1	Structural color in <i>Junonia</i> butterflies evolves by tuning scale lamina thickness
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18	Abstract
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20	In diverse organisms, nanostructures that coherently scatter light create structural color, but
21	how such structures are built remains mysterious. We investigate the evolution and genetic
22	regulation of butterfly scale laminae, which are simple photonic nanostructures. In a lineage of
23	buckeye butterflies artificially selected for blue wing color, we found that thickened laminae
24	caused a color shift from brown to blue. Deletion of the <i>optix</i> wing patterning gene also altered
25	color via lamina thickening, revealing shared genetic regulation of pigments and lamina
26	thickness. Finally, we show how lamina thickness variation contributes to the color diversity that
27	distinguishes sexes and species throughout the genus <i>Junonia</i> . Thus, guantitatively tuning one
28	dimension of scale architecture facilitates both the microevolution and macroevolution of a
29	broad spectrum of hues. Because the lamina is an intrinsic component of typical butterfly

- 30 scales, our findings suggest that tuning lamina thickness is a readily accessible mechanism to
- 31 create structural color across the Lepidoptera.

Introduction 32

33

Structural colors are both visually delightful and abundant in nature. Organisms deploy 34

35 structural colors to display hues for which they lack pigments (frequently blues and greens), to

create specific optical effects such as iridescence or light polarization, and to mediate ecological 36

37 interactions, including intraspecific signaling and camouflage. Unlike pigmentary color, which is

38 caused by molecules that selectively absorb certain wavelengths of light, structural colors result

39 from the constructive and destructive interference of light as it interacts with nanoscale.

40 precisely-shaped physical structures that are made of a high refractive index material (e.g.

41 keratin, chitin, or cellulose).

42

43 Despite the clear importance of structural color for living systems, the biological production of

44 structural colors has long eluded characterization [1]. Many experimental techniques depend on

45 harnessing variation to dissect biological processes, but photonic structures are so small that

46 guantitatively measuring variation in their dimensions is technically demanding, especially for

47 high-throughput sampling, detecting subtle variation that may segregate within populations, or

48 analyzing over developmental time in vivo. The color itself is easier to quantify, but has limited

49 utility as a proxy for nanostructural dimensions, since structural colors and pigments often co-

50 occur and covary. While recent studies [2-4] have made early headway toward describing

51 genetic regulation of structural colors, much work remains to decipher the evolutionary,

52 developmental, and genetic bases of structural coloration, and lab-tractable systems with

53 intraspecific variation in structural coloration are needed. We present a promising system, the

54 butterfly genus Junonia, with extensive variation in a simple structural color, and show how

55 structural simplicity is a tactical advantage when seeking to unravel mechanisms for the

- 56 biological production of nanostructures.
- 57

58 In butterflies, photonic nanostructures occur within the architecture of scales. Scales are the 59 fundamental coloration unit on butterfly wings and have a Bauplan consisting of a grid of ridges

60 and ribs, supported by a lower lamina that is a simple plane (Fig. 1A). Scales are composed of

61 chitin and may also have embedded pigments. Intricate architecture and a high refractive index 62

make scales a pliable substrate for photonic innovations, and indeed scales have been

63 evolutionarily elaborated in many ways for impressive optical effects [5]. Even the simplest

64 butterfly scales can produce structural color, via the lower lamina acting as a thin film reflector. 65 Thin films are the simplest photonic structure and consist of a layer of high refractive index

66 material, on the order of hundreds of nanometers thick, surrounded by a material with a

67 contrasting refractive index, i.e. air (Fig. 1B). Light is reflected from each surface of the film, and

68 these two reflections interfere with each other. If the two reflections remain in phase, which

69 depends on the extra distance traveled through the film and the wavelength, then they interfere

70 constructively to produce observable color [6,7]. Conversely, wavelengths (colors) that undergo

- 71 destructive interference have decreased brightness.
- 72

73 While it is known that the thickness of the lower lamina is one parameter that controls structural 74 color wavelength [8] and that thickness can respond to artificial selection in the laboratory [9], it 75 is not known how general this mechanism is in natural evolution. It is also unknown how lamina structural colors are genetically regulated and whether any recognized butterfly wing patterning

77 genes regulate lamina thickness. Here, we use mutants with deletions in the optix wing

patterning gene, artificial selection on wing color, and genus-wide wing color variation to test the

role of lamina thickness in generating butterfly color. We show that butterflies in the genus

- 30 Junonia thoroughly exploit the relationship between film thickness and color, using the thin films
- 81 necessarily present in their scales to produce a broad spectrum of hues by tuning lamina
- 82 thickness. These lamina colors work in tandem with pigments to define the wing pattern
- 83 elements that distinguish populations, sexes, and species, indicating that the ability to vary
- 84 lamina thickness has been an important microevolutionary and macroevolutionary tool in this
- 85 group, and likely in butterflies more broadly.
- 86

87 **Results**

88

89 Artificial selection for blue wing color increases lamina thickness

90 Here we describe a novel instance of rapid, artificially selected color shift from brown to blue 91 wing color in *J. coenia* buckeye butterflies (Fig 1D-E) and identify the structural changes that 92 enabled the color shift. Edith Smith, a private butterfly breeder, began selectively mating 93 buckeyes with a few blue scales on the costal margin of the dorsal forewing (E. Smith, personal 94 communication, Sep. 2014). After five months of selective breeding, blue spread to the dorsal 95 hindwing of some individuals. By eight months, there was a noticeable increase in blue surface area, and within roughly 12 months (on the order of 12 generations), most butterflies in the 96 97 breeding colony were visibly blue over the majority of their dorsal wing surface. On the forewing, 98 areas proximal to M1 were visibly blue, except the discal bars (Fig. S1). On the hindwing, blue 99 shift did not include the distal-most wing pattern elements, i.e. EI-EIII and eyespots. At its 100 strongest, the phenotype may include blue scales cupping the posterior forewing eyespot and/or 101 a blue sheen in all distal elements of the forewing. Smith maintained the blue colony for several 102 years, introgressing a few progeny from crosses to wild-caught buckeyes about once per year to 103 maintain genetic diversity. Over time, she noted the emergence of a variety of short-wavelength 104 colors, ranging from purple to green. Two years after focused selection, she estimated that the 105 population was 85% blue, 8% green, 2% purple, and 5% brown. Like many familiar examples of 106 human selection (e.g. domesticated animals, crop plants), outcomes are informative even 107 without complete experimental documentation of the selective process [10,11]. These selected 108 blue buckeyes provide a previously unexploited opportunity to study structural color. They 109 demonstrate rapid and extensive evolutionary color change, and are a stark contrast to wild-110 type brown populations with which they are still interfertile. Conveniently, the artificially selected 111 taxon, J. coenia, is a recognized model species for butterfly developmental genetics [12,13]. 112 The selected blue individuals resemble naturally evolved color variants in the sister species, J. evarete (Fig. 1F), and offer a useful comparison to a previously reported artificial selection 113 114 experiment in butterflies [9].

