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TECHNICAL NOTE

Pathology/Biology

Morphometric analysis for determination of larval instars in *Dermestes frischii* Kugelann and *Dermestes undulatus* Brahm (Coleoptera: Dermestidae)

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Abstract

Dermestes frischii Kugelann, 1792 and *Dermestes undulatus* Brahm, 1790 are the most abundant species worldwide at outdoor or indoor crime scenes during the dry and skeletal stages of decomposition. The attribution of larval age in these beetles is problematic due to the variable number of instars, which is influenced by environmental factors. In this study, a morphometric approach was used to look for potential morphological features as evidence of larval stages. Breeding and monitoring were performed for both species in an incubator with a preset temperature of 28°C ± 0.5 without a photoperiod. Morphometric measurements were made on 10 larvae per instar for each species using length, width, and thickness parameters. Linear discriminant analysis was then used to generate decision boundaries that clearly separated larval stages. The cross-validation procedure demonstrated that the morphometric approach successfully discriminated adjacent larval stages in both species with high values of sensitivity and specificity. This less-invasive approach could improve the ability to estimate minPMI in forensic studies of Dermestidae beetles. Future studies may extend this approach to other species and establish good practices for collecting and storing specimens for morphometric analysis.

KEYWORDS
Dermestes frischii, *Dermestes undulatus*, Dermestidae, Dermestidae larvae, instar aging, morphology, morphometric analysis, post-mortem interval (PMI)

Highlights

- Morphometric analysis, a non-invasive examination method, preserves the structures of larvae.
- Morphometric analysis provides a wealth of data and insights in a relatively short timeframe.
- *Dermestes frischii* and *Dermestes undulatus* are excellent indicators of minPMI in advanced decomposition stages.
- Morphometric analysis on these species can discriminate larval stages with great accuracy.

1 | INTRODUCTION

Determining larval instar in the holometabolous Hexapoda is important when it is necessary to distinguish developmental time differences and duration of the juvenile stage.

It is well known that only a small percentage of the holometabolous Hexapoda species have morphological differences that allow entomologists to determine the larval instar rapidly. In contrast, for the other species much longer and more sophisticated procedures are needed, such as analysis of hormones and related genes, as well as histological analysis [1]. However, such methods can be costly, and in addition, they only provide information on the specific sample tested and not on the entire population, unless the whole population is examined, which results, for many methods, in the loss of all samples.

Morphometric analysis proves crucial in certain instances for determining larval stages in insects where morphological differences among various larval instars are not readily apparent (e.g., Dermestidae, Chironomidae, and Tenebrionidae). This analytical approach involves quantifying and comparing the measurable characteristics of larvae, providing valuable insights into developmental stages that might otherwise go unnoticed [2]. In cases where traditional morphological distinctions fall short, morphometric techniques emerge as indispensable tools, unraveling the intricate progression of insect larvae through their various developmental phases [3, 4].

The Dermestidae family includes more than 1000 species divided into 50 genera. The study conducted by Charabidze et al. [5] shows that *Dermestes frischii* and *Dermestes undulatus* are the most frequently found species on the crime scene, particularly in indoor cases in which their association is the most common [6].

These beetles are associated with the dry and skeletal stages of decomposition because they feed on dry organic substances on which they lead their life cycle in their entirety [7]. Notably, only certain species within this family, all belonging to the *Dermestes* genus, prove valuable for forensic applications, serving as a tool for evaluating the post-mortem interval (PMI) by determining the minimum amount of time the body has been exposed. This minimum exposure period is thus referred to as minimum PMI (minPMI).

For flies of forensic importance, the presence of morphological markers and the constancy of the number of larval instars make less difficult the larval age determination, for Dermestid beetles the attribution of the larval age appears to be problematic since no morphological markers are known to allow the determination of larval instars. This variability in the number of larval stages and their duration, even within the same species, is influenced by numerous environmental variables [8, 9].

The scientific literature on Desmestidae is lacking in methods for determining the larval stage to provide a more accurate minPMI. The existing studies [10–13] focus solely on the presence of Dermestidae evidence but do not consider their developmental stage. Previous research [14–17] has primarily concentrated on exploring the life cycle duration of these beetles at various constant temperatures, occasionally assessing the number of larval stages. An enormous variability emerges from these studies, which makes it necessary to explore further whether these beetles can be used, like dipterans, to estimate minPMI, especially in cases of highly decomposed remains.

As Fratczak and Matuszewski [18] did on the Silphidae larvae, in this study a morphometric approach was adopted with the aim of identifying morphological features unaffected by environmental factors which would be useful for detecting the larval instars.

2 | MATERIALS AND METHODS

2.1 | Breeding and monitoring

To avoid the beetles' diapause, the experimental period runs between spring and summer.

