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DISCUSSIONS
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Optics and photonics in nature: general discussion

Hans Arwin, Pascal Barla, Adam James Blake, Aleca Borsuk, Melanie Brien, Stephanie Burg, Yin Chang, Pascal Freyer, Mike Hardy, Amanda Holt, Akhil Kallepalli, Gea Theodora van de Kerkhof, Mathias Kolle, Christian Kuttner, Mathieu Ladouce, Amina Matt, Sébastien R. Mouchet, Nicola J. Nadeau, Daniel Osorio, Andrew Parnell, Primož Pirih, Anupama Prakash, Giselle Rosetta, Lukas Schertel, Diana Skigin, Doekele Stavenga, Silvia Vignolini, Pete Vukusic and Ming Xiao

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Christian Kuttner opened a general discussion of the paper by Pete Vukusic: You have pointed out the differences between ordered, quasi-ordered and disordered systems. But how does this relate to the short- and long-range order? For example, some densely packed arrangements may have very uniform distances to the nearest neighbour, but poor order at longer distances. Would you consider a random close-packed structure as quasi-ordered or disordered (chaotic)?

Pete Vukusic replied: Thank you for this really useful question Christian. We do see many systems that have a strong "local" order, for instance in localised domains that are a few tens or more, lattice constants in size: but then these neighbouring domains are multiply oriented. In these ways we have the short range order you describe, but this does not extend very far. Examples include the 3D structures in the domains of *Parides sesostris*, or in the antireflective nipple-arrays found on the ommatidial surfaces of lepidopteran and other arthropod compound eyes. Should we describe these as ordered, or quasi ordered? Much of your question relates to semantics I think: for me I think the arc leading from ordered through quasi-ordered to disordered is a genuine sliding scale, across multi-variable space. The point at which we decide definitively whether something transitions from having the label ordered or quasi-ordered, is a little subjective.

Christian Kuttner addressed Pete Vukusic: How about using the radial distribution function (RDF) for the evaluation of order? The RDF(r) is a pair-correlation function that describes the probability of finding another particle at distance r from each particle.[1,2]

1 M. B. Müller, C. Kuttner, T. A. F. König, V. V. Tsukruk, S. Förster, M. Karg and A. Fery, *ACS Nano*, 2014, **8**, 9410–9421.
2 J. Sindram, K. Volk, P. Mulvaney and M. Karg, *Langmuir*, 2019, **35**, 8776–8783.

Pete Vukusic replied: Hi Christian, thanks for mentioning this. The RDF would certainly be an effective approach that could be taken to gain a good sense of the nature of the particle distribution. It would bring a very useful perspective to these analyses.

Giselle Rosetta asked: Would this kind of method be applicable to non spherical particles, *e.g.*, ellipsoids? What about if these particles are not aligned in one direction, is there capacity for angular distribution quantification?

Pete Vukusic answered: Thanks for this great question. The shape of particles would certainly be a variable that would contribute to the optical properties of the whole system. Our 2D modelling so far assumes circular particles, but we recognise that applying, say, ellipticity to the particles, especially if the particles' long axes were oriented in a similar direction, would generate directionally preferential scattering. The effect of specific particle shape, in addition to the other described variables such as particle size, particle size distribution, particle spacing *etc.*, is certainly one of the variables that would be valuable to investigate on these systems. We know for sure that for these natural biological photonic systems, in the same way particle size and spacing is not single-valued, particle shape is also variable.

Silvia Vignolini said: Why not use the structure factor and form factor to better characterize the interplay between order and disorder? This can also be applicable to 3D photonic systems, and it is ideal for estimating the effect beyond nearest neighbor interactions.

Pete Vukusic responded: Thanks for the contribution here Silvia. Good point. The structure factor has been invaluable as a core part of scattering since the beginning. It could certainly be applied in a useful way to this area.

Nicola J. Nadeau addressed Pete Vukusic commenting: When considering costs from a biological perspective we need to know more about how these structures are produced and used. In some cases producing a more ordered structure might be "easier" or less costly and there might be adaptive benefits of having a particular level of order/disorder to produce a particular colour effect. We often assume that having a more highly ordered structure is physiologically costly to produce, but it may not be, depending on the self-assembly process involved. This is something we still know remarkably little about.

Sébastien R. Mouchet answered: Very interesting comment. Thank you. It is true that our contribution focused mostly on the costs and benefits of disorder from the point of view of the optical properties. Other aspects such as development and evolution tend to be usually and unfortunately disregarded.

Primož Pirih addressed Pete Vukusic, Gerd Schröder-Turk and Doekele Stavenga: You have identified the problem with boundary conditions which is especially prominent with the Fourier transform approach, somewhat less so with segmentation approaches. Apart from using 2DFT, another useful technique with a 2D window may be wavelet transform (2DWT), a localized analysis which does not assume the wrap-around continuity. I would encourage the theoreticians in the field to have a peek into these methods. A standard textbook is "A wavelet tour of signal processing" by Stéphane Mallat, and there is a toolbox called "ltfat".[1] It seems that unfortunately for the coloration field, much research on 2DWT has focused on image compression and AI classification rather than on statistics and feature analysis. Another approach described by Liu and Picard[2] uses Wold transform to extract three orthogonal components, related to image periodicity, directionality and randomness, respectively. Lastly, a tangent from the vision field: Dyakova and Nordstrom[3] have reviewed the tools used for natural image statistics related to insect vision.

1 S. Mallat, *A Wavelet Tour of Signal Processing*, ScienceDirect, 2009.
2 F. Liu and R. W. Picard, *IEEE Transactions on Pattern Analysis and Machine Intelligence*, **18**(7), 722–733.
3 O. Dyakova and K. Nordstrom, *Current Opinion in Insect Science*, 2017, **24**, 7–14.

Ming Xiao asked: The particle pair–pair correlation function $g(r)$ gives more complete information about distances between particles, including nearest neighbour and second nearest neighbours. Why didn't you use $g(r)$ here? Does that mean only the nearest neighbor distance is relevant to the optical properties?

