UC Davis

UC Davis Previously Published Works

Title

Reflectance-based determination of age and species of blowfly puparia.

Permalink

<https://escholarship.org/uc/item/0b55c79n>

Journal

International journal of legal medicine, 131(1)

ISSN

0937-9827

Authors

Voss, Sasha C
Magni, Paola
Dadour, Ian
et al.

Publication Date

2017

DOI

10.1007/s00414-016-1458-5

Peer reviewed

Reflectance-based determination of age and species of blowfly puparia

Sasha C. Voss¹ · Paola Magni² · Ian Dadour³ · Christian Nansen^{4,5}

Received: 23 May 2016 / Accepted: 5 October 2016
© Springer-Verlag Berlin Heidelberg 2016

Abstract Forensic entomology is primarily concerned with the estimation of time since death and involves determination of the age of immature insects colonising decomposing remains. Accurate age determination of puparia is usually accomplished by dissection, which means destructive sampling of evidence. As part of improving abilities to correctly identify species and developmental age, it is highly desirable to have available non-destructive methods. In this study, we acquired external hyperspectral imaging (HSI) data (77 spectral bands, 389–892 nm) from the dorsal and ventral sides of individual puparia of two species of blowfly (Diptera: Calliphoridae), *Calliphora dubia* Macquart 1855 and *Chrysomya rufifacies* Macquart 1842. Puparia were dissected to determine the presence/absence of eight internal morphological development characteristics (legs, wings, labella, abdominal segments, antennae, thoracic bristles, orbital/facial bristles and eye colour and arista). Based on linear

discriminant analysis and independent validation of HSI data, reflectance features from puparia could be used to successfully (1) distinguish the two species (classification accuracy=92.5 %), (2) differentiate dorsal and ventral sides of puparia (classification accuracy *C. dubia*=81.5 %; *Ch. rufifacies*=89.2 %) and (3) predict the presence of these morphological characteristics and therefore the developmental stage of puparia (average classification accuracy using dorsal imaging: *C. dubia*=90.3 %; *Ch. rufifacies*=94.0 %). The analytical approach presented here provides proof of concept for a direct puparial age relationship (i.e. days since the onset of pupation) between external puparial reflectance features and internal morphological development. Furthermore, this approach establishes the potential for further refinement by using a non-invasive technique to determine the age and developmental stage of blowflies of forensic importance.

Keywords Forensic entomology · Post-mortem interval · Pupae · Hyperspectral imaging · Age estimation

✉ Sasha C. Voss
sasha.voss@uwa.edu.au

Introduction

Forensic entomology is a field of research which applies knowledge of insect biology to legal matters and is commonly employed as a means of establishing time since death or minimum post-mortem interval (_{min}PMI) [1]. Typically, blowflies (Diptera: Calliphoridae) are the predominant taxa used to indicate _{min}PMI, as they are cosmopolitan and among the first insects colonising remains after death [2, 3]. Estimates of _{min}PMI are based on determination of the age of immature insects associated with remains and the predictable sequence of insect colonisation of remains for a given set of abiotic and biotic conditions [4–6]. Consequently, accurate species identification is important but morphological approaches are

¹ Centre for Forensic Anatomy and Biological Sciences, Anatomy, Physiology and Human Biology, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

² Department of Molecular and Biomedical Sciences, School of Veterinary and Life Sciences, Murdoch University, 90 South Street, Murdoch, WA 6150, Australia

³ Program in Forensic Anthropology, Department of Anatomy & Neurobiology, Boston University School of Medicine, 72 East Concord St (L 1004), Boston, MA 02118, USA

⁴ Department of Entomology and Nematology, UC Davis, Briggs Hall 367, Davis, CA 95616, USA

⁵ State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control, Zhejiang Academy of Agricultural Sciences, 198 Shiqiao Road, Hangzhou 310021, China

often challenging. Molecular-based identification involves specimen modification and sample preparation that can inhibit additional forms of analysis [7]. The age of the oldest immature specimen collected from decomposing remains provides an indication of the minimum time that the decomposing remains were available for insect colonisation and thus minPMI .

The developmental duration of blowfly offspring and other forensically relevant insects is strongly correlated with temperature [8–12]. Specimen age is determined using growth data detailing temperature-dependent developmental timeframes for specific life stages encompassing egg, larval instars, pupation and eclosion [13–15]. The availability of species-specific and highly detailed development data is essential for the accurate estimation of minPMI .

Current approaches to age estimation of immature blowflies collected from human remains are limited to the timeframes associated with the start and end of the life stage collected, such as the onset and completion of an instar or pupation. The duration of early life stages (i.e. egg, 1st larval instar) is generally short, so estimates of minPMI based on such life stages are relatively accurate [16]. More advanced immature life stages (i.e. puparial stage), however, have a comparatively greater duration accounting for >50 % of total development time, and as such, the generally practiced use of life stage landmarks (i.e. onset of pupation and eclosion) as indicators of specimen age results in wide confidence intervals associated with arising minPMI estimates [16–18]. Several approaches have been proposed that allow more precise estimation of developmental stage including morphological examination of external and internal developmental changes using a conventional light microscope [19], histological staining [20, 21], scanning electron microscopy (SEM) [22, 23], gas chromatography mass spectrometry (GC-MS) [24–26], micro-computed tomography [18] and optical coherence tomography [27]. Advances in gene expression analysis are also proving useful in improving the precision of puparial age estimation [16, 28, 29]. For instance, puparial age can be indicated by differential changes in the up- and downregulation of genes throughout metamorphosis [30, 31]. Several issues exist, however, in regard to these methods including evidence modification, destructive analysis and underdeveloped reference data.

Importantly, there exists a preference in many legal systems for non-invasive techniques whereby evidence remains ‘unchanged’ following examination [32]. Morphological assessment methods, including tomography, typically involve evidence modification for specimen preparation such as dissection, slide sectioning or histological staining to provide high contrast images for analysis [18, 21]. Similarly, analysis of the quantitative composition of cuticular hydrocarbons extracted from the blowfly puparium using GC-MS and molecular-based methodologies encompasses destructive sample preparation for analysis [29, 30]. In the case of limited

specimen collection, destruction of evidence can greatly inhibit investigative practice. In addition, these techniques are often labour intensive, require expensive analysis equipment and involve a high degree of specialist expertise to interpret relevant morphological changes as these are often based on subjective measures such as colour changes [27, 33]. Furthermore, accurate aging of advanced immature life stages typically requires specially trained technicians, which may not always be available. While current approaches to pupae/puparial aging offer improved accuracy in minPMI estimation, most are associated with disadvantages such as evidence modification, destructive sampling, labour-intensive analysis and/or expensive equipment.

