Springtail coloration at a finer scale: mechanisms behind vibrant collembolan metallic colours

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The mechanisms and evolution of metallic structural colours are of both fundamental and applied interest, yet most work in arthropods has focused on derived butterflies and beetles with distinct hues. In particular, basal hexapods—groups with many scaled, metallic representatives—are currently poorly studied and controversial, with some recent studies suggesting either that thin-film (lamina thickness) or diffraction grating (longitudinal ridges, cross-ribs) elements produce these colours in early Lepidoptera and one springtail (Collembola) species. Especially the collembolan basal scale design, consisting of a single lamina and longitudinal ridges with smooth valleys lacking cross-ribs, makes them an interesting group to explore the mechanisms of metallic coloration. Using microspectroscopy, Raman spectroscopy, electron microscopy and finite-difference time-domain optical modelling, we investigated scale colour in seven springtail species that show clear metallic coloration. Reflectance spectra are largely uniform and exhibit a broadband metallic/golden coloration with peaks in the violet/blue region. Our simulations confirm the role of the longitudinal ridges, working in conjunction with thin-film effects to produce a broadband metallic coloration. Broadband coloration occurs through spatial colour mixing, which probably results from nanoscale variation in scale thickness and ridge height and distance. These results provide crucial insights into the colour production mechanisms in a basal scale design and highlight the need for further investigation of scaled, basal arthropods.

1. Introduction

Colours can be divided into two broad categories. Pigments produce colours by light-selective absorption of some light wavelengths while nanostructured tissues produce structural colours by light scattering and diffraction. In many arthropods, these structures are found in cuticular scales that produce an intense metallic and iridescent coloration [1]. Scales are thought to have evolved from modified setae and typically consist of a chitinous basal lamina (either a single lamina or separated into upper and lower laminae with an intermediate air lumen) adorned with longitudinal, continuous ridges. The valleys between
these ridges can be smooth, or the ridges can be connected by cross-ribs that can be overlaid by herringbone crests [2,3].

Structural colours in insect scales [4,5] (e.g. by thin films [6–8], photonic crystals [9,10], multilayers [6]) are well studied. However, most of these studies have focused on scales of extant, derived butterflies and beetles with distinct hues (i.e. colours of the rainbow), although there is increasing interest in the colour production mechanisms of scales with a broadband, metallic reflection (especially in butterflies). These studies reveal the importance of thin-film effects of upper/lower laminae and of the cross-ribs [8,11–17]. By contrast, colour production of metallic scales of early Lepidoptera and basal arthropods which have many scaled representatives (e.g. jumping bristletails (Archeaognatha), silverfish (Zygentoma), springtails (Collembola)) remains largely unknown. The light reflectance of silverfish scales has been attributed to the underlying integument, though a contribution of melanized ridges was also found [18,19]. It is only in more recent studies that the colour mechanisms in early Lepidoptera have been explored. A study of both fossil and extant members of the Micropterigidae (Lepidoptera), which have a single lamina with longitudinal ridges and cross-ribs, revealed a remarkably conserved pattern of scale nanostructures [20,21], with the striking golden coloration resulting from a combination of a thin film (scale thickness) and a diffraction grating through cross-ribs and longitudinal ridges [21]. Kilchoer et al. [22] found that the thickness of the melanin-pigmented scale (thin film) was critical to color variation, while D’Alba et al. [20] demonstrated that the nanoscale cross-ribs between the longitudinal ridges produced golden coloration through a diffraction grating (most likely in an interaction with scale lamina thin-film effects). This last study also investigated the colour of golden scales of the springtail (Collembola) Tomocerus vulgaris, whose scales are characterized by a single lamina and continuous, longitudinal ridges with clear valleys lacking cross-ribs. A three-dimensional model incorporating both scale laminae and longitudinal ridges produced a consistent broadband golden coloration, while thin-film effects alone were insufficient. This suggests an interesting role for the ridges in metallic colour production. It is therefore clear that the main colour mechanisms in basal scale design are thin films and diffraction gratings, but that their relative contribution and importance remains elusive. This needs to be further clarified for a full understanding of early scale colour evolution, particularly in non-Lepidopteran taxa. For this, the Collembola scale design is most suitable as it allows investigation of the importance of the scale lamina and longitudinal ridges, in the absence of cross-ribs.

Here, we explore the colour-producing mechanisms of collembolan scales. A key component of the soil fauna, they are basal hexapods [23], of which some species are fully covered in body scales [24] that display metallic, iridescent coloration varying from distinctly golden to golden violet to silvery white/grey and towards dark, black scales (see T. vulgaris). These scales have attracted attention since the early days of microscopy [25,26] and their presence and morphology are considered important taxonomic characteristics [27]. Interestingly, scales have multiple independent origins [27,28] and, in addition to the typical scale design with longitudinal, continuous ridges, certain Collembola species exhibit a unique scale type with interrupted ridges in the form of triangular sail-like structures (spinulate scale type; figure 1) [29]. As ridges have been identified as a crucial colour-producing structure in one species of springtail [20], it is worthwhile investigating the effect of ridge morphology as it is likely to directly impact on colour production.

