The evolution of structural colour in butterflies

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Abstract

Butterflies display some of the most striking examples of structural colour in nature. These colours originate from cuticular scales that cover the wing surface, which have evolved a diverse suite of optical nanostructures capable of manipulating light. In this review we explore recent advances in the evolution of structural colour in butterflies. We discuss new insights into the underlying genetics and development of the structural colours in various nanostructure types. Improvements in -omic and imaging technologies have been paramount to these new advances and have permitted an increased appreciation of their development and evolution.

Introduction

In nature some of the most conspicuous colours come not from pigments but instead from physical structures within the integument of some animals and plants [1,2]. These structures, on the order of a few hundred nanometres or less, selectively reflect light to create a vivid repertoire of colours known as 'structural colours' (**box 1**). A diverse range of organisms produce structural colours, including birds [3], plants [2], fish [4] and invertebrates [5–8]. Structural colours have evolved to fill diverse roles including camouflage [9] and intra- [10] and interspecific communication [11].

Butterflies have long been subjects of study for their pigmentary colours, and they have also been firmly at the forefront of our understanding of structural colouration. Vivid displays of structural colours are found across the butterfly phylogeny and utilize diverse optical mechanisms (**Figure 1**)[12••]. Nevertheless, all butterfly structural colours originate from tiny (approximately 100μm long) scales which adorn the wing surfaces. Each scale is a flattened cuticular extension, with an intricate upper lamina (layer) of parallel ridges connected transversely by crossribs which extend down through a lumen to a flat lower lamina (**Figure 1a**)[13,14]. Remarkably, each highly-intricate scale is a result of the expansion of a single cell [14]. Pigments are also crucial for scale colour, with spectral purity of structurally coloured scales achieved by including pigments, such as melanin, into the scale structure [15,16••]. Additional mechanisms such as scale stacking can further modify and enhance the colour [17].

Varied ecological pressures and the underlying malleability of the arthropod cuticle has contributed to the evolution of diverse structural colour mechanisms in butterflies [18,19]. While the vivid blue scales of *Morpho* are a classic example of a complex scale reflector [20], bright colouration also occurs in other species, such as metalmarks (Riodininae), which

46 have a comparatively simple optical nanostructure [12••]. Indeed, even within a single genus

47 several divergent optical nanostructures may be found [21], highlighting the flexibility of the

48 underlying cellular mechanisms governing the evolution and development of structural

49 colour.

50   In this review we aim to highlight the recent advancements in understanding butterfly

51 structural colour evolution from a genetic and developmental perspective. We group butterfly

52 optical nanostructures into three major types based on their location within the scale and

53 discuss each one in turn.   The seminal works of Ghiradella [22–24] have served as a

54 foundation for understanding both the optics of butterfly structural colours as well as their

55 development *in vivo*. Following a period of relatively little activity, the last five years has

56 seen tremendous advancements, including new insights into the genes controlling structural

57 colour [16••,25••,26]. These insights have been aided by the molecular revolution of the past

58 decade, with tools such as CRISPR-Cas9 pioneering our understanding of the underlying

59 genetics. Furthermore, advances in microscopy technologies have reaffirmed our appreciation

60 for wing scale formation at the nanoscale.

61

62 **Surface nanostructures – ridge reflectors**

63 Several features of the upper scale surface can be modified into reflective structures (**Figure**

64 **1**). The parallel ridges, which all butterfly scales possess, can become elaborated into a series

65 of layered lamellae, creating an intense reflection through constructive inference (**Box 1**)

66 [24]. Remarkably, this can result in around 75% of the light being reflected in cases such as

67 the blue *Morpho* scale [27]. However, the light reflected by these structures is not solely

68 limited to the human visible spectrum, for example UV reflectance is present in species such

69 as *Eurema lisa* [24]. Ridge reflectors have evolved independently in multiple lineages and in

70   some cases several times within a lineage, for example in *Heliconius*, driven by convergent

71   evolution for mimicry [28•].

72   Ridge reflectors form during wing scale development, with alternating air and chitin layers

73   attaining optically precise spacing through drying post-eclosion [19]. Electron micrographs

74   by Ghiradella [23] led her to propose that ridge multilayers form by elastic buckling of the

75   cuticle in response to intracellular stresses. Notably though, this hypothesis draws on a

76   purportedly similar process Locke [29] proposed for the formation of taenidial folds in the

77   tracheae of insects; where cuticle folds formed through mechanical buckling [23,29].