Recently, new developments in proximal hyperspectral imaging (HSI) spectroscopy have given rise to potential applications in forensic science. Forensic applications of HSI have previously encompassed identification of chemical signatures in bioagent materials [34, 35], age estimation of blood stains [36, 37], drug analysis [38], document analysis [39–41], facial recognition [42] and forensic assessment of food quality and safety [43]. However, to our knowledge, HSI has never been applied to issues within the area of forensic entomology.

Proximal HSI consists of the acquisition of detailed reflectance profiles (reflectance data in hundreds of narrow wavelength bands) under controlled laboratory conditions (with constant lighting, spatial resolution and projection angle) and subsequent processing, calibration and classification to separate objects that appear indistinguishable to the human eye [44–46]. Using this technology, it is assumed that different target objects, such as blowfly puparia of different age or species, will reflect light differently based on their difference in physical structure and biochemical composition [47].

Changes in the biochemical profile of the insect cuticle and puparium over time have previously been demonstrated using GC-MS [48–50]. Potentially, corresponding changes in reflectance profiles of the puparium over time may be evident, providing age markers for within-stage estimation of minPMI [48–50]. The insect cuticle is highly dynamic in its composition, particularly once it forms a puparia, playing a critical role in water regulation, thermo-regulation, aspects of communication and defence against pathogens [51]. The various complex roles of the insect cuticle support the view that the composition of the cuticle is tightly associated with complex internal physiological processes and that detectable changes in the biochemical composition of the cuticle of blowfly puparia can aid in differentiation between species and determination of age [25, 26, 52]. Consequently, we predict discernible differences, using HSI, in the reflectance profiles of blowfly puparia of different ages and species.

Additionally, as HSI encompasses a component of the infrared spectrum (>700 nm), which can potentially penetrate several millimetres into tissue, there is the possibility that the epicuticular structure of HSI-imaged insects is not the only

determinant of acquired reflectance profiles. Physiological and biochemical composition of internal tissues may also contribute [46]. For instance, internal morphological changes, commonly used as markers for puparial age, may be linked to any changes in the acquired reflectance profile over time. It is therefore proposed that differences in images acquired using HSI of blowfly puparia of different ages and species may be linked to internal morphological changes.

HSI has numerous potential advantages over previously proposed methods providing enhanced discrimination potential that is non-destructive and non-invasive and can be applied to both live and preserved specimens. HSI technologies are relatively inexpensive, the camera system is portable thus having the potential for images to be captured at a crime scene and there is scope for classification automation [47, 53]. Finally, as reflectance profiles provide a non-subjective basis for discrimination, the potential exists for the development of supporting software for image analysis, reducing the level of expertise required for interpretation.

As proof of concept, this study aimed to demonstrate that HSI can be used to discriminate between puparia of different ages in relation to daily changes in puparial development. Two blowfly species (Diptera: Calliphoridae), *Calliphora dubia* Macquart 1855 and *Chrysomya rufifacies* Macquart 1842, were reared at two developmental temperatures (24 and 30 °C), and hyperspectral images were acquired daily until adult eclosion. The presence/absence of eight morphological characteristics was assessed through daily dissection of subsamples of puparia reared at the two developmental temperatures to establish day/age indicators for puparial development of both species under these conditions [27]. Data were then compared to daily reflectance profiles of corresponding puparial age using HSI technology to determine proof of concept for discrimination of puparial age based on reflectance data. Reflectance of both dorsal and ventral surfaces of puparia was assessed to determine if reflectance profiles varied with puparial orientation during imaging. Additionally, the potential to distinguish between puparia of the two species using HSI was assessed.

Materials and methods

Insect cultures

C. dubia and *Ch. rufifacies* colonies were established between November and December 2013 from specimens collected from the Jandakot wildlife reserve (32° 10' S, 115° 50' E), located ~23 km south of Perth, Western Australia. Adult flies were maintained in screened cages and supplied with sugar and water ad libitum. Laboratory conditions (constant temperature room) were maintained at 24±1 °C, 60–70 % relative humidity (RH) and photoperiod of 12L:12D. Bovine liver was

used for oviposition/larviposition [54]. Deposited eggs and larvae were transferred to a meatmeal (Mirco Bros, WA, Australia) food substrate (slaughterhouse by-product consisting of rendered mixed animal offal) and placed in a rearing cabinet within the laboratory until emergence. Field-collected individuals were regularly introduced into the established colonies. Experimental females were exposed to bovine liver once per week for 1 h prior to experiments to obtain protein for egg development [15].

In all treatment groups, 3-week-old, sugar-fed, mated female blowflies were used. At the start of each trial, females were exposed to bovine liver for 1 h. Resulting eggs (*Ch. rufifacies*) and larvae (*C. dubia*) were subsequently set up on pre-prepared trays of meatmeal in batches of 200 specimens, placed within meshed rearing containers above a layer of sterilised sand (required for pupation) and transferred to environmental chambers (Thermoline Scientific Pty. Ltd., NSW, Australia) for development. Replicates were placed in one of two environmental chambers set at either 24 or 30 °C, a relative humidity of 65±10 % and a photoperiod of 12L:12D. Average environmental chamber temperature was measured using three data loggers (Tinytag Plus®, Hastings Data Loggers, NSW, Australia) distributed within each cabinet on the top, middle and bottom shelf. The resulting means differed from chamber set values by no more than ±0.25 °C. Rearing containers for both study species were assessed twice a day (0800 and 1700 hours) for the onset of puparial development. Specimens were considered to have entered the puparial stage at the point where movement had ceased and the external puparium (white) was evident [55]. Two study species (*C. dubia* and *Ch. rufifacies*) and two constant rearing temperatures (24 and 30 °C) were assessed to allow comparison of puparial surface structure, size, associated reflectance profiles and internal morphology over time.

