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Autofluorescence and Fourier transform infrared analyses trace dietary fluorophores and reveal plastic contamination in the gut of mosquito larvae

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Abstract

Understanding the nutritional physiology of mosquito larvae is crucial for optimizing mass-rearing practices and improving control strategies. Here we combined complementary optical and spectroscopic techniques, including fluorescence microscopy, confocal spectral imaging and Attenuated Total Reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy, to trace food ingestion in *Aedes albopictus* larvae. Differences in autofluorescence (AF) signal intensity and spatial distribution were observed in the gut of larvae reared in polystyrene (PS) or glass (GL) containers, suggesting that AF may serve as a relative proxy for evaluating

ingestion efficiency. Chlorophyll-derived AF was detected outside the gut within the larval body, indicating systemic distribution of food-derived fluorophores. Spectral analysis of rearing water before and after larval development revealed changes in flavin-associated fluorescence profiles, consistent with flavin metabolism. Moreover, ATR-FTIR spectroscopy of larval gut samples revealed differences in chemical functional groups between larvae reared in PS or GL containers, suggesting ingestion of PS-derived material released from rearing containers. Despite the spectroscopic differences observed between rearing conditions, no clear effects were detected on standard mosquito life-history traits. Taken together, these findings highlight the potential of fluorescence- and ATR-FTIR-based techniques as sensitive tools to explore mosquito larval biology and nutrition. These approaches can reveal subtle yet biologically relevant effects of the rearing environment, a key factor in vector control programmes.

Keywords: *Aedes albopictus*, feeding, confocal spectral imaging, spectroscopy, chlorophyll, polystyrene.

Introduction

Autofluorescence (AF), or native autofluorescence, is a common phenomenon across the tree of life and consists in the emission of light in the ultraviolet (UV)-visible, near-infrared (IR) spectral range upon excitation with light of suitable wavelengths [1,2]. In general, the overall AF signal depends on various endogenous fluorophores, typical of the biological substrates. The close involvement of the fluorescing biomolecules in functional activities or structures can affect their emission properties, making them intrinsic diagnostic biomarkers. For this reason, AF is

considered as an important *in situ*, real time tool for *in vivo* or *ex vivo* diagnostic applications in biomedicine [3]. A remarkable example is the use of AF-based applications in hepatology, where endogenous fluorophores such as NAD(P)H, flavins, lipofuscins, collagen, vitamin A, fatty acids and bilirubin are regarded as biomarkers for clinical diagnosis and the development of therapeutic strategies in experimental models [4].

While AF has been extensively exploited in biomedical research to probe tissue composition and metabolic processes, the underlying principles are not restricted to vertebrate systems. Autofluorescence originates from endogenous and diet-derived fluorophores whose spectral properties reflect biochemical composition and physiological state across biological taxa. In insects, and particularly during larval stages characterized by intense feeding activity, AF therefore represents a conceptually well-founded yet largely underexplored approach to investigate nutritional physiology and interactions with the rearing environment.

Consistent with this view, to date AF in insects has been explored for various applications ranging from taxonomy to the development of sensors for energy accumulation, as well as tools for environmental remediation and health monitoring [5,6]. In mosquitoes, AF properties have been studied for the characterization of body structures [7–10], as well as to trace metabolism of specific fluorophores (e.g., pteridines, tryptophan) involved in metabolic pathways with relevance for insect biology [11–13].

Mosquito larval food in lab-scale and mass-rearing facilities is usually a complex matrix composed of various biological materials, including arthropod-, plant- and algal-derived material [14]. Characterizing the AF of larval food particles and tracing them as non-invasive markers within the insect body may provide valuable insights

into nutrient internalization, processing, absorption, and excretion. In recent years, increasing attention has been directed toward exploring natural compounds with bioactive potential against mosquito larvae. Among these chemicals, plant-derived molecules, including chlorophyll and its derivatives, have shown promise due to their ability to act as photosensitizers, potentially inducing lethal oxidative stress upon light activation [15]. Initial investigations on chlorophyll processing in the larvae of phytophagous insects have been reported [16], while little is known about how chlorophyll derivatives are processed by mosquito larvae. Moreover, tracing fluorescent food components in the gut and other organs and tissues, and understanding how external conditions (e.g., laboratory rearing) affect their AF profiles, is relevant not only for basic biological research but also for applied purposes. Historically AF has been considered an obstacle in experiments involving exogenous fluorophores for insect genomic manipulations [17]. However, AF characterization may enable researchers to investigate the intrinsic fluorescence emission signals associated with specific endogenous molecules that are typical of the structural and functional features of insect organs and tissues [6].

Mosquitoes, particularly species such as *Aedes albopictus* (Skuse, 1894)(Diptera, Culicidae), are major vectors of numerous infectious diseases, making their control a global public health priority [18]. Understanding mosquito feeding biology is essential for improving environmentally sustainable control strategies, especially during the larval stages in the breeding habitats, before the adult emergence. To our knowledge, AF of food components to trace their intake has not yet been explored in insects, although it may be a powerful tool for estimating food intake and for optimising insect mass-rearing in the context of the Sterile Insect Technique (SIT) [14,19]. The SIT is an environmentally-friendly approach for pest and vector control

that has been successfully adopted worldwide against several detrimental species [20–22]. This approach involves multiple critical components such as strain development and mass-rearing, sex-separation, sterilization and release in the field [23]. Cost-effective and optimised feeding methods are constantly being established [24–27], and different types of rearing containers have been reported to support mosquito larval development [28], with plastic equipment being more commonly used due to lower costs and ease of handling. However, the use of plastic materials for insect rearing warrants careful consideration, as microplastic contamination from plastic containers, including drinking bottles and food packaging, has been widely reported [29,30]. Notably, plastic-derived chemicals were found in *Protaetia brevitarsis* Lewis, 1879 (Coleoptera: Scarabaeidae) larvae reared in plastic cages [31].