115

116 To pinpoint the cause of blueness in artificially selected butterflies, we characterized cover

- 117 scales from the dorsal hindwing (Fig. 2A-D). Butterfly wings have two classes of scales
- arranged in alternating rows that form two layers: superficial cover scales and underlying ground
- scales. Cover and ground scales frequently have contrasting size, shape, and color, and their

120 juxtaposition can be important for wing color [8]. When isolated and laid in the abwing 121 orientation they occupy on the wing, cover scales were blue (Fig. 2B). However, when flipped 122 over and viewed in adwing orientation, which exposes only the lower lamina, scales appeared 123 more brightly blue and iridescence was more apparent (Fig. 2B', 2D). We tested whether the 124 blue was structural rather than pigment-based by immersing the full scale in oil with a refractive 125 index matched to that of chitin (Fig. 2B"). Index-matching eliminates the possibility of reflection 126 and structural color, leaving only pigment-based coloration. We measured the scale's 127 absorption spectrum under these conditions (Fig. 3A), which revealed that blue scales did have 128 some pigment, presumably a brown ommochrome [14], but this pigment cannot account for 129 blueness. The pigment was located in the scale ridges (Fig. S2 B). Lepidopteran structural colors may occur in the lamina, lumen, ridges, or cross-ribs. To isolate which of these features 130 131 had the nanostructure responsible for blue structural color, we dissected the scales (Fig. 2B", 132 Fig. S2 A). After removing all other scale components, we found that the bare lower lamina was 133 sufficient for blue structural color. We also examined regions with all scale components except 134 the lamina and found that these pieces of lamina-less scale were not blue (Fig. S2 C). We thus 135 focused on investigating nanostructure in the lamina. To discern between a single or multilayer 136 lamina and take precise measurements, we cross-sectioned the lamina and viewed it with 137 Helium Ion Microscopy (HIM) (Fig. 2C). HIM imaging indicated the lower lamina was a simple 138 monolayer of chitin with a thickness of 187 ± 13 nm (SD, Fig. 1C), which is a reasonable 139 thickness to reflect blue as a dielectric thin film [8].

140

141 We next investigated whether ground scales also contributed to blueness after artificial 142 selection. In artificially selected buckeyes, the ground scales generally had similar architecture 143 to the cover scales, but with less uniform lamina color: ground scales exhibited a color gradient 144 from the stalk outward (Fig. 4A-B'). Correspondingly, ground scales had a similar mean 145 thickness but more variability than cover scales (190 ± 29 nm). Ground scales were much more 146 heavily pigmented than cover scales (Fig. 3B, Fig. 4B"), such that the abwing surface was black 147 (Fig. 4B). The extra pigmentation in ground scales enhances spectral purity by absorbing light 148 transmitted through the cover scales, thus reducing backscatter and making the observed blue 149 color more saturated (similar to [15]). We conclude that cover scale laminae are the major 150 source of blueness in artificially selected buckeye butterflies, while melanic ground scales 151 secondarily enhance spectral purity.

152

153 For comparison, we tested the source of color in wild-type brown scales and found that they 154 also had structural color (Fig. 2E-H). Brown cover scales had the same general architecture and 155 no more brown pigment than did blue cover scales (Fig. 3A, Mann-Whitney U, Table S1). The 156 salient difference was lamina thickness: brown scales were markedly thinner, measuring only 109 ± 12 nm (Analysis of Variance (ANOVA), $p < 2x10^{-16}$, Fig. 1C, Fig. 2G). A 109 nm chitin thin 157 film reflects a desaturated golden color due to reflectance of many long wavelengths. This 158 159 golden structural color was confirmed by the adwing scale color, the color of the bare lamina in 160 dissected scales, and the adwing reflectance spectra of brown scales (Fig. 2F'-F", H). 161 Therefore, though brown coloration is often attributed to pigmentation, wild-type brown cover 162 scales also had a structural color, one simply tuned to enhance different wavelengths.

163

Artificial selection also altered the absorption and lamina thickness of the ground scales (Fig. 4A-D). The wild-type (brown) ground scales were thinner than the blue ground scales $(151 \pm 30 \text{ nm}, \text{ANOVA}, \text{p} = 2x10^{-6}, \text{Fig. 1C})$. However, the mean difference was less extreme than in cover scales: blue cover scales were on average 78 nm thicker than wild-type, while blue ground scales were on average 39 nm thicker. Selected ground scales were markedly more absorbing than wild-type ground scales (Fig. 3B, Mann-Whitney *U*, Table S1), which is consistent with

- 170 increased pigmentation that decreases backscatter in blue wing regions.
- 171

172 We conclude that the artificially selected buckeye butterflies rapidly evolved blue wing color via

- a 71% mean increase in lamina thickness in cover scales and a similar but less pronouncedeffect in ground scales. The effect was further amplified by increased pigmentation in ground
- scales, but without removing brown pigment from cover scales. Our results show that structural
- color can evolve quickly by modifying one dimension of an existing structure, and the process is
 facilitated by the initial presence of previously unrecognized structural color in wild-type brown *J*.
- 178 coenia.
- 179

180 Since the artificially selected *J. coenia* wing pattern resembles natural iridescent variants in the

- 181 sister species, *J. evarete* (Fig. 1F), we obtained hindwings of brown and blue *J. evarete*
- 182 individuals from different geographic locations and tested whether blue cover scales in this
- species were also associated with increased lamina thickness (Fig. 2I-P). We found that the
- same mechanism explained color differences between geographic color variants: blue scales
- had 78% thicker scale laminae (blue 199 \pm 14 nm; brown 112 \pm 13 nm; ANOVA, p < 2x10⁻¹⁶, Fig. 1C) and no appreciable difference in pigmentation, compared to brown individuals (Fig. 3C,
- 187 Mann-Whitney U, Table S1). Furthermore, in blue J. evarete, the ground scales were darkly

188 pigmented. Thus, the artificially selected blue buckeyes faithfully recapitulate natural variation at 189 the level of scale coloration between sister species.