Sampling protocol

At the onset of pupation, samples of 18 puparia were removed from the rearing containers of each of the four treatment groups (2 species×2 rearing temperatures). For each treatment group, puparia were affixed to a paper card within a 2×9 sample grid using the natural adhesive, gum arabic (Henrietta's Beading Glue, CA, USA). To determine an optimal sampling protocol, the ventral and dorsal surfaces of puparia were imaged for comparison. Within each treatment grid, nine puparia were affixed ventrally along the upper row (dorsal surface imaged) and dorsally along the lower row (ventral surface imaged) (Fig. 1). Orientation was determined by visual assessment of the location of the posterior spiracles and/or dorsal spines [56, 57].

Puparia affixed to each of the four grids (N=9 replicate puparia×2 orientations/grid) were subsequently imaged and returned to allocated treatment rearing conditions for

Fig. 1 Representative image of a single treatment grid of nine replicate puparia prepared for daily hyperspectral imaging. Puparia are affixed to the grid either dorsal side (*Dors*) or ventral (*Vent*) side up. In this instance,

4-day-old *Calliphora dubia* (Diptera: Calliphoridae) puparia are shown following rearing at 24 ± 0.5 °C, relative humidity of 65 ± 10 % and a photoperiod of 12L:12D

continued development. Thus, the same nine pupae were scanned per treatment until emergence. Hyperspectral images (see below) were captured daily between 0800 and 1000 hours until completion of the puparial stage. During the daily hyperspectral image acquisition, puparia were only removed from the allocated rearing conditions for <15 min. Scanned pupae developed and emerged in the same timeframe as the cohort from which they were sampled, which remained in the allocated environmental chamber. The entire procedure was repeated over time (2 weeks apart), so that HSI data were obtained from a total of 144 puparia image captures (2 species $\times$ 2 rearing temperatures $\times$ 18 puparia $\times$ 2 time series).

Starting on day 1 of puparial formation, five puparia were removed daily from the original rearing containers of each of the four combinations of species and rearing temperature and preserved for morphological analysis. Puparial specimens were pierced twice along the ventral surface (head and abdomen regions), immersed in hot water for 10 s and then preserved in 70 % ethanol according to standard protocol [58]. Original rearing containers were maintained under allocated treatment rearing conditions with morphological samples collected between 0800 and 1000 hours daily until emergence.

Puparia of preserved specimens were then removed according to Brown et al. and examined under an Olympus Stereo Microscope SZ6 (Olympus Australia Pty. Ltd.). Image records were obtained using an Olympus DP70 digital microscope camera attachment. Based on descriptive and categorical measures [17], presence/absence of eight morphological characteristics of puparia was recorded for each combination of species and rearing temperature: (1) legs, (2) wings, (3) labella, (4) abdominal segments, (5) antennae, (6) thoracic bristles, (7) orbital/facial bristles and eye colour and (8) arista (Figs. 2 and 3).

Hyperspectral imaging

The HSI system consisted of a push broom hyperspectral camera (PIKA II, www.resonon.com) which used a Firewire (IEEE 1394b) interface (12-bit digital) with a 7° angular field of view processed 240 spectral bands from 392 to 889 nm with a spectral resolution of 2.07 nm (spectral) by 640 pixels (spatial). The objective lens was optimised for the near-infrared and visible near-infrared spectra and consisted of a 35-mm focal length (maximum aperture of F1.4). HSI data were acquired with the spatial resolution of 5 by 5 pixels per mm².

Reflectance data were captured under controlled laboratory conditions consisting of 2 $\times$ 15 W, 12 V LED artificial light bulbs which were mounted on either side of the lens in a room with 24 ± 2 °C temperature and 30–40 % relative humidity. White calibration was achieved using a piece of white Teflon (K-Mac Plastics, USA), and ‘relative reflectance’ refers to proportional reflectance compared to reflectance obtained from Teflon (white = 1) and complete darkness (dark = 0). Additional calibration measures were employed to ensure consistent data acquisition over time. This included the acquisition of daily average reflectance profiles using a green plastic card as a standard. Average reflectance from the green plastic card before and after each daily imaging acquisition event was used to confirm consistency of imaging conditions, and less than 3 % variation within and among days was observed. As such, we were confident that significant changes observed in reflectance profiles could be attributed to changes in puparial reflectance. With hyperspectral images being acquired daily from puparia over 4–11 days (depending on species and rearing temperature), a total of 1224 average reflectance profiles (774 from *C. dubia* and 450 from *Ch. rufifacies*) were included in this study.

Data analysis

All data analyses were conducted in PC-SAS 9.4 (SAS Institute, USA) and based on average reflectance profiles from individual puparia. Separate analyses were conducted across temperature regimes for each species. The first 10 spectral bands were omitted from each hyperspectral data file, as these were considered to be associated with stochastic noise. Consequently, 230 spectral bands from 411 to 889 nm were included in the analysis. After acquisition, we conducted 1 $\times$ 3 spectral binning (decreased the spectral resolution from 2.1 to 6.3 nm), which resulted in 77 spectral bands being included in the analyses. This pre-processing step of spectral binning was included, as it has been shown to increase the classification accuracy in similar analysis of HSI data [59, 60]. Daily reflectance data for individual puparia were examined visually using non-metric multidimensional scaling ordination (MDS), based on a Euclidean distance matrix in the software package, PRIMER v.6.0 (PRIMER-E Ltd, Plymouth, UK) [61, 62].

The first day of appearance for each of the eight morphological characteristics was determined such that if the formation of legs became noticeable on day 2 and puparial development was

Fig. 2 Development of *Calliphora dubia* (Diptera: Calliphoridae) at 30 °C, dorsal view (*left*), ventral view (*centre*) and lateral view (*right*), after removal of the puparial case. Day 2 of pupation (**a–c**); day 7 of pupation (**d–f**); day 9 of pupation (**g–i**). Characteristics considered in

this study included the following: *L* = legs, *W* = wings, *La* = labella, *AS* = abdominal segmentation, *A* = antennae, *TB* = thoracic bristles, *OFB* = orbital/facial bristles and eye colour and *Ar* = arista

Fig. 3 Development of *Chrysomya rufifacies* (Diptera: Calliphoridae) at 30 °C, dorsal view (*left*), ventral view (*centre*) and lateral view (*right*), after removal of the puparial case. Day 2 of pupation (**a–c**); day 3 of pupation (**d–f**); day 4 of pupation (**g–i**). Characteristics considered in

this study included the following: *L* = legs, *W* = wings, *La* = labella, *AS* = abdominal segmentation, *A* = antennae, *TB* = thoracic bristles, *OFB* = orbital/facial bristles and eye colour and *Ar* = arista

350 completed in 6 days, then the dichotomous values for the
 351 variable, legs, would be 0, 1, 1, 1, 1, 1. Furthermore, we
 352 compared average reflectance profiles from day 1 with those
 353 from days 2 to 6 as part of determining the relative difference
 354 between morphological stages with/without appearance of leg
 355 development.