In this study, we aimed to evaluate whether AF can be used as a tool to trace food ingestion and distribution in *Ae. albopictus* larvae reared under laboratory conditions, and whether complementary spectroscopic approaches can reveal rearing-related chemical signatures. To this end, we combined epifluorescence microscopy, spectral confocal imaging and spectrofluorimetric analyses, and Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy to characterize food-derived fluoroscence and chemical functional groups in larval food and gut samples from larvae reared in polystyrene or glass containers.

Materials and methods

Mosquito samples

Eggs of the *Ae. albopictus* RER strain [32] were hatched in a glass jar with 200 ml of autoclaved water. Fifty first-instar larvae were then individually transferred using

disposable glass Pasteur pipettes into 50 ml of autoclaved water in polystyrene (PS) Petri dishes, with three independent biological replicates. Larvae were maintained under controlled insectary conditions (*i.e.*, 26±1 °C, 70% relative humidity, and a 12:12 h light:dark photoperiod). Larvae were fed daily with one commercial food pellet (Tetra Goldfish Granules, Tetra GmbH, Melle, Germany; mean pellet mass of 3.22 ± 0.34 mg, mean ± SD) per container. The entire experiment was independently replicated three times. The same experimental set up, including larval density, water volume, and feeding regime, was used to rear larvae in borosilicate glass (GL) containers. Larvae reared in polystyrene and glass containers are hereafter referred to as larva-PS and larva-GL, respectively.

Sample preparation for epifluorescence and confocal microscopy analysis

For each rearing condition, larvae from three independent biological replicates were analysed. For each replicate, multiple larvae (n = 5-10) were observed; all images presented in the Results and Discussion section were selected as representative of the recurring AF patterns consistenly observed across independent larvae and biological replicates. Individual third instar larvae from both polystyrene and glass rearing containers (larva-PS and larva-GL, respectively) were rinsed twice in autoclaved water. Each larva was moved to a microscope glass slide, fixed (20 µl 4% w/v paraformaldehyde PBS solution, 10 min), mounted with a coverslip and sealed with clear nail polish. In addition, one food pellet was hydrated with a drop of PBS on a glass slide and mounted as described above. All these samples were observed both under epifluorescence and confocal microscopy.

Bright field and AF images of unstained samples were acquired with an Olympus BX53 fluorescence microscope (Olympus Optical Co. GmbH, Hamburg, Germany)

using a X-Cite 120Q (120 W Hg vapour short arc lamp) as the excitation source. Fluorescence was selected by means of optical cubes with the following filter combinations: 340–390 nm excitation, 410 nm dichroic mirror, 420 nm longpass ("cyan"-observation); 405±5 nm excitation, 450 nm dichroic mirror, 455 nm longpass suitable for tetrapyrrole-ring-based fluorophores ("red"-observation). Images were acquired using a Canon EOS 1300D camera (Canon, Tokyo, Japan).

Confocal imaging and spectral analysis were performed using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), equipped with a tunable White Light Laser (WLL), and a 20x oil immersion objective (Leica HC PL APO CS2 20x/0.75 IMM). Larval gut images were acquired using the 405 nm laser line to capture AF signals in two spectral ranges: 415-600 nm (shown in cyan false colour) and 650-750 nm (shown in red false colour), besides the acquisition of brightfield images. "Cyan" images were converted into false colour green to facilitate the visualization (in yellow) of colocalization of blue and red emissions to select areas for spectral confocal imaging. Spectra were derived from AF images collected from the same field, using the lambda scan tool (405 nm laser line excitation, 40 steps, 10 nm detection bandwidth). In this study, AF intensity is used as a relative, comparative descriptor of the recorded signals rather than as a linear quantitative measure of fluorophore concentration. All imaging parameters, including excitation light intensity, detector gain, pinhole size, spectral bandwidth, and acquisition settings, were kept strictly constant across all samples and experimental conditions to ensure direct comparability of AF signals. Images were processed using the FiJi/ImageJ software (v 1.53t) [33]. Briefly, the AF emission intensity profile for each selected region of interest (ROI) was obtained using the 'Plot X-axis profile' tool in Fiji. No spectral smoothing was applied to confocal AF

profiles; intensity values were extracted directly from raw emission data and used for relative comparisons without further normalization beyond identical acquisition settings. The ROIs were selected based on predefined anatomical landmarks and the localization of fluorescent particles, without prior knowledge of the rearing condition (polystyrene or glass) to minimize potential selection bias.

In this study, confocal imaging was purposely used to minimise potential optical artefacts arising from tissue thickness, out-of-focus fluorescence, or overlap of optical planes. The confocal pinhole configuration allowed selective acquisition from defined focal planes, thereby reducing signal contamination from adjacent structures. The use of an oil-immersion objective further limited refractive index mismatch. Spectral profiles were collected from multiple ROIs located at different distances from the gut lumen and within the alimentary canal.

Data were exported to Microsoft Excel (v. 16.98) for graph generation, and spectra shown are representative examples. For presentation purposes, fluorescence and confocal images were adjusted for contrast and brightness using Adobe Photoshop CC 2017 (v. 21.0) and assembled into panels using Adobe Illustrator CC 2017 (v. 21.0).

Cryostat sections

Cryostat sections were prepared from fourth instar larvae (n=5 larvae) based on the protocol by Parenti and colleagues [34]. Larvae were rinsed three times in autoclaved water and fixed in 500 µl of 4% w/v paraformaldehyde in PBS. After a 3h incubation at 4°C, the samples were washed in PBS (3 washing steps of 15 min each), and 500 µl of 15% sucrose was added. After 30 min at room temperature (RT), another 500 µl of 30% sucrose was added for an overnight incubation at 4 °C.