Pete Vukusic replied: Thanks Ming. Good point and you're absolutely right about the usefulness of the $g(r)$ function. The nearest neighbour isn't the only spatial variable that affects optical properties, just as you say. The approach/technique has great value.

In our current approach, the statistics associated with analysis of nearest neighbour distributions was especially useful and it was for this reason that we took the approach described. We and others will look at the full $g(r)$ in due course I think.

Pascal Barla communicated: Are there multiple dimensions to disorder? And if yes, is it known how each dimension affects the produced colors?

Pete Vukusic communicated in reply: Hi Pascal, thanks for your great question. I'd agree that there are multiple disorder "dimensions". My feeling is that there are several variables associated with disorder in each typical system: such as particle size, particle shape, particle separation and combinations of these, which will also then affect filling fraction *etc.* There would be others too.

The initial modelling I presented yesterday was just the initial, and comprised only looking at the effect of nearest-neighbour distance disorder. For us, more time and people are needed to do a multi-dimension job on the work, which hopefully will happen in the next few months, or year or two.

Certainly though, disorder is absolutely multidimensional.

Hope this helps to answer your question. Very happy to discuss further.

Diana Skigin communicated to Pete Vukusic and Sébastien R. Mouchet: How do you manage to build the nearest neighbour histograms when you have images of slices of the 3D structures? Usually sections of particles appear and it is difficult to take them into account in a proper manner.

Pete Vukusic communicated in reply: Hi Diana, thanks for your question. The best data come from good quality TEMs which I know aren't always easy to acquire. Sometimes the way to overcome this with "ordinary" quality TEMs is to do some pre-processing/editing of images, but this can sometimes come with the introduction of artefacts.

I'll contact my colleague Sébastien R. Mouchet, who can talk you through the protocols of doing the image analysis. Do feel free to ask further questions of me though if it would be helpful.

I'll send a separate email to Sébastien R. Mouchet, and copy you in, so that we can discuss this together further.

Sébastien R. Mouchet further communicated in reply: Observing 3D structures with a TEM always brings some challenge. So far, the structures we have used for our analyses were (quasi-) periodic along one or two dimensions. The way the TEM cross-sections (including the angle of the cut) were performed influences a lot of parameters such as the size of the observed elements, their shapes and distances between them. In our study (DOI: 10.1039/d0fd00101e), we mainly selected figures from the literature (Prum and Torres[1]; Prum and Torres[2]; Trzeciak and Vukusic[3]; Kientz *et al.*[4]; Yoshioka and Kinoshita[5]). We assumed the sections were perfectly perpendicular to *e.g.*, the collagen fibres of bird caruncles.

With the TEM images of the 2D systems we selected, we had to go through preliminary image processing steps such as removal of the background and binarisation of the images. This gave us images of white particles in a black background. For each particle, we calculated the centroid coordinates. With these coordinates, we used Delaunay triangulation to compute the nearest neighbour distances.

In addition, we normalised the measured distances by their median values. This helped us to get rid of systematic errors and put forward the extent of disorder.

I hope this answers your question. Do not hesitate to come back to me, should you have further questions. But indeed, such a task is much more complex to implement and time-consuming than how it may sound.

1 R. O. Prum and R. Torres, *J. Exp. Biol.*, 2003, **206**, 2409–2429.
2 R. O. Prum and R. H. Torres, *Integr. Comp. Biol.*, 2003, **43**, 591–602.
3 T. M. Trzeciak and P. Vukusic, *Phys. Rev. E*, 2009, **80**(6), 061908.
4 B. Kientz, S. Luke, P. Vukusic, R. Péteri, C. Beaudry, T. Renault, D. Simon, T. Mignot and E. Rosenfeld, *Sci. Rep.*, 2016, 19906.
5 S. Yoshioka and S. Kinoshita, *Forma*, 2002, **17**, 169–181.

Ming Xiao remarked: Which polarization did you count for the calculated reflectance of 2D hexagonal synthetic PCs – TE or TM or averaged? Have you tried to look at simulated 3D structures?

Pete Vukusic communicated in reply: Hi Ming, thanks for this question. We originally calculated both linear polarisations, but I presented the average polarisation for speed due to limited time. We thought for a long time about 3D structures since for many people these are the most interesting. Unfortunately the computing power needed for 3D systems occupying large volumes was not available so we limited our current models to 2D. 3D models is the way of the future though.

Amina Matt commented: In the scattering angle waterfall plot. The *z*-axis is the average reflectance. How many simulations were used to compute the average reflection?

Pete Vukusic responded: Hi Amina. Each scattering angle comprised the summed/averaged reflections from a minimum of 5 separate calculations/models. Ideally, we would have pushed this figure much higher but the models were computationally heavy to run. We felt that minimum of 5 was sufficient to reduce or eliminate the contributions of spurious outliers. I would be happy to chat further about the detail if interested.

Yin Chang communicated: Dear Professor Vukusic, Thank you for the great talk showing the ways to quantify disorder in 2D models. I was wondering if you have quantified the contribution of disorder on the spectra or visual colour?

For living organisms, imperfectness might always exist in the structures. For a non-scattering system, such as the quasi-ordered weevils, do you think the disorder in such 3D structures still affects the coloration somehow? If so, how do you measure the contribution from disorder with such a quasi-ordered structure? And how do you quantify the contribution from disorder in this case?

Pete Vukusic replied: Thanks so much for your great question.

Glad you enjoyed the talk.

We've looked pretty closely at the variation in the spectra as a result of the disorder evident in the systems. We then plotted these spectra on the classic CIE chart to provide an indication of visual colour quality in the classic sense. There wasn't really time to present this in the talk but we're including them in the paper. I hope you'll be able to look at it then.

For the quasi-ordered systems such as in the weevils scales I mentioned *etc.*, the quasi-order manifests itself as a reduction in colour saturation and an increase in angular spread. The peak wavelength signal/background noise ratio of the reflection also decrease, in line with poorer colour saturation *etc.*

Hopefully you can pick this up in the paper but please do feel free to reach out to me again if further discussion would be useful.