190

191 Color phenotypes in optix mutants include altered lamina thickness

192 Recently, Zhang et al. used CRISPR/Cas9 to generate mosaic knockout mutants of optix [16], a 193 gene previously associated with pigment variation in butterfly wings [17]. Surprisingly, in 194 addition to pigmentation phenotypes, optix mutants in J. coenia gained blue iridescence in wing 195 scales. We tested phenotypically mutant blue scales from mosaic butterflies to determine what 196 structural or pigmentary changes created the color change (Fig. 2Q-T). Where blue scales 197 occured in the background region of the dorsal wing, blueness was due to similar factors as 198 identified in artificially selected buckeyes. Lamina thickness of blue cover scales was substantially increased compared to wild-type brown scales (212 \pm 11 nm, ANOVA, p < 2x10⁻¹⁶, 199 200 Fig. 1C). The concentration of brown pigment in the cover scales was significantly reduced 201 relative to wild-type scales within the same mosaic wing (Fig 5A, Mann-Whitney U, Table S1) 202 but comparable to selected animals (Fig. 3A, Table S1). Ground scales (Fig. 4E-F") were 203 likewise similar to those of selected blue animals, having thick and variable laminae (199 ±31 nm, ANOVA, p=5x10⁻⁵ versus wild-type. p=0.36 versus selected, Fig. 1C) and significantly 204 205 increased pigmentation (Fig. 5B, Mann-Whitney U, Table S1). Overall, blue scale identity in 206 optix mutants was caused by similar mechanisms as artificially selected blue. 207

208 optix mutant phenotypes also affected structural colors and pigments differently across wing pattern elements. As originally postulated [16], excess melanin was produced in some ventral 209 wing regions (Fig. 6A-D, Fig. 5C). We also observed regions where both pigment and structure 210 211 were dramatically changed. For example, discal bars on the dorsal forewing, which are normally 212 orange, gained blue scales through both converting lamina structural color to blue and replacing 213 orange with brown pigment (Fig. 6E-H, Fig. 5D). The kinds of pigmentation effects were diverse: 214 optix mutation increased the quantity (Fig. 5B, C), decreased the quantity (Fig. 5A), or switched 215 the identity (Fig. 5D) of the pigment in different scales (Mann-Whitney U, Table S1). Because 216 the butterflies were mosaic mutants, some of this phenotypic variability could be due to 217 genotypic differences between clones (i.e. mono- versus biallelic gene deletion, as well as the 218 exact size of the deletion) [16]. However, much of the variation in outcome could also be 219 observed within single clones that spanned multiple wing pattern elements (defined by the 220 Nymphalid ground plan [18], Fig. S1), suggesting that the patterning roles of optix are quite 221 context specific. 222

In summary, *optix* knockout can have varied effects in a single scale by altering pigmentation,
nanostructures, or both. These findings are consistent with *optix*'s described role as a
developmental patterning gene that determines gross switches between discrete scale fates,
and which, directly or indirectly, can regulate diverse downstream factors [19]. Since appropriate
coloration critically depends on the proper combination of pigment and structural colors in both
cover and ground scales (e.g. [20,21]), it is of particular interest that *optix* can regulate all of
these components simultaneously. *optix* mosaic knockout mutants demonstrate that lamina

- thickness can be experimentally perturbed and highlight a multifunctional candidate genetic
 pathway for coordinated color evolution.
- 232

233 Lamina thickness consistently predicts structural color wavelength

234 Relatives of J. coenia exhibit extensive color and pattern diversity, and blue structural colors in 235 particular show patterns of variation that hint at ecological relevance (e.g. sexual dichromatism, 236 seasonal polyphenism) (Fig. 7A). To assess the importance of lamina thickness variation in 237 macroevolutionary color diversity, we sampled cover scales from nine species in the genus 238 Junonia and a tenth species, Precis octavia, which belongs to the tribe Junoniini and exhibits 239 seasonally polyphenic wing coloration. We prioritized large pattern elements that distinguish 240 color forms within species. We compared scales using optical imaging, immersion index-241 matching, spectrophotometry, and Helium Ion Microscopy. All scales sampled had typical 242 Nymphalid scale structure with a single plane of chitin forming the lower lamina.

243

244 We tested whether the relationship between lamina thickness and color that we observed in 245 experimental contexts applies more broadly. We sought to address two questions; First, does 246 lamina thickness reliably predict lamina color, as measured from the adwing surface? While it is 247 known that the thickness of a dielectric film controls the film's reflectance, other variables such 248 as refractive index, surface roughness, and pigmentation within the film also factor into 249 reflectance, and these could plausibly vary among taxa. Second, how variable is lamina 250 thickness? What range of thicknesses occur, and is there evidence for either quantized or 251 continuous thickness variation? To address these questions, we measured reflectance spectra

from the adwing surface of disarticulated cover scales from the 23 wing regions indicated in Fig.
7A. We then cross-sectioned scales, imaged with HIM, and measured thickness.

254

We found that lamina thickness varied continuously between 90-260 nm, indicating that all 255 256 thicknesses over a more than 2.5-fold range are accessible (Fig. 8A). To better visualize the 257 relationship between thickness and lamina color, we clustered similar samples into five color 258 groups (Methods). Lamina colors in these groups could be described as gold, indigo, blue, and 259 green, with a fifth variable group that included magenta, copper, and reddish colored scales 260 (labeled as "red" in Fig. 8). Thickness differed significantly between all color group pairwise comparisons (Fig. 8A, ANOVA: $p < 2x10^{-16}$, with *post hoc* Tukey's Honestly Significant 261 Difference test: $p < 2x10^{-6}$ for all pairwise comparisons). The color groups were also associated 262 263 with different reflectance profiles (Fig. 8B). In some cases, we obtained variable measures 264 within individual specimens, which reflects biological color variation between adjacent scales, as 265 well as varying color within individual scale laminae along their proximal-distal and lateral axes. 266 A particularly striking example of the latter came from J. atlites. While the wing appeared light 267 grey, at higher magnification individual scales could be seen to be multicolored (Fig. 7G'), and 268 thickness measures from J. atlites overlapped the ranges of all color groups (Fig. 8A, see 269 further analysis below).

