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***Original article (Orijinal araştırma)***

# Tracing time through cuticular clues: The role of rearing conditions and generational divergence in *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae)

Kütiküler ipuçlarıyla zamanın izlenmesi*:* *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae)'da yetiştirme koşullarının ve nesiller arası farklılaşmanın rolü

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## Abstract

The chemical profiles of the cuticle of adult flies are highly influenced by environmental factors and generational variation, although the extent and mechanisms of these influences are still poorly understood. This research rigorously investigates the influence of rearing environment and generational changes on the cuticular hydrocarbon (CHC) profiles of adult flies collected from the natural environment in Swindon (UK), in June 2019. Gas chromatography-mass spectrometry (GC-MS) was used to analyze the hydrocarbon profiles. Then, chemometric analysis was applied to determine the chemical variation patterns, allowing the samples to be classified according to their chemical fingerprints. Significant differences in hydrocarbon composition were found between laboratory-maintained and field-collected specimens, underscoring the impact of environmental conditions on CHC expression. Additionally, gradual modifications in hydrocarbon content were detected across generations raised in the controlled environment, suggesting the involvement of adaptive physiological or epigenetic mechanisms. These findings contribute valuable insights into cuticle plasticity, highlighting its relevance in forensic entomology, chemical ecology, and insect evolutionary biology. The implications also extend to forensic investigations, where cuticular hydrocarbon profiles (CHCs) demonstrate potential for enhancing postmortem interval (PMI) estimation accuracy and species identification in criminal cases. By demonstrating quantifiable differences in CHC composition across rearing conditions and generations (AUC values ≥0.92 for all comparisons), this study provides a foundation for the broader application of chemical markers in forensic investigations.

**Keywords:** Generation differences, hydrocarbons, rearing conditions

## Öz

Yetişkin sineklerin kimyasal profilleri, çevresel faktörlerden ve nesiller arası değişimlerden etkilenmektedir, ancak bu etkilerin derecesi ve mekanizmaları yeterince anlaşılmamıştır. Bu araştırma, yetiştirme ortamlarının ve nesiller arası değişimlerin, Haziran 2019'da Swindon'daki (Birleşik Krallık) doğal ortamdan toplanan yetişkin sineklerin kutiküler hidrokarbon profilleri üzerindeki etkisini titizlikle incelemiştir. Hidrokarbon profillerini analiz etmek için gaz kromatografisi-kütle spektrometrisi (GC-MS) kullanılmıştır. Ardından kimyasal değişim desenlerini belirlemek için kemometrik analiz uygulanmış, bu sayede örnekler kimyasal parmak izlerine göre sınıflandırılmıştır. Laboratuvarda muhafaza edilen ve doğal ortamdan toplanan örnekler arasında hidrokarbon bileşiminde önemli farklılıklar belirlenmiş ve çevre koşullarının hidrokarbon yapısı üzerindeki etkisi vurgulanmıştır. Ayrıca, kontrollü bir ortamda yetiştirilen nesiller arasında hidrokarbon içeriğinde tespit edilen kademeli değişiklikler, bu durumun adaptif fizyolojik veya epigenetik mekanizmalarla ilişkili olduğunu düşündürmüştür. Bu sonuçların etkileri aynı zamanda adli soruşturmalara da uzanmaktadır; kutiküler hidrokarbon profilleri (CHC'ler) kriminal vakalarda ölüm sonrası zaman aralığı (PMI) tahmin doğruluğunu ve tür tanımlamasını geliştirme potansiyeli göstermektedir. Yetiştirme koşulları ve nesiller arasında CHC bileşiminde ölçülebilir farklılıklar (çoğu karşılaştırmada AUC değerleri ≥0.92) sergileyerek, bu çalışma kimyasal belirteçlerin adli soruşturmalarda daha geniş uygulanması için bir temel sağlamaktadır.

**Anahtar sözcükler:** Nesil farklılıkları, hidrokarbonlar, yetiştirme koşulları

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## Introduction

Examining insects has been a well-established method for decades to determine the minimum post-mortem interval (PMImin) in criminal cases (Benecke, 2001). Blowflies (Diptera: Calliphoridae) are the first group of insects to arrive at decaying materials (i.e., corpse), and they inhabit the decomposing remains and start laying their eggs just a few minutes after death (Krikken & Huijbregts, 2001). If a body is found outdoors at an advanced stage of decomposition, it is important to determine whether this is the actual place where the death occurred or a deposition site. The location of a body impacts colonization timeframes and (consequently) PMImin predictions (Reibe & Madea, 2010). If the body is in a closed room or has limited access to necrophagous flies, or the decomposition takes a long time at lower temperatures, female adult flies might use the same body for several oviposition cycles. In such circumstances, more than one generation may be associated with the body, leading to a lapse in PMImin predictions (Paula et al., 2018). Thus, determining generational diversities is necessary for accurate PMI estimation.

Traditional entomological methods typically include morphological analysis used to define the physical characteristics of the insect to identify the species and determine the life stages (Amendt et al., 2004). Cuticular hydrocarbon (CHC) analysis is a novel approach that has gained popularity in recent years, which involves analyzing the compounds on the insects' cuticles to identify their species and age (Pechal et al., 2014). The cuticle layer includes free lipids such as hydrocarbons, fatty acids, waxes, and alcohols (Gibbs & Crockettj, 1998). Cuticular hydrocarbons are the dominant compounds, with more than 100 different compositions, and various combinations of linear and branched alkanes and alkenes (Blomquist & Bagnères, 2010; Drijfhout et al., 2010). The cuticle layer is very stable structures (Moore et al., 2014) and plays a crucial role in protecting insects against desiccation (Gibbs, 1998).

Forensic entomological studies have successfully demonstrated that combinations of CHCs have been used as taxonomic characteristics (Lockey, 1991) for bees (Lavine & Vora, 2005), ants (Martin et al., 2008), termites (Haverty et al., 1997), flesh flies (Moore et al., 2021), cockroaches (Everaerts et al., 1997; Brown et al., 2000), wasps (Bernier et al., 1998), beetles (Baker et al., 1979), mosquitos (Hugo et al., 2006) and grasshoppers (Tregenza et al., 2000) and have more recently been used to identify forensically important blowflies species (Ye et al., 2007; Drijfhout et al., 2010; Moore et al., 2014). Besides enhancing current techniques in forensic entomology, CHC analysis has the additional benefit of ascertaining age to the precise day (Roux et al, 2006, 2008; Moore et al., 2013, 2017; Pechal et al., 2014). Nonetheless, assessing the impact of environmental circumstances on chemical profiles beyond existing reports will provide novel insights.