Hans Arwin remarked: In our modeling of circular Bragg filters in beetle cuticle we need to include a disorder in pitch in terms of smearing (pitch distribution).[1] The band of reflected circular polarization then becomes slightly wider. We do not know if the layer is chirped or if the variation in pitch is random. Is this disorder an effect of natures limitation to make a perfect Bragg filter, or is it a survival advantage for the beetle?

1 H. Arwin, T. Berlind, B. Johs and K. Järrendahl, *Opt. Express*, 2013, **21**, 22645–22656.

Pete Vukusic communicated in response: Hi Hans, hope all is well with you.

You make a good point. I'm unsure if anyone really knows how deliberate or how unintentional the deviation is away from seriously high quality ordered systems. My mantra is that these systems are (generally) as good (in terms of colour purity, intensity, signal quality *etc.*) as they have to be in order to deliver the function(s) for which they've evolved. I feel that the pursuance of "better" order is unnecessary for the required function(s) and it would certainly "cost" more in terms of investment in morphological processes *etc.* Whether nature is limited in how good it can make its ordered systems is a good question. My own view is that in some systems (like *Pherusa* sp.), the order is very high quality, so in principle, it should be possible in other systems if the selection pressures are strong enough *etc.*

Lots of answers still needed for all this. Thanks for asking the good questions.

Primož Pirih addressed Pete Vukusic and Sébastien R. Mouchet: Do you find a qualitative difference in the outcome and informativeness of the gray-valued *versus* binary (segmented) image analysis approach?

Sébastien R. Mouchet responded: In our study (DOI: 10.1039/d0fd00101e), we did not compare *per se* the two approaches that you mention. The statistical analyses of all selected 2D scattering structures observed by TEM were performed on binarised images. Such an image process step allowed us to discriminate the scattering particles from the background and to compute the centroid coordinates. In order to calculate the layer thicknesses of the 1D structures observed by TEM, we defined the layer interfaces as the mean grey scale value between two consecutive extrema within a single profile. We believe these were the most suited approaches to measure the nearest neighbour distance and the layer thicknesses.

Daniel Osorio commented: This is a variant of the question I put on the chat during the talk, as the original was answered within the talk itself. It is partly suggested by a question from Nicola J. Nadeau, I think after a different talk:

In his talk Pete highlighted the importance of partially ordered structures, and showed that the degree of order is reflected in the colour of a material, so that order and colour purity are positively correlated. This leads to a question: 'What is the relation between nanoscale structural order and energetic cost of production in living organisms?'. The answer is of interest because it bears on the question of how structural colours evolve and function as communication signals, especially in the (numerous) cases where they are sexually selected. One might expect more orderly structures to be more costly to produce, for example because of the need for 'developmental stability'. It is then easy to see how structural colours can evolve as honest signals of quality, with the receivers of these signals preferring more pure colours. The opposite scenario where a high degree of order and hence pure colours are 'cheap', and greyer (and less iridescent/directional) structural colours are more costly, giving a negative correlation between colour purity and quality, would pose a problem for those interested in the evolution of signalling. To be clear, by 'energetic cost of production' I am referring broadly to colour as an

honest signal of quality, and it is a separate issue to whether energy is a universal currency in this context.

Akhil Kallepalli opened a general discussion of the papers by Pascal Freyer: This is a cross-domain question but I am curious to know (1) how do melanosomes compare between those of peacock feathers and human skin? What about parameters such as size, structure, *etc.*? (2) Have you considered using Monte Carlo methods for optical attenuation modelling? If so, how would they compare with multilayer modelling?

Pascal Freyer responded: (1) The melanosomes are more spherically shaped in most (non-structurally coloured) organisms and I think they vary widely in size and shape, but I have not studied this in particular. The various melanosome shapes and sizes that are found in feathers are documented.[1–2]

(2) No, I have not compared this to Monte Carlo methods, but we did previously compare our effective-medium multilayer modelling with FDTD modelling. This yielded surprisingly similar outcomes, even for light with large angle of incidence.[3]

1 H. Dürrer, *Schillerfarben der Vogelfeder als Evolutionsproblem*, PhD thesis, University of Basel, 1977.
2 H. Dürrer, in *Biology of the Integument*, ed. J. Bereiter-Hahn, A. G. Matoltsy, K. S. Richards, 1986, pp. 249–247.
3 P. Freyer, B. D. Wilts and D. G. Stavenga, *Interface Focus*, 2019, **9**(1), 20180043.

Anupama Prakash said: Would you be able to comment on the development of these different colors within a feather? I'm assuming that the color changes in barbules along the length of a barb. Would you be able to guess how melanosome properties and other properties that determine these different colors are controlled with such spatial finesse?

Pascal Freyer answered: Yes, the average colour of a barbule varies along a single barb, creating the macro-pattern of the feather. I did not study the feather development in particular, but it seems like there is still a lot to be learnt here. Recent work suggests depletion attraction during keratinization as a mechanism.[1] However, this does not explain many other photonic structures in feathers such as the unique two-dimensional spacing of the peacock's melanosomes or the cortex layer (which is essentially a spacing of the first layer of melanosomes from the outer barbule interface). Dürrer has also highlighted the importance of the keratinization phase in his very elaborate report on the development and layout of the peacock tail feather.[2]

See also ref. 3 and 4 on the development of such photonic structures in feathers, and ref. 5 and 6 on the development of melanosomes.

1 R. Maia, R. H. F. Macedo and M. D. Shawkey, *J. R. Soc., Interface*, 2012, **9**, 734–743.
2 H. Durrer, *Rev. Suisse Zool.*, 1965, **72**, 263–412.
3 H. Durrer and W. Villiger, *Zeitschrift für Zellforschung*, 1967, **81**, 445–456.
4 J.-P. Iskandar, C. M. Eliason, T. Astrop, B. Igic, R. Maia, and M. D. Shawkey, *Biol. J. Linnean Soc.*, 2016, **119**(2), 477–287.
5 H. Dürrer, in *Biology of the Integument*, ed. J. Bereiter-Hahn, A. G. Matoltsy, K. S. Richards, 1986, pp. 249–247.
6 L. D'Alba and M. D. Shawkey, *Physiol. Rev.*, 2019, **99**(1), 1–19.