356 In each analysis of the morphological characteristics, linear
 357 discriminant analysis was conducted (proc discrim) [63] to
 358 differentiate between average reflectance profiles from
 359 puparium before (without) and after (with) appearance of the
 360 morphological trait. For each classification, stepwise linear dis-
 361 criminant analysis (proc stepwise) was used initially to only
 362 select the spectral bands with significant contribution to each
 363 classification model. In other words, spectral bands with low or
 364 negligible identification contribution were omitted. Subsequently,
 365 a linear discriminant classification model was generated on the
 366 basis of 75 % of the data (a randomly selected training data set)
 367 and the remaining 25 % of the data was used for independent
 368 validation. The division of reflectance data into training and
 369 validation was repeated five times, and the average classification
 370 accuracy was calculated on the basis of the five independent
 371 validations of each classification model.

372 Results

373 Developmental morphology

374 Puparial development of *C. dubia* was completed in 11 days at
 375 24 °C and 9 days at 30 °C (Fig. 4a). Regarding *Ch. rufifacies*,
 376 puparial development at 24 and 30 °C was completed in 5 and
 377 4 days, respectively (Fig. 4b). None of the morphological
 378 characteristics assessed through dissection of individual pu-
 379 paria were present until after day 1. Legs and wings appeared
 380 on days 2 and 3 in both species. Bristles on thorax and face
 381 and presence of arista were the last of the morphological
 382 characteristics to appear in the puparial ontogeny.

383 Reflectance response to ontogeny

384 Within species, reflectance response of puparia demonstrated
 385 a similar trend as a function of time (day). Corresponding to
 386 observable colour changes with puparia darkening over time,
 387 young puparia typically demonstrated greater reflectance than
 388 older puparia at wavelengths >600 nm (the orange and red
 389 light spectrum). As puparia aged, relative reflectance of
 390 increasing variation was observed (in spectral bands beyond
 391 600 nm) under both temperature treatments. There was negli-
 392 gible effect of time on reflectance values in spectral bands in
 393 the blue and green light (Figs. 5, 6, 7 and 8).

394 Reared at a constant temperature of 24 °C, puparia of
 395 *C. dubia* demonstrated markedly higher reflectance of wave-
 396 lengths >600 nm on day 1 compared to all other days. Gradual

397 variation was evident in reflectance profiles over day 2
 398 through day 8. Relative reflectance at wavelengths >600 nm
 399 was lowest on day 9 before increasing again on days 10 and 11
 400 to levels closer to days 2–8 (Fig. 5). This pattern was similar
 401 for *C. dubia* puparia reared at 30 °C with the highest reflec-
 402 tance of wavelengths over 600 nm occurring on day 1 and the
 403 lowest on day 8. On day 9, relative reflectance increased at
 404 wavelengths over 600 nm to levels more consistent with
 405 earlier days 2 through 6 (Fig. 6).

406 Spectral reflectance profiles of *Ch. rufifacies* puparia were
 407 uniquely distinct from *C. dubia* profiles with comparatively
 408 lower reflectance of *Ch. rufifacies* at wavelengths greater than
 409 700 nm (Figs. 7 and 8). Consistent however with *C. dubia*, the
 410 reflectance profiles of *Ch. rufifacies* puparia reared at 24 and
 411 30 °C again demonstrated markedly higher reflectance of
 412 wavelengths >600 nm on day 1 compared to all other days
 413 (Figs. 7 and 8). Gradual variation was evident in reflectance
 414 profiles over proceeding days with lower reflectance in
 415 spectral bands beyond 600 nm. Relative reflectance at
 416 wavelengths >600 nm was lowest on the last day prior to
 417 emergence of *Ch. rufifacies* under both temperature
 418 conditions (day 5 at 24 °C and day 4 at 30 °C).

419 Reflectance-based classification

420 Significant differences in reflectance responses of *Ch. rufifacies*
 421 and *C. dubia* were evident (Fig. 9). Linear discriminant classi-
 422 fication modelling identified that species could be distinguished
 423 throughout puparial development based on reflectance response
 424 with a classification accuracy of 92.5 %. In the linear discrim-
 425 inant classification of the presence/absence of the eight
 426 morphological characteristics, 10–25 of the 77 spectral
 427 bands were selected through forward stepwise selection,
 428 and based on five independent validations, the presence/
 429 absence of all morphological characteristics could be de-
 430 tected with over 82 % accuracy (Fig. 10). The absence of
 431 legs and wings was detected with 90–95 % accuracy in
 432 both species and would be an indication that a puparium
 433 is less than 24 h old. The absence of thoracic bristles was
 434 detected with about 85 % accuracy in both species and
 435 would be an indication that a *C. dubia* puparium is 0–
 436 6 days old and a *Ch. rufifacies* puparium, developing at
 437 less than 30 °C, is 0–4 days old. As the presence/absence
 438 of each morphological characteristic is clearly associated
 439 with a combination of temperature and time, we are pro-
 440 posing that reflectance data from puparia can be used to
 441 run all eight classifications, and based on the presence/
 442 absence of the eight morphological characteristics, it
 443 may be possible to apply a logic framework to predict
 444 the days of puparial development. For instance, in a logic
 445 framework analysis of *C. dubia* puparia (Fig. 4a), where
 446 the analyses suggest the presence of legs, wings, labella
 447 and abdominal segments and the absence of all other

Fig. 4 Timeline of the appearance of key morphological characteristics during puparial development of the blowflies (Diptera: Calliphoridae), *Calliphora dubia* (a) and *Chrysomya rufifacies* (b), reared under two different constant temperature treatments, 24 and 30 °C. Morphological

characteristics examined included the following: *L* = legs, *W* = wings, *La* = labella, *AS* = abdominal segmentation, *A* = antennae, *TB* = thoracic bristles, *OFB* = orbital/facial bristles and eye colour and *Ar* = arista

characteristics, and it is known that ambient temperatures have been around 24 °C, then the puparium is likely 3–4 days old.