Numerous chemometric techniques are available in the literature for interpreting chemical data, of which principal component analysis (PCA) is the most popular multivariate tool used in conjunction with CHC analysis (Martin & Drijfhout, 2009; Lim et al., 2019; Levada, 2020; Lee & Jemain, 2021). Its capacity to reveal the hidden characteristics of complex data sets without prior knowledge makes it valuable for data exploration. It is an indispensable tool when analyzing a large number of samples.

Throughout the life cycle of a blowfly, the CHC profiles of the same species may vary depending on a variety of parameters, such as the environment they were exposed to over the duration of their life cycle (Drijfhout et al., 2009; Paula et al., 2017; Otte et al., 2018), and even the number of generations (Toolson & Kuper-Simbron, 1989; Toolson et al., 1990; Tissot et al., 2001; Vaníčková et al., 2015). Aagaard et al. (2024) investigated thermal tolerance plasticity in the social spider *Stegodyphus dumicola (*Pocock, 1898) (Araneae: Eresidae), a species with overall low genetic variation across populations. Their multiomic approach demonstrated that significant adjustments in temperature tolerance were predominantly driven by changes in gene expression and metabolite profiles, rather than alterations in DNA methylation or the microbiome. Previous studies have shown that CHC diversity can be used as a characteristic to determine the evolutionary development and diversification of species. It is clear that CHC analysis is an effective method for identifying and determining the age of species. It is essential to recognize that the CHC profiles of wild-caught and laboratory-reared insects may vary considerably. Consequently, it is essential to authenticate the CHC analysis for each species before to its use in forensic entomology casework.

Despite the advancements in forensic entomology, there is still a need to understand how rearing conditions and generational changes affect CHC profiles to enhance the accuracy of PMI estimates. While previous studies have shown environmental influences on insect cuticle chemistry in other species, the specific effects on *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) remain unclear. To address this knowledge gap, the present study investigates whether rearing environments (indoor vs outdoor) affect CHC profiles in *L. sericata* adult flies, whether laboratory rearing across multiple generations alters hydrocarbon composition, and whether wild-caught specimens exhibit distinct chemical signatures compared to laboratory-maintained populations. The CHC profiles were examined using gas chromatography-mass spectrometry (GC-MS), and principal component analysis (PCA) was used to display the results. This research aims to improve the precision and reliability of CHC analysis in forensic entomology, ultimately contributing to more accurate PMI estimations.

## Materials and Methods

### Insect materials

Adult flies were collected from the wild (Swindon/United Kingdom) in June 2019 and were placed in outdoor rearing cages, labelled as "F0-wild-caught". They were provided with a diet consisting of lamb's blood, sugar, water, and milk powder. As an oviposition substrate, 200 g of fresh lamb liver used. The Petri dish with the eggs and lamb liver was relocated to small rearing boxes situated in an outdoor rearing cage in the field under natural environmental conditions (from June to August, 2019), while another set of eggs was positioned in an indoor rearing cage in the laboratory maintained at a constant temperature of 25±1ºC and a 12/12 hour light/dark cycle (from June 2019 to January 2020) (Figure 1). Both sets of larvae were reared until they became adult flies and fed on lamb liver. Those colonies were labelled as "F1", since they were the first generation of adult flies obtained from the eggs of wild-caught adult flies. The first-generation adult flies reared outdoors were labelled as "F1-outdoors" adult flies, and those reared indoors were labelled as "F1-indoors". While F2 and F3 generations were also maintained under controlled conditions, preliminary analysis revealed no significant differences in CHC profiles compared to F1 generation, suggesting consistency in chemical composition across these early generations. Therefore, F1 and F4 generations were selected for detailed comparative analysis to highlight the most pronounced generational changes that occur with extended laboratory rearing.

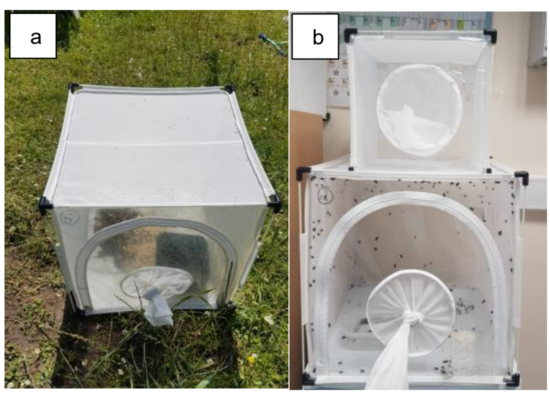


Figure 1. Rearing cages: a) outdoor and b) indoor, respectively.

The fourth-generation adult flies reared indoors was labelled as "F4-indoors". The fourth-generation adult fly colony was obtained in a period of eight months by rearing from one generation to the next. First-and fourth-generation strains were extracted at 10 days post-eclosion. The age of wild-caught specimens was estimated to be approximately 30 days based on their observed reproductive maturity and egg-laying capability, following established protocols in forensic entomology (Amendt et al., 2004). However, this represents a limitation of the study as precise age determination of field-collected specimens remains challenging without controlled rearing from known oviposition dates.

The ambient environmental temperatures were measured by a data logger (Elitech RC-51 waterproof USB temperature and humidity data logger) during the whole developmental stages.

**Sample preparation and GC-MS analysis**

We followed the procedure detailed by Moore et al. (2017) for the liquid extraction of CHCs. Ten adult fly samples were used to form replicate groups for each of the four sets of experiments. Adult fly CHCs were extracted individually, which allowed the GC-MS to identify the chemicals at an appropriate concentration. Each fly was completely submerged in 500 µL of GC-MS grade hexane in a 2 mL GC vial and left undisturbed for 15 minutes. The resulting hexane extract was then transferred to a clean 2 mL vial and allowed to partially evaporate. Next, the extract was transferred into a 300 µL spring-bottom glass insert and left to dry completely. The dried extract was sealed and stored at 4ºC until GC-MS analysis. For analysis, the extract was reconstituted in 30 µL of hexane.

We analyzed the reconstituted samples on an Agilent Technologies 6890N Network GC system equipped with a split/spitless injector (set to 250ºC) and a Restek Rxi-1MS capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature was programmed as follows: held at 50ºC for 2 minutes, increased to 200ºC at 25ºC/min, then to 260ºC at 3ºC/min, and finally to 320ºC at 20ºC/min (held for 2 minutes). The mass selective detector operated in electron ionization mode (70 eV) and scanned from 40 to 500 amu at 1.5 scans per second.

Prior to sample analysis, the GC-MS system was calibrated using a standard n-alkanes (C25:H, C29:H) to establish retention time windows and verify mass spectral patterns. Quality control measures included: (1) hexane blank analysis every 10 samples to monitor contamination, (2) carryover assessment through blank injection following high-concentration samples (no carryover >0.1% detected). Quantitation was performed using relative peak area percentages, with all CHC compounds expressed as percentages of total identified hydrocarbon content within the C20-C32 range.