Amanda Holt asked: How did you model the effective refractive index in 1D, (referring to the plot in Fig. 4 in your paper) and how does this barbule "shape" affect the reflectance?

Pascal Freyer answered: We calculated the 1D effective refractive index profile from the 3D model that is based on the literature anatomical data. To do this, we calculate the volume fractions of the various materials for every multilayer layer (1 nm slices from the model, not to be confused with the 'layers' of melanosomes and air channels). These volume fractions are our weighting factors (f_i), which are used together with the wavelength-dependent refractive indices (n_i) to calculate the effective refractive index using eqn (1).

The second part of your question addresses the barbule shape that is curved. Since our measurements are performed at normal incidence (bifurcated probe) and very locally (micro spectra), the signal that arises from the barbule curvature or barbule cell curvature is not included in our spectra. It therefore does not affect the spectra that we measure here. This curvature would be more important for the macroscopic appearance of the feather. Perhaps to reduce the macroscopic iridescent effect and create a more constant and less angle-sensitive signal? Yoshioka and Kinoshita have studied the effect of the barbule cell curvature on the angular optical spectra.[1] Their calculations show that the curvature reduces the peak reflectance variation when tilting the sample.

1 S. Yoshioka and S. Kinoshita, *Forma*, 2002, **17**, 169–181

Mike Hardy communicated: Dear Pascal, thank you for your paper. Some of the melanosomes appear less than round in the TEM image. Have you modelled any variations to the actual melanosome shape?

Pascal Freyer communicated in response: Dear Mike, thank you for your question. We have looked at the extreme case of perfectly rectangular melanosomes in order to compare different refractive index profile shapes. We find that the shape of the refractive index profile (*i.e.* the melanosome shape) is not very relevant to the spectral characteristics at visible wavelengths (above 400 nm). When using such multilayer approximations, the interpretation of the optical spectrum boils down to the consideration of optical path length (interference) and refractive index contrast (reflectance) of the multiple layers of alternating refractive index materials (see for instance Land (1972)[1] and Kinoshita *et al.* (2008)[2]). We have previously shown in collaboration with Bodo Wilts, however, that these multilayer results agree very well with FDTD modelling, and that random variations in the diameter of the melanosomes have only a very small effect.[3]

1 M. F. Land, *Prog. Biophys. Mol. Biol.*, 1972, **24**, 75–106.
2 S. Kinoshita, S. Yoshioka and J. Miyazaki, *Rep. Prog. Phys.*, **71**, 076401.
3 P. Freyer, B. D. Wilts and D. G. Stavanga, *Interface Focus*, 2018, **9**(1), 20180043.

Stephanie Burg opened a general discussion of the papers by Sébastien R. Mouchet: In the section of the paper entitled "Prediction of the UV response using Mie scattering theory" calculations are performed using a sphere radius of 145 nm as determined *via* SEM images and the Analyze Particles subroutine in ImageJ. By