Then, the larvae were individually transferred using fine forceps (Dumont #5SF, Fine Science Tools, Foster City, CA, USA) to a Petri dish lined with white Whatman filter paper (# 1004 124, Maidstone, England) and allowed to dry. Larvae were transferred to plastic holders, embedded in Tissue-Tek OCT mounting medium (Sakura Finetek Europe B.V., Alphen aan de Rijn, the Netherlands), and stored at -80°C until sectioning using a Leica CM1850 cryostat (Leica Microsystems, GmbH, Wetlzar, Germany). Transverse sections, 30 µm thick, were collected on glass slides and dried for 24 hours before microscopic observation.

Spectrofluorimetric analyses

Rearing water containing larval food was analysed before and after mosquito development using a Luminescence Spectrometer LS50B (Perkin Elmer, Waltham, MA, USA). This experiment was performed using water derived from either PS or GL larval rearing containers. For each of the three conditions (water with food only; water derived from PS rearing containers; water derived from GL rearing containers), three different samples were analyzed, and each sample was measured in duplicate. The effective comparative value of the spectral results was ensured by measurements performed with a conventional spectrofluorometer using standard 10-mm path-length quartz cuvettes under identical instrumental settings. Emission and excitation spectra were shown as real measured intensity values, without smoothing.

ATR-FTIR spectroscopy

To investigate the chemical functional groups characterising larval food and guts of larvae reared in polystyrene or glass containers (larva-PS and larva-GL, respectively), the alimentary canal of fourth instar larvae was dissected in PBS and

individually transferred to microscope glass slides. The samples were dried in the dark at RT, keeping them separated to avoid cross-contamination. Each ATR-FTIR biological replicate consisted of pooled dried guts from three larvae; three independent biological replicates were analyzed per condition (*i.e.*, PS or GL). Clean PS and GL rearing containers (*i.e.,* without larvae) were also analysed as controls. To further control for potential larval ingestion of PS-derived material, fourth instar larvae reared in the presence of PS beads (mean particle size 0.5 μm, 50 μg/ml concentration in rearing water; cat. n. L3280, Sigma-Aldrich, Milan, Italy) were also analysed.

Infrared spectra were recorded using a Perkin-Elmer Spectrum 100 Fourier transform infrared (FTIR) spectrometer equipped with an attenuated total reflectance (ATR) accessory with a diamond crystal (PerkinElmer, Waltham, MA, USA). Spectra were collected after pressing the samples against the ATR crystal at RT (20 °C) in the 4000-450 cm^{-1} range (64 scans, resolution of 4 cm^{-1}). Larval food was powdered and subjected to the same analysis. All measurements were performed in triplicates. The resulting spectra were exported to Microsoft Excel for graphical presentation.

To assess potential differences among the different samples, data from a selected spectral range were processed using PeakFIT software (v4.00, Jandel Scientific Software, AISN Software Inc, Erkrath, Germany).

Second-derivative spectra were calculated from the mean ATR-FTIR profiles obtained from the three independent biological replicates per condition described above. Prior to derivative calculation, spectra were subjected to linear baseline correction. Transmittance values were normalized between 0 and 100 to facilitate qualitative comparison among their profiles. Second derivatives were calculated in

the 1500–800 cm^{-1} spectral range, selected on the basis of the differences observed in the transmittance profiles at wavenumbers below 1500 cm^{-1}. No spectral smoothing was applied, as the signal-to-noise ratio of the mean spectra was sufficient and smoothing could potentially alter peak positions and/or profile shapes. FTIR spectra and second-derivative profiles shown in the Results and Discussion section represent means derived from multiple biological replicates.

Scanning electron microscopy

To examine the internal surface of the PS Petri dishes used for larval rearing and to exclude any potential structural damage caused by larval grazing, a FEG-SEM Tescan Mira3 XMU (Tescan, Brno, Czech Republic) located at the Arvedi Laboratory, CISRiC (University of Pavia, Italy) was employed. The analysis was performed on two types of Petri dishes: unused controls and dishes used for larval rearing, which were gently washed three times with autoclaved milliQ water to remove larval exuviae and food-derived material. In both cases, the dish was carefully cut to obtain pieces of about 2x2 cm appropriate for the analytical instrumentation, away from the cut edges, for analysis. The inner surface of the dish fragment was coated with a thin layer of graphite using a Cressington 208 high vacuum carbon coater. Observations were made at 3-5 KV, in high vacuum mode, through a SE detector (working distance 15 mm), at different magnifications.

Effect of rearing conditions on mosquito biological traits

For both larvae reared in polystyrene and glass containers (larva-PS and larva-GL, respectively), mortality was monitored daily by counting dead individuals until adult emergence. The duration of both larval (until pupation) and pupal (until adult emergence) stages was recorded over 14 biological replicates (each one consisting of ten first instar larvae monitored until adulthood) for both PS- and GL-conditions. In addition, potential effects of the different rearing conditions on mosquito size were investigated measuring wing length and width as indicators of body size. To do so, mosquitoes (2-3 days old) from seven biological replicates (each one consisting of ten first instar larvae monitored until adulthood) for both PS- and GL-conditions, were aspirated into 96% ethanol. Before wing measurements, each mosquito was allowed to dry for about 16 hours at RT and then gently transferred to a glass slide. After removing the legs with fine forceps (Dumont #5SF) and sterile disposable syringes (gauge 25), each mosquito was placed dorsally and both wings were removed under a stereomicroscope (Olympus SZ40, Olympus Optical Co. GmbH, Hamburg, Germany). Each wing was separately transferred to a glass slide, where 3 μl 10% sucrose solution was added to flatten and improve wing adhesion to the slide surface. The slides were allowed to dry at RT for 10 minutes. For each mosquito, wings were observed using a Leica DMLB microscope with a 2.5x magnification. Photographs were acquired of both left and right wings using a Nikon D5000 camera (Nikon, Tokyo, Japan) tethered to Darktable software version 4.6.1 (www.darktable.org). An image of the microscope calibration scale was captured at the same settings for size calibration of the wing images. The FiJi/ImageJ software was used to draw a rectangle from the distal notch of the alula to the wing apex,