**Statistical analysis**

We analyzed the chromatogram datasets using the PCA multivariate statistical approach to enable the display of potential patterns; more information on this method are provided by Martin & Drijfhout (2009). In the analysis, only CHC components with percentage peak areas above 0.5% were considered, as trace-level compounds (those below this threshold) often exhibit high variability due to instrumental noise and variations in extraction efficiency. These minor peaks, while potentially informative in some contexts, can introduce disproportionate amounts of scatter into multivariate statistical analyses such as PCA by adding noise relative to the more abundant and stable compounds. Forensic applications require reproducibility and accuracy for PMI estimations and species identification, so focusing on more abundant compounds ensures robust, consistent chemical profiles driven by signals with less background noise and methodological inconsistencies. Six principal components were used since they accounted for 94.4% of the total variance in the dataset, providing adequate representation of the chemical variation patterns. For PCA, chromatographic peak areas were determined using Agilent Chemstation software, concentrating only on CHCs ranging from C20:H to C34:H. PCA results were visualized using both scatter plots and 95% confidence ellipses to display within-group variance and potential overlap regions. Centroid distances were calculated as Euclidean distances between group means in principal component space.

Classification performance was evaluated using Receiver Operating Characteristic (ROC) curves and Area Under the Curve (AUC) values for binary comparisons between groups. Four binary classifications were performed: (1) Wild-caught vs Laboratory-reared (all laboratory generations combined), (2) F1-outdoors vs F1-indoors, (3) F1-indoors vs F4-indoors, and (4) Wild-caught vs F4-indoors.

To minimize overfitting risks associated with small sample sizes, principal component scores PC1-PC3 were used as features for classification using regularized logistic regression (L1 and L2) and linear support vector machines. Model performance was validated using Leave-One-Out cross-validation, which is most appropriate for small sample studies. Multiple algorithms were compared to assess result robustness. AUC interpretation followed established guidelines (McLachlan, 2005; Fawcett, 2006): ≥0.9 outstanding discrimination, 0.8-0.9 excellent discrimination, 0.7-0.8 acceptable discrimination, and <0.7 poor discrimination. Statistical analyses were performed using Python (version 3.9) with scikit-learn library for PCA, logistic regression, and SVM classification. ROC curve analysis and AUC calculations were conducted using the sklearn.metrics module. Chromatographic data processing was performed using Agilent ChemStation software, and compound identification utilized NIST08 mass spectral library.

## Results

A total of 40 individuals were analyzed: 10 F0-wild-caught, 10 F1-outdoors reared, 10 F1-indoors reared and 10 F4-indoors reared adult flies. Table 1 presents the distribution of n-alkanes, alkenes, and branched methyl alkanes, quantified as numbers and percentages among sample sets. It was identified 33 CHC compounds for wild-caught adult flies, 29 compounds for F1-outdoors, 22 compounds for F1-indoors and 29 compounds for F4-indoors. Of the identified compounds, 13 were common across the four sample sets. The profiles mainly included saturated hydrocarbons, including n-alkanes and branched methyl alkanes, with chain lengths spanning from C20 to C32, facilitating differentiation across the sample sets. The wild-caught adult flies had more CHC compounds than the other colonies. In preliminary studies, no significant differences were observed between the CHC profiles of F1 and F2 or F3 generations, suggesting consistency in chemical composition across these early generations under the controlled rearing conditions.

Table 1. Composition of cuticular hydrocarbons in *Lucilia sericata* adult flies

| Hydrocarbon type | Wild-caught | | F1-outdoors | | F1-indoors | | F4-indoors | |
|---|---|---|---|---|---|---|---|---|
| | Number | Concentration (%) | Number | Concentration (%) | Number | Concentration (%) | Number | Concentration (%) |
| n-alkanes | 11 | 39 | 9 | 45 | 10 | 55 | 9 | 23 |
| branched methyl alkanes | 15 | 30 | 13 | 29 | 9 | 27 | 13 | 47 |
| alkenes | 7 | 31 | 7 | 26 | 3 | 18 | 7 | 30 |
| Total | 33 | 100% | 29 | 100% | 22 | 100% | 29 | 100% |

Table 2 presents information on the extracted compounds used for future PCA, including the overall proportion of each component and the % standard deviation for each individual compound. Odd-chain n-alkanes (OLA) were present in higher quantities in the CHC profiles of all generations than even-chain n-alkanes (ELA). Heptacosane (C27:H - compound 19, Table 2) dominated in four generation sample sets. Pentacosane (C25:H - compound 10, Table 2) was the second most prevalent n-alkane across all four sets. Wild-caught adult flies revealed three distinctive compounds, which were 11,13-dimethyltricosane (compound 4, Table 2), 11,13-dimethylpentacosane (compound 11, Table 2), and heptacosene (compound 18, Table 2). For F1-outdoors adult flies, 3-methylhexacosane (compound 15, Table 2) and nonacosene (compound 25, Table 2) were generation-specific. There was no distinctive compound for F1-indoors and F4-indoors adult flies. In addition to the compounds detected in a profile, the compounds not present may also be considered unique to a profile. Thus, 11,13-dimethylhexacosane (compound 14, Table 2), 3-methyloctacosane (compound 24, Table 2), and 11,13-dimethylnonacosane (compound 30, Table 2) were detected in all profiles, except F1-indoors adult flies. These compounds can be considered unique F1-indoors profiles. Furthermore, differences in chemical concentration may also serve as an alternative means of generation distinction. F4-indoors adult flies had a high concentration of 13,15-dimethylheptacosane (compound 20, Table 2) and 11,13-dimethylnonacosane (compound 30, Table 2), which were distinguishable from other generations.

Table 2. Quantitative details of CHC compounds identified in *Lucilia sericata* adult flies

