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The nature and structure of the white-reflecting underside 'scales' on the hind wing of *Pseudolestes mirabilis* (Odonata: Pseudolestidae)

Albert G. Orr[1], Matthew R. Nixon[2] & Peter Vukusic[2]

[1] Environmental Futures Institute, Griffith University, Nathan, Q4111, Australia;
agorr@bigpond.com

[2] School of Physics, University of Exeter, EX4 4QL, UK;
M.R.Nixon@exeter.ac.uk; P.Vukusic@exeter.ac.uk

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Abstract. The Hainanese endemic damselfly, *Pseudolestes mirabilis*, is unique among the Odonata in having brilliant silvery-white reflective areas on the underside of the hind wings in mature males. The light reflected is easily seen to be several times brighter than that from normal white pruinescence. The hind wing upsides have a striking coppery appearance due to the filtering of light reflected from the inside of the reflective area through bright amber tinted wing membranes, colour which results from small amounts of melanin in those parts of the membrane. Visual signals are thus produced from both sides of the wing and may be used to advertise territory occupancy while perched, as well as having an obvious semiotic function in aerial agonistic displays between pairs of males. The structure consists of a deep layer of long, parallel, flat wax fibres, secreted from the faces of cross-veins in individual wing cells over the affected areas. This is a spectacular and novel mode of cuticular wax secretion. The structure adds about 23–27 % to the mass of the hind wing, which may explain its unusual shape and shortness. However this character is also present in females, which lack the wax fibres, hence it may be an unusual example of an epigametic male trait being partially expressed in females.

Further key words. structural colour, cuticle, epicuticule, wax, fibres, disordered, wing density, wing mass

Introduction

The monotypic genus *Pseudolestes* Kirby, 1900 is endemic to the island of Hainan in southern China where it is a common inhabitant of forest streams (Reels & Zhang 2015). Based on molecular evidence, it is presently placed in its own monotypic family, the Pseudolestidae, within the Calopterygoidea *sensu lato* (Dijkstra et al. 2013). This view is endorsed by Yu & Bu

(2011, 2014), who studied its unique larval morphology. Its position within the superfamily remains unresolved but it is clear on the basis of many characters that the single species in the family, the generotype *Pseudolestes mirabilis* Kirby, 1900, is one of the most unusual of all extant Zygoptera and has no near relatives.

Among the more unusual features of *P. mirabilis* is the extremely short, broad, strongly falcate hind wing, which has length ratio of *ca* 3:4 with respect to the fore wing. This is relatively shorter than in any other known odonate and the condition occurs in both sexes. Moreover in mature males, but not females, the hind wing underside also has brilliant, silvery-white reflective patches underlying amber-tinted areas of the wing membrane (Fig. 1). The main patch occupies approximately the submedial third of the wing and there is a similar, smaller patch at the apex. Sometimes in the intervening area where the membrane is dark there is also light to medium density white frosting. Seen from above, the areas overlaid by the transparent amber membrane have a metallic coppery appearance due to the filtering of white light reflected from the patch beneath (Nixon et al. 2017).

The reflective area consists of a distinct surface layer of an apparent waxy fibrous substance which, as the male matures, builds up within the individual wing cells to form what have often been termed 'scales' (Corbet 1999: plate B6). Dull white reflection from extracuticular pruinescence on the wings and body is well known in the Odonata and has been shown to derive from wax crystals of various forms secreted by the epicuticle (Gorb 1995; Gorb et al. 2000, 2009; Schultz & Fincke 2009). However no species studied reflects white light at an intensity comparable to that observed in *P. mirabilis*.

In a study led by the second author (MRN) to elucidate the photonic properties of the reflective patch and the physical basis of the intense scattering observed, the microstructure of the patch was investigated using FIB-SEM. The photonic properties of the system are reported elsewhere (Nixon et al. 2017). This study examines in detail the morphology of the reflective structure and its origin. We investigated the extent to which the structure increases wing mass and confirm its probable wax constitution by testing its solubility in chloroform.

