Molecular basis of wax-based color change and UV reflection in dragonflies

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Abstract

Many animals change their body color for visual signaling and environmental adaptation. Some dragonflies show wax-based color change and ultraviolet (UV) reflection, but biochemical properties underlying the phenomena are totally unknown. Here we investigated the UV-reflective abdominal wax of dragonflies, thereby identifying very long-chain methyl ketones and aldehydes as unique and major wax components. Although little wax was detected on young adults, dense wax secretion was mainly found on the dorsal abdomen in mature males of *Orthetrum albistylum* and *O. melania*, while pruinose wax secretion was identified on the ventral abdomen in mature females of *O. albistylum* and *Sympetrum darwinianum*. Comparative transcriptomics demonstrated drastic upregulation of *ELOVL17* gene, a member of the fatty acid elongase family, whose expression reflected the distribution of very long-chain methyl ketones. Synthetic 2-pentacosanone, the major component of dragonfly’s wax, spontaneously formed light-scattering scale-like fine structures with strong UV reflection, suggesting its potential utility for biomimetics.

(150 = 150 words)
Introduction

Many organisms exhibit a variety of body color patterns for visual communication and environmental adaptation. The diversity of the color patterns encompasses the ultraviolet (UV) range, reflecting the fact that many animals can detect UV light as well as green, blue and/or red (Osorio & Vorobyev, 2008). UV reflection has been reported from numerous organisms, which may be important not only for protection against UV-induced damage but also for visual signaling (Silberglied, 1979; Eaton & Lanyon, 2003; Paul & Gwynn-Jones, 2003; Lee, 2007). Previous studies on biological UV reflection have focused on its optical properties and structural bases such as multilayer surface structures (Sun et al., 2013). In some plants and insects, production and secretion of wax on their surface were reported to increase UV reflection (Clark & Lister, 1975; Pope, 1979; Holmes & Keiller, 2002; Kakani et al., 2003).

Dragonflies (including damselflies) are colorful, large-eyed, diurnal and actively-flying insects, whose body colors often markedly differ between sexes, developmental stages, and closely-related species (Tillyard, 1917; Corbet, 1999; Futahashi et al., 2012; Futahashi, 2016; Bybee et al., 2016; Futahashi, 2017). Because dragonflies are able to perceive UV light (Bybee et al., 2012; Futahashi et al., 2015), it seems plausible that UV color also plays important roles in their mate recognition, male-male competition as well as other ecological conditions such as habitat selection and behavioral differences. Several studies have reported that a pruinose wax layer on the body surface accounts for UV reflection patterns in dragonflies (Silberglied, 1979; Robertson, 1984; Hilton, 1986; Gorb, 1995; Harris et al., 2011), but its biochemical properties, molecular composition, and genes involved in the dragonfly’s wax production have been totally unknown.
Here, we mainly focus on the white-tailed skimmer dragonfly *Orthetrum albistylum* (Selys, 1848), which is one of the most common dragonfly species in Japan (Sugimura et al., 2001; Ozono et al. 2012). As sexual maturity proceeds, adult males of *O. albistylum* show wax-based body color change from light brown to blueish white, whereas adult females remain brownish throughout most of their lifetime, although very aged females become slightly whitish (Figure 1A) (Sugimura et al., 2001; Ozono et al. 2012). Notably, androchrome females, whose body color is very similar to that of adult males, have been recorded, though very rarely, in the field (Figure 1A) (Sugimura et al., 2001; Ozono et al. 2012). Androchrome females can be distinguished from very aged females because the dorsal abdomen is more whitish than the ventral abdomen after semimature stages (Sugimura et al., 2001; Ozono et al. 2012).

In this study, we investigated the ultrastructure, reflectance, wettability, chemical composition, self-organization, and biosynthesis pathway of surface wax in *O. albistylum* and allied dragonfly species. We found that, during the maturation process, adult males secrete a strongly light-scattering wax layer on the body surface, thereby increasing their visibility not only in the blue and green wavelength ranges but also in the UV range. Chemically, the UV-reflective surface wax consisted of very long-chain methyl ketones and aldehydes, which have not been previously identified as major wax components. Comparative transcriptomics identified a gene belonging to the elongation of very long-chain fatty acids (ELOVL) protein family, whose expression was strongly correlated with the distribution of very long-chain methyl ketones on the surface of dragonflies. Notably, chemically-synthesized 2-pentacosanone, the major component of the surface wax, spontaneously formed scale-like fine structures with strong UV reflection. These results provide a previously undescribed
molecular and structural basis of wax-based body color change and UV reflection with ecological and applied relevance.