78   Subsequent re-evaluation of taenidial fold formation now suggests a more active role of the

79   actin cytoskeleton and chitin synthases, rather than mechanical buckling [30,31]. While much

80   work is still needed on the development of ridge reflectors it is plausible that these

81   nanostructures also require an active role for the actin cytoskeleton and chitin synthases in

82   their formation. Indeed, recent studies have highlighted the importance of the actin

83   cytoskeleton in wing scale development [32,33].

84   Brien *et al.,* [25••] present some of the first insights into the genetic basis of ridge reflector

85   evolution. Using phenotypic analyses of crosses between iridescent and non-iridescent races

86   of *Heliconius erato*, they show that structural colour is a quantitative trait not associated with

87   any of the major effect loci previously linked to pigmentary wing patterning in *Heliconius*.

88   Interestingly, a moderate effect locus was detected on the sex chromosome, reminiscent of

89   much older work [34] showing that differences in ridge-reflector-based UV colouration

90   between *Colias* species is due to a sex-linked locus.

91   Perhaps unsurprisingly, given the precise nanostructures involved, ridge reflector

92   formation shows sensitivity to developmental conditions in many species [35,36•]. Together,

93   with a sex-linked genetic architecture, this has interesting implications for the evolution of

94   ridge reflector colour as a sexual signal. Sex-linkage would aid the evolution of sexually

95   dimorphic colour, important if the sexes have different evolutionary optima; while condition

96   dependence could increase the information content of these colour signals to potential mates.

97

98   **Lower lamina reflectors**

99   The scale's lower-most cuticle layer can function as a thin-film capable of scattering light

100   (**Box 1**). Often this nanostructure is accompanied by pigments which function as 'optical

101   filters' by absorbing wavelengths of light to enhance the structural colour [15]. Lower lamina

102   reflectors are often considered an optically and developmentally simple mechanism of

103   achieving structural coloration. From an optical perspective, the lower lamina is a simple

104   interface of contrasting refractive indexes which can scatter light [16••]. This optical process

105   is reminiscent of that present in ancient Lepidopteran structurally coloured scales from the

106   Jurassic (albeit possessing a slightly different scale morphology of a fused lower and upper

107   lamina) [37•]. Lower lamina reflectance is an "evolutionarily accessible" optical phenotype

108   because all scales possess a flat lower lamina. As such, this removes the need to evolve

109   complex ridge or crossrib structures, which may impact on other intrinsic scale properties

110   including thermoregulation, aerodynamics, hydrophobicity or self-cleaning [38–40].

111   Artificial selection experiments in both *Bicyclus anyana* [41] and *Junonia coenia* [16••]

112   have demonstrated that within a short period of time (6 generations and 12 generations,

113   respectively) the colour produced by lower lamina reflectors can be considerably modified

114   (**Figure 2**). Selection in both these species resulted in changes in thickness of the lower

115   lamina, demonstrating the ease with which this scale element can be sculpted. Indeed, in both

116   *Bicyclus* and *Junonia,* between-species variation in structural colour appears to have evolved

117   through tuning the lower lamina thickness. This reinforces suggestions that the lower lamina

118   is a common evolutionary target for selection in diverse species and that relatively small,

119   quantitative changes to just one part of the scale architecture can have profound micro- and

120   macroevolutionary consequences [16••,42].

121   　　Zhang *et al.* [26] demonstrated that knockouts of the developmental patterning gene *optix*

122   also results in a brown to blue colour change in *J. coenia*. Thayer *et al.* [16••] showed this

123   switch in colouration through *optix* deletion was the result of lower lamina thickening,

124   recapitulating what occurs in both the artificial selection experiments and within natural

125   populations (**Figure 2**). This hints at the possibly of *optix* and its associated gene regulatory

126   networks being the target for selection in naturally evolving, structurally-coloured

127   populations. Interestingly, Thayer *et al.,* [16••] also showed that in other coloured scales of

128   *Junonia*, such as gold scales, the lower lamina was tuned to a thickness to produce a

129   complementary reflected wavelength. By regulating lamina thickness, optix and its

130   downstream targets could control the range of wavelengths produced through thin film

131   interference [16••].　Future work on the downstream targets of *optix* should aid our

132   understanding of the regulatory networks and cellular control of structural colouration.