| Peak number | Retention time | Peak ID | F0-wild | F1-outdoors | F1-indoors | F4-indoors |
|---|---|---|---|---|---|---|
| 1 | 13.047 | C20:H | 0.54±0.22 | 0.60±0.26 | tr | tr |
| 2 | 17.384 | Tricosene* | 1.57±1.46 | tr | tr | tr |
| 3 | 18.160 | C23:H | 9.49±7.26 | 5.98±2.13 | 8.48±3.12 | 1.62±1.02 |
| 4 | 18.719 | 11+13-MeC23 | 4.09±3.27 | tr | tr | tr |
| 5 | 19.334 | 3-MeC23 | 0.53±0.26 | tr | tr | tr |
| 6 | 19.909 | C24:H | 1.50±0.92 | tr | 0.51±0.10 | tr |
| 7 | 20.601 | 3-MeC24 | 1.12±0.78 | tr | tr | tr |
| 8 | 21.430 | Pentacosene* | 9.18±6.38 | 0.71±0.42 | tr | tr |
| 9 | 21.509 | Pentacosene* | 3.81±5.45 | tr | tr | tr |
| 10 | 22.185 | C25:H | 9.48±5.41 | 9.23±3.43 | 11.16±3.61 | 3.93±1.89 |
| 11 | 22.932 | 11+13-MeC25 | 10.04±4.70 | 2.78±0.95 | 2.49±0.61 | 4.22±2.35 |
| 12 | 23.501 | 3-MeC25 | 1.01±1.39 | tr | tr | tr |
| 13 | 24.102 | C26:H | 1.51±0.68 | 0.97±0.30 | 1.10±0.29 | 0.82±0.28 |
| 14 | 24.768 | 11+13-MeC26 | 0.64±0.29 | 0.57±0.17 | tr | 0.69±0.31 |
| 15 | 25.312 | 3-MeC26 | tr | 2.61±3.38 | tr | tr |
| 16 | 25.507 | Heptacosene* | tr | 4.97±3.59 | tr | 4.51±3.36 |
| 17 | 25.631 | Heptacosene* | tr | 3.15±1.55 | 6.36±1.64 | 3.08±2.36 |
| 18 | 25.817 | Heptacosene* | 12.14±8.58 | tr | tr | 5.32±3.65 |
| 19 | 26.571 | C27:H | 12.01±6.49 | 17.82±6.06 | 21.06±4.91 | 9.05±3.33 |
| 20 | 27.134 | 13+15 MeC27 | 5.01±5.27 | 3.10±1.26 | 2.53±0.58 | 8.72±3.22 |
| 21 | 27.823 | 4-MeC27 | 0.70±0.55 | tr | tr | 2.56±0.83 |
| 22 | 27.945 | 3-MeC27 | tr | 1.00±0.46 | 0.85±0.32 | tr |
| 23 | 28.320 | C28:H | 0.85±0.41 | 0.91±0.29 | 0.87±0.16 | 0.85±0.35 |
| 24 | 29.234 | 3-MeC28 | 0.74±0.50 | 4.49±1.34 | tr | 1.36±0.45 |
| 25 | 29.325 | Nonacosene* | tr | 12.39±12.02 | tr | tr |
| 26 | 29.374 | Nonacosene* | 3.00±3.00 | 2.62±2.28 | 6.71±1.49 | 9.20±5.00 |
| 27 | 29.424 | Nonacosene* | tr | 0.83±0.51 | tr | 5.33±1.80 |
| 28 | 29.682 | C29:H | 2.21±1.17 | 7.02±2.27 | 8.35±1.15 | 2.85±1.44 |
| 29 | 29.859 | 13+15 MeC29 | tr | 5.14±1.93 | 6.92±0.65 | 3.23±0.84 |
| 30 | 29.977 | 11+13 MeC29 | 1.58±1.17 | 0.81±0.67 | tr | 9.67±3.19 |
| 31 | 30.256 | 4-MeC29 | tr | 0.50±0.22 | tr | 2.05±1.36 |
| 32 | 30.323 | 3-MeC29 | 0.66±0.34 | 0.52±0.18 | 1.58±0.72 | 1.60±1.43 |
| 33 | 30.510 | C30:H | 0.52±0.10 | 0.52±0.11 | 1.15±0.42 | 0.53±0.07 |
| 34 | 30.741 | 4-MeC30 | tr | tr | 0.67±0.34 | 1.26±0.48 |
| 35 | 30.983 | 3-MeC30 | 0.71±0.35 | 3.37±1.47 | 4.28±1.41 | 6.16±3.00 |
| 36 | 31.059 | Hentriacontene* | 0.76±0.50 | tr | 4.64±0.60 | 1.91±0.96 |
| 37 | 31.210 | C31:H | 0.52±0.20 | 1.93±0.45 | 2.27±0.33 | 1.72±0.81 |
| 38 | 31.427 | 13+15 MeC31 | 1.15±0.57 | 2.04±0.54 | 3.79±0.86 | 2.41±0.99 |
| 39 | 31.495 | 11+13 MeC31 | 0.68±0.87 | 1.28±0.34 | 3.14±0.47 | tr |
| 40 | 31.650 | 3-MeC31 | 0.86±0.44 | tr | tr | 1.08±0.39 |
| 41 | 31.714 | C32:H | 0.84±0.57 | tr | 0.51±0.18 | 0.83±0.37 |
| 42 | 32.403 | Tritriacontene* | 0.56±0.32 | 1.59±0.38 | tr | 0.72±0.34 |

tr: Detected in trace amounts (percentage peak area <0.5%);

* Double bond positions were not determined.

Figure 2 displays the GC chromatograms of F0-wild-caught, F1-outdoors, F1-indoors, and F4-indoors adult flies. The F0-wild-caught adult flies exhibited substantial quantities of CHC compounds with chain lengths ranging from C23:H to C27:H, and these compounds had elevated overall concentrations. Conversely, in some profiles, the concentrations of C23:H to C27:H compounds were decreased, and the concentrations of chain lengths from C29:H to C31:H were elevated. While the CHC profiles of F1-outdoors and F1-indoors adult flies were comparable, the F1-indoors adult flies exhibited a reduced overall number of compounds. Nonetheless, their concentrations were shown to be much higher than those seen in F1-outdoors.

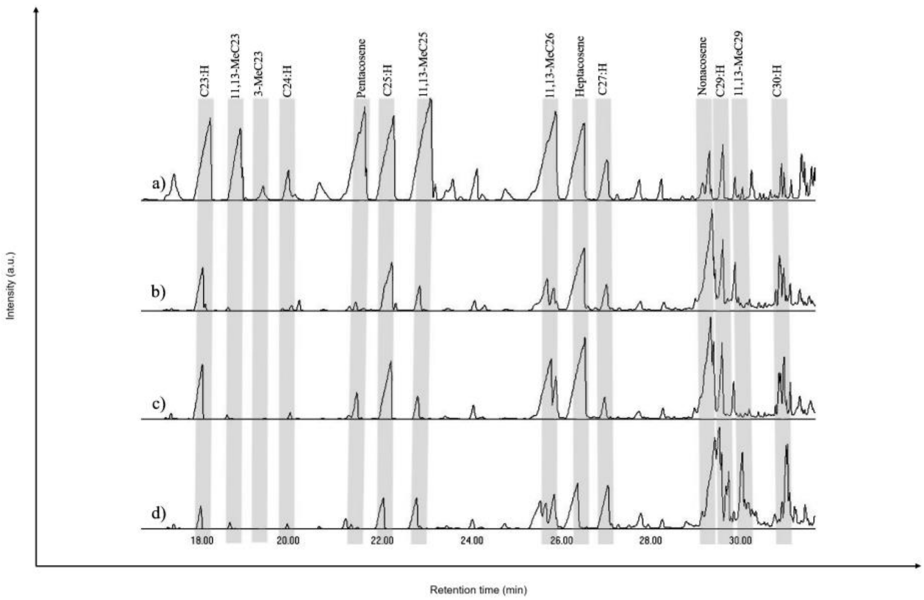


Figure 2. GC chromatograms of adult *Lucilia sericata* from different rearing conditions. Four representative chromatograms are displayed corresponding to adult flies originating from) a) F0-wild-caught, b) F1-outdoors, c) F1-indoors, d) F4-indoors.