We selected seven species of springtails to represent colour and scale ridge type variation: Lepidocyrtus cyaneus and Lepidoctenus lignorum (golden violet, spinulate), Cyphoderus albinus and Heteromurus nitidus (silvery white, spinulate), Heteromurus major (silvery grey, spinulate), Pogonognathellus flavescens (silvery grey, continuous) and T. vulgaris (black/golden bands, continuous). We investigated colour production using optical and electron microscopy, Raman spectroscopy, spectrophotometry and optical modelling to improve our understanding of basal scale design and its colour implications. For all species, we predicted that both longitudinal ridges and scale thickness play important roles.

2. Material and methods

To determine essential scale parameters, we used scanning (SEM) and transmission (TEM) electron microscopy. A subset of the SEM and TEM pictures of T. vulgaris have been used in a previous study [20]; for this study, we expanded the imaging, remeasured the scale parameters and increased the number of analysed scales. This ensures an identical methodological approach for all species in the current study and enables direct comparison with previous ones. We used Raman spectroscopy to determine the presence of pigments. We inputted these parameters into three-dimensional models in finite-difference time-domain (FDTD) optical modelling to investigate the contribution of each parameter to colour production. We compared these simulated reflectance spectra with measured spectra obtained by microspectrophotometry to determine the importance of thin-film and diffraction grating effects.

2.1. Sample collection

Individuals were collected from two private gardens and two forest areas in the north of Belgium in the spring and summer of 2018 and 2020 (see electronic supplementary material, table S1). Individuals from T. vulgaris and L. cyaneus originated from a laboratory-reared population in Ghent University. These individuals were kept in plastic containers (18 × 12 × 5 cm for T. vulgaris; 12 × 5 × 5 cm for L. cyaneus) with plaster added to the bottom. Springtails were fed with dried yeast in overabundance and sprayed with water once a week.

2.2. Scale morphology

The entire body of scaled Collembola is covered with scales, including head, thorax and abdomen and in some species extending onto the legs and antennae. In this study, we focused on the scales on the dorsal side of the abdomen (for L. cyaneus we only had scales situated towards the side of the animal for TEM measurements). Using SEM and TEM, we measured five scale parameters that potentially influence colour production: scale thickness, ridge base, ridge height, ridge distance and ridge length (figure 1). For the SEM and TEM protocols [30], see the electronic supplementary material. For some species (e.g. L. cyaneus; see electronic supplementary material, figure S1), we observed structures in between the ridges. As these were not discernible in TEM cross-sections, they were not used in the design of scale models.

2.3. Scale pigment

We used Raman spectroscopy to determine pigment presence and identity. A previous study found melanin in golden scales of
2.4. Scale coloration

We measured the specular reflectance in the visible spectrum (350–700 nm) of the dorsal surface of scales on the body and of single scales on glass slides at normal incidence using an AX10 UV–visible micro-spectrophotometer (CRAIC Technologies, Inc., USA) with Teflon tape as the white standard. Up to two measurements were taken per scale as some scales were too small for multiple measurements. For *T. vulgaris*, scales from golden and dark bands on the body were separately measured. For single scales originating from a black band, the melanin-rich upper half of a scale was measured separately from the lower half. For *T. vulgaris*, *L. lignorum* and *C. albinus* reflectance values below 380 nm were highly erratic and fluctuating, and therefore these measurements were discarded. For *L. cyaneus*, *H. major*, *H. nitidus* and *P. flavescens* measurements were taken using a ×15 objective (10 × 10 µm area); for *L. lignorum*, *C. albinus* and *T. vulgaris* this was performed using a ×10 objective (15 × 15 µm area).

Analysis and visualization of the reflectance spectra were performed in R v. 4.0.2 [32] using the pavo package [33]. For sample sizes, see electronic supplementary material, table S2. We plotted the reflectance spectra of single scales/body scales per species as well as calculated averages ±95% CI. We modelled the reflectance spectra of single scales and body scales in colorspace to investigate the extent of the similarity of the curves (pavo package [33]). We used colorspace based on the segment classification system by Endler [34] as this provides an intuitive visualization in four used colorspace and is especially useful when the visual system of the observer is ultraviolet, short-, medium- and long-wavelength photoreceptors equally spaced segments. This system does not assume a specific Endler [34] as this provides an intuitive visualization in four used colorspace based on the segment classification system by Endler [34] as this provides an intuitive visualization in four.

For *T. vulgaris* [20], we further investigated the presence of melanin in the scales of *T. vulgaris* by mapping across part of a golden and black band and in dorsal scales of *L. cyaneus*. A *Sepia* melanin standard (Sigma-Aldrich) was used as a reference. For the Raman spectroscopy protocol [31], see the electronic supplementary material.