parallel to the costa (wing length, in µm), and from the costa to the hind margin excluding the fringe (wing width, in µm) (Supplementary Fig. S1). Seven biological replicates were performed for both PS and GL rearing conditions. For each replicate, the adults emerging from a batch of ten larvae were observed, and those with both wings damaged were excluded from the sample set. The left wing was used; if damaged, the right wing was measured.

Prior to inferential statistical analyses, larval and pupal development duration data were subjected to descriptive statistical analysis, including formal assessment of data distribution. Normality was evaluated using both the Shapiro–Wilk test and the D'Agostino–Pearson test. As the assumption of normality was not consistently met, non-parametric Mann–Whitney U tests were applied to compare rearing conditions. The same analytical approach was used for mortality data and for adult wing size measurements (length and width). Statistical comparisons were planned *a priori* and limited in number; therefore, no multiple-comparison correction was applied. Data were analysed in the Real Statistics Resource Pack (8.9.1; Release 8.9.1; Copyright 2013-2023 - Charles Zaiontz, www.real-statistics.com).

Analytical scope and limitations

Microscopy-based AF analyses and ATR-FTIR profiles were conceived as comparative and exploratory spectroscopic approaches aimed at detecting qualitative and semi-quantitative differences between experimental rearing conditions, rather than providing full statistical inference on biological effects. Quantitative life-history traits were instead analyzed using formal statistical tests.

Results and Discussion

Food autofluorescence is detected in the gut of mosquito larvae

Brightfield microscopy revealed the presence of heterogenous structures in the powdered mosquito larval food (Fig. 1A). Epifluorescence microscopy further confirmed this heterogeneity, highlighting marked variability in fluorescence emission patterns (Fig. 1B,C). In some particles, a predominant light-blue emission was observed against a more diffuse reddish fluorescence emission (Fig. 1B). Based on the declared composition of the food, which includes plant-derived and algal materials, we also applied observation conditions more suitable for exciting fluorophores such as chlorophyll, a tetrapyrrole-based derivative (Fig. 1C). Under these conditions, the AF distribution pattern appeared more defined, revealing both bluish and reddish amorphic structures, as well as red spherical particles approximately 0.8-2 μm in diameter, likely corresponding to microalgae [35]. Observation of the gut of intact third instar larvae reared in PS containers (hereafter, larva-PS) (Fig. 1D) revealed an alimentary canal exhibiting high AF intensity within the blue body cavity (Fig. 1E). In the gut, the fluorescence distribution pattern can be related to the ingested food components, which exhibited heterogeneity in size, shape and emission colors, including distinct red-emitting particles (Fig. 1E). Excitation conditions suitable for tetrapyrrole derivatives enhanced the red AF intensity, in contrast to the darker greenish appearance of the surrounding larval body cavity (Fig. 1F). Similarly, in cryostat transverse sections of fourth instar larvae, the morphological and AF features of food components were clearly discernible (Fig. 1G-I). Images shown are representative of consistent patterns observed across multiple larvae and independent biological replicates.

To further characterize the AF properties of both the larval food and gut content, a spectral confocal imaging approach was adopted. Images collected under the “cyan”

and “red” conditions and displayed in false colours were merged to identify regions exhibiting predominantly one or the other emission, as well as overlapping areas. An image from the same field was also acquired in the spectral modality to obtain emission profiles from regions selected based on the AF distribution pattern observed in the merged images. In the case of the food, AF images were consistent with the epifluorescence observations (Fig. 2A-E). Structures with cyan and red AF signals displayed distinct and uneven spatial distributions (Fig. 2B,C), without appreciable overlap (Fig. 2D). Small red fluorescent spherical structures were again visible, resembling the morphology of microalgae [35]. Spectra derived from ROIs selected on the merged images reveal two main emission bands: a broad band spanning the 415-615 nm spectral range, and a narrower band peaking at approximately 675 nm (Fig. 2E). In general, the AF intensity (amplitude) of both emission bands varied depending on the selected area, and within each spectrum the relative amplitude of the two bands depended on the localization of each ROI. As expected, the red-emitting band displayed a relatively higher AF intensity in spectra collected from ROIs with a predominant red pattern. The broad band in the 415-615 nm interval was consistent with emission from proteinaceous material [4,6]. The narrower red band was consistent with chlorophyll-related compounds, based on its peak position around 675 nm [36]. While chlorophyll a is a likely contributor, closely related degradation products such as pheophytin or pheophorbide may also emit in this spectral region and cannot be distinguished solely on the basis of the emission peak position [37].