Material and methods

Specimens of *Pseudolestes mirabilis* for this study were collected in the field, killed and fixed in acetone: 2♂ mature, 1♂ early post-teneral; Wuzhishan, Hainan Province, P. R. of China, 16-iv-2008, collector unknown; 3♂ (two mature, one semi-mature), 2♀, Yinggeling Nature Reserve, Hainan Province, P. R. of China, 27–28-iv-2009, leg. Hao-miao Zhang. All were examined initially and their maturity assessed using a Zeiss Stemi 2000-C dissecting microscope.

The wings of five males and two females were removed and weighed individually, using a Mettler Toledo UMX2 Ultra-Microbalance. Wings were photographed and their lengths, from the base of the costa to the apex, were recorded to an accuracy of ± 0.1 mm. Wing area was estimated for the right

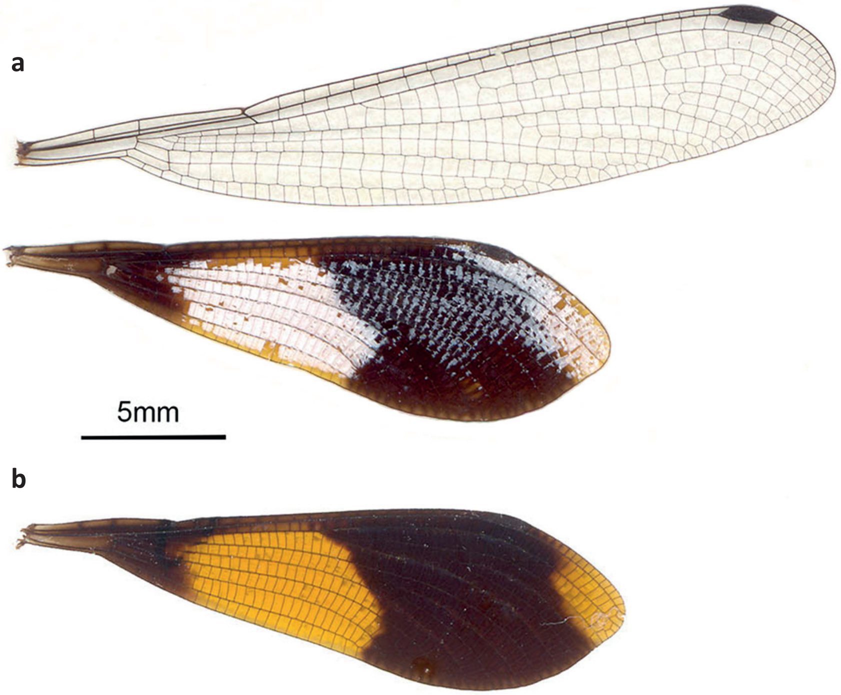

Figure 1. a – Underside of left fore and hind wing of a mature male *Pseudolestes mirabilis;* b – upper side of right hind wing of same individual. Photos: AGO

pair of wings of one mature male, all wings of the post-teneral individual and the right wings of one female by manual integration using a grid overlay on Adobe Photoshop, yielding a result accurate to *ca* ± 1 %. Wing areas of other wings were estimated from their size relative to the measured wings. Although this assumes that wing shape was constant within each sex, it is considered the degree of error introduced was acceptable for the purpose of comparing wing densities.

Two mature male specimens and the post-teneral male were then examined using SEM. The remaining three were treated in various ways with chloroform and mechanical abrasion to remove the wax reflective structure.

SEM-imaging

SEM-images of the white reflecting under-surface of the wing were obtained using an FEI Nova 600 NanoLab Dual-beam system. Samples of *ca* 10 mm^2 were cut from the wings of a mature male *P. mirabilis*. These were mounted on an SEM stub with electrically conducting epoxy resin and sputter coated with approximately 5 nm AuPd then examined using an electron beam voltage of 10 kV, beam current of 7.5 pA and a working distance of 5 mm.

Initial attempts to section the reflective structure and the wing membrane using an ultramicrotome were unsuccessful as the reflective structure shattered. Therefore the reflective layer (and in places parts of the wing in the region of the veins) were sectioned using focussed ion beam (FIB) milling. FIB-milling of regions through the *P. mirabilis* reflective structure was performed with the previously described Nova 600 NanoLab Dual-beam system. A suitable region of the sample was identified (namely an undamaged region of the fibre-structure) using the electron beam and aligned so that the region to be milled was perpendicular to the fibres. To mill the rectangular section, a beam voltage of 30 kV and a beam current of 1 nA was used at a working distance of 5 mm. These sections were then examined by SEM as above.