2.5. Integument coloration

We investigated the potential effects on colour of the integument underneath the scales by determining the reflectance spectra of three *T. vulgaris* individuals through microspectrophotometry using the same methods as above. Similar to the scale banding pattern, the integument of *T. vulgaris* exhibits a dark/light banding pattern. Five measurements were taken in a light band and five in a black band per individual (10 in total). We used TEM pictures to assess the presence of integumentary pigment for all species.

2.6. Optical modelling

To determine the contribution of thin films (lamina thickness) and diffraction gratings (ridge parameters), we used FDTD modelling using a commercial Maxwell equation solver (Lumerical Solutions, Inc.). We used the dimensions for scale parameters obtained from SEM/TEM (table 1) and the results of the Raman spectroscopy indicating the presence of melanin in the ridges to construct simplified three-dimensional models in SketchUp (Trimble Inc.). As we only measured two scales for *C. albinus*, we used the actual scale parameters.

For each species, three possible mechanisms for colour production were tested (figure 2a). Lamina thickness only (thin film), ridges only (diffraction grating) and lamina + ridges (thin film + diffraction grating). Following the TEM and Raman spectroscopy results, the laminae and ridges consisted of chitin and melanin, respectively, with the refractive index obtained from previously reported studies [35,36]. As we observed potential differences in ridge melanin concentration between species, we also simulated ridge-only and lamina + ridge models, with ridges consisting of chitin, to determine the effect of melanin in the ridges. The simulation space was consistently chosen to contain four ridge units wide and two ridge units deep (figure 2b) for the spinulate type to include ridge variation. For the continu-ous ridge type, four ridge units were equally chosen. Simulation depth in this case does not influence the simulation as the height of the ridges is constant. Perfectly matched layers [37] were used to absorb electromagnetic waves from both the top and bottom...
of the simulation area and periodic boundary conditions on the side to simulate infinite periodic structures. The light source covered a 300–750 nm wavelength range and consisted of an unpolarized plane wave at normal incidence. Simulated reflectance spectra were visualized using the pavo package [33] in R (v. 4.0.2) and plotted together with measured scale reflectance spectra (average ± 95% CI) to illustrate (dis)similarity.

To investigate the importance of nanoscale variation in ridge dimensions, we simulated *T. vulgaris* ridge-only and lamina + ridge models of varying ridge height (250, 300, 400 and 500 nm) and distance (700, 800, 900 and 1000 nm) while keeping scale thickness (450 nm) constant.

### 2.7. Polarization-dependent reflectance

As diffraction gratings typically are polarization dependent [21], we tested for polarization effects by observing scales under unpolarized incident light, parallel polarized (TM) and perpendicular (TE) light on a Leica DM 1000 microscope for all species.

### 2.8. Collembola cladogram

To illustrate the phylogenetic relationship between our study species and to examine the distribution of scale presence/absence in Tomoceridae, Orchesellidae and Entomobryidae we constructed a cladogram using a Bayesian phylogeny constructed by Zhang *et al.* [28] (available online as electronic supplementary material, Tree 1 in Newick format). *H. major* and *H. nitidus* are included in this phylogeny, and we manually added other study species to species of the same genus in the Newick file. We added *C. albinus* to *Ascocyrtus* sp. and *P. flavescens* to *Tomocerus* based on previous phylogenies [27,38]. We constructed a cladogram using the package ape [39] in R (v. 4.0.2) by setting all distances to 0.

### 3. Results

#### 3.1. Scale colour

Reflectance spectra for all species fall into two broad categories with spectra increasing towards the higher wavelengths resulting in a broadband silver to golden/metallic colour and spectra showing distinct peaks in the violet/blue/green region (figure 3; see electronic supplementary material, figure S2a–g). Indeed, individual scales seem to be characterized by metallic/golden areas next to clear colour.

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**Figure 2.** (a) Simplified three-dimensional models of scale and ridge types (spinulate (i, ii, iii) and continuous (iv, v, vi)) that were used as objects for FDTD models in three scenarios: scale thickness only (i, iv), ridge only (ii, v) and scale thickness + ridge (iii, vi). (b) Width and depth of the simulation area.

**Table 1.** Scale parameters for seven springtail species. Means (nm) ±95% CI are given with (number of scales/number of individuals). Number of individuals = 1 when not indicated. *T. vulgaris* and *P. flavescens* have uninterrupted ridges, therefore ridge length is designated as ‘continuous’.