133   　　*Optix* also plays a conserved role in pigmentation and scale structure in butterflies

134   [26,43]. This suggests that the evolution of pigments and some nanostructures may be more

135   intertwined than previously thought and may be controlled by a few 'adaptive hotspots' such

136   as *optix* [26]. Indeed, several recent studies have highlighted a regulatory link between both

137   scale structure and pigments [16••,26,44,45••,46]. Future studies should continue to address

138   the link between scale structure, pigments and nanostructures.

139   **Internal nanostructures – lumen multilayers and photonic crystals**

140   Diverse nanostructures may be present within the scale lumen, ranging from simple

141   multilayers to complex 3D photonic crystals (**Figure 1**) [21]. While often considered as

142   highly-ordered structures, lumen nanostructures may also encompass a degree of disorder, for

143   instance in the lumen multilayers of *Ornithoptera* (birdwing) scales [47].

144   Focusing on photonic crystal formation, Ghiradella *et al.* [22] provided the first insights

145   into the role of the smooth endoplasmic reticulum (SER) as a templating network for highly

146   ordered chitin deposition within the developing scale. Subsequently, electron microscopy

147   deduced a gyroid structure of the photonic crystals, consistent with a process of templating by

148   intracellular membranes **(Box 1)**[48]. Recently, techniques such as SAXS and X-ray

149   tomography have permitted deeper understanding of not just the optical properties of such

150   highly-ordered structures but also the underlying cellular processes guiding their formation

151   [49,50]. Indeed, recent insights by Wilts *et al.* [50] showed that the photonic crystals in the

152   scales of *Thecla opisena* were arranged in a size gradient over the scale's proximo-distal axis.

153   Investigations over this gradient demonstrated a time-dependent growth process of crystal

154   formation and that crystals do not form in the same orientation. The authors concluded that

155   rather than a pre-folding template of SER, gyroid formation more likely involves

156   simultaneous membrane templating and chitin deposition.

157   Characterising the optical properties of diverse luminal structures while considering the

158   underlying phylogenetic relationships has provided deeper insights into the evolution of such

159   structures. For example, gyroid structures and multilayers are found in closely-related species

160   of Cattlehearts (*Parides*). This may suggest an underlying commonality in the developmental

161   pathways and cellular effectors governing such structures. Wilts *et al.* [21] suggest that minor

162   deviations in developmental parameters may shift scale cell fate between multilayers or

163   gyroid structures within the scale lumen.

164   Similarly, Ren *et al.,* [12••] used comparative studies across butterfly families to

165   understand the diversity of scale ultrastructures underlying metallic reflectance. Scales of

166 Lycaenids were able to produce metallic, silver scales through an internal multilayer-type

167 architecture. The authors suggested that differences in the number of chitin layers and

168 perforation of the upper lamina could have led to a transition from blue to silver scales. The

169 next major breakthrough will be in identifying the molecular switches involved in these

170 evolutionary transitions between nanostructure types.

171

172 **Beyond butterflies – evolutionary insights from other systems**

173 We are beginning to gain an appreciation of the underlying evolutionary development of

174 structural colours in many systems. Knowledge from these systems may present unique

175 insights into the evolution of structural colour in butterflies. For example, photonic crystals

176 within weevils evolved only once, involving a transition from a hollow scale to one in which

177 the spongy network within the lumen becomes increasingly ordered [51•]. This bears

178 similarity to the evolution of photonic crystals in butterflies, which likely required a transition

179 from a hollow lumen to one with an increasingly ordered multilayer [21]. In weevils,

180 photonic crystal evolution was associated with shifts in feeding strategy and the need for

181 crypsis [51•], raising the question as to whether similar ecological pressures also shaped

182 butterfly structural colour evolution.

183 While much focus has been placed on structural colouration in animals, diverse structural

184 colours are also present in the petals, leaves and fruits of plants [2]. Remarkably, despite

185 disparity in cell types and cuticular materials used for nanostructures, many of the underlying

186 cellular principles are likely conserved between animals and plants. For example, multilayer

187 reflectors in fruits form through microtubule cytoskeleton guided deposition of cellulose into

188 layered, helicoidal structures [2]. In butterfly scales, the actin cytoskeleton guides chitin

189 deposition on the ridges and may also play a role in nanostructure formation [32].