Figure 3 illustrates the temperature data on the outdoor circumstances experienced by F1-outdoors in the UK. The temperature range experienced throughout the procedure ranged from a low of 7.1ºC to a high of 41.3ºC, with an average temperature of 20.7ºC.

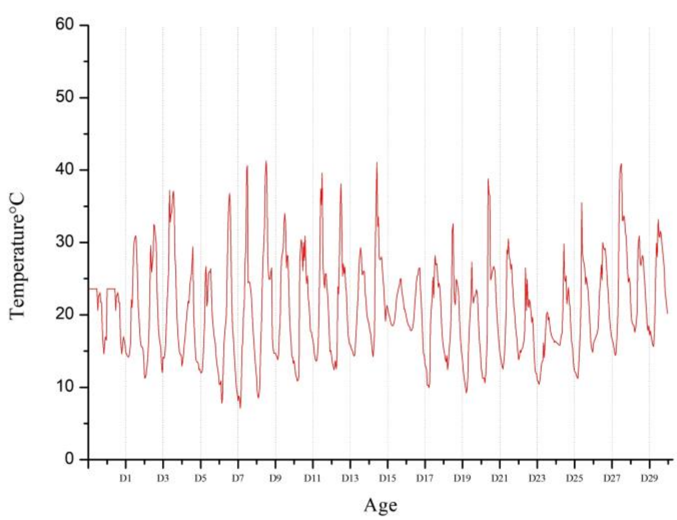


Figure 3. Temperature profile recorded for F1-outdoors flies during development.

Upon comparing the total concentrations of CHCs across the experimental groups (Figure 4), it was observed that the concentrations of n-alkanes were significantly higher than those of branched methyl alkanes in the profiles of F0-wild-caught, F1-outdoors, and F1-indoors adult flies, with the latter exhibiting the highest total concentration of n-alkanes. In F4-indoors adult flies, there was a transition from a predominance of n-alkanes to a profile dominated by branched methyl.

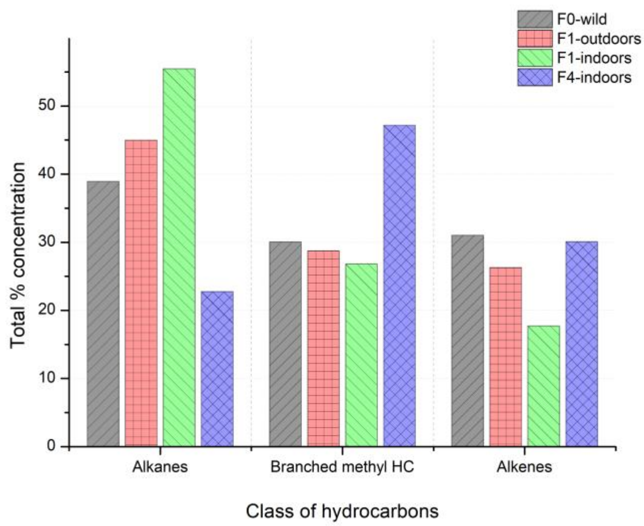


Figure 4. Overall percentage concentrations of CHC classes in adult *Lucilia sericata* flies.

A discriminant analysis was used in the following investigation to visualize any similarities among the samples and to identify clusters, if any existed. PCA was used to discern the chemicals for the separation and grouping of sample sets. The chemicals were then used to generate the 3D plot. The PCA was conducted including n-alkanes, alkenes, and methyl-branched alkanes. Table 2 delineates the chemicals used for PCA, including the overall proportion of each component and the percentage standard deviation for each sample.

To validate the approach, it was examined the cumulative variance explained by the principal components; the analysis showed that the first six components accounted for 94.4% of the total variance, with the first four components contributing 70.4%, 11.2%, 8.2%, and 4.6%, respectively. The PCA plots (Figure 5) display group clustering with objective measures of separation quality. Centroid distances between groups ranged from 2.8 PC units (F1-indoors vs F4-indoors) to 5.2 PC units (Wild-caught vs F4-indoors), providing quantitative assessment beyond visual inspection. The 95% confidence ellipses reveal within-group variance while confirming clear separation between all groups, with no apparent overlap between confidence regions. This suggests strong discrimination power, though the small sample size (n=10 per group) requires cautious interpretation of multivariate results. Centroid distances between groups in PC space ranged from 2.8 to 5.2 units, with wild-caught vs laboratory-reared specimens showing the greatest separation.

Compounds with large loadings are influential for separation. The main compound which has substantial PCA score values is tricosane (C23:H). Other compounds exhibiting a high score are 11+13-methylnonacosane, heptacosene and nonacosene in the PCA dataset.

ROC curve analysis demonstrated outstanding discrimination capabilities between experimental groups, with AUC values ranging from 0.92 to 1.00 across different models and comparisons (Table 3, Figure 6). Wild-caught versus laboratory-reared specimens achieved perfect classification across all three algorithms (AUC=1.000), confirming that CHC profiles provide reliable chemical markers for distinguishing natural versus controlled rearing environments.

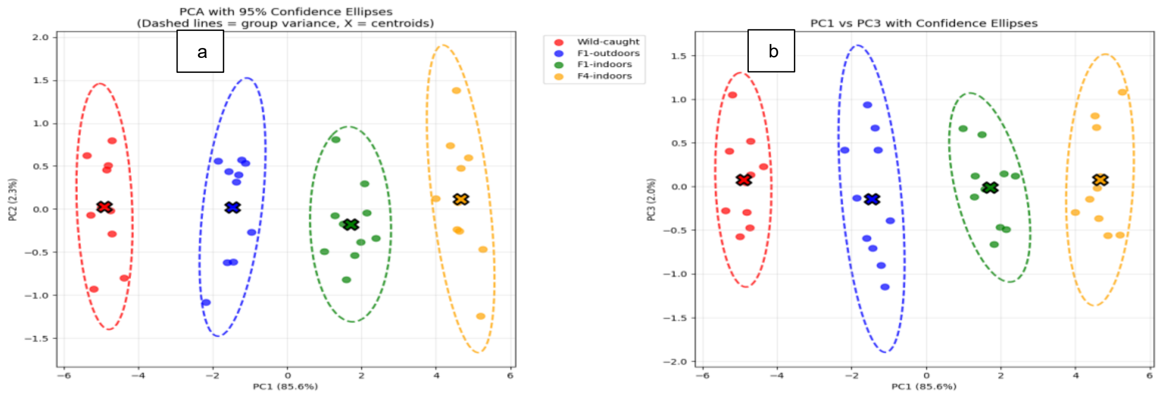


Figure 5. PCA analysis of CHC profiles with quantified group separation: a) PC1 vs PC2 and b) PC1 vs PC3 scatter plots with 95% confidence ellipses (dashed lines) showing within-group variance and potential overlap regions. X markers indicate group centroids with inter-centroid distances ranging from 2.8 to 5.2 PC units.