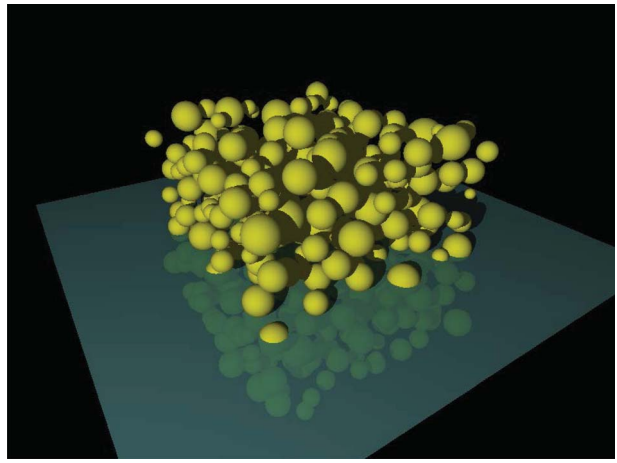

Fig. 1 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

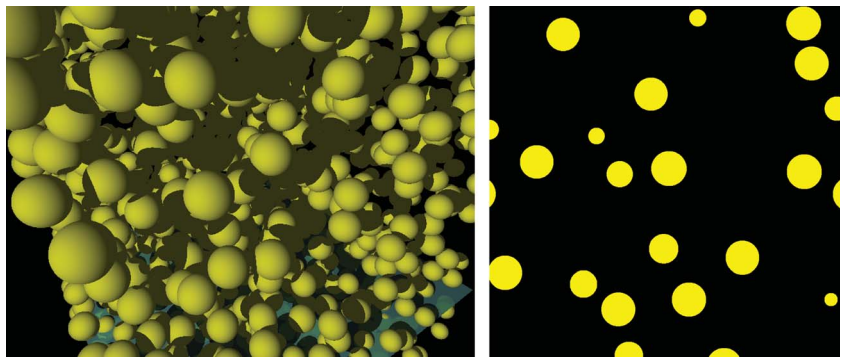

Fig. 2 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

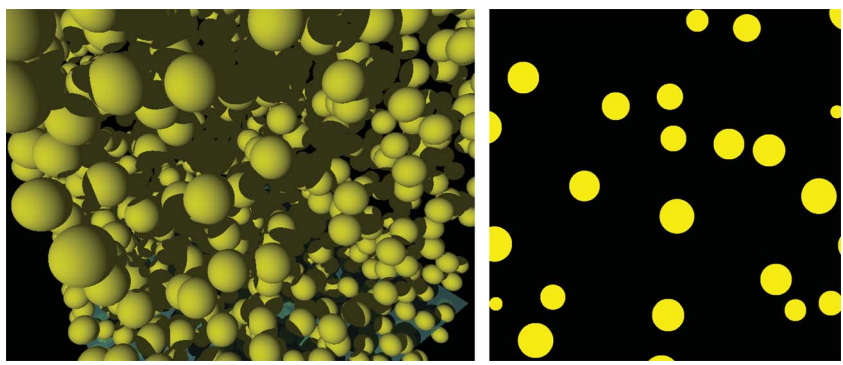

Fig. 3 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

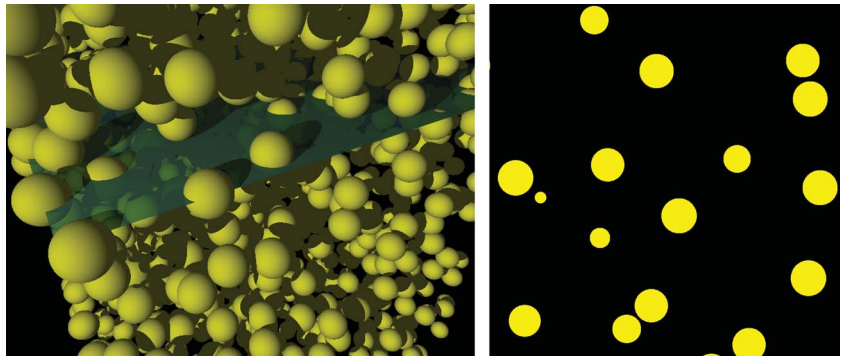

Fig. 4 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

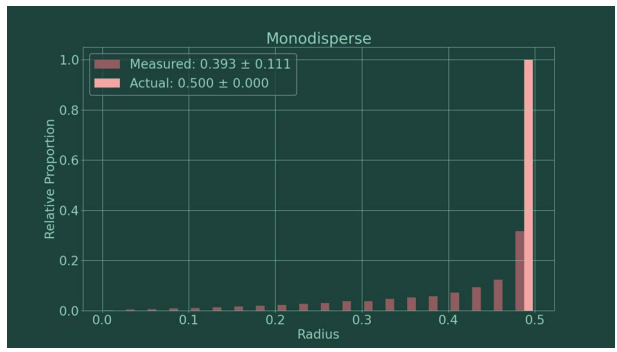

Fig. 5 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

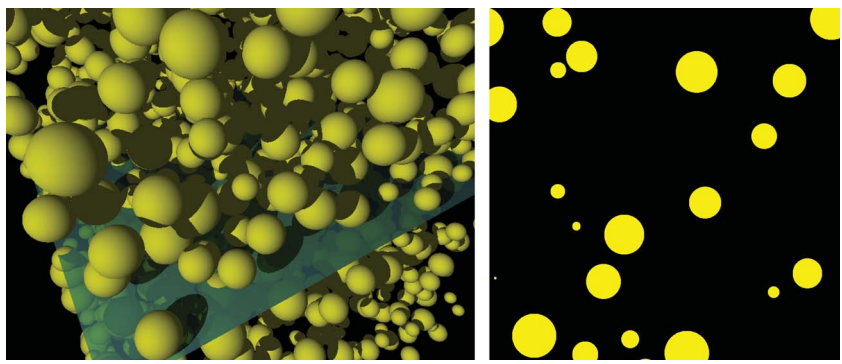

Fig. 6 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

running a series of simulations in which 2D slices are taken through a cube filled with spheres of monodisperse radii, and spheres with a Gaussian distribution of radii, it is possible to show that radii values determined *via* SEM will always be an

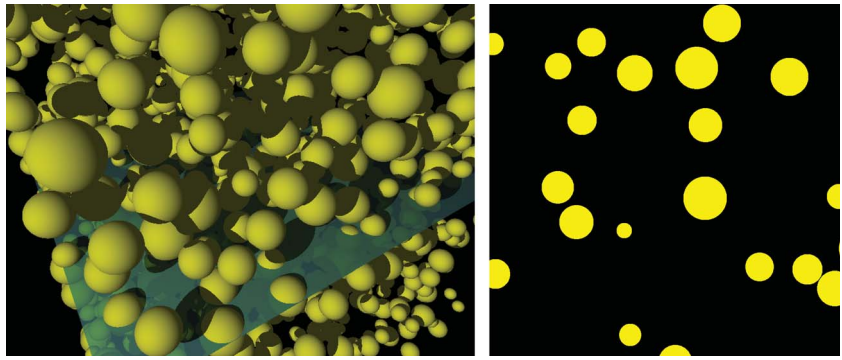

Fig. 7 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

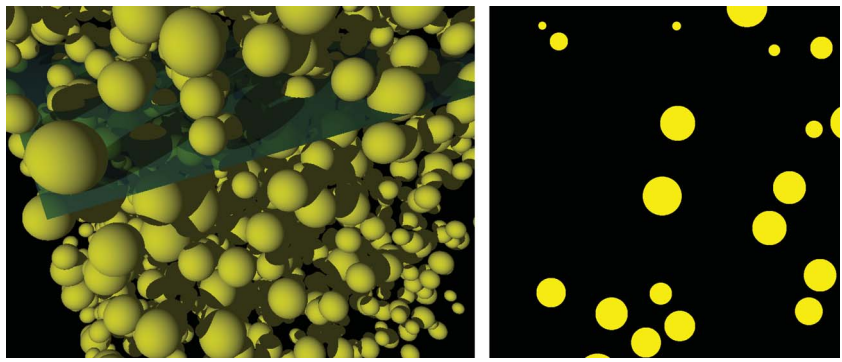

Fig. 8 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

underestimate of the correct value (Fig. 1–11). Therefore, assuming your calculations have been carried out with a lower value of sphere radius than present in the egg shells, how does this effect your results? Additionally, if these calculations are not particularly sensitive to the chosen value for sphere radius, why would this be the case, and is the model still considered reliable if the radius of the pores is accurately known?

