A SIMPLE METHOD FOR CHARACTERIZING IRIDESCENCE

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Iridescence is the change in hue of a surface with varying angles of illumination and/or observation; it is generated by optical diffraction resulting from subwavelength features on the specimen's surface.[1, 2] This form of structural coloration enhances various biological processes (e.g., mate selection, species recognition, defense, and photosynthesis) for a wide variety of animal and plant species.[1, 3, 4] The invention of the electron microscope is responsible for many of the major breakthroughs in the ultrastructural characterization of iridescence, and electron microscopy is among the most commonly cited methods used.[2] The goal of this project is to present a simple method for characterizing iridescence that overcomes cost and portability limitations associated with commonly used methods.

While iridescence is typically characterized using electron microscopy,[1, 3, 5-11] such methods often involve the use of expensive equipment that may be inaccessible to biologists in the field or to student researchers; keeping this in mind, the procedure presented in this paper is designed to be easily performed by individuals interested in researching iridescence. Various forms of microscopy, spectroscopy, and cytophotometry require the use of expensive, typically non-portable equipment that is often unavailable to students completing research or to biologists interested in characterizing iridescent phenotypes in the field. The methods and materials presented in this

Pictured: Scientist with petri dishes, ca. 1960s

paper are comparatively inexpensive (<500 USD) and portable, and the protocols are easily performed. Further, this unique experimental design generates qualitative results comparable to published quantitative results.

The presented project uses angle dependent optical microscopy to generate qualitative information that characterizes iridescence, using the wing of a *Morpho* butterfly as a standard biological specimen; the presented methods and experimental design can be applied to any iridescent material in biology or in other fields.

In the setup used here (Figure 1), a color digital camera and white light source are arranged at controllable angles relative to the sample surface, and data are recorded at various illumination angles. The results observed are qualitatively consistent with results generated from other studies of iridescence in the *Morpho* butterfly and, interestingly, in studies of the *Selaginellawilldenowii*, a blue-green iridescent fern [3, 4]. The following summary of recently published papers on iridescence and its proposed biological functions contextualizes the data presented in this paper.

Iridescence has been characterized in a variety of insects, amphibians, and birds, and plants.[3] Scientists from various disciplines are interested in iridescence, indicating the relevance and potential applications of improved understanding of this phenomenon. Iridescence is produced by optical diffraction resulting from a combination both regular and irregular micro and nano-sized structural features on the surfaces of various animal and plant species.[12]While some structural similarities exist

between iridescent species in the plant and animal kingdoms, its proposed functions differ.[13] The recently published review by Doucet and Meadows provides a concise outline of the proposed functions of animal iridescence. Among these functions is the visual communication of information between animals (e.g., age and sex).[4, 14-18] Structural color in animals is also thought to aid animals in eluding predators, either by camouflage or by mimicry.[19-22]

Plant and floral iridescence, though not as widely characterized as animal iridescence, has been observed in various plant species. Suggested functions of floral iridescence in pollinating flowers are related to the attraction of pollinating animals.[1] It is also hypothesized that plants growing in low-light environments evolve structural features that enable them to capture light within the micro-structures in their leaves;these microstructures that are believed to be responsible for the iridescence of various plant species (e.g., *S. willdenowii*).[13, 19]

An important next step in the continued characterization of plant iridescence is the investigation of the various kinds of plant species that exhibit this structural color property. Characterization of floral iridescence will have to extend beyond structures that are exclusively iridescent in the visible light range, as the optical properties of pollinating animals (e.g. bees) vary greatly from those of humans, thereby enabling some animals to perceive UV-iridescence exhibited in some floral plant species. It was recently demonstrated for the first time that the red rose is UV-iridescent.[22] Similar observations are likely to be found in various species of flowering plants.[22]

Plants also rely on structural color for various purposes related to display and defense. Plants, however, are interested in communicating with pollinating animals rather than with other plants. A likely function of floral iridescence and iridescence in various pollinating species is to assist plants in communicating with pollinators.[4, 23] Plant iridescence is also thought to defend plants from animal predators and from potentially harmful levels of light.[4]

While some forms of structural coloration are chemically produced, iridescence can only be derived from physical properties.[6, 24] Structural colorin butterfly wings is derived from periodically spaced sub-micrometer structures. The formation mechanisms of these biological structures are extremely complex, as each individual scale's nanoscopic properties contribute to this physical color.[2] Various attempts at the biomimetic replication of these nanostructures have been made.[24] Computer technology has also been integral in the characterization and replication of these structures.[2]

Materials and Methods

Some previously reported methods for characterizing iridescent structures in various animal and floral species include various forms of microscopy and spectroscopy (i.e. transmission electron microscopy (TEM), scanning electron (SEM), and atomic force microscopy (AFM)) and various forms of spectroscopy, such as angle-resolved spectroscopy.[1, 3, 13, 22] This paper reports an experiment using optical and light microscopy, thereby providing researchers with a simple method for qualitatively characterizing biological iridescence.