Chemical treatment and physical removal of reflective patch

Treatment A: For two mature specimens the left fore and hind wings were submerged in chloroform at 60°C for one hour. They were then removed, dried and weighed as above.

Treatment B: The right hind wing of the same insects was subjected to rinsing of the underside with a jet of chloroform produced from a syringe at *ca* 50°C for about a minute, after which the wing was placed in a petri dish in 95 % ethanol. Traces of the white material were removed manually using a fine synthetic 'sable' artists brush. Treated wings were reweighed as above. The object of the second treatment was to avoid the possible removal of epicuticular wax from the upside and elsewhere on the wing not associated with the reflective structure.

Results

The development of the white reflective structure

The progression of fibre development from post-teneral to sub-mature and mature individuals of *Pseudolestes mirabilis* is shown in Figure 2. In the post-teneral individual no intracellular fibres are present and the wing membrane is unpigmented in those areas which later develop an amber tint. In older individuals white fibres are gradually extruded from the proximal and distal faces of the cross-veins bounding the wing cells throughout the affected area of the wing. This is particularly clear in the more peripheral cells where the two advancing sets of fibres have not yet met. In fully mature individuals, in the main reflective areas, the fibres meet broadly in the centre of the cell forming a clear central dividing line where they overlap (Figs 3a, c). As the insect matures the wing membrane becomes darker until it is deep amber. On the other hand, the deep melanin in the post median area is well developed and the basal dark area is already plainly discernable. No definite information is available on the timing of this process but it seems likely to take from one to two weeks, based on what we know of the phenology of the insect (G.T. Reels pers. comm.)

The structure of the reflective patch: General views

A general view of the reflective structure is shown in Figure 3a. Even using a low-power dissecting microscope it is evident that flat strands of white material arise from the proximal and distal margins (i.e., the cross-veins) of individual cells and eventually coalesce in the middle where the opposing ends pile upon one another creating a distinct ridge. In all mature male individuals examined (n = 4), the white substance coalesced strongly in the centre of all cells except those situated around the margins of the white patch. Cross-veins were also covered. In some mature individuals cells between the main median and minor apical white patch also contained lesser amounts of white matter, which did not however meet in the middle of the cell. In the single post-teneral individual examined there was no white matter at all on the hind wing under-surface. SEM images (Figs 3b, c) demonstrate that multiple fibres form a deep bed. Individual fibres are strap like and range from 30–40 µm in length, and 2–3 µm in width.

