlapping patches of iridescent feathers may be problematic, and we argue against methods that do not carefully control viewing geometry. We recommend measuring iridescence at maximal reflectance angles using an apparatus that allows for sample rotation, and we compare this technique to some other commonly used methods using iridescent gorget and crown feathers from Anna's hummingbirds (*Calypte anna*). Our apparatus allows for the quantification of angular color change, and we found that maximal reflectance measurements using single feathers are highly repeatable both within feather samples and among samples within an individual.

Keywords Iridescence · Color measurement · Bird coloration · Repeatability · Anna's hummingbird · *Calypte anna* · Structural coloration

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Introduction

Objective characterization of animal colors has been of interest to researchers for at least the past half century, especially for those who study intraspecific communication and sexual selection (Bennett et al. 1994; Cuthill et al. 1999; reviewed in Hill and McGraw 2006a, b). Over the past several years, ultraviolet–visible spectrometers have become common lab equipment, and well-accepted techniques have been developed to measure most animal colors (reviewed in Anderson and Prager 2006; Montgomerie 2006; Vukusic and Stavenga 2009). For most types of color, a spectrometer and light source can be connected to a bifurcated fiber-optic cable, or separate cables held together in a holder or "block," to measure color of intact animals or samples of integument (Andersson and Prager 2006). These techniques work well for most diffusely reflective materials,

but special methodological and analytical consideration is needed for traits that reflect light specularly or change in appearance with viewing angle, such as iridescent colors.

Iridescent colors are involved in some of the most dazzling displays in animals, such as the flashing wings of blue morpho butterflies, the opalescent elytra of beetles, and the elaborate plumage of birds of paradise (Meadows et al. 2009). Iridescence is a subset of structural colors in which layered or crystalline organization of nanoscale optical structures causes the wavelengths of light that constructively interfere to vary with viewing geometry (determined by the spatial relationship between incident light, viewer, and the iridescent object). This causes aspects of perceived coloration, including hue and brightness, to be changeable (Prum 2006). Iridescent colors have been implicated in an intriguing variety of functions (e.g., thermoregulation, predator deterrence, quality signals), but research that quantifies intraspecific variation in these colors is comparatively scarce (Doucet and Meadows 2009). However, the few examples that do exist provide interesting and significant results that compel further research, particularly studies that attend to the uniquely dynamic nature of the iridescent colors of moving animals (Cuthill et al. 1999; Loyau et al. 2007; Kemp et al. 2008; see more below). The aims of our methodologically focused paper here were to (1) review several characteristics of iridescent color that present measurement challenges and have not been well addressed in the literature to date and (2) advocate and test new standardized techniques for quantifying iridescent colors.

Broadening the color metrics for quantifying iridescence

Because of the angle-dependent changeability of iridescent colors, their appearance must be measured in ways distinct from those appropriate for diffusely reflecting colors (i.e., pigment-based colors), which have much less complex spatial reflectance properties and can be measured using traditional methods (e.g., Cuthill et al. 1999; Doucet et al. 2006; Kemp et al. 2006, 2008; Loyau et al. 2007; Biro and Vigneron 2011). Iridescent colors are by definition directional, so minute changes in viewing (and measurement) geometry can produce large changes in the observed color (e.g., Huxley 1968; Land 1972; Osorio and Ham 2002; Vukusic et al. 2004; Kinoshita et al. 2008). When incident light is from a point source (i.e., the sun or a focused beam from a light source), reflectance from iridescent surfaces is restricted to a specific solid angle whose dimensions depend on the physical properties of the iridescent tissue. Reflectance is maximized at an angle of reflectance equal to the angle of incident light (Osorio and Ham 2002). Away from this angle of maximal reflectance, the intensity or brightness of iridescent colors can decrease dramatically (Fig. 4 in Osorio and Ham 2002; Vukusic et al. 2002). Thus, one means by which to obtain comparable metrics of iridescent coloration among individuals is to measure reflectance at the angle at which reflectance is at a maximum. Complicating the above principle, the structures responsible for iridescent reflectance are often not parallel to the main surface of an iridescent tissue. For example, in bird feathers, iridescent barbules are often twisted at the base, such that the reflective surface of the barbules is not in the same plane as the main feather surface (e.g., in magnificent hummingbirds, *Eugenes fulgens*, and common pheasants, *Phasianus colchicus*; Osorio and Ham 2002). Consequently, iridescent colors should be measured with the iridescent surface—not the main surface of the sample—normal to the line that bisects the angle of incidence and the angle of measured reflectance (see Fig. 2a in Osorio and Ham 2002). Although the tissue elements that make up the iridescent surface may not be in perfect alignment with one another, measuring at the angle of maximum reflectance should represent the angle at which the majority of component structures are oriented. Any device used for quantifying aspects of iridescent coloration should ideally allow for continuous, measurable variation in the angular orientation of the surface of the specimen, especially when differences among individuals within a species are important.