In contrast to the methods used in

previous experiments, the methods presented herein are simply performed and the materials are easily obtained and comparatively inexpensive.

The wing from a blue iridescent *Morpho* butterfly is the specimen chosen for this project; iridescence in *Morpho* butterflies is widely characterized.[2, 5, 7, 25] The specimen imaged is supplied by JourdanJoly, Tallahassee, FL. Figure 1 shows the apparatus used to image the butterfly wing and Figure 2 shows the butterfly wing, imaged at two different angles of incidence. The images are split into the three channels, red, green, and blue, which are then analyzed to produce the data in figures 3 and 4.

The sample is imaged using a Dino Scope Pro (The Microscope Store, L.L.C., at a magnification of 17x). The microscope is three inches above the sample at a 90° angle relative to the plane of the sample. The white light source used is a 500-watt Fiber-Lite, High-Intensity Illuminator Series 180 (Dolan-Jenner Industries, Inc.). The lowest intensity setting of the lamp is used to image the sample.

An image of the setup (Figure 1) is taken using a standard digital camera; the camera lens is parallel to the plane of the sample and perpendicular to the beam of light. The angle between the beam of light and the plane of the sample is measured using Screen Protractor software (Iconico, Inc.), and the optimal distance between the light source and the sample is identified as three inches. A ruler is used to measure the distance from the light source to the sample at each angle of illumination, and the distances from the light source to the sample range from 3 to 3.5 inches.

The images photographed with the Dino Scope are analyzed using ImageJ (Research Services Branch, National Institute of Mental Health). The butterfly wing remains stationary while the light source is adjusted according to the desired angle. The data from the analysis of each image in its entirety is reported in Figure 3. The same circular region of the sample is isolated in the nine images taken at varied angles of incidence. These data are reported in Figure 4.

Results and Discussion

Each photograph taken is analyzed twice. Figure 3 data is from the analysis of the circular portion of the center of the image. This region clearly demonstrates the change of the wing's coloration as the angle of incidence changes. The data in Figure 4 are from the analysis of the entire wing. These data are included as the entire photograph of the winghas some regions that are in shadow. Rather than discarding these regions as artifacts, the function of the shadow in *Morpho*'s natural environment is considered. As suggested in previously published literature on *Morpho* structural color, iridescence in this butterfly might function as a defense mechanism; the shadowy regions of the wing as seen at various angles of incidence might serve the same function.[4]

The specimen is placed on the stage underneath the microscope and the angle of incidence between the light source and the specimen is varied. The specimen is imaged at various angles of incidence, and the corresponding angle is measured and recorded. The intensity values of red, green, and blue (reported in gray scale values) are measured in each image and compared as a function of the angle of incidence. Though the

distance of the light from the surface of the specimen varies some as the angle is adjusted, the light source is consistently between 3-3.5 inches from the sample. It can be seen that the intensities of red, green, and blue vary as the angle of incidence is adjusted (See Figure 2 and Supplemental Video 1).

A graph providing the Dino Scope camera's relative spectral responsesto the colors blue, green, and red is available on microscope manufacturer's website; this graph indicates that the maximum spectral responses for these three wavelengths are 470, 540, and 615 nm, respectively. The intensities observed in the data reported in both Figures 3 and 4 indicate that blue is the most intense color observed in the images taken at an angle of incidence less than 41°. This observation is consistent with previous characterizations of *Morpho* iridescence.[2, 26]The relative intensities of green and red are different between the two figures. In the analysis of the circular region of the image indicated in figure 2, as presented in Figure 3 data, the intensity of green generally increases as the angle of incidence is increased. The intensity values measured at lower angles of incidence are also consistent with its striking blue color, which is easily observed when looking at the *Morpho*butterfly's wings.