190 Additionally, light-reflecting ridges on the epidermal layer of petals form through buckling of

191 the cuticle [52], similar to the suggested mechanism of ridge reflector formation in

192 butterflies, which could involve stress-mediated buckling of the chitin cuticle [24]. Overall,

193 different systems may give universal insights into the underlying principles governing

194 structural colour evolution.

195

Conclusions

197 Butterflies have evolved a diverse suite of optical nanostructures to produce vivid displays of

198 structural colour. Advances in molecular genetic tools, such as CRISPR, in addition to much

199 improved resolution of microscopy techniques have provided tantalising new insights into the

200 evolution and development of optical nanostructures in butterflies. Whilst much of the

201 underlying cellular dynamics and developmental pathways remain unknown, the next few

202 years is set to see major advances in our knowledge of structural colour evolution. By its

203 very nature, the study of structural colours is a highly interdisciplinary topic, involving

204 collaborations between physicists, material scientists and biologists alike. Such collaborations

205 will not just give unique insights into evolutionary processes governing structural colouration

206 but will open the doors to a whole range of biomimetic technologies, taking inspiration from

207 the photonic structures which evolution has been finely sculpting over millennia.

208

209

217 **Conflict of interest statement**

218 The authors declare no conflict of interest.

219

220 **References and recommended reading**

221 Papers of particular interest, published within the period of review, have been highlighted as:

222 • of special interest

223 •• of outstanding interest

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Special interest

•• Ren et al., 2020

This study provides insights into how metallic colouration in diverse butterfly families has convergently evolved. Using analyses of spectral properties as well as investigations of scale ultrastructure the authors gain insights into the underlying mechanisms governing metallic colour production, demonstrating a convergence in optical principles such as spatial colour mixing and an unperforated upper lamina. They describe several different nanostructure modifications resulting in the production of metallic colouration, including a potential transition from a blue producing multilayer to a silver producing multilayer in Lycaenidae.

•• Thayer et al., 2020

This article provides insights into the structural and genetic control of lower lamina reflectance. Using investigations of scale architecture in artificially-selected *Junonia coenia* the authors show changes to lower lamina thickness is responsible for dramatic shifts from brown to blue colour. The authors further show that CRISPR-Cas9 deletion of *optix* results in

396   changes to lower lamina thickness, also resulting in blue colouration. They further show that

397   natural variation in colour throughout the *Junonia* genus is controlled by lower lamina

398   thickness, linking scale structure alterations and evolution.

399   •• **Brien et al., 2018**

400   The authors use phenotypic crosses of iridescent and non-iridescent races of *Heliconius erato*

401   to determine the underlying genetic control of structural colour. They show that in *Heliconius*

402   *erato* structural colour is a quantitative trait, controlled by a moderate number of loci. In

403   addition, they show evidence for structural colour being sex-linked.

404   • **Parnell et al., 2018**

405   This paper characterises the ultrastructures underpinning structural colouration in *Heliconius*

406   butterflies. Within the genus, ridge reflectors are responsible for producing structural colour

407   with differences in ridge overlap, curvature and density controlling the brightness.

408   • **Fenner et al., 2019**

409   This study explores UV structural colouration in two closely related species of Dogface

410   butterfly (Pieridae). Using changes to larval diet the authors explore the impacts on the

411   ultrastructures responsible for structural colouration and find plasticity in structures

412   governing UV colour production.

413   • **Zhang et al., 2018**

414   This study describes the architecture of fossil wing scales from the Jurassic period, which

415   have an overall scale morphology similar to extant basal Lepidopterans. Through scale

416   ultrastructure measurements and optical modelling, the authors determine that the scales

417   would have produced a metallic colouration, similar to those produced by many extant basal

418   Lepidopterans today.

419 •• **Matsuoka and Monteiro, 2018**

420 This article demonstrates a link between scale colour and morphology. By harnessing

421 CRISPR-Cas9 the authors show that knockouts of melanin pathway genes in the butterfly

422 *Bicyclus anyana* not only cause effects on pigmentary colour but also various effects on scale

423 structure.