Pitfalls of commonly used techniques for measuring iridescent color

As noted above, commonly used spectrophotometric methods are not ideal for measuring iridescence unless there is some ability to rotate a sample until maximal reflectance is reached. In most published cases where coincident probes, blocks, or even goniometers have been used, samples have not been rotated in this manner (e.g., Cuthill et al. 1999; Doucet 2002; Doucet et al. 2006; Lim and Li 2006; Loyau et al. 2007; Bitton et al. 2007, 2008). As a result, reflectance peaks may have been missed altogether (for example, some hummingbird feather spectra are flat at coincident normal geometry; Osorio and Ham 2002; Meadows, personal observation), and at best, measurement errors have been amplified. Additionally, when probes or blocks are placed directly on samples, very small variations in the angle of the probe or contact pressure on moveable iridescent structures (such as the barbules of bird feathers) may add to measurement errors. This is likely less problematic for non-iridescent colors because reflectance characteristics are not dependent on minute changes in viewing angle.

In the case of bird plumage, many researchers have measured color directly from the bird or from a stacked group of plucked feathers that simulate a feather patch on the animal (e.g., Safran and McGraw 2004; Shawkey and Hill 2005), including some studies on iridescent bird

coloration (Costa and Macedo 2005; Doucet et al. 2006; Bitton et al. 2007, 2008; Bitton and Dawson 2008; Santos et al. 2009). In these cases, small changes in feather arrangement can drastically alter the perception and measurement of iridescent colors, and it can be difficult to control the orientation of all feathers in a group such that they are as well-ordered as they appear on an intact bird. The consequences of these methods for color quantification and repeatability have not been explored, although there are reasons to think that they may produce undesired variation in color measurements.

Another common method for measuring color that can be used for iridescence is to use integrating spheres set up in one of two ways. In these spheres, either the sample is illuminated from many directions and reflectance measures taken from a fixed angle or the sample is illuminated with a point source and reflectance is gathered from many angles (e.g., Osorio and Ham 2002; Papke et al. 2007). In either instance, such a method averages reflected light over multiple angles of incident light or multiple angles of reflection, which in the case of iridescent colors will likely underestimate the reflectance seen by a viewer (e.g., comparing Fig. 4a and d in Osorio and Ham 2002; comparing UV1 and UV2 measures of UV brightness in Table 2 in Papke et al. 2007). This creates special problems when the contributions of iridescent and non-iridescent colors are being compared between samples because non-iridescent colors are not less intense under diffuse lighting (e.g., Fig. 6 in Osorio and Ham 2002). Furthermore, the use of an integrating sphere is not often coupled with specimen rotation (but see Osorio and Ham 2002). In addition to these measurement concerns, the use of integrating spheres provides data that are fundamentally divorced from how animals perceive color in nature, especially in instances when reflected light is collected at all angles.

Despite the issues we mention here, many studies of iridescent signals have found biologically and statistically significant variation in signal properties using methods that we describe as limited or problematic. We suggest that the color measurement used in these studies limited the power of their findings and that using improved methods could add more information, resolution, and accuracy to new studies. Some studies that failed to find or explain variation in iridescent color signals (likely including many that were never published) may have suffered from less rigorous color measurement techniques. Perhaps this has contributed to the paucity of studies on iridescent color in intraspecific communication thus far.

Lessons from the literature to date

While iridescence has been measured using inappropriate methods in some cases, there are research groups who have employed methods that contributed greatly to the ideas presented here. Some of the most exciting contributions to this literature to date have shown that unique visual properties of iridescence—such as various axes of color change—can be measured and convey information. For example, female alfalfa butterflies (*Colias eurytheme*) that mate with males whose UV iridescence is visible over a smaller angular breadth exhibit higher fecundity (although the causal relationship underlying this pattern requires further study; see Kemp et al. 2008). Changeable aspects of peacock (*Pavo cristatus*) iridescence predict female visitation and mating success (Loyau et al. 2007). Male and female European starlings (*Sturnus vulgaris*) also differ in their degree of hue shift with variable viewing angles, suggesting a new axis for considering sexual dichromatism (Cuthill et al. 1999). Clearly, more studies should attempt to measure the changeable properties of iridescent colors in animals in order to understand the function of iridescence per se as opposed to non-iridescent coloration in particular organisms.

Some reported methods have permitted either the measurement of variation in iridescent coloration and/or the rotation of specimens to maximal reflectance geometries. For example, Osorio and Ham (2002) developed an apparatus that allows both the angle of incidence and angle of measurement to vary, and they described the effects of varying these on spectra from iridescent and non-iridescent bird feathers. Biro and Vigneron (2011) have recently described a similar apparatus. Rutowski et al. (2007) examined the effects of changes in wing surface, illumination, and receiver angles on iridescent coloration in orange sulfur butterflies (*C. eurytheme*). Furthermore, Rutowski et al. (2007) describe wide variation in the angular span of iridescence (3° to 24°) and wing angle of peak reflectance (−30° to −50°) among individuals. This demonstrates the importance of collecting accurate data on unique aspects of iridescent coloration and of collecting spectra at maximal brightness angles to account for variation in the angle of iridescent structures. Collecting spectra at maximal reflectance has become common in studies on intraspecific variation in butterfly iridescent coloration (e.g., Kemp 2006a, b, 2008b; Kemp and Macedonia 2006; Kemp et al. 2006; Kemp and Rutowski 2007; Papke et al. 2007; Rutowski et al. 2010), but has only been utilized in one study on an iridescent bird (Madsen et al. 2007), and to our knowledge, this method has never been employed in comparative iridescence measurements from any other taxa. Furthermore, a single apparatus that takes into account both the ability to quantify unique qualities of iridescence and sample rotation as detailed above has not previously been built and described in the literature such that it is highly accessible to others wishing to study iridescent coloration.