As observed in the food, the content of the alimentary canal of larva-PS samples exhibited structures heterogeneous in both size and AF emission (Fig. 2F-J). Consistent with the brightfield image (Fig. 2F), cyan AF signal highlighted the entire

body cavity and outlined the larval cuticle (Fig. 2G). In agreement with epifluorescence microscopy observations, the gut and its contents were visible with higher AF intensity under excitation conditions selective for red emission (Fig. 2H). The merged image revealed regions with predominantly red AF and others where red and cyan signals clearly overlapped (Fig. 2I). Spectra from selected ROIs again revealed a broad emission band in the 415-615 nm interval, and a narrower band peaking at approximately 675 nm (Fig. 2J). As in the food, the broader band at shorter wavelengths in the larva-PS gut likely reflects the combined contribution of multiple fluorophores. Given that biomolecules such as proteins and some vitamins are known to be contributors to blue-range emission, it is important to consider the possible presence of additional fluorophores originating from both the food and the larval tissues, whose chemical identity and specific AF profiles are not yet fully defined. In this context, particular attention was therefore focused on the red-emitting band, which can be more specifically discriminated. Notably, this band did not show detectable changes in peak position when comparing food and larval gut AF (Fig. 2J, ROIs n. 3 and 4) under the spectral resolution of our approach. It should be noted that spectra obtained using confocal spectral imaging (spectral resolution: 8.067 nm) exhibited a red-emitting band with a peak position consistent with that measured using the spectrofluorometer (spectral resolution: 0.5 nm), further supporting the view that the responsible fluorophore does not undergo changes resulting in remarkable spectral shifts. In any case, we can not exclude that subtle chemical modifications of chlorophyll-derived compounds, such as pheophytin or pheophorbide, may occur without producing a detectable change in emission peak position [37]. Even more interestingly, the red-emitting AF band was also detected in the larval body cavity (Fig. 2J, ROI n. 2), whereas no red signal was observed in the

larval cuticle (Fig. 2J, ROI n. 1). The use of confocal spectral imaging reduces the likelihood that this signal arises from optical artefacts related to tissue thickness or overlap of optical sections. In addition, comparable spectral profiles were consistently obtained from regions located at different distances from the gut lumen and within the alimentary tract across independent larvae, reducing the likelihood of scattering effects and supporting the robustness of the detected extra-gut AF signals. Our findings suggest that food-derived tetrapyrrole fluorophores can persist through digestion and reach internal compartments, although their precise chemical identity and metabolic fate remain to be fully resolved [16]. While these data support the presence of food-derived fluorophores outside the gut lumen, further quantitative spatial analyses across multiple time points will be required to elucidate the mechanisms and dynamics underlying their transfer. This is particularly relevant given the growing interest in chlorophyll-derived photosensitizers for insect control in aquatic ecosystems, including mosquitoes [38,39].

Food autofluorescence in the gut of mosquito larvae changes according to rearing conditions

When mosquito larvae were reared from hatching to pupation in GL containers (larva-GL), epifluorescence microscopy revealed a lower presence of food particles exhibiting AF signals in the gut compared to larvae reared in PS containers (Fig. 3A-C). Confocal spectral imaging supported these epifluorescence observations and also revealed a more evident colocalization of cyan- and red-emitting areas, as indicated by the prevalence of yellow regions in merged images, compared to PS-reared larvae (Fig. 3D-G). Despite these differences in AF signal abundance and spatial distribution, spectral analysis showed that, in larva-GL samples, the

characteristic emission features observed in PS-reared larvae were retained. In particular, in addition to the broad band in the 415-615 nm spectral region, the chlorophyll-associated red band peaking at approximately 675 nm was detected both in the alimentary canal (Fig. 3G, ROIs n. 1 and 4) and in the larval body cavity (Fig. 3G, ROI n. 2). As observed in the larva-PS samples, spectra collected from the cuticle showed no detectable red AF signal (Fig. 3G, ROI n. 3). Moreover, similarly to larva-PS samples, the AF intensity of both emission bands varied depending on the selected ROIs.

Comparison of spectra from larva-PS (Fig. 2J) and larva-GL (Fig. 3H) samples generally revealed lower AF intensity for both emission bands in GL-reared larvae. This difference was further supported by mean AF intensity values calculated from spectra extracted from the overall gut area (Supplementary Fig. S2; Supplementary Fig. S3).

Taken together, these observations suggest reduced food intake and/or differences in food processing in larvae reared in GL containers compared to those reared in PS containers. At present, AF intensity should be regarded as a qualitative, relative proxy for ingestion or accumulation rather than a linear measure of ingested quantities. The relationship between AF intensity and absolute intake is likely non-linear and warrants dedicated experiments to be fully resolved.

Spectrofluorimetric analyses show AF changes in breeding water depending on larval development

To further investigate the marked difference in AF intensity observed between the two rearing conditions, we analysed the AF profiles of larval breeding water before

444 and after larval development, with the aim of assessing the presence of food-derived
445 fluorophores and potential changes associated with food processing.
446 In water supplemented with food but without larvae (control condition), excitation at
447 366 nm resulted in two emission bands peaking at approximately 450 and 525 nm,
448 respectively. In contrast, water collected after larval development exhibited a single
449 emission band peaking at about 450 nm (Fig. 4A). Larval rearing conditions did not
450 markedly affect overall spectral shapes but were associated with lower AF intensity
451 in larva-GL compared to larva-PS samples. The two emission peak values were then
452 used to examine excitation profiles. Under observation at 450 nm, spectra displayed
453 a main band peaking at about 360 nm, with no appreciable differences in the
454 spectral shapes among the three tested media samples.
455 When analysing excitation profiles under observation at 525 nm, the control medium
456 exhibited two bands peaking at about 380 and 445 nm, respectively. Excitation at
457 450 nm, in turn, resulted in a well-defined band with a maximum at approximately
458 530 nm (Fig. 4B). In both larval rearing conditions, the breeding water showed a
459 single excitation band in the 360-390 nm range when observed at 525 nm, and an
460 emission band peaking at about 515-520 nm under excitation at 450 nm.
461 According to the literature, these spectral features, particularly the excitation profiles,
462 are typical of flavins, which play key roles as cofactors in the electron transport chain
463 and in lipid metabolism [40]. Indeed, flavins typically display a distinctive profile with
464 two peaks at approximately 380 and 445 nm [41,42]. In this context, the observed
465 reduction in AF intensity and the alteration of the excitation profile at 525 nm in larval
466 breeding water are consistent with the conversion of flavin into lumichrome [43–45].
467 Finally, excitation at 405 nm of the control medium did not reveal any AF signal
468 ascribable to chlorophyll, while a characteristic narrow emission band was clearly

detected in larval breeding water (Fig. 4C). This emission feature was observed without detectable shifts in peak position with respect to that observed following confocal spectral imaging. The presence of chlorophyll-releated AF signal in the breeding water is therefore consistent with the release of chlorophyll-derived compounds during larval development. Notably, this observation parallels the detection of chlorophyll-associated AF in larval tissues outside the gut (*i.e.*, in the haemolymph) (Fig. 2I,J; Fig. 3G,H).