Results and Discussion

Stage- and Sex-Dependent Body Color Change and UV Reflection in *O. albistylum*. We compared wax-based body color change and UV reflection patterns of adult insects of *O. albistylum* using a high sensitivity camera with a UV filter. In immature males and females, UV reflection was hardly detected on their body surface (Figure 1B, D, G and I; Video 1). As sexual maturation proceeded, males accumulated whitish wax mainly on the dorsal abdomen, which strongly reflected UV (Figure 1C, F, H and K; Video 1). It should be noted that, in mature females, UV-reflective whitish wax was secreted on the ventral abdomen only (Figure 1D, F, I and K; Video 1). As adult aging proceeded further, not only males but also females developed pruinose wax on the entire body surface (Figure 1A), which resulted in considerable UV reflection even in females. The adult insects of *O. albistylum* were subjected to optical measurements for quantitative evaluation of sex- and stage-dependent changes in reflectance on both the dorsal and ventral abdominal regions. Immature males and females mainly reflected light above 500 nm in wavelength, and did not exhibit remarkable UV reflection (Figure 2A and D-figure supplement 1A and D; Figure 2-source data 1; Figure 2-source data 5). In mature males, reflectance increased, in particular below 600 nm, which resulted in strong UV reflection on the dorsal abdomen and moderate UV reflection on the ventral abdomen (Figure 2B-figure supplement 1B; Figure 2-source data 2; Figure 2-source data 6), whereas in mature females, moderate UV reflection was observed on the ventral abdomen only (Figure 2-figure supplement 1E; Figure 2-source data 2; Figure 2-source data...
In aged males and females, reflectance increased to some extent on both dorsal and ventral sides of the abdomen (Figure 2C and F-figure supplement 1C and F; Figure 2-source data 3; Figure 2-source data 7). Microspectrometry of small areas (10 μm x 10 μm) on the dorsal abdomen of a mature male indicated that the surface wax is responsible for overall reflectance, in particular in the short wavelength range including UV (Figure 2G; Figure 2-source data 4): strong reflection was found in the wax-covered white micro-areas (Figure 2G; a, c, d, e and f) whereas little reflection was detected in the blackish micro-areas where the surface wax was lost (Figure 2G; b, g and h).

**Surface Fine Structure of *O. albistylum***. Sex- and stage-dependent changes in the surface fine structure of *O. albistylum*, with special attention to the surface wax, were observed by scanning electron microscopy (SEM). In mature males, the dorsal abdomen was covered with scale- or plate-like fine structures (2–3 μm wide, 50 nm thick), which represented the secreted wax layer (Figure 3A-D and K). The depth of the wax layer reached up to some 6 μm from the cuticle surface (Figure 3D). In mature females and immature individuals, by contrast, only tiny nanopillar-like structures (100 nm wide, 200-300 nm high) were seen on the dorsal abdomen (Figure 3E-J and L). In both mature males and females, on the other hand, small plate-like structures (up to 2 μm wide) were observed on the ventral abdomen, which presumably represented the pruinose wax secretion (Figure 3O and P) that were not conspicuous in immature males and females (Figure 3M and N). Here we suggest that these fractal surface structures consisting of randomly-arranged fine wax platelets are responsible for the whitish structural color due to light scattering, as observed on the dorsal abdomen of mature males and the ventral abdomen of mature males and females. The idea that the
secreted wax layer produces structural color was confirmed by a simple experiment: the whitish color disappeared when light scattering was disturbed by acetone application, and the whitish color instantly recovered upon evaporation of the applied acetone (Video 2). Note that the sizes of the wax platelets (up to 2–3 μm) are larger than the wavelength of UV and visible light and thus capable of light scattering, while the sizes of the nanopillar structures (up to 200-300 nm) are smaller than the wavelength of UV light and thus incapable of light scattering (Vukusic & Sambles, 2003), and thus the reflectivity at the surface may decrease (Kinoshita & Yoshioka, 2005).

**Cuticular Fine Structure of O. albistylum.** In diverse insects, specialized glands or structures for wax secretion have been characterized (Pope, 1979; Ammar et al., 2015), but such wax-producing structures have not been described from dragonflies. Transmission electron microscopic (TEM) observations of the abdominal ultrathin sections of *O. albistylum* identified a number of fine ducts penetrating the cuticle layer in both immature and mature individuals (Figure 3Q-X). Particularly on the dorsal abdomen of mature males, the cuticular ducts were well-developed and full of electron-dense material, probably reflecting the active wax secretion there (Figure 3S).

**Chemical Composition of Dragonfly’s UV-Reflective Wax.** To investigate the biochemical properties and molecular composition of dragonfly’s wax, the surface wax of *O. albistylum* was tested for solubility in organic solvents with reference to surface fine structure and wettability. We found that the secreted wax is insoluble in ethanol (Figure 4B), partially soluble in hexane (Figure 4C and E), and completely soluble in chloroform (Figure 4D and F).
While the untreated abdominal surface exhibited strong water repellency (Figure 4A), removal of the wax by hexane or chloroform treatment resulted in drastically reduced water repellency or increased wettability (Figure 4C and D). As reported in a variety of plants and insects (Hadley, 1981), the strong water repellency conferred by the surface wax may be important for dragonflies with aerial lifestyle to reduce water loss. On the basis of these results, we extracted the surface wax of *O. albistylum* with hexane or chloroform, and analyzed its chemical composition by gas chromatography and mass spectrometry. In the hexane extract from the dorsal abdomen of mature males, only three very long-chain methyl ketones, namely 2-pentacosanone (C$_{25}$H$_{50}$O), 2-heptacosanone (C$_{27}$H$_{54}$O) and 2-nonacosanone (C$_{29}$H$_{58}$O), were identified (Figure 5A-figure supplement 1). In the chloroform extract from the dorsal abdomen of mature males, in addition to the three very long-chain methyl ketones, four very long-chain aldehydes, namely tetracosanal (C$_{24}$H$_{48}$O), hexacosanal (C$_{26}$H$_{52}$O), octacosanal (C$_{28}$H$_{56}$O), and triacosanal (C$_{30}$H$_{60}$O), were detected (Figure 5B-figure supplement 1). It should be noted that, in mature males, the very long-chain methyl ketones were major on the dorsal abdomen whereas the very long-chain aldehydes were dominant on the ventral abdomen (Figure 5B and C). By contrast, only the four very long-chain aldehydes were identified in the chloroform extract from the ventral abdomen of mature females (Figure 5E), while neither the very long-chain methyl ketones nor the very long-chain aldehydes were found in the chloroform extract from the dorsal abdomen of mature females (Figure 5D). Such very long-chain methyl ketones and aldehydes have been identified, although not existing as major components, from surface wax of plants (Kolattukudy & Walton, 1972; Post-Beittenmiller, 1996; Yamamoto et al., 2008; Kunst & Samuels, 2003) and skin lipids of snakes (Ahern et al., 1974). Similar ketones were
characterized as sex pheromones of cockroaches (Nishida et al., 1976) and snakes (Parker & Mason, 2014). In the light of previous studies on wax secretions of various insects, in which hydrocarbons, long-chain esters, alcohols, and/or free fatty acids were identified as major components (Brown, 1975; Blomquist & Jackson, 1979; Hadley, 1981), the chemical composition of the dragonfly’s abdominal UV-reflective surface wax seems unique among insects.