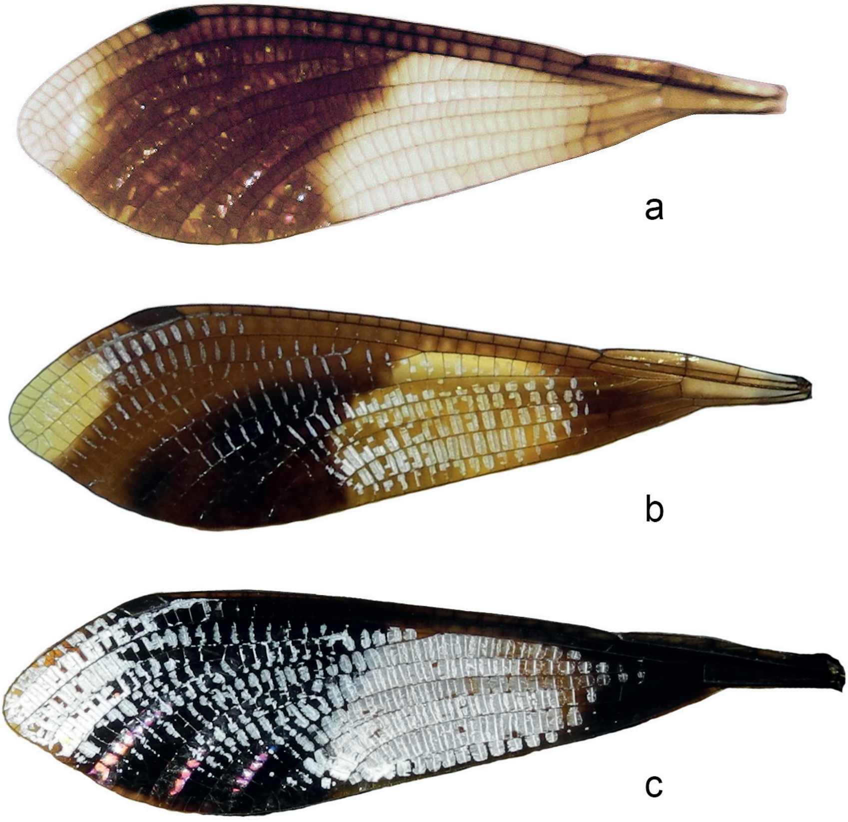

Figure 2. Development of wax layer on underside of right hind wing of *Pseudolestes mirabilis* individuals. a – Post-teneral male; b – sub-mature male; c – mature male. Photos: MRN (2a), AGO (2b, c)

The structure of the reflective patch: Cross sections

High magnification SEM sections also show that the fibres originate from the face of cross-veins and are clearly secreted (Fig. 4a). In the lumen of the cross-vein is agglutinated substance, which may well be wax or wax precursor. Normally such structures are filled with haemolymph, which drains away during preparation leaving a hollow lumen. By contrast, in a section through a cross-vein of the post-teneral male (Fig. 4b), the lumen is empty and there is no evidence of developing fibres. In a view of a longitudinal vein of a mature individual in which the fibrous structure is largely removed (Fig. 4c), the remaining fibres can be seen to lie parallel to the longitudinal vein and not originating from it, although there appear to be un-associated, amorphous agglutinations of wax on the surface of the vein. Seen in section (Fig. 4d), the fibres from a mature specimen appear to be 80–250 nm thick and rather chaotically arranged but with a tendency for the flat fibres to lie

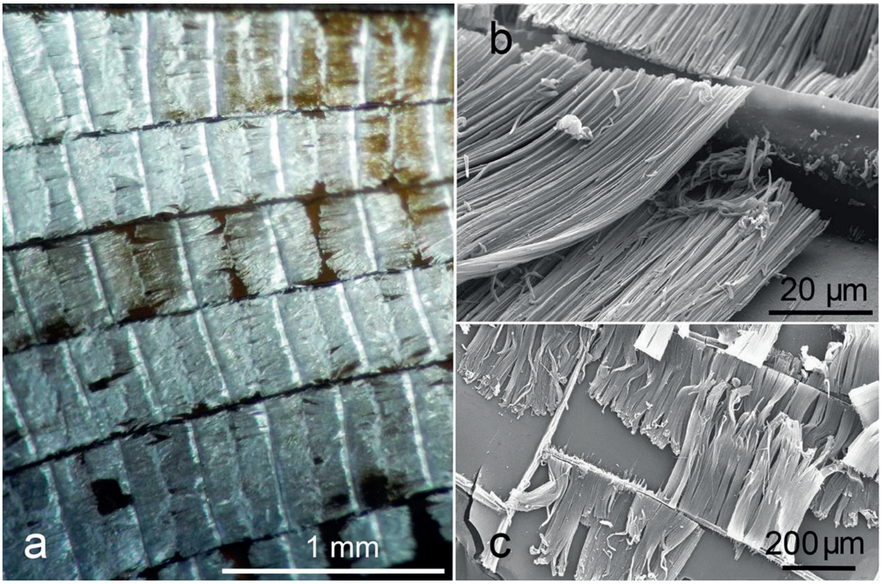

Figure 3. Details of reflective structures of *Pseudolestes mirabilis* wings. a – Detail of reflective area under light microscope; note fibres in third row from top do not meet in centre; b – oblique SEM image of cross-vein with emerging fibres showing depth of secretion; c – near orthogonal SEM view of fibres, many broken, possibly by handling. Photos: AGO (3a) MRN (3b, c)

closer to horizontal than to vertical. It is not clear if the fusion of fibres at the point of sectioning is natural or an artefact of the FIB milling process, hence the true estimate of their thickness may be closer to 80–120 nm. The fibres lie on the wing membrane which is covered with a completely separate thin layer of probable fine wax crystals (Fig. 3b).