Additionally, while measurements taken at a maximal reflectance orientation of a specimen should provide the most accurate and comparable measurements of individual coloration, specimen rotation may increase the potential of other sorts of error, which could lower the repeatability (Lessells and Boag 1987; Zar 1999; described in "Methods" below) of such measurements. The relative repeatabilities of measurements made with these and other techniques have not been carefully explored; several of Kemp's studies provide a repeatability estimate for individual coloration on different wings, but not repeatability of the method for the same spot or relative to those obtained from other methods (e.g., Kemp 2006a, 2008a; Kemp and Rutowski 2007). We also caution the reader that, although we attempt here to make logical and tested arguments for some types of measurements versus others, it is difficult to confirm absolutely that our methods offer the best possible mimicry of natural conditions. However, the field could be strengthened by adopting a standard technique for measuring iridescent colors within and across taxa.

Development and testing of a new method for quantifying iridescence

Our goal was to develop a method to quantify iridescent coloration in a way that (1) allowed the angles of incident light, measurement probe, and specimen to be altered with known precision for the purposes of measuring aspects of color change and the approximation of a variety of potential viewing scenarios relevant to diverse signaling contexts; (2) allowed a sample to be rotated to measure maximal reflectance of the signal while maintaining alignment of the sample, measuring spot, and light source; (3) was repeatable, both across multiple measurements of the same sample (i.e., a repeatable technique) and across multiple samples of the same individual (i.e., an accurate approximation of the color of a full color patch on the basis of only a few measurements); and (4) allowed a relatively small area to be measured, given the small size of some iridescent tissue samples or color patches. Based on these ideas, we developed a technique that can be used to quantify the iridescent coloration of a variety of specimens. We tested this technique using iridescent gorget and crown feathers from Anna's hummingbirds (*Calypte anna*). Here, we demonstrate the ability of a device to quantify color change in iridescent coloration and assess the efficacy of measuring color from feather samples rotated to maximal reflectance by comparing repeatability estimates with those of color metrics using other common techniques (fixed orientation measurements and measurements from overlapping feather "patches"). We also examine the repeatability of measurements of different feathers within individuals in a large sample to determine our ability to capture color of an entire color patch using a few feathers and to characterize individual variation in color parameters.

Methods

Study specimens

We used iridescent crown and gorget feathers from adult male Anna's hummingbirds captured in Tempe, Arizona, in January 2007, December 2008, and January 2009. At the time of year collected, these birds have recently completed their yearly molt (completion varies from September to December), and importantly, feathers were collected during the early breeding season when they would be used as a sexual or social signal. Male Anna's hummingbirds have iridescent magenta crowns and gorgets that they display during agonistic and courtship interactions, which are often oriented toward the sun (Hamilton 1965; Stiles 1982). Linear arrays of hollow melanin platelets inside a keratin matrix in distal feather barbules produce the iridescent color via multilayer interference (Greenewalt et al. 1960), and the barbules are twisted at the base, presumably to direct specular reflectance toward an observer (Osorio and Ham 2002; Doucet and Meadows 2009). Plucked feathers from the gorget and crown were taped to matte black cardstock, either singly or in overlapped groups (as they appear on the bird), by the non-iridescent proximal end of the feather. The iridescent parts of the feathers were not touched during plucking, taping, or measurement to avoid damage to or alteration of color-producing microstructures.

Spectrophotometry and measurement geometry

We developed an instrument, drawing from the desirable aspects of previously used techniques (e.g., Osorio and Ham 2002; Rutowski et al. 2007), that allows systematic and measureable control and precise alignment of specimen stage, light source, and spectrometer probe angles. The resulting apparatus is similar to the gonioreflectometers employed to create bidirectional reflectance spectra (e.g., Baribeau et al. 2009), but simplified for the needs of biological studies (see Vukusic and Stavenga 2009). Further details about the specific instrument that we developed to achieve these goals are available in the Electronic supplementary material (ESM). The instrument attaches to standard spectrometry equipment; for our study, we used a USB2000 spectrometer and PX-2 pulsed xenon light source (Ocean Optics, Dunedin, FL, USA). The spectrometer and light connect via separate fiber-optic cables to quartz collimating lenses mounted on rotatable arms, resulting in a measurement spot diameter of ~2 mm. Using our setup, spot diameters from approximately 2 to

30 mm can be measured by simple adjustments to the device dimensions and/or changes to the focal length of the collimating lenses.