|SEM| TEM |
|---|---|
|ridge distance| ridge length| scale thickness| ridge height| ridge distance| ridge base|
|Lepidocyrtus cyaneus| 526 ± 60 (8/2)| 2032 ± 123 (8/2)| 136 ± 14 (6)| 208 ± 19 (6)| 595 ± 57 (6)| 213 ± 14 (6)|
|Lepidocyrtus lignorum| 544 ± 81 (2)| 2260 ± 114 (2)| 131 ± 18 (7)| 491 ± 251 (4)| 642 ± 76 (8)| 231 ± 47 (4)|
|Cyphoderus albinus| 287 ± 36 (7)| 2118 ± 471 (7)| 203 ± 20| 317 ± 33| 263 ± 42| 186 ± 7|
|Heteromurus major| 914 ± 83 (4)| 5129 ± 162 (4)| 1265 ± 34 (13)| 429 ± 65 (5)| 1210 ± 120 (8)| 328 ± 27 (9)|
|Heteromurus nitidus| 945 ± 89 (3)| 5071 ± 725 (3)| 233 ± 40 (6)| 797 ± 137 (6)| 1050 ± 59 (6)| 352 ± 44 (6)|
|Tomocerus vulgaris| 663 ± 82 (10/3)| continuous| 519 ± 91 (13/3)| 309 ± 42 (13/3)| 918 ± 91 (13/3)| 273 ± 35 (13/3)|
|Pogonognathellus flavescens| 787 ± 59 (12)| continuous| 663 ± 188 (4)| 674 ± 68 (4)| 881 ± 109 (4)| 210 ± 64 (3)|
Figure 3. Measured reflectance spectra of single scales on glass slide (left) and scales on body (right). Orange lines with shaded area represent the mean ± 95% CI. Grey lines depict a representative subset of individual scale measurements. For T. vulgaris, scales from golden (yellow) and dark (brown) bands on the body and single scales, originating from a golden band (yellow) and from the upper part (melanin-rich, brown line) and the lower part (low amount of melanin, orange line) of a dark band, were measured separately.
patches and transversal bands. As such, the average reflectance spectrum typically has a peak around the 450 nm region and increases towards higher wavelengths. An exception to this general pattern is *L. cyaneus*, which exhibits a dip around 500 nm with a more pronounced purple colour and, to a lesser extent, *C. albinus* with a peak around the 500 nm region with a more blue colour. For *T. vulgaris*, we measured scales from golden and black bands separately. Golden scales show higher reflectance than the reflectance of black scales, which is probably caused by a lower melanin content in the ridges (figure 3). Similarly, a higher reflectance is observed in the lower half of a scale from a black band (low melanin content) than in the upper half (high melanin content). Normalized and averaged reflectance spectra for single scales and body scales can be found in electronic supplementary material, figure S3.

Single scales exhibit higher reflectance than scales on the body, most likely because of reflectance from the glass slide. Otherwise, scales on the body and single scales generally display similar reflectance spectra (figure 3; electronic supplementary material, figure S2a–g), but with some slight differences. *L. cyaneus* and *L. lignorum* seem to display a more pronounced peak at lower wavelengths when single scales are measured than when body scales that exhibit a more golden (longer wavelength) coloration are measured. This situation is reversed in *P. flavescens*,

**Figure 3.** (Contd.)
Figure 4. Scale ultrastructure in Collembola. Macrophotograph (a), SEM (b) and TEM (c) pictures of the seven springtail species included in this study. All springtail pictures (except P. flavescens) were taken by Marie Louise Huskens.
which has a more pronounced peak around 425 nm in scales on the body.

This pattern is also visible when plotted in segment color-space as single scales are more distinct from body scales for *L. cyaneus*, *L. lignorum*, *C. albinus* and *P. flavescens* than for *H. nitidus*, *H. major* and *T. vulgaris*. Moreover, for both single scales and body scales, the colour spectra of *L. cyaneus*, *L. lignorum* and *C. albinus* are generally non-overlapping, while *H. nitidus*, *H. major*, *P. flavescens* and *T. vulgaris* show extensive overlap (electronic supplementary material, figure S4).

Scale colour for *L. cyaneus* and *T. vulgaris* did not exhibit a strong angle-dependent response as scale colour was largely maintained over various lighting angles (electronic supplementary material, figure S5).

### 3.2. Scale morphology

All investigated species are characterized by a full covering of scales on the dorsal side of the abdomen. Scales show a clear roof-tile-type stacking and vary both between individuals of different species and within one individual in size and shape (electronic supplementary material, figure S6). In SEM pictures, scales sometimes appeared to have overturned edges or to be fully curled. This is likely to be an artefact of drying during the SEM procedure (see Material and methods).

Spinulate scales have ridges in a herringbone pattern composed of numerous sail-like, triangular structures (electronic supplementary material, figure S7). The uninterrupted rib type consists of continuous longitudinal and parallel ridges. Both types are oriented along the longitudinal axis of a scale and are, on cross-section, characterized by a wide base that narrows towards the top. Ridges are generally electron-dense (figure 4), indicating the presence of pigment (melanin, figure 5), although melanin quantities seem to vary between species (e.g. they are lower in *C. albinus* and *H. nitidus*) while the basal lamina generally lacks pigment.