Morphometrics, wing mass and chemical treatment

There was no significant difference in the mean fore or hind wing length of males and females, which ranged from 27.8–32.1 mm and 21.0–24.2 mm,

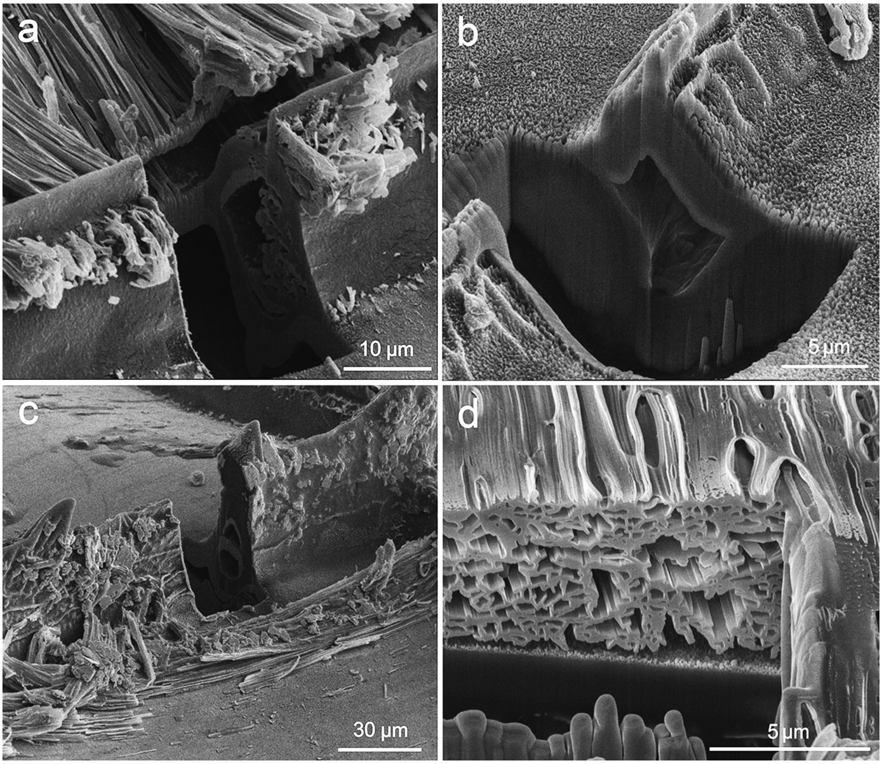

Figure 4. SEM images of FIB milled sections of *Pseudolestes mirabilis* wings. a – Mature male, section of cross-vein; b – mature male, section of longitudinal vein; c – post-teneral male, section of cross-vein, note wax deposits accumulated in lumen; d – section of reflective fibrous structure. Photos: MRN

Table 1. Morphometric statistics of specimens of *Pseudolestes mirabilis* from Wuzhishan, Hainan Province, P.R. of China, showing means ± s.d. * – Includes interpolated values based on wing length.

	Males (n=5)	Females (n=2)
Mean fore wing length [mm]	29.8 ± 1.8	29.5 ± 2.3
Mean hind wing length [mm]	22.1 ± 1.5	22.1 ± 1.6
Mean ratio hw/fw	0.74 ± 0.01	0.75 ± 0.01
Mean fore wing area* [mm²]	112.8 ± 13.1	111.8 ± 17.7
Mean hind wing area* [mm²]	85.6 ± 10.9	92.4 ± 13.1

respectively. Morphometrics are summarised in Table 1. Females have slightly broader and less falcate hind wings which undoubtedly results a greater relative wing area but the present data set is too small to show a significant difference.

Wing mass in males varied from 396–794 μg in the fore wing and 289–797 μg in the hind wing. The lower figures were associated with a post-teneral individual. Females ranged from 593–750 μg (fore wings) and 504–599 μg (hind wings). All measurements are given in Table 2. To allow for variation in wing size, the most reliable statistic for drawing comparisons is wing density, also provided in Table 2. In mature specimens of both sexes this ranges from 6.0–6.2 μg mm^{-2} in the fore wing, but is a remarkably low, 4.0 μg mm^{-2} in the post-teneral male, suggesting that it increases its mass by 50 % during maturity, possibly by the acquisition of surface wax secreted by the pore canals in the wing membrane. The wing density of the hind wing is almost the same as of the fore wing in mature females (5.9–6.1 μg mm^{-2}) and also in the post-teneral male.