The adult specimens of *D. frischii* and *D. undulatus*, which formed the main colonies for this study, were collected manually from pig carcasses, placed in a wooded area of Lombardia (northwest Italy, Europe) used for experimental purposes. The insects were bred in a transparent case on the bottom of which was placed on a sheet of colored paper. This provided the dermestid beetles with a place to hide as they demonstrated negative phototaxis and chromatic contrast to enhance the visibility of any eggs laid. The adults were fed with dried pork and water ad libitum that was supplied by plastic disks with cotton wool inside soaked daily. To avoid their hatch, the eggs were collected every day with a thin brush and placed in smaller PVC containers with the colored paper, pork, and water ad libitum; to allow the passage of air between the ambient and the container these containers were closed with netting and a perforated cap. Each container contained about 7 to 30 eggs, depending on the number of eggs laid daily.

The colony of adults and the containers with eggs were placed inside an incubator (F.lli Galli, model GTEST-CL070SD), at a predefined temperature of 28°C ± 0.5; relative air humidity in the incubator was maintained at 75% and without photoperiod. Every day, at the same time, the containers with the eggs were inspected to establish the number of hatched ages together with the possible presence of exuviae as an index for the ecdysis.

Subsequently, 50 containers were set up for each species.

For the morphometric study, 10 larvae were fixed in 75% EtOH, for each instar. Morphometric measurements were performed using the Leica S9i stereomicroscope with LasX software.

The evaluated parameters per each larval instar were the length, the width, and the thickness, measured as follows: the dorsal length from the cephalic apex to the end of the last abdominal segment, the ventral width, and lateral thickness of the second thoracic tergite.

Mean and standard deviation were calculated for each parameter and for every larval instar.

The samples are stored at the Forensic Entomology Laboratory of the University of Pavia.

2.2 | Statistical analyses

In both species, length, width, and thickness values were normally distributed in each larval instar, as assessed through one-sample Kolmogorov–Smirnov tests for normality (length: $D > 0.198$, $p > 0.41$; width: $D > 0.184$, $p > 0.51$; thickness: $D > 0.168$, $p > 0.63$).

To evaluate whether and how these three measures vary among larval instar within each species, we used a linear discriminant analysis (LDA) [19], a powerful statistical method that can be used to project high-dimensional datasets onto a lower-dimensional space while preserving the class separation between the data points. However, it is important to note that LDA is sensitive to outliers, which can affect the accuracy of the classification. This dimensionality reduction is essential to analyze morphometric data that could contain many irrelevant features, potentially leading to overfitting and inaccurate classification. LDA extracts the most discriminating features among the three morphometric measures, allowing us to focus on the most relevant characteristics for separating the larval instar classes.

After identifying the most informative features, we calculated decision boundaries [20] for each pair of morphometric measures. Decision boundaries are mathematical constructs that represent the dividing line between two or more classes of data points. In the case of larval instar classification, these boundaries separate the larvae into different developmental stages based on their morphometric measurements. To evaluate the decision boundaries, we first selected a pair of variables (e.g., length and width) and we generated a grid of 300×300 points within the ranges of the two variables. Then, for each point of the grid, we estimated the value of the third measure using a linear model on the original data. Finally, we used LDA functions to classify points and generate the regions for which a new observation would be assigned to the larval instar. Following this procedure, were generated three maps for each species showing decision boundaries for each combination of the three morphological measures. Reflecting the operative priority of recognizing the larval instars of "unknown" individuals collected in the field, the capability of the LDA model

TABLE 1 First discriminant functions for larval instar classification in *Dermestes frischii* and *Dermestes undulatus*.