Within species, differences were evident in reflectance response of dorsal and ventral puparial orientation. Puparial orientation could be distinguished with 81.5 % accuracy for *C. dubia* and 89.2 % accuracy for *Ch. rufifacies* based on corresponding reflectance profiles. The classification accuracy of individual traits with corresponding reflectance response of either dorsal or ventral orientated puparia was consistently above 80 % for both species (Table 1). For all morphological traits, either dorsal or ventral imaging performed better than data combining reflectance response of both orientations (Table 1). On average, imaging the dorsal surface of puparia outperformed ventral imaging but not for all combinations of species and traits (Table 1).

Discussion

Determination of the age of developing insects collected from decomposing remains is an important indicator of how long the remains have been colonised by insects and consequently min PMI [64]. Issues currently exist in respect to aging specimens within the lengthy puparial stage, as only the start and end of the life stage is used as a time marker with any certainty [27]. The use of HSI to age insect specimens within life stages for use in forensic applications offers many advantages over currently proposed methods including rapid, non-destructive, in situ analysis with enhanced discrimination potential [43, 53]. Additionally, as reflectance data provides a non-subjective basis for discrimination and can be incorporated into supporting software for image analysis, refinement of the technique can ultimately reduce the level of expertise required for interpretation.

Fig. 5 Average daily reflectance response acquired from puparia of the blowfly, *Calliphora dubia* (Diptera: Calliphoridae), on key developmental days during development from puparial formation to emergence at a constant temperature of 24 °C (*N*=36; 2 trials × 9 replicates × 2 orientations)

Fig. 6 Average daily reflectance response acquired from puparia of the blowfly, *Calliphora dubia* (Diptera: Calliphoridae), on key developmental days during development from puparial formation to emergence at a constant temperature of 30 °C (*N*=36; 2 trials × 9 replicates × 2 orientations)

Fig. 7 Average daily reflectance response acquired from puparia of the blowfly, *Chrysomya rufifacies* (Diptera: Calliphoridae), on key developmental days during development from puparial formation to emergence at a constant temperature of 24 °C ($N=36$; 2 trials $\times$ 9 replicates $\times$ 2 orientations)

This work aimed to demonstrate the potential of HSI technology as a tool to age blowfly puparia developing under different temperature conditions. Acquisition of daily reflectance profiles of the two blowfly species investigated yielded a wealth of robust data allowing for confidence in predictive modelling. Subtle differences in daily reflectance profiles of puparia were evident and strongly associated with puparial age. Daily differences in reflectance generally corresponded to the puparia darkening over time; however, relative reflectance increased in the days prior to eclosion indicating additional contributing factors to changes in reflectance. Reflectance profiles of 1-day-old puparia, corresponding with undifferentiated tissue upon dissection, were markedly different to profiles of older puparia where

Fig. 8 Average reflectance response acquired from puparia of the blowfly, *Chrysomya rufifacies* (Diptera: Calliphoridae), on key developmental days during development from puparial formation to emergence at a constant temperature of 30 °C ($N=36$; 2 trials $\times$ 9 replicates $\times$ 2 orientations)

the appearance of key internal morphological characters, such as legs and wings, occurred. As internal morphological changes correspond to changes in external reflectance, it is likely that these key morphological changes are linked to associated changes in the external puparium. Such gradual changes over time are thus evident in the reflectance profile of the puparia and can indicate age.

Using an independent validation of classifications models, we demonstrate that the presence/absence of all eight established internal morphological characteristics could be determined with >82 % accuracy. As such, the use of HSI technology has great potential in the application of the development of temperature-dependent predictive models for determining puparial age in forensically relevant species. Notably, within days, differences in ventral and dorsal reflectance profiles were evident and thus orientation is important during image capture and needs to be considered. Overall, however, these findings highlight the potential of the technique in forensic entomological applications and strongly support the value of further research and development of the approach.

On the basis of this work, it is evident that reflectance profiles of the puparium change over time in synchrony with internal developmental changes; however, as reflectance is also externally driven, the cause itself requires further investigation. In refining the technique, future directions should address not only the comprehensive acquisition of HSI data for predicting temperature-dependent development of forensically relevant species but also the cause of the observed relationship. The biochemical profile of the puparium is known to change over time as the insect develops in sync with internal physiological processes and likely morphological changes [25, 26, 48]. This has been demonstrated in respect to the success of hydrocarbon profile analysis as a tool in discriminating blowfly puparial age. Thus, it is hypothesised that differences in biochemical composition of the cuticle, as previously demonstrated in regard to hydrocarbon composition [25, 26, 52], will equate to differences in reflectance profiles of blowfly puparia of different ages and species. In regard to this, there is a need to establish the relationship between hydrocarbons and reflectance profiles to improve our understanding of the cause of external developmental changes. Refinement of HSI techniques in predictive models of blowfly development will further establish associated error rates.

Of the two approaches, HSI and hydrocarbon analysis, both require further development, but hydrocarbon analysis is also destructive in respect to evidence processing. Further refinement efforts would therefore best be served by focussing on HSI techniques for the non-destructive analysis of living specimens for age estimation as this is highly preferred as it accommodates additional forms of analysis and/or

Fig. 9 nMDS (non-metric multidimensional scaling) ordination of average daily reflectance profiles from puparia of two blowfly species (Diptera: Calliphoridae), *Calliphora dubia* and *Chrysomya rufifacies*, reared under two different constant temperature treatments, 24 and 30 °C. For ease of interpretation, the daily average of the reflectance profiles of individual puparia within each treatment is presented. Data

were normalised prior to ordination. The relative distances apart of the points are the same rank order as the relative dissimilarities of the reflectance profiles. Thus, points that are close together represent puparial reflectance profiles that are similar, while points that are further apart are more dissimilar

547 preservation for later evidence review [18, 58]. Additionally,
548 although the main aim of this study was to investigate the
549 potential of HSI as a discriminative tool in respect to puparial
550 age, the technique also has applications in regard to species
551 identification. Based on the obtained reflectance profiles, there
552 was a clear indication of discrimination between the two spe-
553 cies investigated with >92 % accuracy. As predicative models
554 for aging entomological specimens are species specific,

Fig. 10 Linear discriminant classification accuracy (%) for the detection of the presence/absence of eight morphological characters throughout puparial development based on daily reflectance profiles from puparia of two blowfly species (Diptera: Calliphoridae), *Calliphora dubia* and *Chrysomya rufifacies*, reared under two different constant temperature treatments, 24 and 30 °C. Morphological characteristics examined included the following: L = legs, W = wings, La = labella, AS = abdominal segmentation, A = antennae, TB = thoracic bristles, OFB = orbital/facial bristles and eye colour and Ar = arista

accurate species identification is paramount to the resulting
accuracy of associated age-determined $\min$ PMI estimation
[65]. As the majority of forensic entomologists currently prac-
ticing are not taxonomic specialists, the advantages of using
HSI to both identify and age specimens collected from
decomposing remains are high. Much like gene expression
analysis and hydrocarbon analysis for aging pupae/puparia,
DNA-based species identification is time consuming, destruc-
tive and cost prohibitive compared to a refined and further
developed HSI approach.