Mounted feathers were placed on the specimen stage, with the proximal tip of the feather pointing toward the azimuth of the light source (Fig. 1). For all measurements, the angle between the light source path and the collecting beam path was 45° (Fig. 1). The orientations of feather specimen, light, and probe angles were chosen to simulate realistic display conditions, with the sun at a 45° elevation (approximate solar elevation in Phoenix, AZ, at mid-morning in mid-March, during the breeding season of Anna's hummingbird; Cornwall et al. 2009) and an observer directly in front of the signaler looking at a feather in the gorget ventral to the bill. We then tilted the feather around the axis in the plane of the feather and perpendicular to its long axis (feather rachis) until a maximal reflection was recorded as assessed using real-time spectrophotometer output (i.e., the specimen is oriented so that, on average, the line bisecting the angle between the light and probe arms is normal to the surface of the reflecting barbules). We checked the alignment of the illuminating spot of light and the desired measurement area on the specimen surface (a 2- to 3-mm diameter area of iridescence on a feather) by shining a laser pointer through the collecting probe fiber-optic cable. To prevent misalignment during stage rotation, the stage surface was lowered so that the surface of the specimen was at the axis of rotation for the stage and light and probe arms (see ESM Fig. S1).

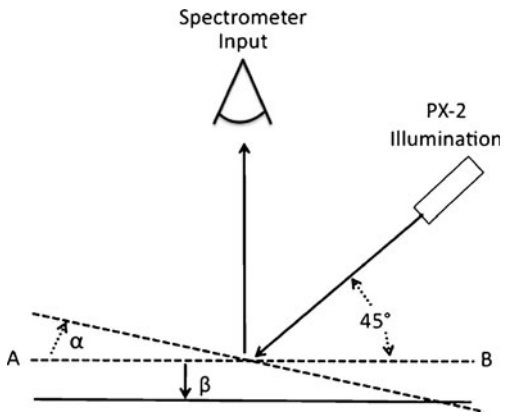

Fig. 1 Schematic showing spectrometry setup for hummingbird feathers. A PX-2 light source illuminated the specimen at a 45° angle relative to the horizontal and a USB2000 spectrometer collected reflected light at 90°. The rachis of the feather was situated on line *AB* such that the proximal end was towards *B*, the azimuth of the light source. The translational stage, calibrated so that the surface was at the center of rotation for the stage, light, and probe, was lowered by the measured thickness of the sample to the nearest 0.01 mm (β) to place the surface of the specimen at the center of rotation and maintain alignment. The stage was then rotated around an axis perpendicular to the plane defined by the light beam and collecting beam to the angle at which maximal reflectance was achieved (α). At this point, the plane of the structures producing the iridescence are taken to be normal to the line bisecting the angle between the light and probe, and the angles of incidence and measured reflectance were equal (22.5°)

All color measurements were done in a dark room and taken relative to a magnesium oxide white standard (Rutowski et al. 2005). Data were collected at wavelengths from 300 to 700 nm, the avian visual range (Cuthill 2006; Mullen and Pohland 2008). Reflectance was binned in 1-nm increments, and brightness, intensity, red chroma, and hue were extracted from the spectra using CLR 1.05 (Montgomerie 2008); abbreviations here correspond to those in Montgomerie (2006), with further details given in CLR 1.05 (Montgomerie 2008). For hue (H1), we used the wavelength of maximum reflectance. For chroma, we used red chroma (S1R), the sum of reflectance in the red portion of the spectrum 605–700 nm divided by the sum of the total reflectance 300–700 nm. We calculated mean brightness (B2), the average percent reflectance between 300 and 700 nm. Mean brightness, which does not rely on the spectral range used or wavelength bin increments, is a standardized index of brightness that can be readily compared among studies and individuals (Montgomerie 2006). However, we also examined the repeatability of intensity (B3), a less commonly used brightness metric, as the maximum percent reflectance value. We use this metric here because our rotational technique specifically maximizes this value, but we caution that it may not be an appropriate brightness metric to use in many studies, as it is sensitive to spikes in reflectance curves that could be caused by noise (Montgomerie 2006).

Quantifying iridescent color change

To evaluate the ability of our instrument to measure angular color changes, such as hue and brightness shifts, we collected spectra from a single gorget feather at a variety of viewing geometries, a process similar to that undertaken to arrive at bidirectional reflectance distribution functions (Baribeau et al. 2009; Vukusic and Stavenga 2009), but simplified to be relevant to the perception of colors by receivers and for the needs of ecologists conducting studies that require measurements on many individuals. First, the apparatus was set up as above, and the feather was rotated on the stage about an axis in the plane of the feather and perpendicular to the feather rachis until it reached maximal reflectance. To obtain spectra demonstrating the reduction in brightness with small deviations from this maximal reflectance geometry, we then rotated the feather 5°, 10°, 15°, 20°, and 25° away from the maximal reflectance position and recorded spectra, holding the angles of the light and probe constant. To capture changes in hue, we first set both the probe and light arms at 80° elevation relative to the horizontal (the closest possible to coincident

normal geometry, limited by the width of the probe and light holding arms) and rotated the feather until maximal reflectance was reached. For this particular feather, the average angle at which maximum reflectance occurred was 29° away from horizontal (which indicates that barbules are twisted approximately 29°; Osorio and Ham 2002). The angles of incidence and measured reflectance were increased symmetrically in increments of 5°, while the feather remained in a fixed position. While it is possible to measure hue changes by keeping the probe angle constant and moving only the light source (Osorio and Ham 2002), the limited angular breadth of iridescent reflectance (high directionality) of these feathers required both light and probe to be moved to produce measurable reflectance at all angles desired.