In the analysis of the circular portion of the wing, the peak intensity value for red is observed between 0-40 °, whereas the peak intensities for blue and green are observed at higher angles of illumination. In the analysis of both figures 3 and 4, it can be seen that blue and green generallyhave similar intensity measurements. Red intensity values

remain comparatively constant between the two figures.

As animal iridescence has been suggested as a way for animals to communicate with each other and to defend themselves against predators, it is conceivable that *Morpho* iridescence might be an evolved defense or communication method; Frederiksen and co-workers provide an analysis of the *Morpho*'s optical properties that might explain the observed trends between the data presented in Figures 3 and 4.[27] The co-development of the coloration systems of predator and prey imply their interconnected nature and interdependence; the characterization of iridescence further develops an understanding of the fundamental biological relationships and mechanisms responsible for the construction of these evolved structural details.

In bright light, the blue-green iridescence of the *Selaginellawilldenowii* becomes reddish brown. This observation is consistent with the shift in coloration of the *Morpho* data reported in this experiment.[13] The lower angles shine light more directly on the specimen than the higher angles. The diversity of natural photonic structures in the anima and plant kingdoms indicates the degree to which light functions as a significant selective pressure in various species. Vukisic and Sambles propose arm that the sensitivity to shadow observed in the iridescent ossicles in a light-sensitive species of brittlestar (*Ophiocomawendtii*) functions as a warning in the presence of predators.[5] Perhaps the same is true in the *Morpho*.

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APPENDIX

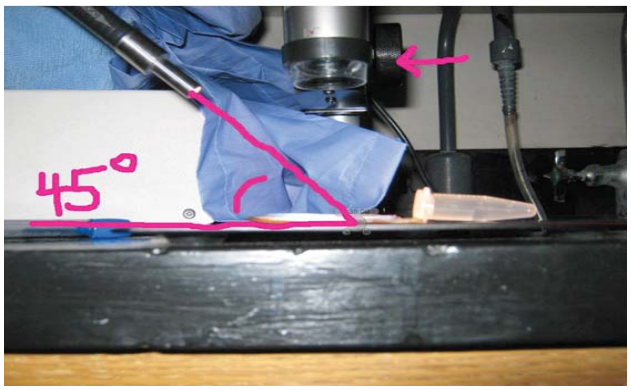
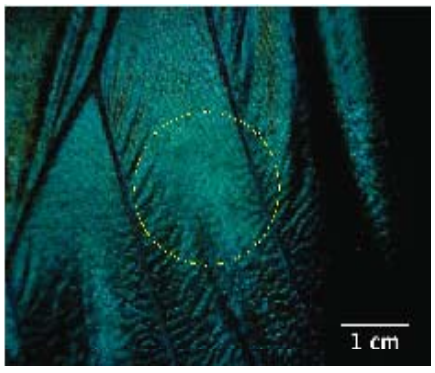
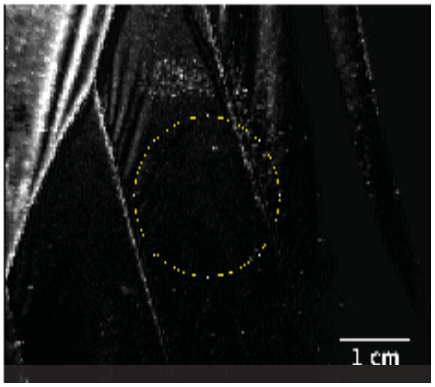


Figure 1: Figure 1 shows how each angle of incidenceis defined and where the camera is positioned relative to the sample. The arrow indicates the position of the camera, which is not altered throughout the course of this experiment.The angle between the beam of light and the surface of the wing is defined. In the following experiments, the "angle" is defined as the point where the beam of light meets the plane of the surface of the wing. The wing is held stationary by a microcentrifuge tube, which is resting on the edge of the specimen.The microcentrifuge tube is 3.81 cm long.