270

271 Lamina thickness had a consistent relationship with adwing scale reflectance for the taxa and 272 color range we sampled. The order of color shift as lamina thickness increased followed 273 Newton's series, which is the characteristic color sequence for thin films [6,23]. This sequence 274 can be understood in terms of an oscillating thin film reflectance function, which shifts toward 275 longer wavelengths as film thickness increases (Fig. 8C-G). The thinnest films appeared gold 276 due to reflectance of all the longer wavelengths (Fig. 8C). In mid-thickness laminae, a mix of two 277 oscillations determined color: reflectance of the first oscillation was shifted toward far red 278 wavelengths, while a second reflectance peak rose in the ultraviolet (Fig. 8D). Visible 279 reflectance of thicker laminae was dominated by the peak of the second oscillation as it moved 280 from indigo to green (Fig. 8E-G). That the trend between thickness and reflectance holds 281 broadly suggests that color changes in Junonia butterfly scales have recurrently evolved via 282 lamina thickness adjustments. Moreover, the consistency of the relationship between thickness 283 and reflectance is useful. For example, structural variation could be rapidly surveyed by 284 extracting fitted thickness estimates from reflectance measurements, a much less laborious 285 process than sectioning for electron microscopy.

286

287 Lamina structural color influences wing color throughout the genus Junonia

288 We next tested whether the extensive variation in lamina structural color among Junonia 289 butterflies, explained by lamina thickness, also drives variation in overall wing color. An 290 alternative hypothesis would be that composite wing color is usually dominated by pigmentation, 291 particularly by pigments distributed on the outward-facing abwing surfaces of cover scales. 292 above the lamina thin film. We measured pigmentation in cover scales from the same regions 293 (Fig. 7A) to test the relative importance of pigments and lamina structural colors for wing color. 294 (Structural colors and pigments are listed per each specimen in Table S2 and representative 295 examples are shown in Fig. 7B-M".)

296 Pigmentation was highly variable among Junonia species (Fig. 7B-M", Fig. 9, Table S2). This 297 included marked differences in pigmentation between regions of a single wing (e.g. yellow and 298 blue regions in J. hierta, Fig. 7B-E", 9A) and also variation between color forms and species 299 throughout the genus (e.g. between sexes in J. orithya, Fig. 9C, and seasonal forms in P. 300 octavia Fig. 7H-M", 9B). Absorbance spectra varied in both shape and magnitude. Variation in 301 magnitude, such as between the red band and the wet season morph of *P. octavia* (Fig. 9B), 302 represents differences in pigment abundance. We also observed distinct absorbance spectral 303 shapes, which can indicate the identity of the pigment (for example, contrast the spectral shape 304 of the vellow pigment in J. hierta, Fig. 9A, versus red pigment in P. octavia, Fig. 9B, and brown 305 pigment in J. orithya, Fig. 9C).

306

307 Notwithstanding the clear importance of pigmentation among Junonia butterflies, pigment 308 variation was insufficient to explain the breadth of wing color diversity, and lamina structural 309 colors made up the shortfall. The importance of lamina structural color was most obvious in 310 scales that entirely lacked pigments. For example, the blue basal aura regions of male J. 311 westermanni, J. hierta, and J. oenone wings had unpigmented cover scales with structurally 312 blue laminae (Fig. 7B-C", Fig. 9A). Most of the pigmentless scales we sampled were blue, with 313 the notable exception of J. atlites scales (Fig. 7F-G", Fig. 9D). These scales had rainbow 314 gradient laminae, which presumably create the overall light grey by additive color mixing [24]. J. 315 atlites demonstrates that lamina structural color can fundamentally drive wing color even in 316 neutrally colored wing regions that are not obviously iridescent, and also that thickness can be patterned at fine spatial resolution within a single lamina. 317

318

319 In most wing regions, color was determined by the interaction of both lamina structure and 320 pigments. For example, in the cover scales of J. hierta (Fig. 7D-E", Fig. 9A), the yellow lamina 321 structural color and yellow pigment were mutually reinforcing, with the lamina sensibly reflecting 322 wavelengths that the pigment does not absorb. Other examples help delineate how much 323 pigment is required to overpower the lamina color. In blue J. evarete, pigments in the cover 324 scale ridges absorbed approximately 0.2 AU (Absorbance Units, i.e. 37% of light not 325 transmitted, Fig. 3C) of the blue wavelengths that the lamina reflected most brightly (Fig. 2L). 326 With this ratio, wing hue was still driven by the lamina structural color. The cover scales of J. 327 orithya were similar (Fig. 9C), having a neutral dark pigment (i.e. a pigment that absorbs all 328 visible wavelengths) in the scale ridges. Perhaps dark pigment in the ridges functions like a 329 Venetian blind to limit iridescence, so that at high viewing angles, where iridescence would be 330 most pronounced, light from the lamina is guenched.

331

332 Because of their range of pigment concentrations, P. octavia specimens were also useful to test 333 the tradeoff between pigment abundance and lamina color influence. When viewed at high 334 resolution, scales from the wet season morph of *P. octavia* contained red pigment in the ridges 335 and ribs (max absorbance 0.12 ± 0.02 , Fig. 7K,K', Fig. 9B), while reflected light from the blue 336 lamina spilled through the windows between ridges. Viewed macroscopically, this combination 337 made a lightly saturated red. To display a richly saturated red, much more pigment was 338 required, as seen in the red band of the dry season morph (max absorbance 0.38 ± 0.04 , Fig. 339 9B, Fig. 7 L-M"). These reddest scales also had thinner, structurally magenta and copper

colored laminae that may further reinforce redness (Fig. 8A, Fig. 7M'). The concentration of red

pigment was the most important driver of the color difference between *P. octavia* seasonal

342 morphs. The blue and red morphs had only a subtle difference in lamina thickness (Fig. 8A),

and the laminae of both were blue (Fig. 7 l', K'), but the blue morph lacked any red pigment (Fig.9B).

345

Overall, *Junonia* wing color was determined by complex mix-and-matching of different lamina
thicknesses and pigments. A thin film lower lamina was present in all scales, but its influence on
wing color was adjusted by the amount and placement of pigment, especially in the upper
surface of the scale. Pigments can mask lamina structural color at high enough density,
depending on the placement and color of the pigment as well as the color of the lamina. In our
tests, when pigmentation absorbed ≤ 0.2 AU of the relevant wavelengths, it did not cancel out
lamina structural color.