Despite the higher AF intensity of the red band observed in the gut of larva-PS compared to larva-GL samples, corresponding differences were not detected in the rearing water. In addition, previous *in situ* microspectrofluorometric analyses of the Malpighian tubules of *Ae. albopictus* larvae and adults did not reveal an emission band peaking at approximately 675 nm [11]. Taken together, these observations raise additional questions regarding the mechanisms by which mosquito larvae process chlorophyll-derived compounds and highlight the need for further targeted experiments, including studies relevant to larval control strategies in the field [39].

ATR-FTIR analyses of chemical functional groups show the presence of PS material in mosquito larvae

To further investigate the differences between larva-PS and larva-GL samples highlighted by our AF-based analyses, an ATR-FTIR analytical approach was adopted. Transmittance spectra were acquired from the larval food as well as from the guts of larvae reared under the two conditions to compare their chemical profiles (Supplementary Fig. S4). Overall, the spectra showed similar profiles, with peaks corresponding to biochemical functional groups shared among the three sample types. An exception was represented by a peak around 1742 cm^{-1}, detected in the

494 food and compatible with the presence of the carbonyl group (C=O). This peak is
495 consistent with contributions from the acetyl and uronic ester groups present in
496 hemicellulose, or the ester linkage of the carboxylic group of ferulic and p-coumeric
497 acid of lignin. Hemicellulose and lignin residues are commonly found in plant
498 materials together with cellulose and can therefore be present in the larval food [46–
499 48](Supplementary Fig. S4; Supplementary Table S1).
500 In addition, FTIR spectra derived from larval-PS or -GL gut samples were compared
501 with spectra acquired from the material of the respective rearing containers
502 (Supplementary Fig. S5). The PS spectrum was characterized by numerous peaks,
503 consistent with published FTIR references spectra [49,50]. The larva-PS profile
504 generally exhibited broader peaks than those of PS and at different positions, except
505 for the two evident peaks in the interval 2853-2920, consistent with stretching
506 vibration of -C-H in CH, CH_2 and CH_3 groups (Table 1). The GL spectrum displayed
507 only a limited number of peaks at positions shorter than 1400 cm^{-1}, corresponding to
508 borosilicate glass [51]. Among these, only the peak centred around 1120 cm^{-1}
509 partially overlapped with the 1000-1230 cm^{-1} transmittance interval observed in larva-
510 GL samples and ascribable to cellulose (Table 1).
511 Comparison of the mean spectra from larva-PS and larva-GL samples revealed
512 some differences in transmittance at wavenumbers below 1500 cm^{-1} (Fig. 5A). This
513 observation prompted a more detailed analysis of the FTIR spectra of the two larval
514 samples by inspecting the second derivative of the profiles in the 1500-800 cm^{-1}
515 range. Second-derivative profiles were derived from mean spectra obtained from
516 independent biological replicates, which showed consistent features across
517 replicates for each rearing condition. While the larva-GL profile exhibited two positive
518 peaks at about 1070 and 1020 cm^{-1}, larva-PS samples displayed a shoulder around

519 1070 and a peak around 1037 cm^{-1} (Supplementary Fig. S6). Interestingly, focusing
520 on the 1200-980 cm^{-1} interval, the profile of the food closely resembled that of larva-
521 GL samples. This interval is compatible with functional chemical groups associated
522 with cellulose (conjugated three peaks related to the C-OH) and proteins (C-O
523 stretching corresponding to the COO-) (Table 1). On the other hand, a peak at
524 approximately 1037 cm^{-1} was observed only in larval-PS samples. While this peak is
525 consistent with reported FTIR signatures of PS and was absent in both the food and
526 larva-GL samples, the possibility that overlapping biological compounds with similar
527 vibrational modes may partially contribute to this signal cannot be excluded.
528 Nevertheless, its selective presence in PS-reared larvae, its proximity to reference
529 PS peaks, and its similarity to spectra obtained from larvae exposed to PS beads
530 collectively support a PS-related origin. Indeed, it is noteworthy that the position of
531 the second-derivative peak detected around 1037 cm^{-1} in larva-PS samples closely
532 matched the peak observed around 1027 cm^{-1} in larvae fed with PS beads,
533 consistent with the 1027 cm^{-1} peak detected in the transmittance FTIR spectrum of
534 PS (Fig. 5B). Together, these observations suggest an effect of PS material on the
535 vibrational energy of the chemical functional groups of the larva-PS and PS-bead-
536 exposed larvae, an effect that is absent in larva-GL samples. Taken together, these
537 controls reduce – though do not entirely eliminate – the likelihood that the observed
538 band arises solely from endogenous biological or dietary components.
539 Accordingly, while the ATR-FTIR data support an influence of PS-derived material,
540 definitive chemical identification would require complementary analytical approaches
541 (e.g. Raman spectroscopy or mass spectrometry).
542 To investigate potential sources of PS material in larva-PS samples, the internal
543 surface of PS Petri dishes, both clean (*i.e.,* without larvae) and after larval

development, were examined by scanning electron microscopy (SEM). This analysis did not reveal visible signs of mechanical damage attributable to larval grazing on the internal surfaces of the dishes (Supplementary Fig. S7).