Table 3. Classification performance metrics for binary comparisons of CHC profiles using multiple algorithms]

| Comparison | L1 Logistic AUC | L2 Logistic AUC | Linear SVM AUC | Best Accuracy |
|---|---|---|---|---|
| Wild-caught vs Lab-reared | 1.000 | 1.000 | 1.000 | 1.000 |
| F1-outdoors vs F1-indoors | 1.000 | 1.000 | 1.000 | 1.000 |
| F1-indoors vs F4-indoors | 1.000 | 0.960 | 0.920 | 0.900 |
| Wild-caught vs F4-indoors | 1.000 | 1.000 | 1.000 | 1.000 |

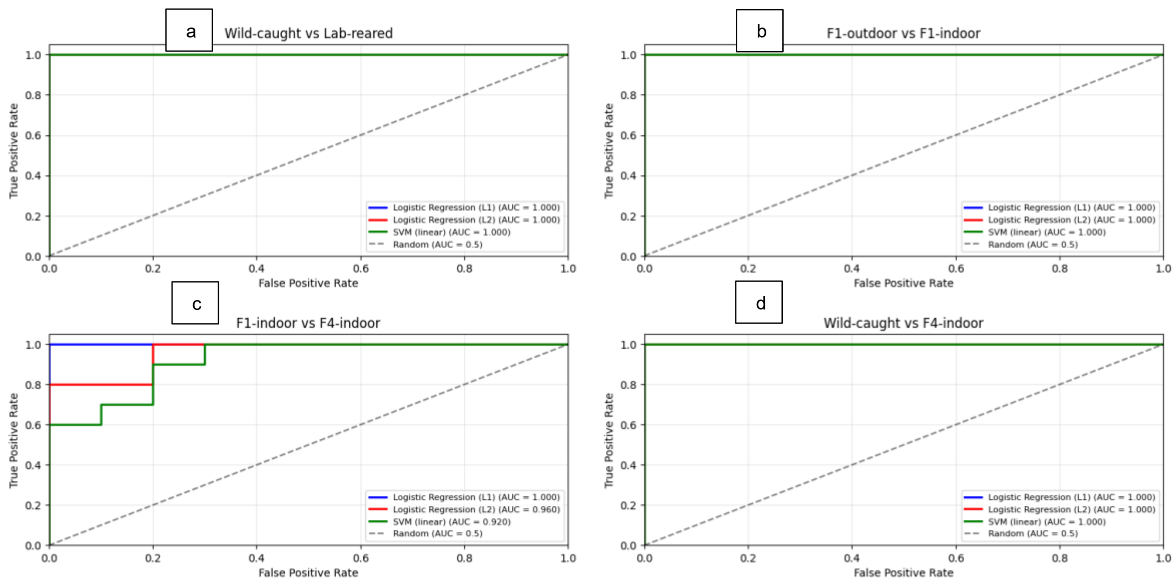


Figure 6. ROC curves for binary classification of CHC profiles showing: a) Wild-caught vs Laboratory-reared, b) F1-outdoors vs F1-indoors, c) F1-indoors vs F4-indoors, and d) Wild-caught vs F4-indoors specimens. AUC values demonstrate outstanding discrimination capabilities (≥0.92) across all comparisons.

Classification between F1-outdoors and F1-indoors groups also showed perfect discrimination (AUC=1.000), demonstrating that temperature variation (7-41ºC outdoors vs. constant 25ºC indoors) creates distinct and measurable differences in cuticular hydrocarbon composition within the same generation. The comparison between F1-indoors and F4-indoors generations revealed model-dependent performance (AUC range: 0.92-1.00), with L2 regularized logistic regression (AUC=0.960) and linear SVM (AUC=0.920) showing more conservative estimates than L1 regression (AUC=1.000). This variation suggests that generational changes, while significant, represent more subtle chemical modifications compared to environmental effects.

Perfect classification was consistently observed between wild-caught and F4-indoors specimens across all models (AUC=1.000), representing the maximum chemical divergence between natural conditions and extended laboratory rearing. The high discriminatory power observed across comparisons reflects the chemical specificity of cuticular hydrocarbons to environmental and generational factors, consistent with their known biological functions in desiccation resistance and chemical communication.

## Discussion

This research examined the impact of rearing circumstances and generational diversity on the chemical profiles of adult of *L. sericata* flies. Generally, it is difficult to determine whether adult flies originate in the wild, or develop within a closed environment (Hall, 2021). In the example of a criminal case, a dead body was discovered in a room with closed windows, and numerous dead *Lucilia* adults were around (Krikken & Huijbregts, 2001). It is uncommon to discover a significant number of dead adult flies in a closed space, as their access to the body is restricted unless they were already inside. The determination of this factor has significant importance in elucidating a forensic case, as it has implications for the assessment of evidence, revealing the location of the crime scene and the computation of the PMI. Developing indoors, where the environment is different from those in the wild, can lead to alterations in physiological and biochemical features; consequently, the prediction of PMImin would naturally be affected if a corpse was relocated from an indoor to a wild environment, or vice-versa (Charabidze et al., 2017).

CHCs are not only crucial for water retention and protection but also serve as reliable markers for species identification and age estimation in necrophagous insects. Their work synthesizes data from the past decade, outlining how intra-species variations driven by factors such as age, sex, geographical origin, and environmental conditions can be harnessed to refine PMI estimations (Steward-Yates et al. 2025). The cuticle must be sufficiently elevated to provide effective waterproofing at ambient environmental temperatures. The boiling point rises in direct proportion to the length of the carbon chain, making longer-chain hydrocarbons more effective in minimizing water loss (Gibbs, 1998; Chown et al., 2011). To disseminate across the cuticle and be exchanged among conspecifics, these chemicals must possess sufficient mobility.

The cuticle also functions in signaling during inter- and intraspecific interactions (Howard & Blomquist, 2005; Butterworth et al., 2018). While branched and unsaturated CHCs are mostly associated with pheromonal communication, n-alkanes are generally used for waterproofing purposes (Toolson & Kuper-Simbron, 1989; Menzel et al., 2017). Recent studies have broadened understanding of these compounds. For instance, Bell et al. (2024) investigated the CHC profiles of the Rhinoceros beetle, *Trypoxylus dichotomus* (L., 1771) (Coleoptera: Scarabaeidae) and demonstrated that chemical signatures vary significantly by sex and correlate with body size. Their GC-MS analyses coupled with multivariate statistics revealed that male beetles exhibit distinct profilesc (particularly elevated levels of heptacosenes) that are positively associated with indicators of body size such as pronotum width and horn length. This work emphasizes that, beyond their role in waterproofing, CHCs serve as reliable indicators of an individual's morphological state and may mediate critical social interactions.