424 • **Seago et al., 2019**

425 This study analyses the diversity of photonic nanostructures in weevils to gain an

426 understanding of the evolution of photonic crystals. The authors predict that structural colour

427 evolved only once in weevils and required a transition from a hollow scale with a spongy

428 network to a photonic crystal with gyroid symmetries.

429

430 **Additional elements**

431 **Box 1. Optical nanostructures in butterflies.**
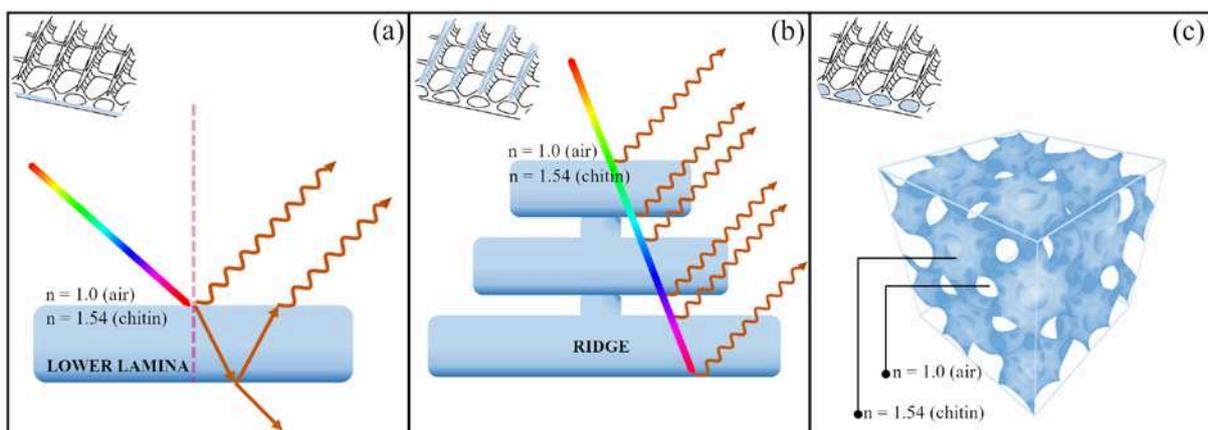
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433 Diverse optical mechanisms in butterflies are governed by the same basic principle – to

434 generate structural colour light must pass through materials of differing refractive indexes

435 (n). The cuticle of butterflies is composed of chitin, which has a refractive index of 1.54 (at

590 nm). To attain a contrasting refractive index, butterfly nanostructures also encompass air spaces which have a refractive index of 1.0 [3].

Thin films are the simplest optical mechanism present in butterfly scales. The lower lamina of the scale can form an optically precise thin-film reflector (a). Light waves are reflected by both the upper and lower surface of the lamina. When these reflected light waves are in phase, so that they peak at the same time, they can interfere constructively to accentuate particular wavelengths of light. The colour produced depends on the thickness of the chitin layer as well as the viewing and incidence angles [16••].

Thin film reflectors may also be stacked upon each other to form a multilayer-type nanostructure [3]. Examples of multilayers in butterfly scales may be found on both the upper surface as well in the lumen. The same principle of differences in refractive indexes applies, with the alternating air and chitin layers forming the contrasting indexes necessary to generate the phase change required for light interference. In the example of the ridge reflector multilayer (b) light is reflected and transmitted by each layer, producing a colour through constructive interference. Changing the number of layers governs the intensity of reflection while modifying the spacing of the air and chitin layers changes the colour produced [1].