Synthetic “Dragonfly Wax” Exhibits Structural Self-Organization, Strong Reflection and Water Repellency. We chemically synthesized 2-pentacosanone, the main UV-reflective wax component identified from the dorsal abdomen of mature males of *O. albistylum* (Figure 5F), and attempted to recrystallize it on glass plates using three methods, namely dropping, quenching, and slow cooling (see Materials and Methods). The dropping method yielded numerous wax platelets randomly arranged on the substratum (Figure 6B and F), which were quite reminiscent of the fine structure of the surface wax on the dorsal abdomen of mature males (Figure 6A and E). By contrast, the quenching method and the slow cooling method resulted in larger wax platelets (Figure 6C, D, G and H), which looked structurally dissimilar to dragonfly’s surface wax (Figure 6A and E). The 2-pentacosanone sheets made by the three methods showed qualitatively similar reflectance patterns across UV to visible range, which were also similar to the pattern of dragonfly’s surface wax (Figure 6I-L-source data 1). Notably, however, the 2-pentacosanone sheets made by the dropping method yielded stronger light reflectance and lower wettability than those made by the quenching method and the slow cooling method (Figure 6I-P), which are probably due to the smaller wax platelets made by the dropping method that better mimics the fine structure of dragonfly’s surface wax.
These results strongly suggest that the light-scattering nanostructure spontaneously formed by
the secreted very long-chain methyl ketones, including 2-pentacosanone, should play a pivotal
role in formation of the wax layer strongly reflecting UV and visible light on the dorsal
abdomen of mature males of *O. albistylum*.

**UV-Reflective Wax Production in Other Dragonflies.** Diverse dragonfly species are known
to secrete whitish or bluish wax on their body surface (Tillyard, 1917; Corbet, 1999;
Sugimura et al., 2001; Ozono et al., 2012). In a comparative perspective, we investigated wax
production, reflectance, and chloroform-extracted wax composition of the abdominal body
surface of three other dragonfly species, *Orthetrum melania*, *Sympetrum darwinianum*, and
*Crocothemis servilia* (Figure 7). The blue-tailed skimmer dragonfly *O. melania*, which is
closely related to *O. albistylum*, prefers shady habitats in contrast to *O. albistylum* that tends
to form territories in sunny places (Sugimura et al., 2001; Ozono et al., 2012). Mature males
of *O. melania* develop bluish wax mainly on the dorsal abdomen, whereas mature females do
not secrete wax even on the ventral abdomen (Figure 7A). In *O. melania*, UV reflection was
observed only on the wax-bearing mature males (Figure 7E, I and L-figure supplement 1A
and D; Figure 7-source data 1; Figure 7-source data 4; Video 3). In the red dragonfly *S.
darwinianum*, only mature females secrete whitish wax on the ventral abdomen (Figure 7B),
where UV reflection was clearly detected (Figure 7F, J and M-figure supplement 1B and E;
Figure 7-source data 2; Figure 7-source data 5; Video 3). The scarlet dragonfly *C. servilia*
develops little wax on the body surface throughout the lifetime (Figure 7C and D), and UV
reflection was not detected in both sexes (Figure 7G, H, K and N-figure supplement 1C and
F; Figure 7-source data 3; Figure 7-source data 6). These reflectance data indicated that (i)
presence of the secreted wax on the body surface accounts for the UV-reflecting body regions in all dragonfly species, (ii) meanwhile, the levels of the UV reflection vary among dragonfly species and also across different body regions, and (iii) among them, the dorsal abdomen of mature males of *O. albistylum* exhibits the strongest UV reflection (Figures 2 and 7). The wax composition also varied among dragonfly species (Figure 8). For example, only very long-chain aldehydes were detected from mature males of *O. melania* (Figure 8A and C), while only a very long-chain aldehyde, tetracosanal, was identified from the ventral abdomen of mature females of *S. darwinianum* (Figure 8B and C). Very long-chain methyl ketones were detected only in regions where UV reflection was strong in *O. albistylum* (Figure 8C).