The analysis of n-alkanes, branched alkanes, and alkenes distributions reveals that the predominance of n-alkanes over other CHCs in the F0-wild-caught and F1-indoors samples is attributable to adaptation to fluctuating temperatures. Upon examining Table 2, it can be inferred that the presence of short-chain CHCs in these two sample groups serves as a protection against temperature decreases. In the indoor samples, it can be seen that as adaptation to constant temperature increases, n-alkanes decrease, and other compounds increase. For instance, changes in temperature, humidity, or diet during rearing might have triggered the biosynthesis of heptacosene in F0 and F4 but not in F1. The reappearance of heptacosene in the F4 generation suggests that the compound may play a role in long-term adaptation to laboratory conditions or might be a response to subtle environmental changes experienced by the later generations. The standard deviations associated with individual compound peak areas provide insight into the variability of CHC synthesis under different rearing conditions. Lower variability in laboratory-reared samples compared to field-collected specimens likely reflects a stabilized physiological state under controlled conditions—an observation that may affect the reliability of PMI assessments in forensic investigations. Moreover, the discrepancies in the relative percentages of n-alkanes versus branched methyl alkanes across generations suggest adaptive modulation of CHC biosynthesis in response to environmental conditions, which can serve as an indirect indicator of developmental stage and exposure history. The findings underscore that even subtle shifts in CHC profiles can be effectively harnessed to infer insect colonization dynamics and to enhance the accuracy of forensic time-of-death estimations.

The observed CHC profile changes have direct implications for forensic investigations. When investigators encounter multiple generations of flies at a crime scene, the distinct chemical signatures identified in this study can help determine: (1) Generation identification—The shift from n-alkanes dominance in F1 to branched methyl alkanes predominance in F4 provides a chemical marker for distinguishing generations. (2) PMI refinement—Knowledge of generational differences allows for more accurate PMI calculations, particularly in cases where bodies have been exposed for extended periods. (3) Scene reconstruction—The ability to distinguish between wild-caught and laboratory-maintained profiles can indicate whether flies developed naturally at the scene or were introduced from elsewhere. These chemical markers complement traditional morphological methods and provide additional evidence for forensic timeline reconstruction, particularly in complex cases involving body relocation or extended exposure periods.

The findings of Sharif et al. (2024) offer compelling evidence that environmental conditions critically influence the chemical stability of CHCs, thereby affecting the accuracy of forensic PMI estimations. In their work, significant decreases in the concentrations of n-C27 and n-C29 were observed in both buried and above-ground settings. In contrast, CHC concentrations remained relatively unchanged in indoor conditions, where exposure to these environmental stressors is minimized. This integration of advanced machine learning with traditional chemical analysis not only enhances the precision of age estimation protocols but also reinforces the importance of considering environmental variables when interpreting forensic entomological evidence. Such variations have been observed in CHCs where prolonged laboratory rearing led to shifts in the chemical profiles of insects over generations, possibly due to genetic drift or selective pressures unique to the laboratory environment. These findings suggest that CHCs are not fixed traits but rather dynamic signals that reflect both intrinsic factors and environmental influences. In this particular scenario, further research is needed as this study is a proof of concept.

Developing and maintaining a laboratory colony has many benefits, including the ability to control the life stage, age, number, and quality of insects used and the ability to manipulate some environmental conditions while holding other conditions constant. Nevertheless, it is important to exercise care when assessing the findings since the controlled environment of the laboratory may not accurately reflect circumstances in the natural habitat. Although the sample size (n=10 per group) was adequate for detecting major differences in CHC profiles using GC-MS analysis and was consistent with previous forensic entomology studies using similar methodologies, future studies with larger sample sizes would enhance the robustness of multivariate statistical analyses and improve the generalizability of findings.

Comparing the current findings to previous research, similar generational changes in hydrocarbon profiles have been documented in studies involving other insect species, such as *Drosophila pseudoobscura* (Frolova, 1929) (Diptera: Drosophilidae) (Toolson & Kuper-Simbron, 1989), *Drosophila mojavensis* (Patterson & Crow, 1940) (Diptera: Drosophilidae) (Toolson et al., 1990), *Phlebotomus argentipes* (Annandale & Brunetti, 1908) (Diptera: Psychodidae) (Kamhawi et al., 1992), *Pogonomyrmex barbatus (*Smith, 1858) (Hymenoptera: Formicidae) (Tissot et al., 2001), *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) (Paula et al., 2018) and *Drosophila melanogaster* (Meigen, 1830) (Diptera: Drosophilidae) (Cortot et al., 2022), where specific compounds disappeared and then reappeared across generations due to environmental factors or genetic adaptations. These findings underscore the complexity of CHC profiles and their sensitivity to both environmental and generational influences. It is possible to make a clear distinction between wild-caught and laboratory-reared generations using discriminant function analysis since the CHC profiles show a progressive change over time. In this study, if the colonies had been reared outdoors, generational differences could have been more clearly observable; however, since this study was preliminary, it was preferred to rear the colonies in a controlled environment at a constant temperature.

This research found that rearing environments, whether indoor or outdoor, can affect the CHC profiles of adult flies. Initial findings suggest that adult flies can be distinguished by their chemical profiles between wild-caught specimens (unspecified generation) and first-generation strains (reared in outdoor or indoor environments) (León-Morán et al., 2024). This allows for a more precise PMImin estimation in a scenario suspected of more than one generation of insect succession. The profiles of F1-outdoors exhibited distinctions when compared to those F1-indoors attributable to the rearing environment. The chromatograms indicated that temperature influenced the CHC profiles of F1-outdoors and F1-indoors adult flies. A greater percentage concentration of n-alkanes was noted in F1-indoors. This difference might be due to the temperature fluctuations to which adult flies reared outdoors and indoors were exposed. It should be emphasized that CHC profiles change with age (Moore et al., 2017). Since this study is preliminary, the evaluation was limited to the 10-day age range of the reared adult flies. The age of the wild-caught colony assumed to be 30 days' old, since wild-caught adult flies are known to reach egg-oviposition adulthood. Thus, more research is needed to eliminate the uncertainties.

The research analyzed the impact of generational differences on the CHC profiles by comparing the CHC profiles of first- and fourth-generation strains raised indoors. The amounts of n-alkanes in the F4-indoors adult flies were significantly reduced. They exhibited a greater percentage concentration of branched methyl alkanes and a reduced concentration of n-alkanes relative to wild-caught and first-generation strains. The elevated content of n-alkanes was replaced by branched methyl alkanes and alkenes after four generations of cultivation. Comparable outcomes were also achieved in prior research with fruit flies (Toolson & Kuper-Simbron, 1989; Toolson et al., 1990) and ants (Tissot et al., 2001) and cricket species (Ozdemir et al., 2024).