We calculated the following scale parameters (figure 1 and table 1):

— Ridge distance. The distance between the ridges is similar for *L. cyaneus* and *L. lignorum* (595 and 642 nm, respectively, for TEM), while ridges are more closely spaced in *C. albinus*. The greatest ridge distance was found in *H. major* and *H. nitidus* (greater than 1000 nm) while *T. vulgaris* and *P. flavescens* demonstrated an intermediate ridge distance. Ridge distance calculated from TEM was generally larger (except for *C. albinus*), which is likely to be due to slightly angled SEM pictures.

— Ridge length. This was only calculated for those species with a spinulate scale type as both *T. vulgaris* and *P. flavescens* have continuous ridges. Two main classes of ridge length can be discerned: the ridges of *L. cyaneus*, *L. lignorum* and *C. albinus* are, on average, around 2 μm long, while the ridges of *H. major* and *H. nitidus* measure around 5 μm.

— Scale thickness. Similar to ridge distance, the average thickness of a scale varies markedly between species but is relatively constant within genera. *L. cyaneus* and *L. lignorum* have the thinnest scales, with *H. major*, *H. nitidus* and *C. albinus* having intermediate values of scale thickness. The thickest were found in *T. vulgaris* and *P. flavescens*.

— Ridge height. Height of the ridges seems to be the most variable parameter between species with *L. cyaneus* having the lowest ridges, followed by *T. vulgaris*. *L. lignorum* and *H. major* have intermediate ridge heights. The tallest ridges were found in *P. flavescens* and *H. nitidus*. The two scales of *C. albinus* display a markedly different...
ridge height: one having an intermediate height and one with high ridge height.

— Ridge base. The width of the ridge base varies from around 200 nm for *L. cyaneus*, *L. lignorum*, *C. albinus* and *P. flavescens* while being larger for *T. vulgaris* (270 nm) and *H. major* and *H. nitidus* (over 300 nm).

### 3.3. Scale pigment

The results of the Raman mapping experiment show two distinct areas of different colours in the map, corresponding to different spectra and indicating the golden and black bands for *T. vulgaris* (figure 5). Both of these areas indicate the presence of eumelanin [40,41] with the difference in spectra likely to have been caused by differences in eumelanin concentration. However, we cannot fully rule out the contribution of other molecules present on the scales or a different quality (or crystallinity) of the pigment in the black and gold areas due to the influence of focusing or fluorescence on different measuring points. Moreover, eumelanin was confirmed to be the main pigment in scales of *L. cyaneus*.

The effect of melanin in scales is most apparent in the scales of the golden and black bands of *T. vulgaris*. Golden scales have a lower melanin concentration than black band scales as well as a higher distance between ridges (electronic supplementary material, figure S8). The bases of ridges on black scales meet, leaving approximately 300 nm in between ridge tops. Moreover, black scales exhibit a clear melanin gradient, with higher amounts in the top half. Scales are stacked in such a way that only the high-melanin region is exposed. This results in lower reflectance (figure 3) and a general black appearance.

### 3.4. Scale colour modelling

To understand the importance of lamina thickness (thin film) and ridge structure (diffraction grating) in colour production we designed simplified three-dimensional models (table 2), based on the measured scale values.

We tested three physical bases (thin film, diffraction grating or a combination of both) by simulating the spectral reflectance of lamina thickness only (thin film), ridges only (diffraction grating) and lamina thickness + ridges (thin film + diffraction grating).

These simulations indicate that both lamina thickness (thin film) and ridge structure (diffraction grating) play a role but that their importance differs between species and hence scale types. For the spinulate type of *L. cyaneus* (figure 6a), a scale of 130 nm (averaged across all measured scales) produces a thin-film effect with high chroma in the UV part and an increasing reflectance towards the higher wavelengths. This largely corresponds to the observed reflectance spectra of single scales. Note that the high reflectance of the scale models is a consequence of the normalization process and that the modelled reflectance is lower (see electronic supplementary material, figure S9a–g for simulated reflectance spectra). Moreover, a larger lamina thickness of 160 nm provides an optimal fit, not far from the 128 nm average. The ridges produce a peak in the violet/blue region and increased reflectance towards the higher wavelengths (red region) which is compatible with some of the individual scale measurements. A combination of both lamina thickness and ridges also shows a dip at 500 nm, which is mainly driven by the thin-film effect.

In *L. lignorum* (figure 6b), we also find that the scale thin-film model provides a good fit with the observed spectrum, both having lowest reflectance at 400 nm and being compatible with golden scale measurements. The combination of lamina thickness and ridge shows a similar pattern, albeit more undulatory. However, ridge-only simulations exhibit an increase towards higher wavelengths, together with multiple peaks, especially around 450 nm, presenting a good fit to the observed spectra as well.

For *C. albinus* (figure 6c), the scale thin-film model predicts peaks that correspond with observed peaks in the 425 nm and 500 nm regions (dark grey lines). Strikingly, the ridge-only model also returns peaks in a similar region, albeit slightly shifted to the right. The combination of lamina thickness and ridge models fits the observed spectrum of multiple peaks that corresponds most closely to a broadband metallic colour (light grey line).