Repeatability of measurement methods

We argued above that specimen rotation to maximize reflectance is important for iridescent colors to maximize the consistency of such measurements. We evaluated this assertion using the statistical methods for characterizing repeatability of Lessells and Boag (1987) and Zar (1999). As noted above, rotation of the specimen may introduce measurement error. We compared the repeatability of maximal reflectance measurements with fixed specimen angle measurements (always oriented horizontally), which are expected to be highly repeatable even though less ideal for comparisons of iridescence among individuals (as detailed above). We also compare measurements obtained from single feathers to measurements from overlapped groups of feathers.

Reflectance spectra were collected from a single, centrally located gorget feather from each of five individuals at both fixed 90° (perpendicular to the collecting probe) and maximal reflectance positions. To ensure independence of each measurement from a given feather, we randomized the measurement order of feathers and measured each feather once, standardized with the white standard, and randomized feather order again and repeated this process three times to get three measurements per feather. Measurements were taken blind to individual identification. For maximal reflectance measurements, the specimen stage was carefully rotated until maximal reflectance was reached. From the spectra, we extracted brightness, chroma, and hue as described above, and we examined the repeatability of each color metric separately for the fixed angle and maximum reflectance measurements.

Because prior studies that measured iridescent color from stacked groups of plucked feathers did not determine the repeatability of measurements taken in this fashion, we tested the repeatability of this measurement method here with iridescent hummingbird feathers. Reflectance measurements were taken from a group of three overlapped central gorget feathers three times each for feather groups from five individuals. Care was taken to mount all of the feathers such that they were parallel to one another and that overlap was about the same for each feather group. We used the same spot size for these measurements so that brightness measurements would be comparable. Measurements were done blind to individual identification and were randomized as above. We used the maximal reflectance method for these measurements, and the repeatabilities of color metrics were calculated as described previously. We also examined the correlation and paired differences between color metrics extracted from spectra from groups of feathers and a single feather from each group. This was done to help determine whether the time-intensive process of mounting groups of feathers is warranted.

Repeatability of color among feathers within an individual

To determine whether or not measuring just a few single feathers can characterize color for an entire colored patch, we calculated the repeatability of measurements from different feathers of the same individual. If color among feathers is highly repeatable, it is acceptable to measure color from just a few feathers to approximate color of the full ornament, thus reducing measurement time. This is not an expectation for all species or color patches per se (see Wolfenbarger 1999; Hill et al. 2005; Safran and McGraw 2004), but for many color ornaments, intrapatch color variation is not obvious to the human eye or has not been rigorously tested. Furthermore, high repeatability (a significant effect of individual) would indicate that this method effectively captures individual variation in color metrics. We took spectral measurements from six different feathers (three crown feathers and three gorget feathers from standardized locations: upper center and lower left and right of each color patch) from 55 individual birds and calculated the repeatability of color metrics as above. Because color metrics extracted from gorget and crown feathers were significantly different from one another (M. Meadows, unpublished data), we calculated the repeatability of gorget and crown feather color metrics separately.

Statistical analyses

To examine how changing the angle of feather presentation or the angle between the light and probe affected color metrics, we used Pearson's correlations. We quantified the repeatability of color measurements obtained using different methods using the procedure described in the section above, which incorporates analysis of variance (ANOVA)

with individual as the factor. We used paired *t* tests to examine differences between color metrics from overlapping groups of feathers and a single feather from the group and Spearman's rank correlations to examine the correlation between these measurements. Non-parametric correlations were chosen here due to the small sample size (n=5). We used a standard log transformation to linearize brightness change with feather rotation data; no other data required transformation. Unless otherwise specified, all data met the assumptions of parametric statistics, and we used an alpha level of 0.05.

Results

Color change

Changes in brightness with small changes in feather angle were substantial; feather reflectance was nearly flat when hummingbird gorget feathers were rotated just 25° away from maximal reflectance geometry (Fig. 2a). Brightness (log-transformed) was significantly positively correlated with feather angle (Fig. 2b). Red chroma also decreased significantly as the feather was rotated away from maximum reflectance (r=0.902, N=6, P=0.014), but, as expected, hue was not affected by feather rotation (r=−0.710, N=6, P=0.114; see Fig. 2 in Osorio and Ham 2002).

Hue of Anna's hummingbird gorget feathers did vary with changes in light and probe angle, shifting to shorter wavelengths with increasing angles of incidence and measured reflectance (Fig. 2c). Hue was significantly correlated with elevation angle (Fig. 2d), as were brightness (r=0.979, N=10, P<0.001) and red chroma (r=0.985, N=10, P<0.001).