In respect to further development of the technique,
reflectance of both dorsal and ventral surfaces of puparia
indicated that greater resolution of species and age differ-
ence could be achieved by imaging dorsally oriented
puparia. Further, the development of any predictive model
requires validation under field conditions along with
associated error rates prior to adoption in mainstream foren-
sic practice [3]. Blind test validation in field research and
case work is needed to comprehensively establish the
technique as reputable in regard to delivery during expert
testimony in court proceedings. Results presented in this
paper were executed in a controlled laboratory environment
under two different temperature regimes with successful
age-based identification achieved with incorporation of
temperature in the predictive model. Further investigation
of puparial age-linked reflectance profiles under more var-
iable field environmental conditions is however required to
establish the validity of the proposed method and any asso-
ciated error [3, 5]. Ongoing development of the technique
will test if the practical implications discussed will be an
issue for application of HSI analysis under field conditions.

t1.1 t1.2	Table 1 Linear discriminant classification accuracy (%) of morphological trait identification based on daily reflectance profiles from ventrally and dorsally oriented puparia of two blowfly species (Diptera: Calliphoridae), <i>Calliphora dubia</i> and <i>Chrysomya rufifacies</i> , reared under two different constant temperature treatments, 24 and 30 °C	Species	Characteristic	Classification accuracy (%)			t1.3
				Ventral	Dorsal	Combined	
t1.4	oriented puparia of two blowfly species (Diptera: Calliphoridae), <i>Calliphora dubia</i> and <i>Chrysomya rufifacies</i> , reared under two different constant temperature treatments, 24 and 30 °C	<i>Calliphora dubia</i>	Legs	100	100	95.8	
t1.5			Wings	87.8	89.5	89.4	
t1.6			Labella	84.0	87.6	84.9	
t1.7			Abdominal segments	88.9	87.9	84.5	
t1.8			Antennae	84.5	91.0	89.9	
t1.9			Thoracic bristles	85.1	84.2	84.5	
t1.10			Orbital/facial bristles/eye colour	85.9	86.6	86.8	
t1.11		<i>Chrysomya rufifacies</i>	Arista	96.5	95.3	94.7	
t1.12			Legs	98.1	96.5	91.0	
t1.13			Wings	98.1	96.5	91.0	
t1.14			Labella	98.1	96.5	91.0	
t1.15			Abdominal segments	95.0	98.5	89.1	
t1.16			Antennae	95.0	93.8	90.7	
t1.17			Thoracic bristles	82.4	90.2	83.5	
t1.18			Orbital/facial bristles/eye colour	82.4	90.2	84.2	
t1.19			Arista	82.4	90.2	84.2	

586 Conclusion

587 This study has demonstrated the potential of HSI to discrim-
 588 inate between puparia of different ages that appear similar to
 589 the naked eye. Although factors other than age may influence
 590 reflectance profiles of developing puparia, there is still great
 591 potential for determining $\min$ PMI using daily changes in
 592 reflectance profiles of puparia as a basis for discrimination.
 593 This successful proof-of-concept study identifies the value of
 594 further refinement of the technique in forensic applications
 595 involving entomological specimens and identifies the
 596 considerable potential of HSI in forensic practice.

597 **Acknowledgments** We are grateful to Mike Johnson and Yvette
 598 Hitchen at The University of Western Australia, School of Animal
 599 Biology, for access to a dissecting microscope with image capture
 600 capabilities.

602 References

- Q2 603 1. Amendt J, Richards CS, Campobasso CP, Zehner R, Hall MRJ
 604 (2011) Forensic entomology: applications and limitations.
 605 Forensic Sci Med Pathol 7:379–92
- 606 2. Matuszewski S, Bajerlein D, Konwerski S, Szpila K (2010) Insect
 607 succession and carrion decomposition in selected forests of central
 608 Europe. Part 1: pattern and rate of decomposition. Forensic Sci Int
 609 194:85–93
- 610 3. Baque M, Amendt J (2013) Strengthen forensic entomology in
 611 court—the need for data exploration and the validation of a gener-
 612 alised additive mixed model. Int J Legal Med 127:213–23
- 613 4. Matuszewski S, Madra A (2015) Factors affecting quality of tem-
 614 perature models for the pre-appearance interval of forensically use-
 615 ful insects. Forensic Sci Int 247:28–35
5. Tomberlin JK, Mohr R, Benbow ME, Tarone AM, VanLaerhoven
 SL (2011) A road map for bridging basic and applied research in
 forensic entomology. Annu Rev Entomol 56:401–21
6. Voss SC, Spafford H, Dadour IR (2010) Temperature-dependant
 development of *Tachinaephagus zealandicus* Ashmead
 (Hymenoptera: Encyrtidae), on five forensically important carrion
 fly species. Med Vet Entomol 24:189–98
7. Harvey ML, Dadour IR, Gaudieri S (2003) Mitochondrial DNA
 cytochrome oxidase I gene: potential for distinction between imma-
 ture stages of some forensically important fly species (Diptera) in
 Western Australia. Forensic Sci Int 131:134–9
8. Warren JA, Anderson GS (2013) Effect of fluctuating temperatures
 on the development of a forensically important blow fly,
Protophormia terraenovae (Diptera: Calliphoridae). Environ
 Entomol 42:167–72
9. Ikemoto T, Takai K (2000) A new linearized formula for the law of
 total effective temperature and the evaluation of line-fitting methods
 with both variables subject to error. Environ Entomol 29:671–82
10. Campbell A, Frazer BD, Gilbert N, Gutierrez AP, Mackauer M
 (1974) Temperature requirements of some aphids and their para-
 sites. J Appl Ecol 11:431–8
11. Davidson J (1944) On the relationship between temperature and
 rate of development of insects at constant temperatures. J Anim
 Ecol 13:26–38
12. Damos P, Savopoulou-Soultani M (2012) Temperature-driven
 models for insect development and vital thermal requirements.
 Psyche 2012:1–13
13. Nabity P, Higley L, Heng-Moss T (2006) Effects of temperature on
 development of *Phormia regina* (Diptera: Calliphoridae) and use of
 developmental data in determining time intervals in forensic ento-
 mology. J Med Entomol 43:1276–86
14. Richards CS, Villet MH (2009) Data quality in thermal summation
 development models for forensically important blowflies. Med Vet
 Entomol 23:269–76
15. Voss SC, Cook DF, Wei-Feng H, Dadour IR (2014) Survival and
 development of the forensically important blow fly, *Calliphora*
varifrons (Diptera: Calliphoridae) at constant temperatures.
 Forensic Sci Int 10:314–21