Mathieu Ladouce answered: As described in our manuscript, the scattering mean free path was first calculated from the total transmittance measurement of the beige hen eggshell, using the following equation:[1]

$$T_{\mathrm{tot}}(\lambda) = \frac{l_{\mathrm{t}}(\lambda) + z_{\mathrm{c}}(\lambda)}{t + 2z_{\mathrm{c}}(\lambda)},$$

with (black dashed curve) and without (black solid curve) extinction correction factor to T_{tot}. This experimental assessment of the scattering mean free path was compared to predictions based on a first Born approximation of the scattering medium[2] (blue and red curves):

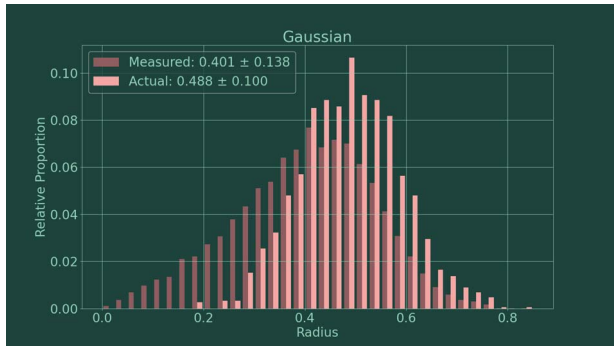

Fig. 9 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

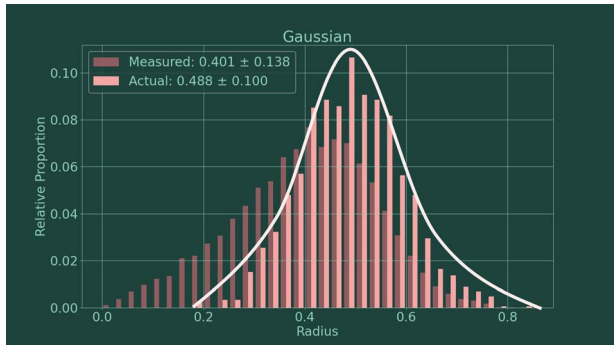

Fig. 10 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

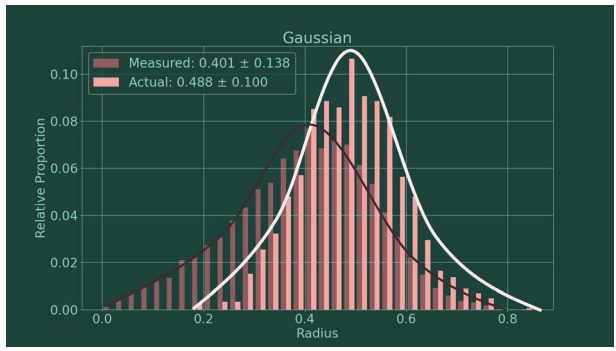

Fig. 11 Simulations by Dr Adam Washington, code at https://gitlab.com/rprospero/sphere-slicing.

$$l_t = \frac{1}{\rho\sigma(\lambda)},$$

where ρ is the density of the scatterers and $\sigma(\lambda)$ is the Mie scattering cross-section of a single spherical volume which depends on the sphere radius a.

Two densities were compared: the density extrapolated from the surface density of the pores measured in the SEM images (red curve) and the density measured by mercury intrusion porosimetry (blue curves) which appear to be more accurate. Using the latter, we compared two sphere radii: the one measured in SEM images (blue solid curve) and the average radius of the gaussian curve fitted to the pore radius distribution measured by mercury intrusion porosimetry (blue dashed curve).

We observe a satisfactory match between the scattering mean free path calculated from Mie theory with the scatterer density measured by mercury intrusion porosimetry (blue curves), and the mean free path calculated from the corrected total transmittance spectra (black dashed curves) in the UV range (Fig. 12 in the discussion). However, the radius a does not seem to affect the scattering mean free path that much in this range.

The mismatch between the mean free path calculated from the pore density assessed by SEM observations (red curve), and the one calculated from the corrected total transmittance spectra (black dashed curve), is due to the cross-section observations performed by SEM that tend to underevaluate pore densities.

1 E. Akkermans and G. Montambaux, *Mesoscopic Physics of Electrons and Photons*, Cambridge University Press, 2007.
2 M. Burresi, L. Cortese, L. Pattelli, M. Kolle, P. Vukusic, D. S. Wiersma, U. Steiner and S. Vignolini, *Sci. Reports*, 2014, **4**, 1–8.

Sébastien R. Mouchet further responded: Many thanks for this question and the few slides you have prepared for the occasion. Really nice illustration of the underestimation of the pore size measured by SEM observation. We are indeed

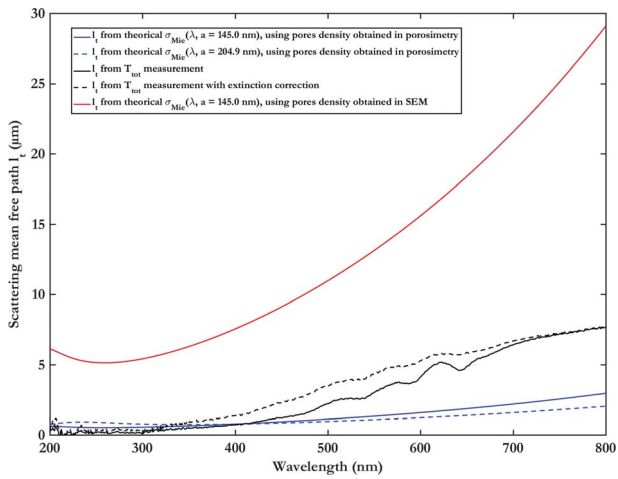

Fig. 12 Scattering mean free path.

fully aware of that experimental error. That is why we also performed mercury intrusion porosimetry in our study to assess the pore size distribution. The related measured mean radius is 204 nm, which is significantly different from 145 nm. However, one drawback of mercury intrusion porosimetry is that only open or connected pores are detected, as also mentioned by Gerd Schröder-Turk in this discussion. The porous region of the eggshell spans a depth of *ca.* 20 μm to *ca.* 220 μm. Therefore, we treated the samples with EDTA solutions. One consequence of such treatment is that they could possibly enlarge the pores a little bit.

In our numerical study, we calculated the scattering mean free paths using the Mie scattering cross-sections of single scatterers with radii equal to 145 nm and 204 nm. Only the former case was shown in the ESI of the manuscript since we regarded the results for both radii as not significantly different. Mathieu Ladouce uploaded here in this discussion a figure comparing these scattering mean free paths (Fig. 12, in the discussion). The computation of the scattering mean free paths was a preliminary approach to assess whether an effective model could account for the optical properties of the scatterers in the UV-visible range.