For both *H. major* (figure 6d) and *H. nitidus* (figure 6e), lamina thickness and the combination with ridge models fit the observed spectra poorly. However, the ridge-only model fits the measured data as the ridges of both *H. major* and *H. nitidus* simulate an oscillating spectrum towards higher wavelengths.

Thin-film effects alone in *P. flavescens* (figure 6f) and *T. vulgaris* (figure 6g) do not fit the observed reflectance spectra while the ridge models (especially for *P. flavescens*) and the combination models (especially for *T. vulgaris*) fit well. For *T. vulgaris*, the model with ridges of 300 nm height and 800 nm distance produces a distinct peak in the 450 nm region, rather than a broadband metallic coloration.

### 3.5. Ridge pigmentation, height and distance

Simulations indicate that the presence of melanin in the ridges has only a limited effect, resulting in a slightly lower reflectance than ridges consisting of only chitin (electronic supplementary material, figure S10a–g), especially for the ridge-only models.
Figure 6. Fit of lamina thickness, ridge-only and combination models (dotted and dashed lines) with measured scale reflectance spectra (orange line, means ± 95% CI). All spectra are normalized, ranging from 0 to 1. Grey lines show a selection of representative, individual scale measurements. All models follow the parameters set out in table 2; when multiple models are constructed, the values of the parameters (scale thickness or ridge distance) are given. For clarity, we represent the simulations offset from the measured values where necessary. All springtail pictures (except *P. flavescens*) were taken by Marie Louise Huskens.
Comparing the simulated reflectance spectra of the ridge and lamina + ridge models of *T. vulgaris* (electronic supplementary material, figure S11) reveals that nanoscale differences in ridge height and distance can produce different reflectance spectra. This is most apparent in the ridge-only models where a shift of the lower wavelength peak towards longer wavelengths occurs with increasing distance between the ridges for a given ridge height. Increasing ridge height seems to mainly result in lower peaks. For the lamina + ridge models, these differences are less apparent.

### 3.6. Integumental coloration

The cuticle of *T. vulgaris* exhibits a metallic silver to golden coloration (electronic supplementary material, figure S12). Moreover, a banding pattern is observed that is opposite to the overlying scale pattern. The anterior and middle parts of an abdominal segment have a golden coloration (overlying scales are black), while the posterior part is darker and characterized by a lower reflectance (with overlying golden scales; electronic supplementary material, figures S12 and S13). Both the cuticular surface ornamented with microtubercles interconnected by ridges and the cuticular multilayer structure itself could influence colour production. Moreover, similar to melanin in scales, the presence of a dark pigment (most likely melanin) appears to affect the general appearance with a silvery grey (*H. major, P. flavescens*) to black/golden (*T. vulgaris*) and purple (*L. cyaneus*) coloration corresponding to extensive deposits beneath the cuticle (electronic supplementary material, figure S14). In species that are silvery white (*H. nitidus, C. albinus*) to golden (*L. lignorum*), these deposits are largely absent.
3.7. Polarization-dependent reflectance

We observed weak polarization-dependent scale reflectance for all species. There was little difference in scale colour between unpolarized and TM polarized light (electronic supplementary material, figure S15). The effect of TE polarized light seems to be limited for *L. cyaneus* (though a weak violet colour can be discerned), *L. lignorum* and *C. albinus*, while it is slightly more pronounced in *H. major* and *H. nitidus* and strongest in *P. flavescens* and *T. vulgaris*, where scales from the black band exhibit a violet colour while the colour of golden scale bands seems to be reduced. There is some polarization of the integument underneath the scales which is particularly pronounced in those species with a pale colour (*C. albinus* and *H. nitidus*).

4. Discussion

To gain insight into colour production by body scales in basal arthropods, we investigated seven springtail species that differ in scale parameters such as thickness and surface topology (spinulate versus continuous). Despite these morphological differences, all seven investigated species display a metallic golden to silver colour with additional peaks in the violet/blue region. Optical modelling shows that collembolean colour is produced via thin-film effects originating from the scale lamina and diffraction from the longitudinal ridges. Nanoscale variations in scale thickness and ridge dimensions probably cause spatial colour mixing, resulting in a broadband silver to golden coloration.