In the light of our FTIR data suggesting the presence of PS-related signatures in larva-PS samples, it should be considered that plastic containers are reported to release variable amounts of micro- and nanoplastics (MNPLs). Polystyrene, in particular, has been demonstrated to undergo fragmentation, and to release microplastic-sized particles, and monomers depending on oxidation and/or hydrolysis processes, temperature, aging and partition coefficients [52,53].

Plastic release is receiving increasing attention due to its potential hazardous effects across the food chain, from aquatic organisms to mammals and humans [54–57]. In insect rearing, PS, obtained from styrene monomers polymerization, is one of the plastic polymers most commonly found in rearing containers. Although plastic or metal trays are generally used as containers for mosquito larval rearing [58], disposable PS Petri dishes are frequently employed in laboratory experiments involving mosquito larvae [59], potentially introducing unrecognized effects on mosquito life-history traits. Considering that a recent study has shown that commercial PS MNPLs added to *Ae. albopictus* larval rearing water are ingested, cross the larval gut barrier, affect metabolic and hormonal balance and are ontogenically transferred [60], the present findings underscore the importance of assessing plastic leaching and MNPL uptake in laboratory rearing systems used for mosquito mass production and vector control programs.

Rearing container material affects mosquito life-history traits

To assess the potential effects of rearing container material on mosquito biological traits, a set of life-history parameters was analyzed in both larva-PS and larva-GL samples. Mean larval development duration (days, mean ± standard error, SE) differed significantly between the two treatments (7.04 ± 0.08 SE, and 6.69 ± 0.07 SE for GL- and PS-larvae, respectively; Mann-Whitney U test, U = 7282, p=0.0032). In contrast, no significant difference was observed in pupal development duration between the two conditions (2.35 ± 0.04 SE, and 2.42 ± 0.05 SE for GL- and PS-pupae, respectively; Mann-Whitney U test, U = 8544, p=0.2915) (Fig. 6).

Similarly, no statistically significant differences were detected in larval survival (Mann-Whitney U test, U = 77, p=0.3239), or pupal survival (Mann-Whitney U test, U = 85.5, p=0.5946) between the two treatments. Adult wing size, used as a proxy of mosquito body size, was not significantly affected by rearing condition, as indicated by wing length and width measurements in both sexes (female wing length: Mann-Whitney U test, U = 320, p=0.0582; female wing width: Mann-Whitney U test, U = 345.5, p=0.1283; male wing length: Mann-Whitney U test, U = 328.5, p=0.7585; male wing width: Mann-Whitney U test, U = 295.5, p=0.3582; Supplementary Table S2).

Taken together, these results indicate that standard laboratory assays commonly used to evaluate mosquito life-history traits do not reveal striking differences between the two rearing conditions, despite the differences in food ingestion revealed by AF and the variations in the functional groups shown by our ATR-FTIR analyses. This outcome should be interpreted in the context of our experimental design, as biological assays were intended to detect biologically meaningful differences in conventional laboratory settings commonly used to evaluate mosquito performance, rather than subtle physiological changes. Accordingly, while our results

indicate no detectable effects of rearing container material on survival or adult body size under the conditions tested, the presence of more subtle effects cannot be excluded. Detecting such effects may require larger sample sizes, alternative life-history or physiological endpoints, or more sensitive analytical approaches.

Some methodological limitations should be acknowledged, including the use of AF as a qualitative, relative proxy, the composite nature of spectral signals arising from multiple fluorophores, and the restriction of the experimental design to a single laboratory strain, diet, and rearing setup.

Conclusions

In this study, we show that AF can be effectively applied to trace food-derived fluorophores within *Ae. albopictus* larvae, providing a direct and sensitive approach to investigate mosquito larval nutrition and physiology. By combining epifluorescence microscopy, spectral confocal imaging, and spectrofluorimetric analysis, we detected differences in AF signal topological distribution and a higher AF intensity in larvae reared in PS compared to those reared in GL containers. These differences are consistent with variations in food intake and/or processing. The detection of red-emitting AF signals ascribable to chlorophyll-related compounds both within and outside the larval alimentary canal opens new perspectives on the ability to trace naturally fluorescent dietary components with potential photosensitizing properties, offering insights into accumulation mechanisms relevant to environmentally friendly larvicides. Furthermore, analysis of breeding water before and after larval development revealed changes in AF profiles, particularly in flavin-associated signals, consistent with differences in food processing between larvae reared in PS and GL containers.

The present findings support the use of AF-based approaches as sensitive, real-time tools to monitor mosquito larval feeding-related processes, while ATR-FTIR spectroscopy provides complementary chemical information relevant to the detection of material-derived signatures in laboratory rearing systems and, potentially, in field investigations. Together, these spectroscopic techniques represent underutilized tools capable of revealing effects that may not be easily detected using standard laboratory life-history trait assays alone.

Although the present findings were obtained under specific dietary and laboratory rearing conditions using a single mosquito strain, the overall methodological framework is broadly transferable. The combined use of AF imaging, spectral analysis, and ATR-FTIR spectroscopy can be applied to different larval diets, rearing materials, and mosquito species to qualitatively trace food-derived fluorophores and detect rearing-related chemical signatures. However, AF spectral profiles, signal intensity, ingestion and accumulation dynamics, and interactions with material-derived compounds are expected to be strongly system-specific, varying with diet composition, rearing materials, larval feeding behaviour, gut physiology, and species-specific metabolism. Accordingly, interpretation of AF and spectroscopic signatures should be validated on a case-by-case basis.