- 654 16. Tarone AM, Foran DR (2011) Gene expression during blow fly
655 development: improving the precision of age estimates in forensic
656 entomology. *J Forensic Sci* 56:S112–22
- 657 17. Brown K, Thorne A, Harvey M (2015) *Calliphora vicina* (Diptera:
658 Calliphoridae) pupae: a timeline of external morphological devel-
659 opment and a new age and PMI estimation tool. *Int J Legal Med*
660 129:835–50
- 661 18. Richards CS, Simonsen TJ, Abel RL, Hall MJR, Schwyn DA,
662 Wicklein M (2012) Virtual forensic entomology: improving esti-
663 mates of minimum post-mortem interval with 3D micro-computed
664 tomography. *Forensic Sci Int (Online)* 220:251–64
- 665 19. Greenberg B (1991) Flies as forensic indicators. *J Med Entomol* 28:
666 565–77
- 667 20. Barritt LC, Birt LM (1971) Development of *Lucilia cuprina*: cor-
668 relation of biochemical and morphological events. *J Insect Physiol*
669 17:1169–83
- 670 21. Davies K, Harvey ML (2012) Internal morphological analysis for
671 age estimation of blow fly pupae (Diptera: Calliphoridae) in post-
672 mortem interval estimation. *J Forensic Sci* 58:79–84
- 673 22. Sukontason KL, Kanchai C, Piangjai S et al (2006) Morphological
674 observation of puparia of *Chrysomya nigripes* (Diptera:
675 Calliphoridae) from human corpse. *Forensic Sci Int (Online)* 161:15–9
- 676 23. Sukontason KL, Narongchai P, Kanchai C et al (2006)
677 Morphological comparison between *Chrysomya rufifacies*
678 (Macquart) and *Chrysomya villeneuvei* Patton (Diptera:
679 Calliphoridae) puparia, forensically important blow flies.
680 *Forensic Sci Int (Online)* 164:230–4
- 681 24. Feng D-X, Liu G-C (2013) Pupal age estimation of forensically
682 important *Megaselia spiracularis* Schmitz (Diptera: Phoridae).
683 *Forensic Sci Int* 231:199–203
- 684 25. Moore HE, Adam CD, Drijfhout FP (2013) Potential use of hydro-
685 carbons for aging *Lucilia sericata* blowfly larvae to establish the
686 postmortem interval. *J Forensic Sci* 58:404–12
- 687 26. Xu H, Ye G-Y, Xu Y, Hu C, Zhu G-H (2014) Age-dependent
688 changes in cuticular hydrocarbons of larvae in *Aldrichina grahami*
689 (Aldrich) (Diptera: Calliphoridae). *Forensic Sci Int* 242:236–41
- 690 27. Brown K, Harvey M (2014) Optical coherence tomography: age
691 estimation of *Calliphora vicina* pupae in vivo? *Forensic Sci Int*
692 242:157–61
- 693 28. Ames C, Turner B, Daniel B (2006) Estimating the post-mortem
694 interval (II): the use of differential temporal gene expression to
695 determine the age of blowfly pupae. *Int Congr Ser* 1288:861–3
- 696 29. Zajac BK, Amendt J, Horres R, Verhoff MA, Zehner R (2015) De
697 novo transcriptome analysis and highly sensitive digital gene ex-
698 pression profiling of *Calliphora vicina* (Diptera: Calliphoridae) pu-
699 pupae using MACE (Massive Analysis of cDNA Ends). *Forensic Sci*
700 *Int Genet* 15:137–46
- 701 30. Boehme P, Spahn P, Amendt J, Zehner R (2014) The analysis of
702 temporal gene expression to estimate the age of forensically impor-
703 tant blow fly pupae: results from three blind studies. *Int J Legal*
704 *Med* 128:565–73
- 705 31. Zehner R, Amendt J, Boehme P (2009) Gene expression analysis as
706 a tool for age estimation of blowfly pupae. *Forensic Sc Int Genet*
707 *Suppl Ser* 2:292–3
- 708 32. Morris B, Dadour I (2005) Forensic entomology: the use of insects
709 in legal cases. In: Freckelton I, Selby H (eds) *Expert evidence*. Law
710 Book Company, Sydney
- 711 33. Proença B, Ribeiro AC, Luz RT, Aguiar VM, Maia VC, Couri MS
712 (2014) Intrapuparial development of *Chrysomya putoria* (Diptera:
713 Calliphoridae). *J Med Entomol* 51:908–14
- 714 34. Edelman GJ, Gaston E, van Leeuwen TG, Cullen PJ, Aalders MCG
715 (2012) Hyperspectral imaging for non-contact analysis of forensic
716 traces. *Forensic Sci Int* 223:28–39
- 717 35. Brewer LN, Ohlhausen JA, Kotula PG, Michael JR (2008) Forensic
718 analysis of bioagents by X-ray and TOF-SIMS hyperspectral imag-
719 ing. *Forensic Sci Int* 179:98–106
- 720 36. Edelman G, van Leeuwen TG, Aalders MCG (2012) Hyperspectral
721 imaging for the age estimation of blood stains at the crime scene.
722 *Forensic Sci Int* 223:72–7
- 723 37. Li B, Beveridge P, O'Hare WT, Islam M (2013) The age estimation
724 of blood stains up to 30 days old using visible wavelength
725 hyperspectral image analysis and linear discriminant analysis. *Sci*
726 *Justice* 53:270–7
- 727 38. Edelman G, Lopatka M, Aalders MCG (2013) Objective color clas-
728 sification of ecstasy tablets by hyperspectral imaging. *J Forensic Sci*
729 58:881–6
- 730 39. Reed G, Savage K, Edwards D, Daeid NN (2014) Hyperspectral
731 imaging of gel pen inks: an emerging tool in document analysis. *Sci*
732 *Justice* 54:71–80
- 733 40. Malik MI, Ahmed S, Shafait F et al (2015) Hyper-spectral analysis
734 for automatic signature extraction. 17th biennial conference of the
735 International Graphonomics Society
- 736 41. Rémi C, Prévost L, Anquetil E (2015) Drawing, handwriting pro-
737 cessing analysis: new advances and challenges. In 17th biennial
738 conference of the International Graphonomics Society
- 739 42. Uzair M, Mahmood A, Shafait F, Nansen C, Mian AS (2015) Is
740 spectral reflectance of the face a reliable biometric? *Opt Express* 23:
741 15160–73
- 742 43. Wu D, Sun D-W (2013) Advanced applications of hyperspectral
743 imaging technology for food quality and safety analysis and assess-
744 ment: a review—part II: applications. *Innovative Food Sci Emerg*
745 *Technol* 19:15–28
- 746 44. Nansen C (2016) The potential and prospects of proximal remote
747 sensing of arthropod pests. *Pest Manag Sci* 72:653–9
- 748 45. Nansen C, Macedo T, Swanson R, Weaver DK (2009) Use of spatial
749 structure analysis of hyperspectral data cubes for detection of insect-
750 induced stress in wheat plants. *Int J Remote Sens* 30:2447–64
- 751 46. Christian N, Norman E (2016) Remote sensing and reflectance
752 profiling in entomology. *Annu Rev Entomol* 61:139–58
- 753 47. Wu D, Sun D-W (2013) Advanced application of hyperspectral
754 imaging technology for food quality and safety analysis and assess-
755 ment: a review—part I: fundamentals. *Innovative Food Sci Emerg*
756 *Technol* 19:1–14
- 757 48. Roux O, Gers C, Legal L (2008) Ontogenetic study of three
758 Calliphoridae of forensic importance through cuticular hydrocar-
759 bon analysis. *Med Vet Entomol* 22:309–17
- 760 49. Zhu GH, Ye GY, Hu C, Xu XH, Li K (2006) Development changes
761 of cuticular hydrocarbons in *Chrysomya rufifacies* larvae: potential
762 for determining larval age. *Med Vet Entomol* 20:438–44
- 763 50. Butler SM, Moon RD, Hinkle NC, Millar JG, McElfresh JS,
764 Mullens BA (2009) Characterization of age and cuticular hydrocar-
765 bon variation in mating pairs of house fly, *Musca domestica*, col-
766 lected in the field. *Med Vet Entomol* 23:426–442
- 767 51. Chapman RF (2012) *The insects. Structure and function*. 5th ed.
768 Cambridge University Press.
- 769 52. Frere B, Suchaud F, Bernier G et al (2014) GC-MS analysis of
770 cuticular lipids in recent and older scavenger insect puparia. An
771 approach to estimate the postmortem interval (PMI). *Anal Bioanal*
772 *Chem* 406:1081–8
- 773 53. Nansen C, Sidumo AJ, Capareda S (2010) Variogram analysis of
774 hyperspectral data to characterize the impact of biotic and abiotic
775 stress of maize plants and to estimate biofuel potential. *Appl*
776 *Spectrosc* 64:627–36
- 777 54. Grassberger M, Freidrich E, Reiter C (2003) The blowfly *Chrysomya*
778 *albiceps* (Wiedemann) (Diptera: Calliphoridae) as a new forensic
779 indicator in Central Europe. *Int J Legal Med* 117:75–81
- 780 55. Davies K, Harvey ML (2013) Internal morphological analysis for
781 age estimation of blow fly pupae (Diptera: Calliphoridae) in post-
782 mortem interval estimation. *J Forensic Sci* 58:79–84
- 783 56. Fraenkel G, Bhaskaran G (1973) Pupariation and pupation in
784 cyclorrhaphous flies (Diptera): terminology and interpretation.
785 *Ann Entomol Soc Am* 66:418–22