Reflectance spectra of single scales and of scales measured on the body are generally uniform, indicating that a single scale is the unit of colour production. There are, however, subtle differences when scales are measured on a glass slide or on the body. It is noteworthy that those species (*L. cyaneus*, *L. lignorum*) with marked thin-film effects have more pronounced peaks in the lower wavelengths when single scales are measured. This is less the case for those species in which the ridges play a role (*C. albinus*, *T. vulgaris*, *H. nitidus*, *P. flavescens*). It is possible that measuring scales on a glass slide enhances scale thin-film effects. Melanin deposits underneath the cuticle can produce a grey appearance and potentially enhance the purple and golden coloration in *L. cyaneus* and *T. vulgaris*. The cuticle of *T. vulgaris* exhibits a metallic coloration and localized dark pigment deposits, creating a banding pattern. The colour pattern is inverted compared with scales: golden under black scales and dark under golden scales, probably resulting from an absence/presence of melanin that potentially enhances scale colour. This is comparable to silverfish, where the integumentary colour, caused by a chipped multilayer, is similar to the metallic scales and probably produces their metallic coloration [18,19]. Cuticle golden coloration in springtails is likely to arise from a multilayer reflector or even from the microtubercles found on the cuticle surface. These function as a hydrophobic barrier to protect the animal [42,43], but their role in colour production (if any) is currently unknown.

When viewed under sufficient magnification, the scales of all species show colourful patches and bands that generally run transverse to the ridges. The metallic coloration thus is likely to result from spatial mixing, as seen in metallic butterfly scales [8], and explains the observations of both broadband colour reflectance spectra and spectra with pronounced peaks in the violet to blue region of the spectrum. To increase our understanding of the colour production mechanisms, we performed optical modelling using three-dimensional models of lamina-only, ridge-only and lamina + ridge models. We focused on thin-film (lamina thickness) and diffraction grating (ridges) effects as these have been shown to be important in previous studies of Micropterigidae (albeit by cross-ribs [20–22]) and in the springtail species *T. vulgaris* [20]. Our results confirm the role of longitudinal ridges in colour production but also indicate that simultaneous thin-film effects are at play, the importance of which is species dependent. Indeed, especially in *L. cyaneus*, *L. lignorum* and *C. albinus*, which are species with a small lamina thickness, the lamina-only and especially lamina + ridge models match observed spectra. The ridge-only model shows a broadband metallic coloration for *L. lignorum*, but more pronounced peaks for *L. cyaneus* and *C. albinus*. For species with thick lamina, the lamina-only models generally are a poorer fit. This is mainly the case for *H. major* and *H. nitidus*, where both the lamina-only and lamina + ridge models deviate. It is rather the ridge-only models that produce a broadband metallic colour. For *P. flavescens* and *T. vulgaris* the lamina-only models of thin-film effects also seem a poorer fit to the observed values with ridge-only models producing broadband colours in *P. flavescens*. In *T. vulgaris* the ridges produce a broadband colour and a spectrum with a main peak around 450 nm. The scale + ridge models fit the observed reflectance spectra well and are in agreement with previous results on *T. vulgaris* scale colour production [20].

Observing scale colour under differently polarized incident light allows further insights into colour production, specifically about the contribution of diffraction grating effects through ridges. These results support our simulation results, as we find limited polarization effects in *L. cyaneus*, *L. lignorum* and *C. albinus*, indicating a lower contribution of ridges in these species. For *H. major* and *H. nitidus*, the effects of TM polarized light are slightly more pronounced, and stronger in *T. vulgaris* and *P. flavescens*. This suggests that grating effects by scale ridges might play a more important role in these species.

It is, however, important not to overinterpret our results as nanoscale differences in scale thickness, ridge height and ridge distance can have profound effects on the observed colour. For example, ridge models for *L. cyaneus* and *L. lignorum* differ by only 300 nm in height and 50 nm in distance but produce markedly different reflectance spectra. For *T. vulgaris*, simulating varying ridge height and distance while keeping lamina thickness constant confirms this far-reaching effect as spectra with pronounced hues (peaks in the purple/blue region) and more broadband coloration are produced (electronic supplementary material, figure S11). Our simulation results therefore strongly suggest that both scale thickness and ridges play a role in colour production, and these probably work in interplay as evidenced by the accuracy of many of the lamina + ridge models. More precisely, it is the morphological nanoscale variation in ridge and lamina dimensions that produces distinct colour patches that combine, through spatial mixing, in a broadband golden colour.

The scale thickness of a melanized single lamina has been found to play a major role in the metallic coloration of the micropterigid *Micropterix aurantella* (Lepidoptera), which is characterized by purple and golden scales [22]. This situation is reminiscent of our results for *L. cyaneus* (with similar scale thickness around 130–140 nm) and points towards scale thickness as the major contributor to the characteristic purple colour in *L.
cyaneus. Previously found in T. vulgaris [20], we confirm the presence of melanin in the scales of L. cyaneus, though it is not present in the laminae but limited to the ridges. Thin-film effects have been found to contribute in other early Lepidopteran groups together with diffraction grating from cross-ribs and longitudinal ridges [20,21]. Our results also point towards multiple structures on a springtail scale that affect colour production (i.e. interplay between lamina and ridges). Interestingly, the valleys between the ridges are smooth in springtails, indicating that bright metallic coloration can be achieved in the absence of cross-ribs, similar to silverfish scales where longitudinal ridges on scales without cross-ribs are thought to produce a metallic colour [19]. This advocates for a more consistent inclusion of ridge effects in colour mechanism studies. Indeed, longitudinal ridges have been found to function as colour production mechanisms in derived Lepidopteran groups [44,46], albeit with more complex morphological modifications, such as extra layers (i.e. christmas tree structure in Morpho [44]), than the smooth sides of the ridges in Collembola. Ridge melanin clearly plays a role, as indicated by the lower reflectance of the upper half of scales originating from the black bands in T. vulgaris (figure 3) and by the difference in melanin concentration in golden and black band scales, as indicated from Raman spectroscopy (figure 5). Our optical models incorporating the presence/absence of melanin in the ridges confirm the lower reflectance for melanized ridges, though this effect was very limited and more pronounced in the ridge-only models (electronic supplementary material, figure S10a–g). Moreover, not only melanin but also lowering ridge distance seem to reduce reflectance. Black scales of T. vulgaris have ridges of which the bases almost meet, leaving only 300 nm between ridge tops, while the ridges on the larger scales of the golden bands are further apart (approx. 1 µm).