353

354 **Comparison to thin film equation**

We compared our empirical data to Fresnel's classical thin film equations, which model the 355 356 reflectance of an idealized dielectric thin film [7,25]. This model has previously been used to 357 estimate the thickness of butterfly scale laminae based on their adwing reflectance spectra 358 [8,21]. For each sample, we modeled the expected reflectance using our thickness 359 measurements, and then compared to the measured reflectance spectra. We used 1.56 for the 360 refractive index of chitin [26] and a maximal angle of illumination of 30° following [27] (because 361 spectra were measured through an objective lens with a numerical aperture of 0.5). To account 362 for measurement error, we modeled films over all thicknesses within one standard deviation of 363 the measured mean per sample (red envelopes, Fig. S3 A). We also modeled films with 364 Gaussian thickness distributions for each sample, following [15]. This model is analogous to a 365 single uneven film with mean thickness and surface roughness defined by the measured 366 thickness and sample standard deviation (solid red lines, Fig. S3 A).

367

We found that qualitatively the model describes the main behaviors of our data: reflectance oscillates with a given frequency and brightness, and the function shifts toward longer wavelengths as thickness increases. Quantitatively, mean maxima and minima in the

371 reflectance function were offset laterally for every specimen, by about 40-80 nm, with the

372 modeled curves blue-shifted relative to the observed. A similar blue shift has been reported in

373 butterfly scale laminae before [9]. The comparison improves if we assume a higher refractive

index or thickness. However, to align modeled and measured spectra would require either an

impossibly high refractive index (around 1.75) or increased thickness outside the error range of

our measures (20-25 nm thicker than mean measurements). Possibly the lateral offset is due to

a combination of the former. Alternatively, these results could indicate that scales have
 additional properties not fully described by the model. There are a number of differences

between the idealized film and real scales, including curvature of the film and possible

380 birefringence of the ridges. The lamina itself may not necessarily have a uniform material

381 composition or refractive index. For example, contrasting sublayers within the lamina (as in [28])

382 could create extra reflective interfaces. Thus, our data are compatible with the expected

behaviors of thin films, but modeling the specific case of butterfly scale laminae with quantitative
 precision may require additional parameters or calibration to an empirical dataset.

385

386 Discussion

387 This study leverages the simplest photonic nanostructures, thin films, to interrogate the 388 evolution and genetic regulation of structural color in Junonia butterfly scales. While there is a 389 large body of literature attributing optical properties to various biological nanostructures, such 390 claims commonly rest on correlation between mathematical models and spectral 391 measurements. Here, we use two different experimental manipulations of the structure (artificial 392 selection on wing color and knockout of the optix gene) in addition to broad interspecies 393 comparisons to establish that lower lamina thickness quantitatively controls structural color 394 wavelength in Junonia butterfly scales. The relationship between lamina thickness and 395 wavelength holds over a wide range of thicknesses (90-260 nm) that generate Newton's color 396 series for dielectric thin films. Moreover, lamina structural color is one important determinant of 397 overall wing color, including in wing regions that also contain pigments. Lamina structural colors 398 contribute to the color differences that distinguish sexes, species, seasonal variants, and 399 selectively-bred lineages of Junonia butterflies, highlighting that quantitatively tuning lamina 400 thickness is a vehicle for color evolution in both micro and macroevolutionary contexts.

401

402 Because the lower lamina is part of the typical architecture of butterfly scales, our findings have 403 broad implications for future research on adult color in numerous butterfly taxa. Foundational 404 literature drew a distinction between highly derived scales with vivid structural colors and 405 "standard, undifferentiated scales," which conform to the butterfly scale Bauplan, have a simple 406 monolayer lower lamina, and "are not truly iridescent, i.e., they do not produce brilliant structural 407 colors" [29]. However, within the past ten years, individual examples of thin film interference 408 from the lower lamina have emerged in diverse Lepidoptera, including in simple scales 409 [8,9,15,21,28,30,31]. These newer descriptions and our thorough examination of many scales 410 indicate two points: first, although thin films are indeed less brilliant than some other classes of 411 Lepidopteran photonic structures (thin films only reflect around 20% of incident light), they are a 412 consequential source of structural color. Second, thin films occur in many butterfly and moth 413 lineages and likely arose early in Lepidopteran evolution. The lower lamina has a thin film 414 morphology in all scales that resemble the scale Bauplan, meaning that reflectance from the 415 lamina is the shared condition except where it is masked by either heavy pigmentation or a 416 derived structure with higher optical contrast. Because butterflies commonly produce multiple 417 lamina colors across wing pattern elements and scale types, it is probable that the 418 developmental genetic networks for quantitatively varying lamina thickness are deeply 419 conserved as well. Hence, it will be useful to report which lamina colors are present, in addition 420 to identifying pigments, when describing butterfly colors. 421 422 Physical constraints inherent to thin film colors may help explain the division of color space

Physical constraints inherent to thin film colors may help explain the division of color space
between pigments and photonic structures. It is not well understood why certain hues seem to
be more often produced by pigments while others are more often produced by structural colors
(e.g. the abundance of blue structural colors but lack of blue pigments in birds [32] and the rarity
of one class of red structural color in birds and beetles [33]). In *Junonia*, we show that by tuning

427 thickness, thin film laminae can produce nearly all the spectral colors (i.e. yellow, green, blue, 428 indigo), and even light achromatic colors (e.g. light grey in J. atlites) via color mixing across a 429 gradient. Yet thin films are fundamentally incapable of producing certain colors, notably dark 430 brown, black, and pure red. The medium thickness films that most nearly approach red have 431 inherently poor color properties due to the oscillating nature of the thin film reflectance function. 432 Since the colors of mid-thickness films are a mix of two reflectance peaks (Fig. 8C), they are 433 reddish but not pure or well-saturated, and are better described as copper, magenta, and 434 purple. Further, mid-thickness films are not bright: they reflect less total visible light than other 435 thicknesses we observed (compare Fig. 8D to 8C, E-G). By contrast, red, black, and brown are 436 prevalent pigment colors in Junonia, making pigments and thin film structural colors 437 complementary color palettes with little overlap. The optical limitations of thin films may have 438 partially determined how pigment families and scale architecture evolved in early butterfly 439 lineages, which in turn initialized whether pigments or structures provide the most accessible 440 route to evolve specific hues during subsequent diversification.