**On Ecological Relevance of UV-Reflective Wax Production in Dragonflies.** Here we point out that the UV reflection/wax production patterns observed in these dragonflies seem to reflect, at least to some extent, their environmental and behavioral characteristics. In general, body pigmentation and/or wax production are conspicuous in mature dragonflies, especially reproductively-active territorial males (Tillyard, 1917), which may be related to mate recognition, male-male competition, UV protection and anti-desiccation that are fatally important for them (Corbet, 1999). In the closely-related *Orthetrum* species, *O. albistylum* that dominates sunny habitats show stronger UV reflection than *O. melania* that prefers shady habitats: mature males of *O. albistylum* form dense whitish wax layer on the dorsal abdomen (Figure 1C and F; Figure 9A) whereas mature males of *O. melania* develop relatively thin bluish wax layer (Figure 7A; Figure 9B); mature females of *O. albistylum* wear pruinose wax on the ventral abdomen (Figure 1F) while mature females of *O. melania* do not (Figure 7A). Similar patterns are found in other *Orthetrum* species: mature males of *O. luzonicum* prefer
sunny habitats and develop whitish wax (Figure 9C), whereas mature males of *O. glaucum* attach to shady habitats and form bluish wax (Figure 9D). It is intriguing why mature females of some dragonflies produce UV-reflective wax only on the ventral abdomen, as in *O. albistylum* (Figure 1F and K) and *S. darwinianum* (Figure 7B and F). We point out that, in these species, males form territories in sunny places, wait for females that fly in, and chase and copulate with them on nearby plants (Figure 1F; Figure 7B). Therefore, these species usually mate in sunny places, where the female’s ventral abdomen is exposed to direct sunshine for an extended period (Figure 1F; Figure 7B). By contrast, *O. melania* usually mates in shady places (Figure 7A), *C. servilia* quickly mates during flight for only a few seconds (Figure 7D), and the female’s ventral abdomen develops no wax and exhibits no UV reflection in these species (Figure 7A, D, E and H). Based on these observations, we suggest that, although speculative, the female’s ventral wax might be to protect the ventral abdomen, which is less pigmented, less sclerotized and containing ovaries, against UV damage.

**Comparative Transcriptomics of Wax-Producing and Non-Producing Epidermal Regions in *O. albistylum***. What molecular mechanisms underlie the production of dragonfly’s surface wax of unique chemical composition? Notably, rarely-discovered gynandromorphic dragonflies consistently exhibit discontinuous surface wax patterns (Figure 9E and F) (Sugimura et al., 2001; Karube & Machida, 2015), suggesting that de novo wax production may be regulated in a cell-autonomous manner. In an attempt to gain insights into the molecular basis of dragonfly’s wax production, we performed RNA sequencing using samples of the dorsal and ventral abdominal epidermis dissected from immature, semimature, mature, mature-aged, and aged individuals of both sexes of *O. albistylum*. The adult maturity
was judged based on the amount of wax and the wing condition. In addition, we were fortunately able to examine a mature androchrome female (Figure 10A). Figure 10B summarizes the RNA sequencing data. Of 7,790 gene models whose max FPKM values were greater than 10, 1,708 genes exhibited twice or higher expression in dorsal epidermis of mature males (with wax) than in that of mature females (no wax); 518 genes exhibited twice or higher expression in dorsal epidermis of an androchrome female (with wax) than that of mature females (no wax); and 305 genes were commonly identified in these two categories as upregulated genes associated with wax production. Of the 305 genes, 26 genes were highly expressed (FPKM > 100), of which 5 genes exhibited extremely high expression levels (FPKM > 1,000) (Figure 10C-figure supplement 1; accession nos. LC416763- LC416767).

**Drastically Upregulated ELOVL Genes in Wax-Producing Epidermis of *O. albistylum***.

Among the extremely upregulated genes, we identified a gene belonging to the elongation of very long-chain fatty acids (ELOVL) protein family that was drastically and specifically (more than 100-fold) expressed in dorsal epidermis of semimature and mature males and androchrome female (Figure 10C and D-figure supplement 1). ELOVL proteins catalyze the elongation of fatty acids with acyl chains longer than 18 carbon atoms (Kihara, 2012) and also hydrocarbons (Chertemps et al., 2007). ELOVL proteins are conserved from yeast to mammals, and 3, 7 or 20 ELOVL family protein genes are identified in the genome of yeast, mammals or the fruit fly *Drosophila melanogaster*, respectively (Figure 11A) (Szafer-Glusman et al., 2008; Kihara, 2012). In the transcriptomic data of *O. albistylum* and also in the draft genome data of the scarce chaser dragonfly *Ladona fulva* (BCMHGSC: I5K, GenBank accession no. APVN00000000.2), we identified 17 ELOVL genes in total (Figure
11A; accession nos. BR001497-BR001513, LC416747-LC416763), all of which contained a conserved HXXHH motif (Figure 11B). In addition to the drastically upregulated ELOVL gene (= ELOVL17) mentioned above, two ELOVL genes exhibited notable upregulation patterns (Figure 11C). ELOVL14 was highly expressed in dorsal epidermis of semimature and mature males, and also in both dorsal and ventral epidermis of androchrome female (Figure 10E; Figure 11). In aged females, ELOVL17 and ELOVL14 genes were slightly upregulated (Figure 10D and E), which may account for the slight wax secretion on aged females of *O. albistylum* (see Figure 1A). Meanwhile, ELOVL3 gene was preferentially expressed in the ventral abdomen of immature females (Figure 10F), which may be relevant to the preferential accumulation of very long-chain aldehydes on the ventral abdomen of mature females of *O. albistylum* (see Figure 5E). These results strongly suggest that these ELOVL genes are involved in production of the surface wax, mainly consisting of very long-chain methyl ketones and aldehydes, of dragonflies. To confirm this idea, we attempted to knock-down the expression level of ELOVL17 gene by injection of small interfering RNA followed by electroporation, as established in other dragonfly species (Okude et al. 2017). Employing the RNAi treatment, we expected that the wax production on the abdominal surface of mature males would be suppressed. However, because the electroporation damaged the adult cuticle and caused high mortality of the treated insects, we failed to observe the phenotypes expected for the RNAi experiment. Hence, the precise functions of the dragonfly’s ELOVL gene products are to be verified in future studies, for which establishment of stable laboratory rearing system for *O. albistylum* and application of genome editing technology to it are anticipated. In addition to ELOVL17 gene, the following four genes exhibited extremely high expression in wax-producing regions; Acyl-CoA Delta(11) desaturase, ferritin, NPC.
intercellular transporter 2, and uncharacterized protein (LC416764- LC416767), although their stage and region specificity was not prominent compared to ELOVL17 gene. Biological roles of these genes also deserve future studies.