It is striking to note that, despite substantial differences in scale parameters such as scale thickness, ridge height, ridge distance, ridge length (spinulate type) and even ridge type (spinulate versus continuous ridges), the reflectance spectra of these seven species are largely similar and characterized by a broadband metallic golden to silver colour with peaks in the violet/blue region. Small differences between species exist as L. cyaneus, L. lignorum and C. albicus occupy neighbouring yet seemingly distinct areas in the segment colour space while H. major, H. nitidus, P. flavescens and T. vulgaris are largely overlapping. This indicates that similar spectra can be produced by morphologically distinct ridge types (spinulate versus continuous) and points to a certain degree of convergent evolution and similar selection pressures on springtails, with continuous and spinulate ridges, as they form distinct phylogenetic groups belonging to separate families and with spinulate ridges seemingly to having evolved from continuous ridges [28] (electronic supplementary material, figure S16).

While we employed two ridge types (spinulate and continuous) in our analysis, two more scale types are currently recognized in Collembola: the short rib type (interrupted longitudinal ridges of similar length) and the long basal rib type (with long ridges at the base of the scale and interrupted ridges near the apex) [29]. It would be most interesting to perform a similar analysis including these types and to further elucidate the effect of ridge structure. Moreover, expanding our range and including other basal and scaled arthropods such as Archaeognatha and Zygentoma that all have continuous ridges and metallic coloration holds enormous potential to investigate colour production in early scales.

Scale function in springtails is largely unknown. It is not a continuous trait as scale presence depends on the moult cycle [47]. There is evidence that they function as an antipredator mechanism [48] by readily detaching from the body, allowing the animal to escape. Scale structure, and especially ridges, might be involved in mechanical strengthening. Scales seem to deform and bend (most likely because of drying; see, for example, electronic supplementary material, figure S2d). Curling seems to primarily occur from the sides and moving inwards rather than following an anterior–posterior direction, indicating that longitudinal ridges might confer mechanical strengthening. Curling seems more extensive in spinulate ridges, which could indicate a higher strengthening effect of continuous ridges, especially as these are found in larger species with larger scales though we cannot exclude the effects of a smaller lamina thickness. Additionally, in the larger species (P. flavescens and T. vulgaris), structures connecting the ridges (figure 4) are present that potentially indicate the need for additional strengthening in the orthogonal direction. It is striking to note that even the larger Lepidoptera have marked cross-ribs between the ridges. It would be most interesting to investigate the link between scale structures and scale size, within the context of scale rigidity across taxa. Especially Collembola could provide interesting insights as, in contrast with Lepidoptera, juveniles are already scaled, which allows us to track scale morphology across life stages and sizes. To what extent colour plays a role in species recognition or sexual selection remains unknown. Lastly, scales could play a role in thermoregulation by influencing the amount of reflected light or in countering dehydration by retaining a layer of water between cuticle and scales.

5. Conclusion

In this study, we investigated the colour production mechanisms of collemboalan scales. We found that the two main features, a basal lamina and longitudinal ridges, both contribute to the characteristic metallic coloration through thin-film and diffraction grating effects, though the importance of thin-film effects seems to be species specific. It is likely that nanoscale variations in scale thickness and ridge dimensions (height and distance) cause differently coloured surfaces on a single scale, resulting in a broadband silver to golden colour through spatial mixing. Our results give first insights into the coloration mechanisms of basal arthropods, a group with a high proportion of scaled representatives.

Data accessibility. All raw data and code have been uploaded to the Dryad data repository: https://doi.org/10.5061/dryad.dv4ins1zc.

Authors’ contributions. B.V., L.D. and M.S. conceived the study, performed experiments and wrote the manuscript. A.R. and P.V. performed the Raman spectrometer experiments and wrote these sections. T.P., F.J. and J.M. assisted in data collection and data interpretation. All authors critically revised the manuscript.

Competing interests. We declare we have no competing interests.

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