Mature male hind wings were consistently denser than the fore wings by a factor of 1.24–1.27; the corresponding figure for the sub-mature male is 1.22. This suggests that the reflective structure is adding 24–27 % to the wing mass in mature males and 22 % in the sub-mature specimen. This estimate is supported by comparisons of hind wing masses before and after chemical/physical removal of the structure (Table 3).

Table 2. Wing masses and wing densities for individual specimens of *Pseudolestes mirabilis* from Wuzhishan, Hainan Province, P. R. of China.

Specimen	Mass [µg] fore wing (left, right)	Mass [µg] hind wing (left, right)	Density [µg mm^{-2}] fore wing	Density [µg mm^{-2}] hind wing
Mature ♂	743, 748	690, 686	6.1	7.6
Mature ♂	794, 791	797, 795	6.1	7.8
Mature ♂	620, 624	604, 603	6.2	7.7
Sub-mature ♂	641, 638	575, 570	5.7	7.0
Post-teneral ♂	396, 399	289, 291	4.0	3.8
Mature ♀	747, 750	599, 598	6.0	5.9
Mature ♀	595, 593	508, 504	6.0	6.1

Table 3. Changes in wing density for individual specimens of *Pseudolestes mirabilis* from Wuzhishan, Hainan Province, P. R. of China, after soaking in 60°C chloroform for 1 hour (treatment A) and washing underside and removing fibres from wing underside with light chloroform wash and brush (treatment B).

Specimen	Density [µg mm^{-2}] fore wing, treatment A	Density [µg mm^{-2}] hind wing, treatment A	Density [µg mm^{-2}] hind wing, treatment B	Hind wing mass attributed to reflective structure based on treatment B
Mature ♂	5.6	5.9	6.2	+ 25.8 %
Mature ♂	5.9	5.8	6.2	+ 23.3 %

It is evident that both wings increase in mass significantly with maturity, but this effect is much greater in the hind wing of males. Treatment A completely dissolved the fibrous structure, but also resulted in a reduction in mass of the fore wings, suggesting additional surface waxes are being removed, but treatment B appears to remove only the fibrous structure, and gives estimates of its contribution to wing mass very close to those obtained from comparisons of fore and hind wing density.

Discussion

The Bauplan of the insect cuticle, including wing membranes and veins, consists of an inner chitinous procuticle and an outer epicuticle consisting of complex lipoprotein elements (Chapman 1998). The epicuticle is traversed by numerous pore canals secreting wax that covers the surface of the epicuticle, typically very thin but in Odonata often developed into complex crystalline structures (Gorb 1995; Gorb et al. 2000, 2009; Hooper et al. 2006; Schultz & Fincke 2009; Kuitunen & Gorb 2011). In many cases these areas reflect white light, a phenomenon known as pruinescence, which is widespread on both wings and bodies in many families of both Zygoptera and Anisoptera. Pruinescence is known to develop gradually with maturity, typically over several days, after the insect has emerged from its larval exuvia (Corbet 1999). In cases where there is no obvious white colour, the wax overlay may modify structural colours underneath (Kuitunen & Gorb 2011) and it has also been suggested that increasing roughness of the wing surface may minimise aerodynamic surface drag (Hooper et al. 2006) and repel water (Gorb et al. 2009).