Based on your question, it seems like some confusion appeared. The Mie scattering and backscattering efficiencies were predicted for particle sizes corresponding to the pore size distribution measured by mercury intrusion porosimetry. We believe this distribution is more reliable than the mean pore radius measured from the SEM images. In Fig. 7(a and b) in our paper, you can see that both Mie scattering and backscattering efficiencies are affected by the scatterer size.

Lukas Schertel remarked: Aren't the pore sizes observed in the egg-shell cross-sections too large and the filling fraction of pores too small for efficient UV scattering?

Sébastien R. Mouchet replied: To make sure there is no confusion, I would like to stress that the figure related to mercury intrusion porosimetry (Fig. S7, ESI of our paper) exhibits two peaks in the pore size distribution. One corresponds to pores with a diameter equal to *ca.* 2 μm. We believe that it relates to larger channels connecting the egg to the topmost surface, or to surface roughness. The second peak corresponds to pores with a mean diameter equal to 408 nm. I am unsure why you wonder whether the pores might be too large. As a simple comparison, the white (hence visible) colours of clouds, fog, mist and some aerosols are known to originate from Mie scattering by droplets of a few microns in diameter. Of course, the refractive indices are different but this could be an *a priori* intuition that particles with a diameter of *ca.* 0.4 μm could scatter UV light. In addition, predictions of the Mie scattering and backscattering efficiencies in the UV range (200–350 nm) show different modes (Fig. 7 in our paper). Of course, different particle sizes could possibly give rise to higher backscattering and scattering efficiencies. The pore size in eggshells may not have been optimised for UV scattering. We agree that the pore density appears relatively low (*i.e.*, 1.33×10^{19} m^{-3}). However, the porous region of the eggshell is thick, namely *ca.* 220 μm. This implies that there is a very large number of scatterers involved in the measured optical response.

Christian Kuttner remarked: Could you please explain the combination of light scattering and absorption of the shell? It seems to me that the scattering is the dominant effect. What is the significance of light absorption?

Sébastien R. Mouchet responded: Usually, pigments are found in the top layer of the eggshell. These are typically protoporphyrin IX and biliverdin IXα. In our study we treated the samples with ETDA in order to remove the absorbing cuticle. Our chemical analysis showed that the calcified shell contains mainly calcite (which is not the case in the other layers). No pigment seems to be found in this part of the eggshell. Light backscattering is surely dominant in that part. However, when the concentration of pigments in the cuticle is high, this absorption by pigments is probably dominant.

Mathias Kolle asked: In heavily pigmented shells, do the scattering structures still play a role in maximizing interactions with the pigments?

Sébastien R. Mouchet replied: Presumably, the pores could indeed increase absorption by pigments. So far, in our study, we mainly tried to suppress the absorption by pigment in order to explain the UV scattering. The interplay between pigments (located in the top layers) and the underlying scattering pores is surely an aspect we should consider.

Andrew Parnell asked: How does the chemical treatment you carry out affect the eggshell? In particular long exposure time. Does it dissolve the eggshell and does it introduce pores?

Sébastien R. Mouchet answered: This chemical treatment aims to dissolve the topmost 20 μm so that the pores in the calcified shell can be reached by mercury. You are certainly right; treatments with acidic solutions can enlarge the pores and possibly introduce more pores. However, the pores that could be introduced are most likely much smaller than 200 nm or even 100 nm.

Nicola J. Nadeau opened a general discussion of the paper by Primož Pirih: Is there any behavioural evidence that any *Vanessa* species can distinguish red and green? The red colour may not be for signalling to conspecifics but targeted at other species, like predators.

Primož Pirih replied: The behavioural evidence[1] shows that *Vanessa atalanta* can discriminate blue (440 nm) from red (620 nm), while orange (590 nm) and red (620 nm) monochromatic stimuli were discriminated solely by their apparent intensity. *Heliconius erato*, which has red pigments in a subset of ommatidia that likely are the retinal substrate for much better colour acuity in the red range, was able to discriminate very well between orange and red, and less well between red and deep red (640 nm). Our modelling assumed that the green rhodopsin template has a peak at 520 nm – perhaps a more accurate estimate would be 530 nm.[2] In our model, the sensitivity peak of R3-8 shifted from 520 to ~538 nm, and the peak of R9 to ~550 nm. If the Admiral's R9 is functional in colour vision, it should increase colour acuity in the range between, and surrounding, the two peaks in the green-yellow-orange range, 530 to 560 nm. The Red Admiral's red

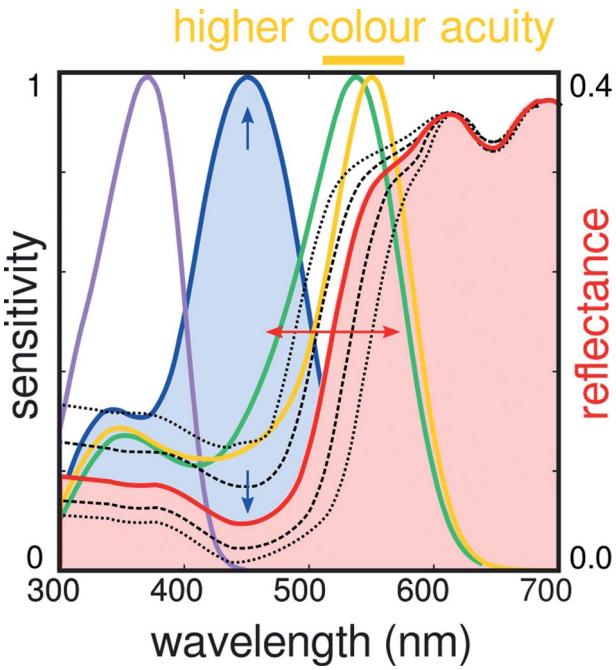

Fig. 13 The peak sensitivity of the Red Admiral's blue receptor is coinciding with the lowest reflectance of red wing patches (blue arrow). The peak sensitivity of the green photoreceptor is coinciding with the spectral range where the reflectance of red wing patches has the steepest slope. If the basal receptor R9 (yellow sensitivity curve) is functioning in color vision, it could increase the butterfly's ability to discern the color changes related to the concentration of the pigment deposited in the red patches (red arrow).