Differentiation in the phylogenetics of F0-wild-caught adult flies has been brought about due to environmental factors such as ecology, diet, evolution, and physiological behaviors in response to abiotic pressures (Alotaibi et al., 2021). This could potentially cause variations in the epicuticular layer. It is possible that this is because the laboratory environment has a low desiccation stress level. This indicates that prolonged upbringing in laboratory settings may result in alterations to the CHC profiles of *L. sericata* adult flies over many generations. These results confirm the hypothesis that insects prevent water loss in warmer conditions by increasing the quantity of straight-chain CHCs in the epicuticular layer (Toolson et al., 1990). It is common practice for adult flies to have alkenes and branched methyl alkane concentrations as high as the alkane concentration, since these compounds are known to be involved in intercolonial communication (Howard & Blomquist, 2005).

In this study, PCA was employed as a chemometric method to reduce the dimensionality of the complex CHC dataset and to reveal underlying patterns of variation among samples. Subtle differences in numerous compounds measured under varying rearing conditions and across generations necessitated the use of an unsupervised method that could extract the most significant variance without a priori group classifications. It was determined that the selected principal components accounted for a high percentage of the total variance, thereby providing a robust and simplified representation of the chemical profiles for subsequent clustering and group comparisons. Both score and loading plots (as described by Lee & Jemain, 2021) were generated to visualize the spatial distribution of the samples and to identify compounds that exerted the most pronounced influence on differentiation. Clustering was performed on the basis of inherent chemical similarities, independent of sample origin, and further comparisons were subsequently restricted to the key substances identified through this process. The application of PCA in this manner demonstrated its effectiveness as an analytical tool for deciphering complex datasets in forensic investigations involving multiple samples. PCA analysis with confidence ellipses provides objective visualization of *L. sericata* group separation while acknowledging inherent variance limitations. The quantified centroid distances (2.8-5.2 PC units) confirm statistically significant separation, though confidence regions indicate that discrimination precision varies between comparison types, with environmental effects (indoor vs outdoor) showing stronger separation than generational effects alone.

The outstanding classification performance (AUC≥0.92 across all comparisons) reflects the profound influence of environmental and generational factors on cuticular hydrocarbon biosynthesis. These results are biologically plausible given the known responsiveness of CHC composition to temperature, humidity, and developmental conditions (Toolson & Kuper-Simbron, 1989; Gibbs, 1998).

The perfect discrimination between wild-caught and laboratory-reared specimens (AUC=1.000) likely reflects the cumulative effects of multiple environmental differences including temperature fluctuation (7-41ºC vs. constant 25ºC), diet composition, humidity variation, and microbial exposure. Such dramatic environmental contrasts are expected to produce correspondingly distinct chemical signatures, particularly given that CHCs serve as the primary waterproofing barrier and are directly influenced by thermal stress (Chown et al., 2011).

The model-dependent variation observed in F1-indoors vs F4-indoors comparisons (AUC=0.92-1.00) provides important methodological insights. The fact that more regularized models (L2 logistic regression, SVM) showed slightly lower AUC values (0.92-0.96) compared to L1 regression (1.00) suggests that generational differences, while statistically significant, represent subtler chemical modifications that may be near the detection limits of the current analytical approach. This gradient of classification difficulty aligns with the expected biological hierarchy: environmental effects > generational effects within the same environment.

These findings have significant implications for forensic entomology applications. The ability to perfectly distinguish wild-caught from laboratory-reared specimens addresses a critical question in criminal investigations: whether insects developed naturally at a crime scene or were potentially introduced from laboratory sources. The consistent discrimination between outdoor and indoor rearing conditions (AUC=1.000) suggests that CHC analysis could help determine whether a body was initially located indoors or outdoors, information crucial for timeline reconstruction.

The generational discrimination capability (AUC≥0.92) provides a novel approach for estimating colonization duration in cases involving extended exposure periods. In scenarios where multiple generations may have colonized a body, CHC profiling could potentially distinguish between early and later generations, refining PMI estimates beyond traditional morphological methods.

Understanding these factors that impact CHC profiles can contribute to more accurate PMI estimations, especially in cases where samples are collected from crime scenes that likely involve multiple generations of species. This study offers valuable insights into validating the CHC method and underscores the importance of considering environmental factors and generational variations. While the current study's observational window was limited to the first 10 days post-eclosion and relied on estimated ages for wild-caught specimens, the findings enhance the previous literature by elucidating the impact of rearing environment and generational diversity on adult fly CHC profiles. While PCA visualization suggests clear group clustering, several methodological limitations warrant consideration. The confidence ellipse analysis reveals potential overlap regions between groups, particularly among laboratory-reared generations, indicating that visual separation may overestimate discrimination power with small sample sizes. The controlled experimental conditions may have maximized chemical differences between groups, and the sample size (n=10 per group) approaches the lower limit for robust multivariate statistics with 42 CHC variables. Real-world applications will likely encounter additional sources of variation including seasonal effects, geographical differences, and intermediate environmental conditions not represented in this binary classification framework.

The preliminary sample size (n=10 per group), while sufficient for demonstrating significant differences and consistent with established forensic entomology protocols, may have contributed to the exceptional classification performance. The use of Leave-One-Out cross-validation helps mitigate overfitting concerns, but independent validation using larger, geographically diverse datasets will be essential for establishing operational thresholds.

All laboratory-reared specimens received a standardized diet (lamb's blood, sugar, water, and milk powder), while field-collected specimens consumed diverse natural food sources. Since dietary composition can influence CHC profiles independently of environmental factors (Howard & Blomquist, 2005; Menzel et al., 2017), the observed differences between wild-caught and laboratory specimens may partially reflect dietary effects. Future studies should employ controlled feeding experiments to separate dietary from environmental influences on CHC composition.

Future studies should extend the observational period beyond the initial 10-day window used in this study to capture later-stage changes in cuticular CHC profiles. Longer-term studies would provide insights into how CHC composition evolves throughout the entire adult lifespan, thereby allowing for a more comprehensive understanding of its temporal dynamics. Outdoor rearing experiments conducted under naturally fluctuating environmental conditions (such as variable temperatures, humidity, and biotic interactions) could further validate the current findings by testing whether the patterns observed under controlled laboratory conditions persist in more complex, real-world scenarios. Additionally, replicating this study with additional forensic species and under a broader range of rearing conditions would help to ascertain the generality of the observed trends. Such expanded investigations would not only refine the applicability of CHC analysis for PMI estimations but also enhance the robustness and universality of the conclusions drawn from this work.

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