There can be little doubt that the fibrous reflective structure in *Pseudolestes mirabilis* is homologous with other surface wax structures as summarised by Gorb et al. (2009). The fact that the fibres shattered when an attempt was made to section them with a microtome, their solubility in chloroform, and the way they shear off cleanly from the face of the wing cross-veins from which they originate (Fig. 4a) indicates that they are not part of the epicuticle but secreted wax. This also agrees with all other studies showing copious secretion of wax from the epicuticle in the Odonata. Gorb (1995) distinguished between pruinescence based on laminar wax structures found in Libellulidae and Lestidae, and filamentous structures in Calopterygidae and Euphaeidae. Considered in this context, the hyper-reflective wax structure of *P. mirabilis* is quite unique, perhaps emphasising its level of specialization and remoteness from other extant taxa. It differs chiefly in (1) the scale (and perhaps the chemical composition) of the wax fibres, which are much larger than in any recorded species; (2) in the concentration of the secretory area, which is confined to the inner and outer faces of cross-veins in certain areas of the hind wing; (3) the degree of order in the structure, which is far more cohesive than any fibrous or laminar structures illustrated, for example, by Gorb (1995) and is best described as 2-D disordered (Nixon et al. 2017); and (4) its reflectivity, which is roughly 4–6 times as intense as white light from the pruinescent wing band of *Megaloprepus caerulatus* (Drury, 1782) reported by Schultz & Fincke (2009). This undoubtedly gives the structure its silvery appearance on the underside; the upside appears brilliant copper because reflected light from the upper (inner) surface of the reflecting structure is filtered by the melanin based amber pigmentation of the wing membrane (Nixon et al. 2017).

Given the way the fibres are extruded from the ventral surfaces of cross-veins, it is probable that they arise from discrete clumps of pore canals arranged over the surface of the vein, although in this study SEM images were not obtained at sufficient resolution to identify the canals. This is in contrast to other species in which wax is secreted by pore canals in the wing membrane, although it is probable that normal crystalline surface wax is also produced, as suggested by the loss of mass in chloroform treated fore wings, but this aspect of wing morphology was not examined in detail in this study.

The 50 % increase in wing mass with maturity due to causes other than the development of the reflective patch seems remarkable. With only a single post-teneral specimen and limited SEM cross-sections it is premature to draw more than tentative conclusions from this. Increased mass may be derived from extracuticular wax but may also result from increased mass of the epicuticle with maturity. This phenomenon may be more widespread than is presently known, as the first author has measured a twofold increase in wing density between teneral and mature individuals in *Austrolestes leda* (Selys, 1862) (AGO unpublished data) and a need for closer investigation in a range of species is indicated.

The reflective patch in males appears to function in two ways. Firstly, males perch on the upper side of streamside leaves in sunlight with their hind wings held flat and swept back in an arrow-head formation (Fig. 5a). In this position they are highly conspicuous and the posture may function as passive advertisement of their occupancy of a territory, deterring rival males and attract-

Figure 5. a – Overhead view of territorial male of *Pseudolestes mirabilis* perched in the ‘arrowhead position’; b – *Pseudolestes mirabilis* male in agonistic display showing upper side gold and underside silver-white. Hind wings are held almost stationary while the insect flies with its fore wings. Photos: Graham T. Reels

ing females. When an intruding male enters the territory the occupant rises to meet him and the two commence a protracted contest during which they face each other, flying mainly with the fore wings, displaying the underside white on the hind wings (Reels 2008; Fig. 5b). It is possible that the whiteness of the underside conveys information about an individual's physiological state and vigour. It is also possible that the two hovering males are displaying the coppery upside of the wings to watching females. Receptive females may be visually attracted by the male display, and may also assess each male as a prospective mate. No courtship behaviour is known in this species and agonistic encounters are protracted, often with no obvious resolution (Reels 2008) hence this display might actually constitute a 'mini-lek', or dual display as is known in certain birds (see, e.g., Trainer et al. 2002). This hypothesis requires further investigation but it is clear that the production of reflective wax fibres on the male hind wing involves a considerable material cost and also adds substantially to the mass of the wing, probably affecting its aerodynamic properties and the energy required to sustain flight. Such a highly specialized, complex, and energetically and materially expensive structure must surely have evolved and is maintained in response to strong selective pressures.

It is unclear why the female also has a shortened hind wing. If males evolved shorter hind wings to accommodate the mass of the heavy reflective wax then this would be an example of an epigametic male trait being partially expressed in females. It is worth noting that females also have a small pruinescent white, apical spot on the underside of the hind wing. This however has no fibrous structure, is not particularly bright and probably consists of surface wax crystals similar to those documented for pruinescent areas by other authors (e.g., Gorb et al. 2009; Schultz & Fincke 2009).

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