colouration is different from the monochromatic stimuli used in the behavioural study of Zaccardi *et al.*[1]: the reflectance spectra of the red patches are those of long-pass filters. The highest slope of the reflectance spectra is between 500 and 550 nm, coinciding well with the region where the higher colour acuity due to R9 is supposed to be. It is also worthwile to note that the lowest reflectance of red patches coincides with the peak of blue photoreceptors. The inflection wavelength of reflectance spectra depends on the pigment concentration (and possibly oxidation state) and might therefore be a useful proxy for detecting the 'freshness' of individuals in intraspecific communication (see discussion figure, Fig. 13). This of course does not preclude the possibility that the red coloration is also being used as a signal for other species. It would indeed be interesting to test whether the Red Admiral can discriminate between different long-pass orange-red colours.

1 G. Zaccardi, A. Kelber, M. P. Sison-Mangus and A. D. Briscoe, *J. Exp. Biol.*, 2006, **209**, 1944–1955.
2 A. D. Briscoe, G. D. Bernard, A. S. Szeto, L. M. Nagy and R. H. White, *J. Comp. Neurol.*, 2003, **458**(4), 334–349.

Adam James Blake remarked: What would you expect to find with your pupil response method when examining the model organism *Pieris rapae* with a more complicated ommatidium that includes screening pigments and multiple types of red receptors? Would you expect this method to identify the multiple different red receptors?

Primož Pirih answered: As a rule, pierids and especially small lycaenids have a much slower pupil than the nymphalids. The pupil however does react, so the sluggishness is merely an experimental nuisance. Willi Ribi reports that the red pigment granules of proximal tier cells R5-8 do travel, albeit less than the dark pigment granules of R1-4.[1] R9 is devoid of pigments. Since the eyeshine of *Pieris rapae* is red, the ORG test light has to be red too, which implies that the measured pupil action due to red pigments will be small. I predict that the ORG in Pieris will have strong peaks corresponding to the spectral sensitivities of R1-4, while the red receptors will contribute much less to the overall response. Assuming that the signal remains detectable, however, the different peaks of red receptors should pop out in the analysis. This also brings another interesting point that goes past the point of experimental ORG: since the red pigments are transparent in the red range, the absolute sensitivity of light-adapted receptors R5-8 around their effective red peaks will not change much due to their own pupil action. The other uncharted territory is the dorsal area, which has a green eyeshine and is without red pigments. All in all, rather interesting and deserving to be put to an experimental test.

1 W. A. Ribi, *Cell Tiss Res.*, 1978, **195**, 299–308.

Melanie Brien asked: Is there any sexual dimorphism in the eye, as in *Heliconius* for example?

Primož Pirih replied: Sexual dimorphism in the eye of Nymphalini butterflies has to my knowledge not been shown. In terms of genetic expression there have been three opsins reported, and there are no reports on sexual dimorphism. In terms of colouration, in Nymphalini, as a rule, the two sexes have very few differences, often limited to subtle differences in the patterns, but not in the presence/absence of specific colours.

There may be, however, more subtle differences in the function of the eye: for instance, the males could have a slightly bigger acute zone, or the photoreceptor transduction speed could be different, or the fractions of ommatidial types may differ – not only between the sexes, but also among the closely related species.

Aleca Borsuk opened a general discussion of the paper by Doekele G. Stavenga: Thank you for a fascinating talk Professor Stavenga. Considering the range of natural variation in conical epidermal cell morphology, do you have insight into what particular traits – such as cell geometry, packing, or pigment spatial distribution – promote the velvety effect? Additionally, since you are finding that light focusing should not be assumed to be a primary function in these types of cells, what comparisons are there to be made between conical epidermal cells that reduce surface gloss *vs.* conical epidermal cells that do promote the focusing of light on plastids?

Doekele Stavenga answered: Dear Aleca, thanks for the interesting question. However, I am just opening this can of worms. There is indeed a rich variety of conical or papillate epidermal cells in flowers. In my opinion the assumed lens function of the conical cells is generally misleading, as it will only hold for normal illumination. For plants, or at least their flowers, that have petals with different orientations in a natural environment with more or less random illumination, even when dominated by sun light, focusing is a red herring. As I mentioned in my comment after my talk, anti-wetting may be another important function. From my limited observations, the velvety appearance distinctly depends on the packing of the papillate cells and the shape of the cones. Why that is so, needs much further study. I hope to be able to give a more informed opinion in due time.

Gea Theodora van de Kerkhof asked: In your manuscript you reject the hypothesis that cone-shaped epidermal cells are formed to increase light focusing onto the pigment, because of the directionality of light that is needed for the lensing to be effective. Instead, you find that their main function is to reduce surface gloss. How would you relate this to the function of the epidermal ridges in the California poppy, which have so far been assumed to function for light focusing? These flowers have a distinct gloss, so surface gloss reduction is out of the question.

Doekele Stavenga replied: The California poppy is a very special case where the epidermal cells deviate from the typical more or less circular symmetric shape, as they are very prolonged along one dimension, so forming longitudinal ridges (as beautifully shown in the Wilts *et al.* paper, as you well know[1]). I presume that a local gloss is created by incident light in a plane more or less parallel to the ridges rather than by light incident in a plane more perpendicular to the ridges. This will not happen with the standard conical epidermal cells. Your statement of 'gloss reduction is out of the question' may therefore not be fully justified, in my humble opinion, because gloss reduction will also hold for the California poppy for most incident angles except for light incident in planes that are approximately parallel to the ridges.

1 B. D. Wilts, P. J. Rudall, E. Moyroud, T. Gregory, Y. Ogawa, S. Vignolini, U. Steiner and B. J. Glover, *New Phytol.*, 2018, **219**(3), 1124–133.

Conflicts of interest

Adam James Blake and Primož Pirih have collaborated on work together in the past, there are no other potential conflicts to declare.