441

442 Our findings uncover a link between artificially selectable responses in lamina thickness and 443 natural butterfly color variation, and expand on a previous artificial selection study on butterfly 444 wing color [9]. In both J. coenia and B. anynana, color shift was accomplished by modifying the 445 dimension of an existing structure, the lower lamina, with pigmentation being less important. 446 Since the selected taxa diverged 78 million years ago [34] this similarity may be informative 447 about evolvability in nymphalid butterflies generally. However, artificial selection in B. anynana 448 primarily increased thickness in the obscured layer of ground scales, which can only weakly 449 influence color, whereas *Bicyclus* species with naturally evolved violet wing color have violet 450 thin films in their cover scales. In our study, artificial selection continued longer (12 vs. 6 451 generations) and elicited a more extreme response (71% vs. 46% increase in lamina thickness). 452 Moreover, in *J. coenia*, we show that lamina thickness increased in the cover scales and fully 453 recapitulated the naturally evolved mechanism of structural color in the sister species J. evarete. 454 The thickness increases caused a stark wing color change plainly visible by eye, with 455 appropriate wing patterning that also resembled *J. evarete* (thickened blue scales filled the 456 background dorsal wing, while evespots, distal pattern elements, and the ventral wing were 457 unaffected). Our results robustly connect a rapid microevolutionary process to 458 macroevolutionary diversity.

459

460 By using butterflies with CRISPR/Cas9-generated knockout of the optix gene, we are able to 461 provide insight into the genetic regulation of lamina thin films. It was previously known that the 462 optix wing patterning gene can regulate a switch between wild-type brown and blue iridescent 463 wing color in *J. coenia* [16], but the mechanistic basis for the color switch remained unknown. 464 Specifically, it was unclear whether optix regulated scale structure itself, or whether optix 465 deletion merely caused the loss of brown pigment, thus unveiling a pre-existing iridescent structure. Here, we show explicitly that in certain wing regions and scale types, optix deletion 466 467 substantially increases lamina thickness. Our findings also amend the earlier conclusion that 468 optix represses structural coloration in J. coenia [16]. Rather, by regulating lamina thickness, 469 optix regulates the wavelength of a photonic structure that exists in both wild types and mutants. 470 This distinction has implications for the likely identities and behavior of downstream genetic

471 factors, as well as the developmental basis of mutant blue coloration. For example, rather than

- 472 preventing a cascade of downstream genes from acting to erect a photonic structure *de novo*,
- 473 *optix* may subtly regulate the expression of a gene or genes that directly regulate lamina
- thickness, such as chitin synthase. Additionally, we uncover disparate effects of *optix* deletion
- on pigmentation, including promoting, suppressing, and switching the identity of pigments in
- 476 different scale types. In aggregate, these results show that *optix*'s functions in *J. coenia* are
- highly context specific, depending on both wing region and scale type (i.e. ground or cover
- scale). Moreover, because *optix* can regulate both pigmentary and structural color, the *optix*
- pathway is an especially interesting candidate for coordinated color evolution, and further workon the detailed regulation of *optix* and its downstream targets is called for.
- 481

In summary, thin film reflectors, a morphologically simple class of photonic structures, are experimentally manipulable and broadly employed in the lower lamina of *Junonia* butterfly wing scales. Lamina thickness explains variation in structural color wavelength, responds to selection on wing color, and is regulated by the *optix* wing patterning gene. Tuning lamina thickness facilitates both microevolutionary and macroevolutionary shifts in wing color patterning throughout the genus *Junonia*, making the buckeye butterflies a promising study system with

- 488 which to decipher the genetic and developmental origins of structural color.
- 489 490

491 Materials and Methods

492 Butterfly specimens

493 Reared J. coenia were fed fresh Plantago lanceolata or artificial diet (Southland Products, Lake 494 Village, AK) as larvae and kept at 27-30 °C on a 16/8 hour day/night cycle. Artificially selected 495 blue J. coenia were purchased as larvae from Shady Oak Butterfly Farm in 2014 (Brooker, FL). 496 Wild-type J. coenia were from an established laboratory colony, originally derived from females 497 collected in Durham, North Carolina [35] (for the comparisons to both optix mutant and selected 498 butterflies) or were collected in California (comparison to selected butterflies only). We acquired 499 preserved specimens from various vendors and collaborators (Table S2). Species-level 500 identification was generally unambiguous. However, relationships among Neotropical Junonia 501 are not well-resolved and the limited molecular data available do not cleanly support current 502 designations [36-38]. Two recognized species, J. evarete and J. genoveva, have large ranges 503 with extensive overlap and many variable color forms, including both brown and blue. We 504 therefore described three Neotropical specimens as belonging to the J. evarete species 505 complex to avoid accidental misidentification. Available diagnostic details, including ventral 506 antenna club color and full collection details, are in Table S2. 507

508 Optical Imaging

509 Scales were laid on glass slides. Optical images of scales were taken with a Keyence VHX-

- 510 5000 digital microscope (500-5000x lens). For refractive index matching, we used immersion oil
- 511 (nD=1.56) from Cargille Laboratories (Cedar Grove, New Jersey), and imaged with transmitted
- 512 light. Scales were dissected by hand using a capillary microinjection needle. Whole wings were
- also imaged on the Keyence VHX-5000, using the 20-200x lens.
- 514

515 Microspectrophotometry

For reflectance spectra, individual scales were laid flat on a glass slide, with the adwing surface 516 facing up. We collected spectra of the adwing surface with an Ocean Optics Flame-S-UV-Vis-Es 517 spectrophotometer mounted on a Zeiss AxioPhot reflected light microscope with a 20x/0.5 518 519 objective and a halogen light source. Measurements were normalized to the reflectance of a 520 diffuse white reference (BaSO₄). Data were recorded with SpectraSuite 1.0 software with 3 521 scans to average and a boxcar width of 7 pixels. The software wizard determined optimal 522 integration time from the reference sample; time was generally about .007 seconds. Spot size 523 was roughly circular, 310 µm in diameter, and centered on the scale. We processed spectra in 524 RStudio 1.0.153 with the package 'pavo,' version 0.5-4 [39]. We first smoothed the data using 525 the procspec function with fixneg set to zero and span set to 0.3. We then normalized the data 526 using the "minimum" option of the *procspec* function, which subtracts the minimum from each 527 sample. Because we use a diffuse standard and scales are specular, raw spectra overestimate 528 reflectance. We therefore followed [8] in dividing spectra by a correction factor. We used a 529 smaller correction factor of only 2.5, because in our setup the scale does not fill the full field of 530 view. Absorption spectra from scales submerged in index-matched oil were collected and 531 processed similarly, but under transmitted light with an integration time of 0.01 seconds, and 532 without the "minimum" option.