- 786 57. Smith KGV (1986) A manual of forensic entomology. Trustees of the 799
- 787 British Museum, Natural History and Cornell University Press, London 800
- 788 58. Amendt J, Campobasso C, Gaudry E, Reiter C, LeBlanc H, Hall M 801
- 789 (2007) Best practice in forensic entomology - standards and guide- 802
- 790 lines. *Int J Legal Med* 121:90–104
- 791 59. Zhang X, Nansen C, Aryamanesh N, Yan G, Boussaid F (2015) 803
- 792 Importance of spatial and spectral data reduction in detection of 804
- 793 internal defects in food products. *Appl Spectrosc* 69:473–80 805
- 794 60. Nansen C, Geremias LD, Xue Y, Huang F, Parra JR (2013) 806
- 795 Agricultural case studies of classification accuracy, spectral resolu- 807
- 796 tion, and model over-fitting. *Appl Spectrosc* 67:1332–8 808
- 797 61. Krushkal JB, Wish M (1978) Multidimensional scaling. Sage, 809
- 798 Beverley Hills
62. Clarke KR (1993) Non-parametric multivariate analyses of changes 799
- in community structure. *Aust J Ecol* 18:117–43 800
63. Fisher RA (1936) The use of multiple measurements in taxonomic 801
- problems. *Ann Eugenics* 7:179–88 802
64. Richards C, Villet M (2008) Factors affecting the accuracy and 803
- precision of thermal summation models of insect development used 804
- to estimate post-mortem intervals. *Int J Legal Med* 122:401–8 805
65. Malewski T, Draber-Monko A, Pomorski J, Los M, Bogdanowicz 806
- W (2010) Identification of forensically important blowfly species 807
- (Diptera: Calliphoridae) by high resolution melting PCR analysis. 808
- Int J Legal Med* 124:277–85 809

UNCORRECTED PROOF