Conclusion and Perspective. In this study, we found that mature males of *O. albistylum* exhibit strong reflection including UV range in a previously uncharacterized way, namely very long-chain methyl ketone production. Plausibly, differences in wax production between sexes, stages and species are important for their signal communications, and may reflect their habitats and behavior. It should be noted that synthesized 2-pentacosanone, a major component of very long-chain methyl ketone, reproduced strong reflection, surface fine structure, and water repellency. Considering that UV reflective materials can be applied in the fields of cosmetics and painting, and *O. albistylum* has been traditionally used as medicine in Asia (Corbet, 1999), the dragonflies’ UV reflective wax may be potentially utilized as a novel eco-friendly biological material.

Materials and Methods

Insects and UV images. Adult insects of *O. albistylum*, *O. melania*, *S. darwinianum*, and *C. servilia* were collected at Tsukuba, Ibaraki, Japan, or Imizu, Toyama, Japan. Photos of UV reflection were taken in the field using a high sensitivity camera (Sony A7S, IDAS UV-VIS mod) through a UV filter (IDAS-U).

Spectrometry and micro-spectrometry. In order to quantitatively investigate the wax-based color change, the dorsal and ventral parts of abdominal segment 5 were surgically divided and
used for reflection measurements. The reflections from small areas (diameter 6 mm) were
taken using a spectrometer (HR2000+, Ocean Optics), and those of micro areas (10 x 10 µm)
were carried out using a micro-spectrometer (CRAIC Technologies) equipped with an upright
microscope (Eclipse E-400; Nikon). The specimens were epi-illuminated with 75 W Xenon
arc lamp (Nikon), and the measurements were obtained from an approximately 10 µm x 10
µm area. The reflected spectral radiances were converted to relative reflectance by
normalization with a white reflectance standard (Spectralon USRS-99-010, Labsphere).

Histology. The microstructural changes due to wax secretion was examined by scanning
electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM
observation, the dissected dorsal and ventral parts of the abdominal segment 5 were coated
with 2-3 nm osmium layer using a hollow-cathode plasma chemical vapor deposition
(HPC-1SW; Vacuum Device), and observed under a scanning electron microscope (H-4800;
Hitachi) with an accelerating voltage of 5 kV. For TEM observation, dissected dorsal and
ventral parts of the abdominal segment 5 were prefixed for 12 h in 2% glutaraldehyde and 2%
paraformaldehyde in 0.1 M cacodylate buffer (pH7.2), post-fixed with 1% osmium tetroxide
for 2 h in 0.1 M cacodylate buffer, and embedded in Quetol 812-Araldite regin mixture
(Nisshin EM). Ultrathin sections (approximately 70nm thick) were cut perpendicular to the
anterior-posterior axis on an ultra-microtome (UC7; Leica) with a diamond knife (DiATOME),
stained with 2% uranyl acetate for 5 min followed by lead citrate solution for 3 min
(Sigma-Aldrich), and observed under a transmission electron microscope (JEM-1400; JEOL,
100 KV).
Measurement of surface wettability and wax solubility. To elucidate the biochemical properties of dragonfly’s wax, surface wettability and solubility was examined. Surface wettability was evaluated based on the contact angle of water micro-droplet on the samples. Each sample was fixed on a glass substrate, and a micro-droplet of distilled water (about 1.0 nL) was placed on the surface of the sample. The shape of droplet was recorded immediately using a high-speed camera (HAS-220; Ditect) with a microscopic contact angle meter (MCA-3; Kyowa Interface Science). Wax solubility was analyzed by treating the dissected abdominal segment 5 with hexane or chloroform for 30 min.

Wax extraction and analysis. To identify the molecular composition of dragonfly’s wax, wax samples were extracted from living specimens with chloroform or hexane. For gas chromatography/mass spectrometry (GC-MS) analysis, to avoid contamination from internal lipids, the solvent was carefully pipetted several times on the abdominal surface of the living individuals. The extracts were analyzed by GC-MS using a 6890N GC coupled with 5973 MSD (Agilent) in the split-less mode, through a DB-5MS fused silica column (30 m x 0.25 mm i.d., 0.25 μm film thickness, Agilent) with helium as the carrier gas at a flow rate of 1.0 mL/min at a temperature programmed to change from 80°C (1 min) to 320°C at a rate of 15°C/min and then held for 3 min. The mass spectrometer was operated in the scan mode with 70 eV ionazation voltage as electron ionization. Histological examinations, wettability tests, and GC-MS analyses were conducted using different samples.