533

534 Helium Ion Microscopy

Surface imaging by HIM provides increased depth of field and enhanced topographic contrast 535 536 compared to Scanning Electron Microscopy for a range of biological and other materials [40], 537 including butterfly wing scales [41]. Samples were prepared for HIM by laying the wing on a 538 glass slide with the region of interest facing down, wetting with ethanol, and freezing with liquid 539 nitrogen. We then promptly cross-sectioned the wing through the region of interest with a new 540 razor blade. After the sample warmed and dried, we used a capillary microinjection needle to 541 transfer individual cut scales onto carbon tape. Scales were placed overhanging the edge of a 542 strip of carbon tape, with one end pressed into the tape. We optically imaged the tape strip as a 543 color reference and then transferred the tape to the vertical edge of a 90° stepped pin stub (Ted 544 Pella #16177). While non-conductive samples can be imaged by HIM using low energy 545 electrons for charge neutralization, we found that the unsupported overhanging edges of our 546 scales tended to bend due to local charging [42]. We thus sputter coated with 4.5 - 13 nm of Au-547 Pd using a Cressington 108auto or Pelco SC5. Images (secondary electron) of the sectioned 548 scales were acquired with a Zeiss ORION NanoFab Helium Ion Microscope using a beam 549 energy of 25 keV and beam current of 0.8 - 1.8 pA (10 µm aperture, spot size 4). We then used 550 the line measurement tool in ImageJ software to measure lamina thickness from the 551 micrographs. We corrected measurements for slight variations in working distance not accounted for by the software scale bar, using $T_{correct} = (T_{raw})/9058 \ \mu m \ x \ d \ \mu m$, where d is the 552 553 measured working distance and 9058 µm is the reference working distance. Thickness of 554 female J. westermanni scales was not measured because specimens were unavailable. 555

556 Even with vertical mounting, the sectioned surface of the scale was not always perfectly

557 perpendicular to the direction of the imaging beam, largely due to the scales' tendency to curve.

- 559 erroneous tilt could cause systematic underestimation of thickness. We therefore tilted the
- 560 microscope stage until the scale lamina was perpendicular at the measurement site, as
- 561 diagnosed by observing an inflection point in lamina curvature (i.e. a switch between the upper
- and lower surfaces being visible). Thickness was only measured at visible inflection points (Fig.
- 563 S3 B-D). We performed a tilt calibration to test the precision of our inflection point criterion and
- 564 determined that an inflection point was only visible if the sample was within 4-5° of
- 565 perpendicular. Since erroneous tilt is limited to 5° , thickness underestimation is limited to 1 nm.
- 566 Slight overestimations are likely, due to the sputter coating.
- 567

568 The sectioned scale shown in Fig. 1A was milled using the gallium ion beam of the Zeiss 569 ORION NanoFab (beam energy 30 keV, beam current 300 pA).

- 570
- 571 Analyses
- 572 Statistical analyses were conducted in R 3.2.2. For Fig. 8 A-B, specimens were grouped
- 573 following the largest natural breaks in the data for two metrics, mean thickness and weighted 574 average reflected wavelength, which were in good agreement.
- 575
- 576 Modeling film thickness
- 577 We modeled the reflectance from chitin thin films as previously described [27], including
- 578 integrating reflectance for values of θ from zero to the maximal angle of illumination (i.e.
- averaging reflectances to simulate the inverted cone of light collected by the objective lens used
- 580 in microspectrophotometry, given its numerical aperture). Specifically, since our objective had
- 581 NA=0.5, we calculated reflectance over values of θ from 0 to 30°, multiplied by $2\pi\theta$, and then
- 582 averaged over the cumulative circular surface area. For the model with Gaussian thickness
- 583 distributions, we followed [15] using n=400 observations from the simulated thickness 584 distribution.
- 585

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1 Figure Legends

2 Figure 1: The lamina of a typical butterfly scale functions as a thin film reflector. (A) Colorized 3 helium ion micrograph of a nymphaline scale, with a window milled using a gallium focused ion 4 beam. Inset at higher magnification, with labels for general architectural components of a scale 5 (R = ridges, r = cross-ribs, L = lamina). (B) Diagram of reflection and refraction in a chitin thin 6 film. White light enters, reflections are produced at each surface of the film, and reflections of 7 select wavelengths remain in phase as a function of film thickness (T). (C) Experimental 8 disruptions of wing color are associated with altered lamina thickness. In J. coenia, artificial 9 selection for blue color increased lamina thickness in both cover and ground scales. In optix 10 mosaic knockout mutants, certain wing regions have similar thickness increases. This trend 11 recapitulates natural variation in *J. evarete*, where blue butterflies have thick laminae relative to brown individuals. (*** = $p < 1x10^{-7}$) Bars show mean thickness, with error bars of one standard 12 13 deviation. (D) Wild-type J. coenia. (E) Blue artificially selected J. coenia. Image by Edith Smith. 14 (F) J. evarete.

15

16 Figure 2: Structure and color of Junonia cover scales. (A-D) Artificially selected blue J. coenia. 17 (E-H) Wild-type J. coenia. (I-L) J. evarete, blue male from Bolivia. (M-P) J. evarete, brown male 18 from Jamaica. (Q-T) optix mosaic knockout mutant (mKO) in J. coenia. (A,E,I,M,Q) Dorsal 19 hindwing, red arrowhead indicates the characterized scale's location. (B,F,J,N,R) Scale in the 20 orientation it would occupy on the wing, showing the abwing surface of the cover scale. Black 21 scale bars are 25 µm. (B',F',J',N',R') Adwing surface of cover scale, showing the underside of 22 the lamina. (B", F", J", N", R") Dissected scale with arrow showing regions where all ridges and 23 ribs are removed to expose the bare lamina. The lamina is sufficient to create iridescent blue 24 and gold structural colors. (*B^{''}*,*F^{'''}*,*J^{''}*,*N^{'''}*,*R^{'''}*) Scale immersed in fluid with a refractive index 25 matched to chitin, thus eliminating reflection to show only pigmentary color. Blue and brown 26 scales have comparable amounts of a brown pigment. (C.G.K.O.S) Helium ion micrograph of 27 cross-sectioned scale. Each lamina is colorized, with approximate thickness indicated by an 28 adjacent red bar (precise measurements were taken at sites chosen as in Methods). White 29 scale bar is 500 nm and applies to all HIM images. (D.H.L.P.T) Reflection spectra for the adwing 30 surface of disarticulated scales. Solid line is the mean spectrum, and blue envelope is one 31 standard deviation; minimum N=6 spectra per graph. 32

Figure 3: Absorbance spectra show the effect of artificial selection on scale pigmentation. (A) Absorbance measures in J. coenia wild-type (brown), and artificially selected (blue) cover scales show that both have comparable pigmentation (Mann-Whitney U, Table S1). (B) Absorbance of selected J. coenia ground scales is nearly doubled relative to brown wild-type scales. (C) Absorbance does not differ between blue and brown J. evarete cover scales, and is similar to pigmentation in J. coenia cover scales. Plots show mean spectra with envelope of one standard deviation, N=6 spectra per sample.