Repeatability of measurement methods

Brightness, intensity, red chroma, and hue variables obtained from a single gorget feather were significantly and highly repeatable whether the measurement was taken at a fixed feather orientation (Table 1) or with the feather rotated to measure at maximum reflectance (Table 1). For groups of three overlapping central gorget feathers, chroma and hue metrics were significantly and highly repeatable, but brightness and intensity were not repeatable (Table 1).

There were no significant differences in measurements collected from single feathers or groups of feathers (paired *t* tests; B2: t_4=−0.02, P=0.850; S1R: t_4=0.61, P=0.576; H1: t_4=0.06, P=0.959; Fig. 3). However, metrics calculated

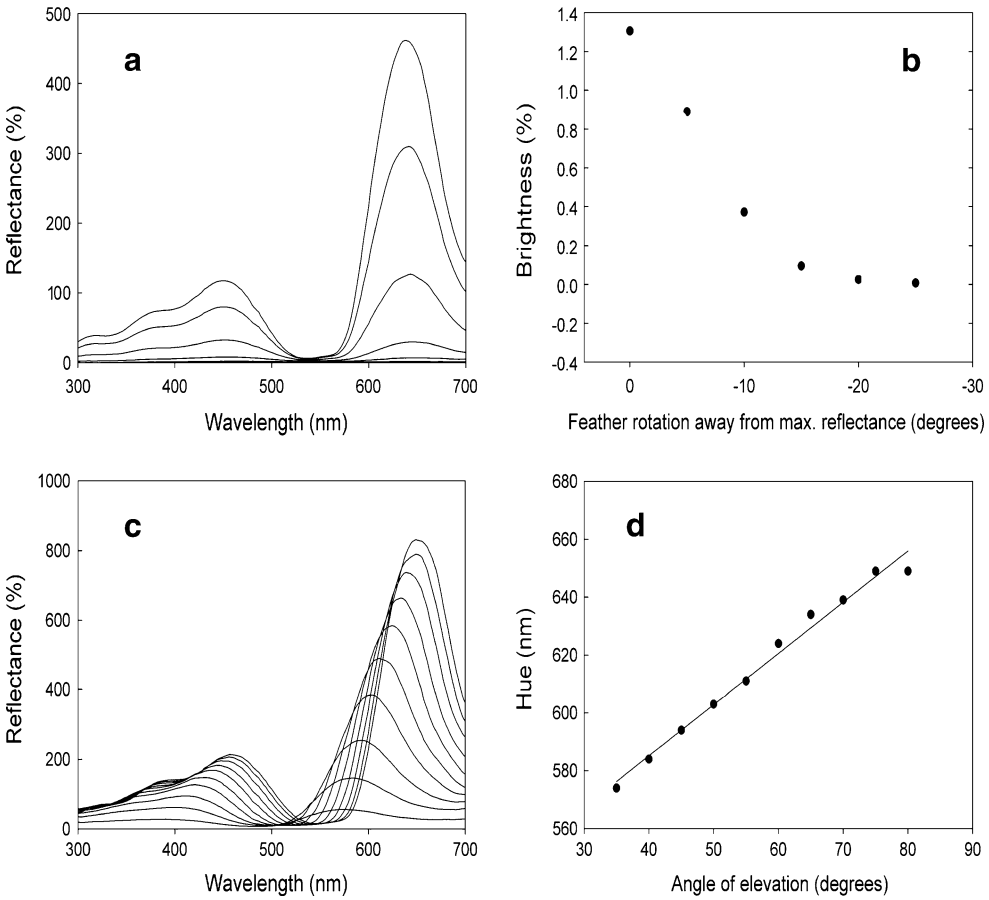

Fig. 2 Effects of changing viewing geometry on reflectance of iridescent feathers in Anna's hummingbirds. (**a**) Light and probe were held at constant geometry and the feather was rotated to maximum reflectance (*top line*) and then was rotated 5°, 10°, 15°, 20°, and 25° away from maximum reflectance, which caused (**b**) brightness to decrease significantly with angle (r=0.987, N=6, P<0.001) (**c**). Light and probe arms were rotated to the minimum angle between probe and light (20°) and feather was rotated to maximum reflectance, producing equal angles of incidence and measured reflectance of 10°. The feather was then held in this position while the light and probe arms were rotated symmetrically to increase the angles of incidence and measured reflectance in 5° increments, which caused (**d**) hue to increase as the angle of elevation increased (r=0.993, N=10, P<0.001)

Table 1 Repeatability estimates for color metrics using various measurement methods