Chemical synthesis of wax components. To confirm whether the very long-chain methyl ketones form the scale-like fine structures, 2-pentacosanone, the major component of
dragonfly’s wax was chemically synthesized from 1-tetracosanol via 1-tetracosanal and 2-pentacosanol. Pyridinium chlorochromate (PCC, 1.29 g, 5.97 mmol) was added to a suspension of 1-tetracosanol (395 mg, 1.11 mmol) and powdered molecular sieves 4A (2.5 g) in dry CH$_2$Cl$_2$ (35 mL), and stirred for 4 h at room temperature. The mixture was filtered through Celite and washed with diethyl ether. The combined filtrate and washings were filtered through florisil (15 g), washed with diethyl ether (200 mL) and concentrated in vacuo. The residue was chromatographed on silica gel (15 g) and concentrated in vacuo to give a white solid of 1-tetracosanal (290 mg, 0.82 mmol, 74%, GC $t_R = 23.7$ min, MS $m/z$ (%) : 352 (M$^+$, 2), 334 (18), 96 (78), 82 (100), 57 (93), 43(72)). A solution of 1-tetracosanal (176 mg, 0.50 mmol) in dry tetrahydrofuran (THF, 10 mL) was cooled in ice bath. When the temperature reached 0°C, 1.4 M CH$_3$MgBr in THF:toluene 1:3 (1 mL, 1.4 mmol) was added dropwise, and stirred for 1.5 h at 0°C and for 1 h at room temperature. The reaction was quenched with saturated NH$_4$Cl (5 mL), and the product was extracted with hexane (3 × 20 mL). The organic layer was dried with anhydrous magnesium sulfate and concentrated in vacuo. The residue was chromatographed on silica gel (15 g, ethyl acetate/hexane, 1:5) to give a white solid of 2-pentacosanol (106 mg, 0.29 mmol, 58%). PCC (335 mg, 1.56 mmol) was added to a suspension of 2-pentacosanol (257 mg, 0.70 mmol) and powdered molecular sieves 4A (1.0 g) in dry CH$_2$Cl$_2$ (20 mL), and stirred for 3 h at room temperature. The mixture was filtered through Celite and washed with diethyl ether. The combined filtrate and washings were filtered through florisil (15 g), washed with diethyl ether (200 mL) and concentrated in vacuo. The residue was recrystallized from hexane to give a white solid of 2-pentacosanone (202 mg, 0.55 mmol, 76%). Nuclear magnetic resonance (NMR) spectra of 2-pentacosanal and 2-pentacosanone were measured with Bruker AV-400 III
Spectrometer (400 MHz) using TMS as an internal standard. Each sample was dissolved in CDCl$_3$ and $^1$H spectrum was acquired. $^1$H-NMR of 2-pentacosanal (CDCl$_3$, 400 MHz) was as follows: $\delta$ 3.79 (1H, sex, $J$=6.0 Hz, H-2), $\delta$ 1.18 (3H, d, $J$=6.0 Hz, H-1), $\delta$ 0.88 (3H, t, $J$=6.0 Hz, H-25). $^1$H-NMR of 2-pentacosanone (CDCl$_3$, 400 MHz) is as follows: $\delta$ 2.41 (2H, d, $J$=7.6 Hz, H-3), $\delta$ 2.13 (3H, s, H-1), $\delta$ 0.88 (3H, t, $J$=6.4 Hz, H-25).

**Preparation of biomimetic wax surfaces.** Biomimetic wax surfaces were composed of micro crystals of 2-pentacosanone. Heated 2-pentacosanone was recrystallize on gold-coated glass plates by different cooling processes; 1) continuous dropping of micro fused material of 1.0 µL under room temperature, 2) quenching from the melting state, and 3) keeping to near melting point at 64°C from the melting state. Micro-spectrometry, surface fine structure, and wettability were analyzed using a micro-spectrometer (CRAIC Technologies), a scanning electron microscope (H-4800; Hitachi), or a high-speed camera (HAS-220; Ditect) with a microscopic contact angle meter (MCA-3; Kyowa Interface Science), respectively, as described above.

**Transcriptome Analysis.** To investigate the genes involved in wax production, total RNA samples were extracted from the freshly dissected abdomens of *O. albistylum* using RNeasy mini kit (Qiagen) or Maxwell 16 LEV Simply RNA Tissue kit (Promega). RNA sequencing was performed as described previously (Futahashi et al., 2015). Using 1 µg of total RNA per sample as template, cDNA libraries were constructed using TruSeq RNA Sample Preparation Kits v2 (Illumina) and sequenced by HiSeq2000, Hiseq2500, or MiSeq (Illumina). The sequence data were deposited in the DNA Data Bank Japan Sequence Read Archive.
(accession numbers are shown in Supplementary File 1). The raw reads were subjected to de novo assembling using the Trinity program (Grabherr et al., 2011) implemented in the MASER pipeline (Kinjo et al., 2018). After automatic assembling, we checked and manually corrected the sequences of highly expressed genes in mature males using the Integrative Genomics Viewer (Thorvaldsdóttir et al., 2013) as reported previously (Futahashi, 2017). After revising the sequences, sequence read mapping was performed using the BWA-MEM program (Li, 2013) implemented in the MASER pipeline, whereby transcript expression levels were estimated in terms of fragments per kilobase per million reads (FPKM) values. ELOVL genes of *L. fulva* were obtained by tBLASTn search against the draft genome sequence (APVN00000000.2) (https://www.hgsc.bcm.edu/).

**Phylogenetic analysis.** To construct the molecular phylogeny of ELOVL family genes, deduced amino acid sequences were aligned using the Clustal W program implemented in MEGA7 (Kumar et al., 2016). Molecular phylogenetic analyses were conducted by the neighbor-joining method and maximum-likelihood method using MEGA7, and by the Bayesian method using MrBayes version 3.1.2 (Ronquist et al., 2012). Bootstrap values for neighbor-joining and maximum likelihood phylogenies were obtained by 1000 resampling. In total 3750 trees were generated for each Bayesian analysis (ngen = 500,000, samplefreq = 100, burn in = 1250).