40

Figure 4: Structure and color of *J. coenia* ground scales. (*A*,*C*,*E*) Wings with red arrowhead
indicating the region from which scales were sampled. (*B*,*D*,*F*) Scale in abwing orientation, i.e.
ridges facing up. (*B'*,*D'*,*F'*) Scale in adwing orientation, i.e. lamina facing up. (*B'*,*D''*,*F''*) Scale
immersed in fluid with a refractive index matched to chitin, thus eliminating reflection to show

only pigmentary color. (*A-B*") *J. coenia* artificially selected ground scales. (*C-D*") *J. coenia* wild type ground scales. (*E-F*") optix mKO mutant ground scales. Scale bars are 25 μm.

47

48 Figure 5: Absorbance spectra show the effect of *optix* knockout on scale pigmentation across 49 wing pattern elements. All comparisons are between wild-type and mutant regions in the same mosaic wing. (A) optix mutation decreases absorption in cover scales from the main background 50 51 region of the dorsal hindwing (Fig. 2Q). (B) Absorbance of ground scales from the dorsal 52 hindwing (Fig. 4E) is increased in mutant scales. (C) Absorbance increases with optix mutation 53 in ventral hindwing cover scales (Fig. 6A.C). (D) In the dorsal discal bars, (Fig. 6E,G) optix 54 regulates a switch between orange and brown pigment. Plots show mean spectra with envelope 55 of one standard deviation, N=6 spectra per sample. Differences for all four comparisons are 56 statistically significant (Mann-Whitney U, Table S1).

57

58 **Figure 6:** Effects of *optix* mutation on structure and color of *J. coenia* cover scales vary by wing 59 region. (*A*,*C*,*E*,*G*) Wings with red arrowhead indicating the region from which scales were

sampled. (B,D,F,H) Scale in abwing orientation. (B',D',F',H') Scale in adwing orientation.

 $(B^{"}, D^{"}, F^{"}, H^{"})$ Scale immersed in fluid with a refractive index matched to chitin to show only

62 pigmentary color. (A-B") Mutant cover scales from an *optix* mKO ventral hindwing have

63 increased melanin. (*C-D*") Wild-type cover scales from an *optix* mKO ventral hindwing. (*E-F*")

Mutant cover scales from an *optix* mKO forewing discal bar have lost orange pigment, gained brown pigment, and increased lamina thickness, resulting in a shift to blue. (*G-H*") Wild-type cover scales from the *optix* mKO forewing discal bar have both orange pigment and an orange lamina structural color. Scale bars are 25 μ m.

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69 Figure 7: Lamina structural colors are an important component of overall wing color throughout 70 Junonia. (A) Phylogeny of color variation in Junonia (based on [22]). Arrowheads indicate the 71 color regions sampled for scale characterization. WT = wild-type. AS = artificial selection. mKO 72 = optix mosaic knockout mutant. DS = winter/dry season form. WS = summer/wet season form. 73 J. evarete variants are from different locations. Female J. hierta image is © Krushnamegh Kunte, NCBS. (B,D,F,H,J,L) Dorsal hindwing, arrow indicates the characterized scales' location. 74 75 (C,E,G,I,K,M) Abwing surface of cover scale. (C',E',G',I',K',M') Adwing surface of cover scale, 76 showing lamina color. (C".E".G".I".K".M") Scale immersed in fluid with refractive index matched 77 to chitin, thus showing only pigmentary color. (B-C") J. hierta basal aura scales are 78 unpigmented and appear blue due to lamina structural color. (D-E") J. hierta has coordinated 79 yellow pigment with a structurally yellow lamina. (F-G") Neutral light grey of J. atlites is 80 exclusively structural, due to additive color mixing of the multicolored lamina. (H-I") Blue scales 81 of dry season P. octavia are structurally colored since no pigment is present. (J-K") Wet season 82 P. octavia has discordant red pigment and blue lamina colors. The red pigment is localized in 83 the ridges and cross-ribs on the abwing surface of the scale, while blue light from the lower lamina spills through the windows between them. (L-M") The red band in dry season P. octavia 84 85 is a more saturated red than in (J), due to the combination of both more red pigment and a 86 structurally reddish lamina. 87

88 Figure 8: Lamina thickness predicts lamina color across the Junonia phylogeny. (A) Thickness measures for the regions indicated in Fig. 7A vary continuously over a 170 nm range (minimum 89 N=3 scales and 12 measures per specimen). To visualize the relationship between thickness 90 and color, we clustered similar specimens into five color groups described as gold, red, indigo, 91 92 blue, and green. Thickness is significantly different between groups (ANOVA and Tukey's HSD, 93 $p < 2x10^{-6}$). J. atlites, which has rainbow color gradients in each individual scale, has especially 94 variable thickness, with measures overlapping the ranges of all color groups. Boxplots show 95 median and inner quartiles, whiskers extend to 1.5 times the interquartile range, outliers are 96 shown as points, and notches show 95% confidence interval of the median. (B) Color groups 97 are associated with different reflectance profiles. Lines are mean spectra and envelopes show one standard deviation. N=6 spectra per specimen from panel A; clusters follow panel A. (C-G) 98 99 Adwing reflectance spectra for representative individual specimens with increasing lamina 100 thicknesses. The color sequence follows Newton's series. Solid line is the mean spectrum and 101 the envelope is one standard deviation; N=3 scales and 6 spectra per graph. 102

103 **Figure 9:** Absorbance spectra show variable pigment concentrations and identities among

104 representative *Junonia* butterflies. Spectra were taken from cover scales from the regions

shown in Fig. 7A. (A) J. hierta pigmentation varies by wing region (Fig. 7B-E"). (B) Extent of red

pigmentation is the most important driver of color difference between seasonal morphs of *P*.

107 octavia (Fig. 7H-M"). (C) Pigment absorbance differs by sex in J. orithya. (D) J. atlites scales
 108 lack pigmentation (Fig. 7F-G"). Plots show mean spectra with envelope of one standard

109 deviation, minimum N=6 spectra per sample.



Fig. 1



Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9