Overall, these approaches provide a useful framework for future investigations focused on optimizing laboratory and mass-rearing protocols in vector control programs, including those based on the SIT [19].

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Author contributions

S.S., M.L.W., A.O., A.G., A.M., and A.C.C. performed the experiments; S.S., M.L.W., A.C.C. M.L. and L.M.G. analyzed the data; F.S. conceived and designed the study, supervised the work and wrote the paper with contributions from all authors. All authors reviewed and approved the final version of the manuscript.

Additional information

Competing Interests Statement: The authors declare no competing interests.

Data availability statement

Raw data corresponding to mosquito larval and pupal development duration, mortality and wing size, spectral data from confocal and spectrofluorimetric analyses, and individual ATR-FTIR spectra with the corresponding mean spectra used for second-derivative analysis are available at https://osf.io/z7gxh/?view_only=6679b05a9106405894e721e5cfc59889.

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Figure legends

Fig. 1. Autofluorescence patterns in the food and in *Ae. albopictus* larval guts. Bright field (A, D, G), and fluorescence images captured at the epifluorescence microscope under cyan (B, E, H), and red (C, F, I) observation conditions. Powdered larval food (A-C); portion of the posterior midgut of a third instar larva reared in polystyrene containers (larva-PS) containing food material (D-F); transverse cryostat section of the whole body of a fourth instar larva reared in polystyrene containers (larva-PS) (G-I); ep, epithelial cells; fb, food bolus. Scale bars: 50 µm (A-C), 200 µm (D-F), 25 µm (G-I).

Fig. 2. Spectral confocal imaging of powdered larval food and of the gut of a larva-PS. Images recorded in bright field (A,F), cyan (B,G) and red (C,H) conditions, together with the corresponding merged images (D,I). Autofluorescence emission

841 spectra from selected ROIs (E,J). For visualization purposes, spectral intervals
842 covering the 450-600 nm and 620-720 nm ranges are displayed using separate
843 scales (J). Scale bars: 100 µm.

844 **Fig. 3. Epifluorescence and spectral confocal imaging of the gut of a larva-GL.**
845 Bright field (A), and epifluorescence images acquired under cyan (B), and red (C)
846 observation conditions. Confocal images recorded in bright field (D), cyan (E) and
847 red (F) conditions, together with the corresponding merged image (G).
848 Autofluorescence emission spectra extracted from selected ROIs are shown (H). For
849 visualization purposes, spectral intervals covering the 450-600 nm 620-720 nm
850 ranges are displayed using separate scales (H). Scale bars: 200 µm (A-C), 100 µm
851 (D-G).

852 **Fig. 4. Spectrofluorimetric analysis of rearing water before and after larval**
853 **development.** Excitation and emission spectra recorded from rearing water
854 containing larval food before (green; control condition) and after larval development
855 in PS (blue) and GL (red) containers. Spectra were acquired under different
856 combinations of excitation and emission conditions (A and B) or under excitation at
857 405 nm (C), as indicated in the respective panels.

858 **Fig. 5. ATR-FTIR transmittance spectra of larval guts and PS material.** Mean
859 ATR-FTIR transmittance spectra of gut samples from larva-PS and larva-GL
860 conditions are shown, as indicated by the colours in the legend. Transmittance
861 values were normalized to the 4000-3700 cm^{-1} wavenumber interval (A). Second-
862 derivative ATR-FTIR spectra of gut samples from larva-PS and larva-GL conditions,
863 as well as from larvae fed with PS beads, are indicated by the colours in the legend,
864 with values referring to the right y-axis. The ATR-FTIR transmittance spectrum of the

PS reference material is also shown (light blue), with values corresponding to the left y-axis (B).

Fig. 6. Life-history traits of *Ae. albopictus* larvae reared in different container materials. Larval and pupal development duration for larva-PS vs larva-GL treatments (n = 134 and 136 larvae for PS- and GL-treatment, respectively; n = 134 and 136 pupae for PS- and GL-treatment, respectively). Only individuals that reached adulthood were included in the analysis. Crosses indicate mean values, horizontal lines within boxes represent medians, and whiskers indicate data dispersion; dots outside the whiskers denote outliers. * $P < 0.05$.

Table

Table 1. Assignment of ATR-FTIR peaks to functional groups of the gut from larvae reared in GL or PS, as well as PS and GL reference materials.

Wavenumber/ transmittance peaks (cm^{-1})	**Functional groups**	**Sample types**			
		GL	**PS**	**Larva-GL**	**Larva-PS**
701-747	Out of plane C-H bending vibration of the benzene ring	-	X	-	-
905	Out of plane C-H bending vibration of the benzene ring	-	X	-	-
1000-1230	Cellulose (stretching vibration, C-O)	-	-	X	X
1027-1037	In plane C-H bending vibration of the aromatic ring/C-H bending vibration of aliphatic groups/C-C	-	X	-	X

	stretching vibration				
1000-1120	Stretching vibration (Si-O-Si)	X	-	-	-
1230	Stretching vibration (C-O related to protein)	-	-	X	X
1390	Stretching vibration (B-O related to borosilicate)	X	-	-	-
1396-1455	Vibration def. (C-H, C-OH)	-	-	X	X
1452-1493	Aromatic C=C stretching vibration	-	X	-	-
1510-1533	Amide II band (-NH_2)	-	-	X	X
1601	Aromatic C=C stretching vibration	-	X	-	-
1636	Amide I band (C=O)	-	-	X	X
2853-2920	Stretching vibration (-C-H in CH, CH_2 and CH_3 groups)	-	X	X	X
3030-3060	Aromatic C-H stretching vibration	-	X	-	-
3272	Stretching vibration (N-H)	-	-	X	X

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