**ACKNOWLEDGMENTS.** We thank Tateo Shimozawa for SEM observation, Hiroyuki Futahashi for insect samples, Mitsutoshi Sugimura and Makoto Machida for photos of gynandromorphic dragonflies in Figure 9, and Mutsuo Tanaka and Minoru Moriyama for...
technical assistance. We would like to acknowledge i5K, Stephen Richards and Oliver Niehuis for allowing us to use the *L. fulva* genome data. This work was supported by the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research Grants JP26660276, JP18H02491, and JP18H04893 (to R.F.), and the Cooperative Research Grant of the Genome Research for BioResource, NODAI Genome Research Center, Tokyo University of Agriculture (to R.F., R.M.K., and S.Y). This research was partially supported by Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)) from AMED under Grant Number JP17am0101001.

**Data deposition:** The sequences reported in this paper have been deposited in the DNA Data Bank Japan Read Archive, www.ddbj.nig.ac.jp (accession nos. BR001497-BR001513, LC416747-LC416767, DRA001687, DRA001690, DRA001693-DRA001694, DRA001697-DRA001698, DRA001700-DRA001703, DRA001706-DRA001707, DRA001709-DRA001710, DRA001712-DRA001713, DRA001716-DRA001717, DRA007015-DRA007018).

**Competing interests:**
We filed an international patent on the synthesis method and application of very long-chain methyl ketones and aldehydes as PCT/JP2018/019559.

**References**


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**Figure Captions**

**Figure 1.** Stage- and sex-dependent adult color change in *O. albistylum*. (A) Adult males and females of *O. albistylum*. (B, G) Immature male. (C, H) Mature male. (D, I) Immature female. (E, J) Mature female. (F, K) Mating pair. Images photographed normally (B-F) or through a UV filter (G-K) in the field.
**Figure 2.** Reflectance on the adult body surface at the 5th abdominal segment of *O. albistylum.*

(A-F) Spectrometry of a round area (6 mm in diameter) in males (A-C) and females (D-F). (A, D) Immature individuals. (B, E) Mature individuals. (C, F) Aged individuals. Solid and dotted lines indicate averaged UV reflectance on dorsal and ventral sides of the abdomen, respectively. The standard deviation is shaded. (G) Micro-spectrometry of the 5th abdominal segment of a mature male. UV reflectance was measured of eight micro-areas (10 µm x 10 µm each) depicted as red squares in the photos. In the photos, white areas are covered with secreted wax whereas black areas are without wax presumably due to accidental scratches and cracks of the adult body surface.

**Figure supplement 1.** Micro-spectrometry (10 x 10 µm) of *O. albistylum.* (A-C) Male. (D-F) Female. (A, D) Immature individuals. (B, E) Mature individuals. (C, F) Aged individuals. Three to eight micro-areas were measured for each individual.

**Figure 3.** Fine structure of the adult body surface at the 5th abdominal segment of *O. albistylum.* (A-P) Scanning electron microscopic (SEM) images of the body surface. (Q-X) Transmission electron microscopic (TEM) images of the sectioned cuticle. Here it should be noted that surface wax was dissolved and removed during the sample processing for TEM observation. (A-H) Dorsal side of mature male (A-D) and mature female (E-H). Panels B and F are magnified images of panels A and E, as indicated by red rectangles. Likewise, panels C and G are magnified images of panels B and F. (D, H) Cross-sectioned images of cuticle and surface wax. (I-L and Q-T) Dorsal side. (M-P and U-X) Ventral side. (I, M, Q, U) Immature male. (J, N, R, V) Immature female. (K, O, S, W) Mature male. (L, P, T, X) Mature female.
Figure 4. Solubility and wettability of the abdominal wax of *O. albistylum*. (A-D) Dorsal side of the 5th abdominal segment of mature males. (A) No treatment. (B) After ethanol treatment. (C) After hexane treatment. (D) After chloroform treatment. (E) Scanning electron microscopic images of dorsal surface of mature male 30 min after hexane treatment. (F) Scanning electron microscopic images of dorsal side of mature male (left) or mature female (right) after chloroform treatment.

Figure 5. Identification and chemical synthesis of surface wax of *O. albistylum*. (A) Chromatogram of hexane extract from the dorsal abdomen of mature male. (B) Chromatogram of chloroform extract from the dorsal abdomen of mature male. (C) Chromatogram of chloroform extract from the ventral abdomen of mature male. (D) Chromatogram of chloroform extract from the dorsal abdomen of mature female. (E) Chromatogram of chloroform extract from the ventral abdomen of mature female. (F) Chromatogram of chemically-synthesized 2-pentacosanone. Asterisks indicate nonspecific peaks also detected with the solvent only. The Y-axis shows the abundance of total ion current.

Figure supplement 1. Mass spectra and extracted ion chromatogram of dragonfly’s wax (A, B, D-H) and synthetic 2-pentacosanone (C).