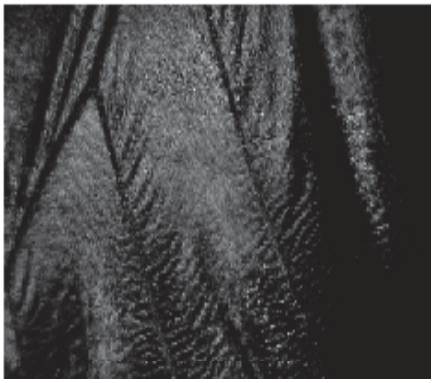


(a)

(b)

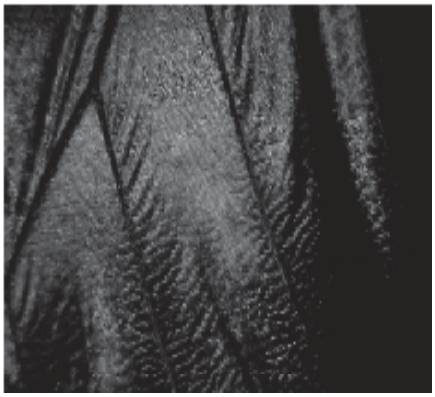


(c) Blue 39.808



(d) Blue 82.38

(e) Green 39.618



(f) Green 87.926

(g) Red 83.279

(h) Red 20.702

Figure 2: Here, the wing can be seen imaged at two different angles of incidence; (a) 17.94° is on the left, and (b) 57.62° is on the right. These two angles are chosen because they clearly demonstrate the changes in color of the wing with the changing angles of incidence. The images are split into blue, green, and red channels (Figure 2 c-h). The intensity corresponding to each channel is provided below each image (reported in gray scale values). The difference in the intensities of each color at different angles of incidence can be seen in this figure. The circular region indicated in the first of these images corresponds to the region that is analyzed in Figure 3 data. Supplemental video 1 shows all nine images analyzed arranged in order of increasing angle of illumination.

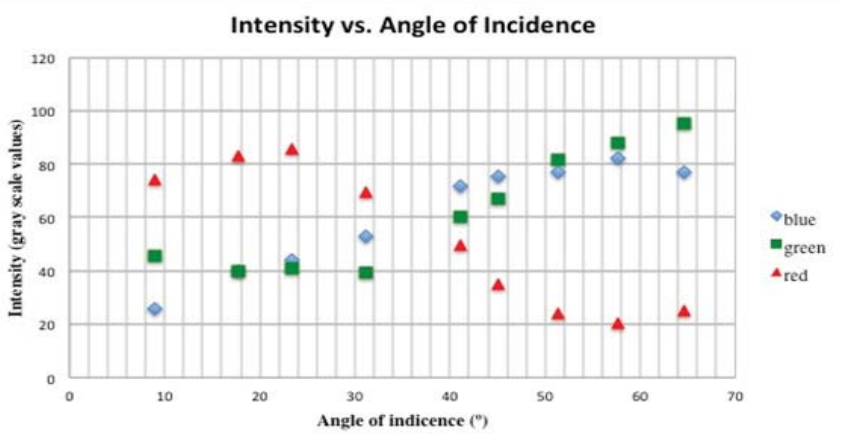


Figure 3: These data demonstrate the changes in intensity of the colors red, green, and blue observed as the angle of incidence is varied. The circular portion of the image of the wing indicated in Figure 2 (a) is analyzed in this figure.

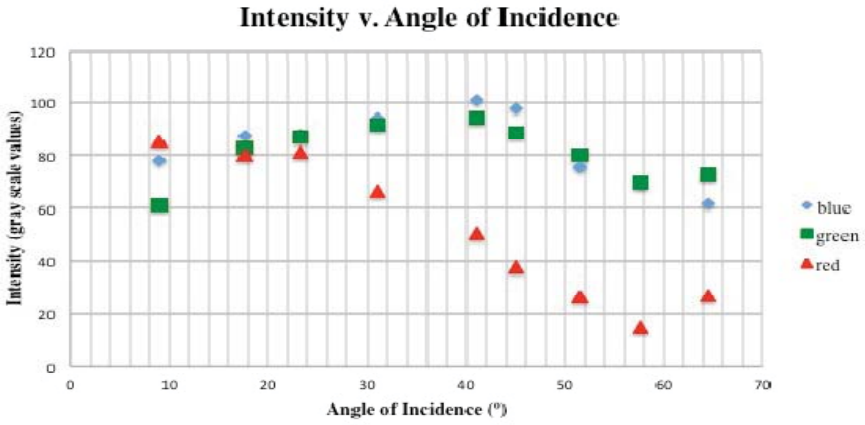


Figure 4: These data demonstrate the changes in intensity of the colors red, green, and blue observed as the angle of incidence is varied. The entire image of the specimen (Figure 2) is analyzed in these data. Figures 3 and 4 are both included as they compare the analysis of a small portion of the image with that of the entire image. The intensity values of blue and green fluctuate more than those of red between the two figures.