3D photonic crystals in butterflies are highly-ordered, repeating structures on the order of the wavelength of light (c). As the light waves enter the structure, only certain wavelengths of light may propagate, producing a specific colour [3]. This reflectance of a certain wavelength occurs regardless of the angle of light entering. Such structures are found in the lumen of butterfly scales, as highly periodic 3D crystals of chitin. One particularly interesting type is the 'gyroid' structure, having a nanoscale bicontinuous structure surrounding air spaces, which can be described by a mathematical concept in which a curved structure is maximally connected through the smallest surface area possible [1].
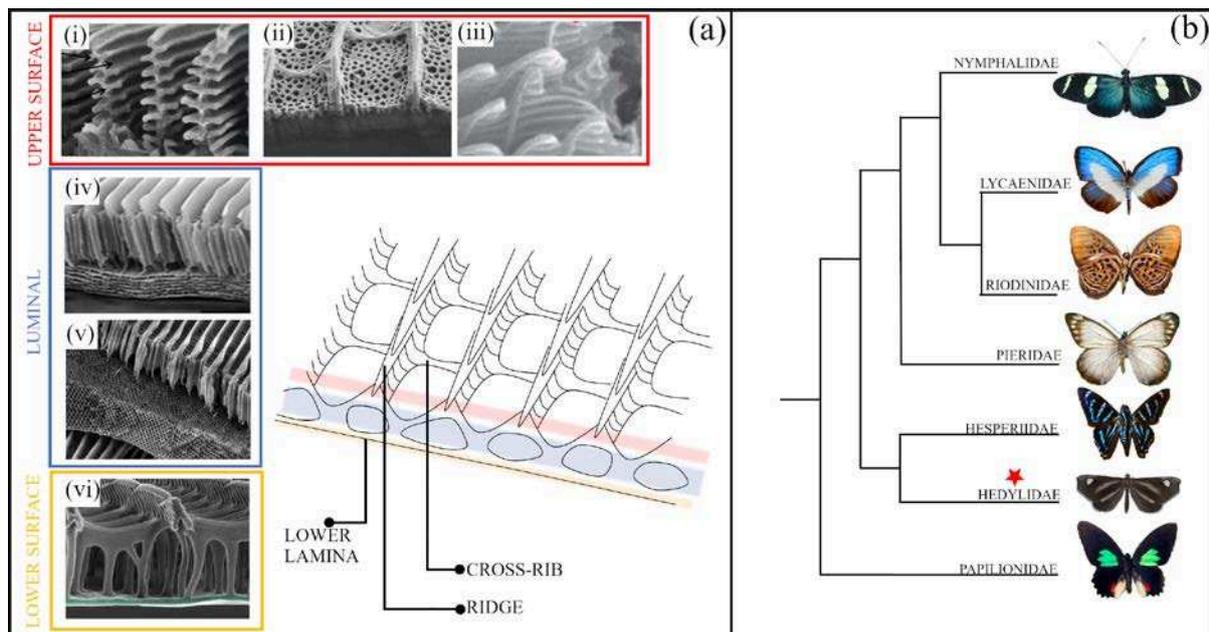
Figures

Figure 1. The diversity of structural colour in butterflies. (a) Morphologically diverse optical nanostructures have evolved through modifications to a basic scale 'ground plan'. Modifications can be grouped depending on their location within a scale (upper surface, lumen, lower surface). Several features of the upper surface may be modified, including the ridges (i), inter-ridge cuticle (ii), and microribs (iii). The hollow lumen may also contain nanostructures, including multilayers (iv) and highly-ordered photonic crystals (v). Changes in cuticle thickness of the lower lamina can produce lower lamina reflectors (vi). (b) Examples of structural colour in the major families of Papilionoidea (butterflies). Structural colour remains undescribed for the Hedylidae (star), but are presumed present due to the existence of white scales in some species. Phylogeny drawn from [53]. SEM images in (a) reprinted from: (i) Potyrailo et al., [54] (iv, v) Wilts et al., [21], (vi) Thayer et al., [16••]. All made available under a CC-BY 4.0 license (https://creativecommons.org/licenses/by/4.0/). Image (ii) reprinted with permission from Trzeciak et al., [55] © The Optical Society. Image (iii) used with permission of The Royal Society (U.K.), from [56]; permission conveyed through Copyright Clearance Center, Inc. Images of Lycaenidae

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Figure 2. Insights into the genetic and structural control of lower lamina structural colour in *Junonia coenia* through artificial selection experiments and *optix* knockouts. Wildtype *J. coenia* hindwings are predominantly brown in colour. A blue colouration is observed in wings which have been artificially selected for blue scales and in CRISPR-Cas9 knockouts of the developmental patterning gene *optix*. Changes in the observed colour of artificially selected and *optix* knockout wings can be seen in reflectance plots, with a shift to the blue end of the spectrum compared to wildtype brown scales. Helium ion microscopy (HIM) cross-sections showing scale morphology, with the lower lamina false coloured in green. The lower lamina is considerably thicker in *optix* knockout wings and artificially selected wings compared to wildtype wings. Images reproduced from Thayer *et al.,* [16••] with permission and under a CC-BY-4.0 license (https://creativecommons.org/licenses/by/4.0/).