Figure 6. Comparison of surface fine structure, reflectance, and wettability among the dorsal abdomen of mature males of *O. albistylum* and synthetic 2-pentacosanone crystallized on glass plates using three different cooling processes. (A-H) Scanning electron microscopic

**Figure 7.** UV reflection patterns in *O. melania*, *S. darwinianum* and *C. servilia*. (A, E) Mating pair of *O. melania* (B, F) Mating pair of *S. darwinianum*. (C, G) Mature male of *C. servilia* with mature male of *O. albistylum*. (D, H) Mating pair of *C. servilia*. Each image photographed normally (A-D) or through a UV filter (E-H) in the field. (I-N) Spectrometry of a round area (6 mm in diameter) on the 5th abdominal segment of *O. melania* (I, L), *S. darwinianum* (J, M), and *C. servilia* (K, N). (I-K) Male. (L-N) Female. Solid and dotted lines indicate averaged UV reflectance on dorsal and ventral sides of the abdomen, respectively. The standard deviation is shaded.

**Figure supplement 1.** Micro-spectrometry (10 x 10 µm) of *O. melania* (A, D), *S. darwinianum* (B, E), and *C. servilia* (C, F). (A-C) Male. (D-F) Female. Five to ten micro-areas were measured for each individual.

**Figure 8.** Comparison of wax components on the dorsal and ventral abdomen of *O. albistylum*, *O. melania* and *S. darwinianum*. (A) Chromatogram of chloroform extract from the dorsal abdomen of mature male of *O. melania*. (B) Chromatogram of chloroform extract from the ventral abdomen of mature female of *S. darwinianum*. Asterisks indicate nonspecific
peaks also detected with the solvent only. (C) Summary of wax components detected from *O. albistylum*, *O. melania* and *S. darwinianum*. Relative amount was judged from the abundance of total ion current. ++++, high amount; ++, moderate amount; +, small amount.

**Figure 9.** Surface wax of *Orthetrum* species and allied dragonflies. (A-D) Mature males of *O. albistylum* (A), *O. melania* (B), *O. luzonicum* (C) and *O. glaucum* (D). (E-F) Gynandromorphic individuals of *O. japonimum* (E) and *Lyriothemis pachygastra* (F). Photos courtesy of Mitsutoshi Sugimura (E) and Makoto Machida (F).

**Figure 10.** Genes associated with UV reflective wax. (A) An androchrome female used for transcriptome analysis. (B) The number of genes upregulated in the dorsal abdominal epidermis of males and/or an androchrome female compared to normal females. (C) The list of genes upregulated in the dorsal abdominal epidermis of both males and an androchrome female (max FPKM > 1000). Gene expression levels are displayed by heat map. The numbers indicate FPKM values. Red and blue indicate high and low expression levels, respectively. D and V indicate the dorsal and ventral abdominal region, respectively. (D-F) Expression level of three Elongation of very long-chain fatty acids (ELOVL) genes in dorsal and ventral parts of epidermis of *O. albistylum*.

**Figure supplement 1.** The list of genes upregulated in the dorsal abdominal epidermis of both males and an androchrome female (max FPKM > 100). Gene expression levels are displayed by heat map. The numbers indicate FPKM values. Red and blue indicate high and low expression levels, respectively. D and V indicate the dorsal and ventral abdominal region, respectively. Andro and Gyno mean androchrome and normal (gynochrome) females,
respectively.

Figure 11. Identification of 17 ELOVL genes in dragonflies. (A) Phylogenetic tree of ELOVL family genes on the basis of their amino acid sequences. A maximum likelihood phylogeny is shown, while neighbor-joining and Bayesian phylogenies exhibit substantially the same topologies. Statistical supporting values are indicated on each node in the order of (bootstrap value of neighbor-joining)/(bootstrap value of maximum likelihood)/(posterior probability of Bayesian). Asterisks indicate support values < 50%. Blue and gray shade indicate *O. albistylum* and *L. fulva* genes, respectively. Accession numbers or annotation identities are shown in parentheses. (B) Alignment of 17 ELOVL genes of *O. albistylum*. The conserved histidine motif is boxed. (C) Gene expression levels of 17 ELOVL genes in *O. albistylum*. The numbers indicate FPKM values. Red and blue indicate high and low expression levels, respectively. D and V indicate the dorsal and ventral abdominal region, respectively.
A (Mature male, dorsal)  
160 ± 1 °

B (Mature male, dorsal + ethanol)  
156 ± 4 °

C (Mature male, dorsal + hexane)  
121 ± 8 °

D (Mature male, dorsal + chloroform)  
99 ± 4 °

E  
Mature male dorsal  
(+ hexane 30 min)

F  
Mature male dorsal  
(before treatment)

Mature female dorsal  
(before treatment)

Mature male dorsal  
(+ chloroform 10 sec)

Mature female dorsal  
(+ chloroform 10 sec)

Mature male dorsal  
(+ chloroform 2 h)

Mature female dorsal  
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### Graphs

**Graph A:** Androchrome female

**Graph B:**

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- Female > 2
- 100 < max FPKM ≤ 1000
- 10 < max FPKM ≤ 100
- Total

**Graph C:**

- Immature
- Seminatute
- Mature
- Mature-aged
- Aged

**Graph D:**

- ELOVL17
- FPKM

**Graph E:**

- ELOVL14
- FPKM

**Graph F:**

- ELOVL3
- FPKM
O. albistylum

**Male**

**Immature**

A  
Reflectance (%)  
Wavelength (nm)

**Mature**

B  
Reflectance (%)  
Wavelength (nm)

**Aged**

C  
Reflectance (%)  
Wavelength (nm)

**Female**

**Immature**

D  
Reflectance (%)  
Wavelength (nm)

**Mature**

E  
Reflectance (%)  
Wavelength (nm)

**Aged**

F  
Reflectance (%)  
Wavelength (nm)

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