Feathers used	Brightness (B2)		Intensity (B3)		Red chroma (S1R)		Hue (H1)	
	ANOVA	r	ANOVA	r	ANOVA	r	ANOVA	r
Single feathers, fixed 90°	$F_{4,10}=15.85$, $P<0.001$	0.83	$F_{4,10}=17.59$, $P<0.001$	0.85	$F_{4,10}=58.92$, $P<0.001$	0.96	$F_{4,10}=16.12$, $P<0.001$	0.83
Single feathers, maximal reflectance	$F_{4,10}=12.85$, $P<0.001$	0.80	$F_{4,10}=14.02$, $P<0.001$	0.81	$F_{4,10}=6.80$, $P=0.007$	0.65	$F_{4,10}=11.83$, $P<0.001$	0.78
Feather group	$F_{4,10}=1.44$, $P=0.290$	0.13	$F_{4,10}=1.21$, $P=0.366$	0.06	$F_{4,10}=221.21$, $P<0.001$	0.99	$F_{4,10}=114.36$, $P<0.001$	0.97
Three different feathers, crown	$F_{54,110}=2.69$, $P<0.001$	0.36	$F_{54,110}=2.91$, $P<0.001$	0.39	$F_{54,110}=4.93$, $P<0.001$	0.56	$F_{54,110}=12.62$, $P<0.001$	0.79
Three different feathers, gorget	$F_{54,110}=2.38$, $P<0.001$	0.31	$F_{54,110}=2.47$, $P<0.001$	0.33	$F_{54,110}=4.69$, $P<0.001$	0.55	$F_{54,110}=3.49$, $P<0.001$	0.45

Repeatabilities for single feathers and feather groups are for central gorget feathers from five individuals. Repeatabilities of color metrics from different feathers are from 55 individuals

from feather group and single-feather spectra were not significantly correlated (B2: $r_s<0.001$, $N=5$, $P>0.999$; SR1: $r_s=0.700$, $N=5$, $P=0.188$; H1: $r_s=0.700$, $N=5$, $P=0.188$; Fig. 3).

Repeatability of color among feathers within an individual

For measurements of three different crown and three different gorget feathers from each bird, all color metrics were significantly repeatable for both crown and gorget feathers (Table 1).

Discussion

The methods we present here show promise for improving the precision with which the reflectance properties of iridescent color patches in animals are quantified. We developed an apparatus that permits quantification of color change while allowing for continuous variation in specimen, light, and probe angles. Though variable-angle probe holders (blocks) allow one to measure reflectance with a probe and light source at a variety of angles (e.g., Doucet et al. 2006), continuous variation in these angles is limited by the number of holes in the block (which depends on the size of the block) and the specimen cannot be rotated to maximal reflectance before varying probe and light angles. We also describe here the utility of measuring unique aspects of iridescent coloration, such as angle of maximum reflectance or color change, and demonstrate that this can easily be done using the described apparatus.

Perhaps most importantly, we reason here that it is necessary to rotate specimens until maximal reflectance is reached to obtain results that are comparable among individuals and that these measurements should be sufficiently repeatable. Such measurements will better assess variation in overall individual coloration rather than variation in other surface characteristics, such as the degree of barbule twisting in some iridescent bird feathers, scale orientation in butterfly wings, etc. While measurements taken at maximum reflectance were slightly less repeatable than measurements taken at fixed orientation, the differences in repeatability were small, and measurements from rotated specimens were still high and significant. Though it is possible that differences in surface coatings such as preen oil or soil could present additional sources of variation, attempts to clean the samples may affect the orientation of color-producing structures (e.g., barbule angle).

We show that measurement repeatability for overlapped groups of feathers, a common method of sample preparation in studies of avian coloration, was high and significant for hue and chroma, but not significant for brightness or intensity. Brightness is likely most affected by feather grouping because it is so greatly affected by small changes in feather orientation. Thus, when grouped feathers are not oriented perfectly with one another during measurement, brightness changes substantially and cannot be repeatably measured. We cannot be sure how broadly this conclusion applies to other iridescent or non-iridescent color systems, but it is likely that the overlap of feathers and deviations in their orientation are unavoidable and cause this problem. Thus, we recommend that grouping should either be avoided in future work when brightness could be a key parameter (as in Anna's hummingbirds) or that researchers should confirm the repeatability of measurements from overlapping feathers. Although it makes sense to overlap feathers as they are arranged on the bird itself prior to measurement, it can be time-intensive, delicate work to mount overlapping feathers in the desired arrangement. Additionally, feather group measurements were not significantly different

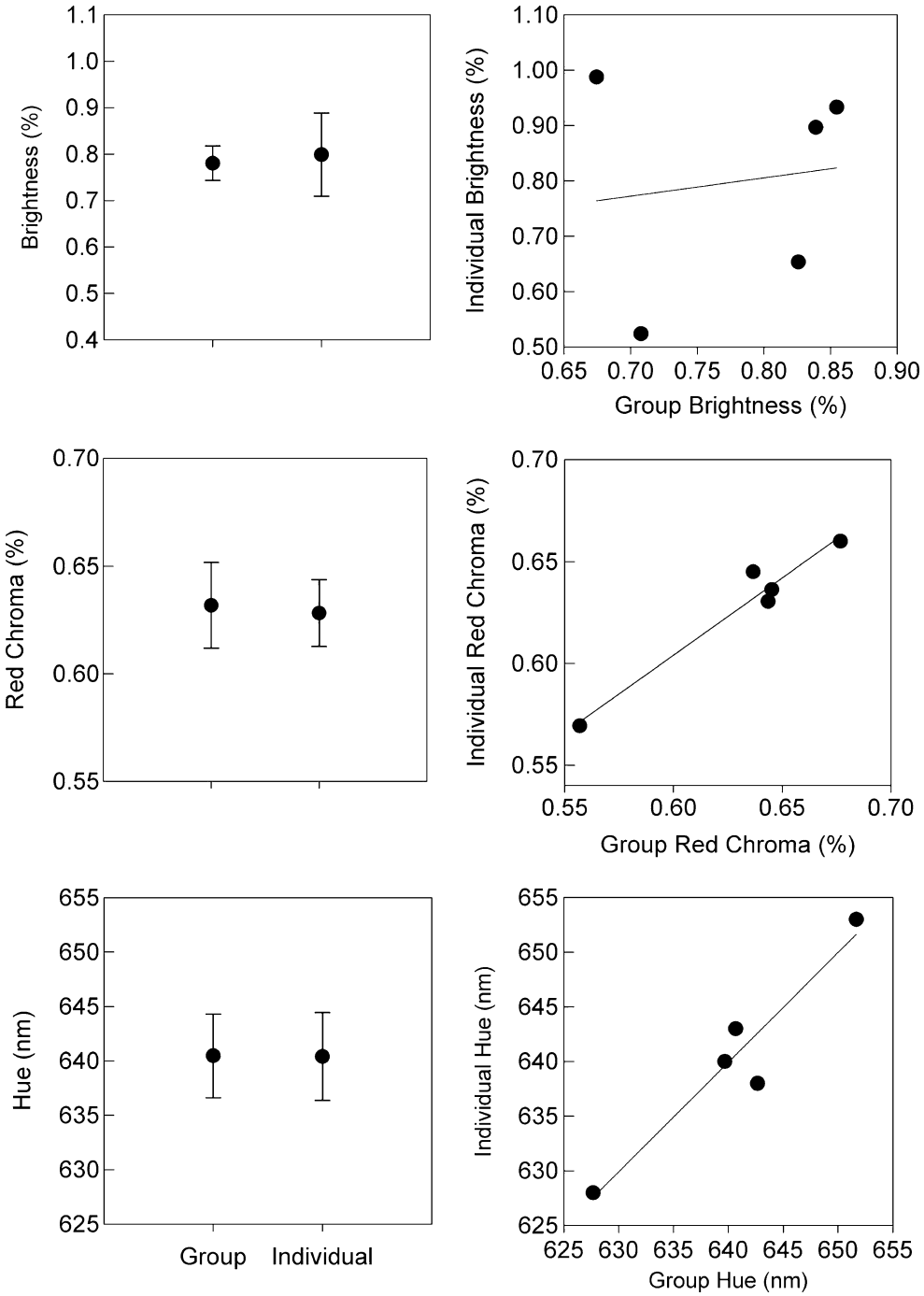

Fig. 3 Color metrics measured from groups of feathers versus single feathers. *Column 1* Mean±SE for groups of three overlapping feathers and individual feathers from five individuals' gorgets. Brightness red chroma, and hue are calculated from spectra from three measurements for each individual. *Column 2* Correlation of average color metrics for groups and single feathers within an individual

from measurements of a single feather from the group. This further supports measurements from single mounted feathers as the best method of collecting color data from Anna's hummingbird feathers, and perhaps other types of iridescent feathers. Grouped feather measurements were not significantly correlated with single feather measurements, but this is perhaps a result of the small sample size and the limited power of a non-parametric test.

We found that sampling three feathers from each ornament from 55 different birds (gorget and crown) provided significant repeatability of color metrics from multiple feathers within an individual. This shows that individual coloration can be approximated using color measurements from a few feathers. While repeatability estimates in this case are lower than repeatabilities from multiple measures from the same feather, this is likely a result of variation among feathers within individuals. However, because repeatability is still significant (ANOVA results in Table 1), we can conclude that measured variation within individuals is less than variation between individu-

als, making this method suitable for characterizing individual variation. Similarly, Kemp (2006a) and Kemp and Macedonia (2006) showed that measuring the same color area on both butterfly wings is repeatable, justifying the use of the average of these measurements to compare color metrics among individuals. We suggest that other researchers, studying iridescent or other types of coloration, consider sampling in a similar way.

In conclusion, we urge researchers to consider the techniques and potential pitfalls we describe here when attempting to quantitatively study variation in iridescent colors. We suggest that studies of iridescent coloration in animals take into account the characteristics that make iridescence unique and make every attempt to measure shifts in color parameters with controlled and measureable changes in viewing geometry. We highly recommend taking reflectance measurements from specimens oriented so that they reflect maximally, rather than at unspecified and uncontrolled orientations. The repeatability of measurement methods and of samples of different areas on colored patches among individuals should be considered and reported, especially when potentially problematic methods are used due to equipment limitations or the particular needs of a project.

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