	D. frischii	*D. undulatus*
	LD1 (99.6%)	LD1 (98.9%)
Length	0.3872	0.2163
Width	4.0279	4.0098
Thickness	0.2757	1.4451

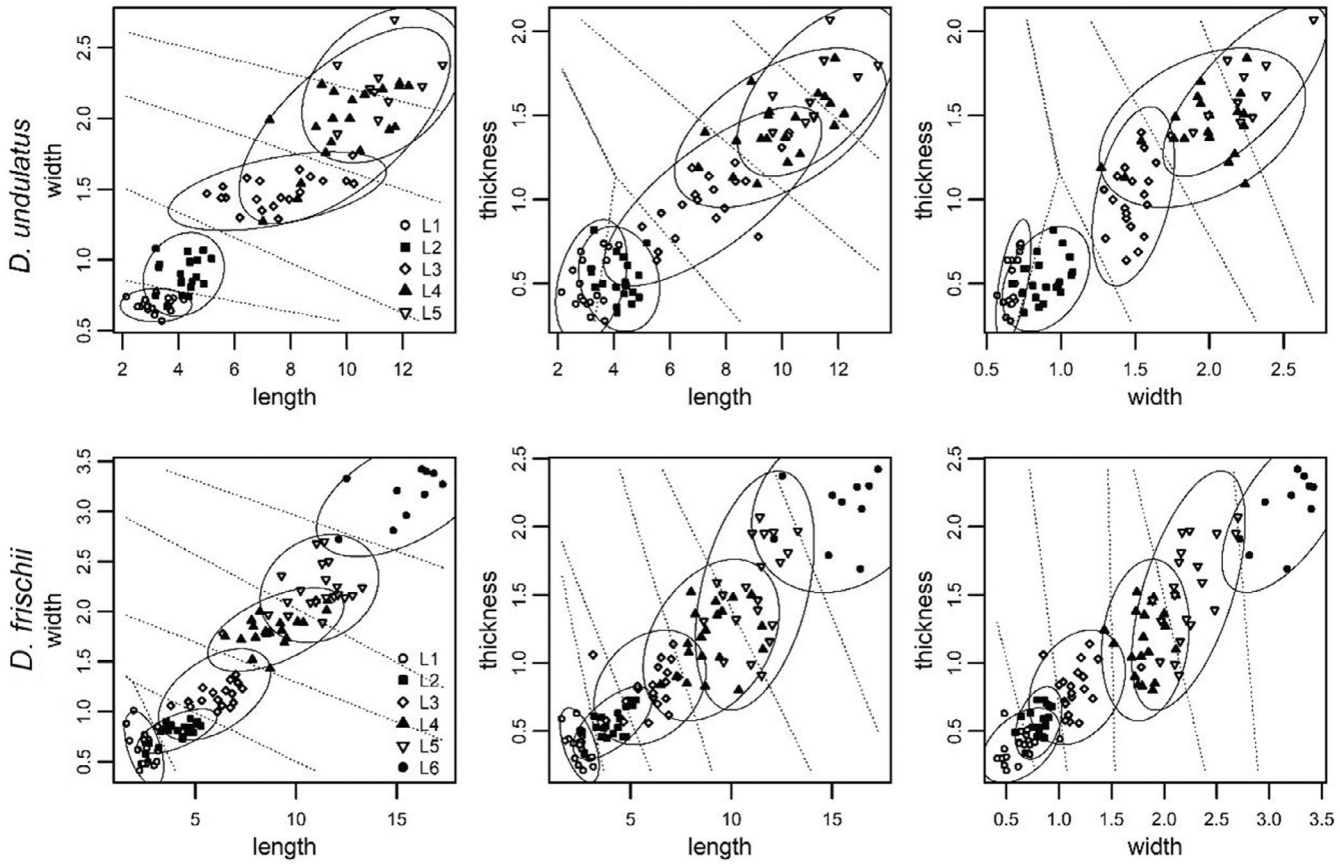

FIGURE 1 Pairwise covariation of morphological variable assessed in the larval instars of *Dermestes undulatus* and *Dermestes frischii*. Values of the axis are expressed in mm. Ellipses contain 95% of the probability for each larval instar shown. The dashed lines are the LDA decision boundaries for each larval instar, delimiting the regions in which a new observation will be assigned to the larval instar.

to classify larval instars was assessed using a cross-folding validation procedure as follows: for each species, the set of samples of two contiguous instars was divided at random into five equal-sized subsets, each having the same distribution of samples in the two contiguous larval instars. In turn, four subsets were used to generate the discriminant function, and the fifth was used for testing. A receiver operating characteristic curve (ROC) assessment was performed to evaluate the overall ability of the model to correctly identify one instar relative to the other; in fact, ROC curves are a way of visualizing the performance of a binary classifier plotting the true-positive rate on *y*-axis against false-positive rate on *x*-axis. The curve should ideally be close to the top left corner of the graph, indicating that the classifier has a high sensitivity and specificity.

Data preparation and analyses were performed in R 3.6.3 [21], using the packages ''ROCR' [22], 'vegan' [23], and 'MASS' [24].

3 | RESULTS

The first discriminant function (Table 1) explained 99.6% and 98.9% of the full variance for *D. frischii* and *D. undulatus*, respectively. The map with LDA decision boundaries (Figure 1) showed that larval instars in both species are clearly separated, especially those in earlier instars. However, a certain degree of overlapping between contiguous larval instars occurred with increasing size. Length and width were more efficient for separating larval instars than the thickness (Figure 1), and in general, a better separation was achieved in *D. frischii*, despite the higher number of larval instars.

The cross-folding validation procedure showed that LDA models could reliably distinguish contiguous larval instars in both species as area under the curve (AUC) values were overall greater than 0.84 and exceeded 0.95 in six out of nine cases (Figure 2, Table 2). Sensitivity and specificity were also high being higher than 0.75 and 0.90, respectively (Table 2), corresponding to a low false-negative rate (FNR) (0.05–0.25) and an even lower false-positive rate (FPR) (0–0.10). In general, LDA performed better with *D. frischii* than with *D. undulatus* and with earlier stages in both species (Table 2).

4 | DISCUSSION

The results of this study showed that a morphometric approach can be used to distinguish larval instars in *D. frischii* and *D. undulatus*, which are important beetles for estimating the upper limit of the PMI in forensic investigations. Discriminant function analysis based on length, width, and thickness measurements allowed the generation of the decision boundaries that clearly separated larval instars, especially in the early stages. The cross-folding validation procedure demonstrated great reliability in distinguishing contiguous larval instars, with high sensitivity and specificity values.

Our results are consistent with previous studies that focused on the life cycle of dermestid beetles and their association with the decomposition process. However, in contrast to the studies that mainly focused on the assessment of various environmental factors affecting life cycle length and larval stage number, our study identified the morphological traits least affected by such factors.

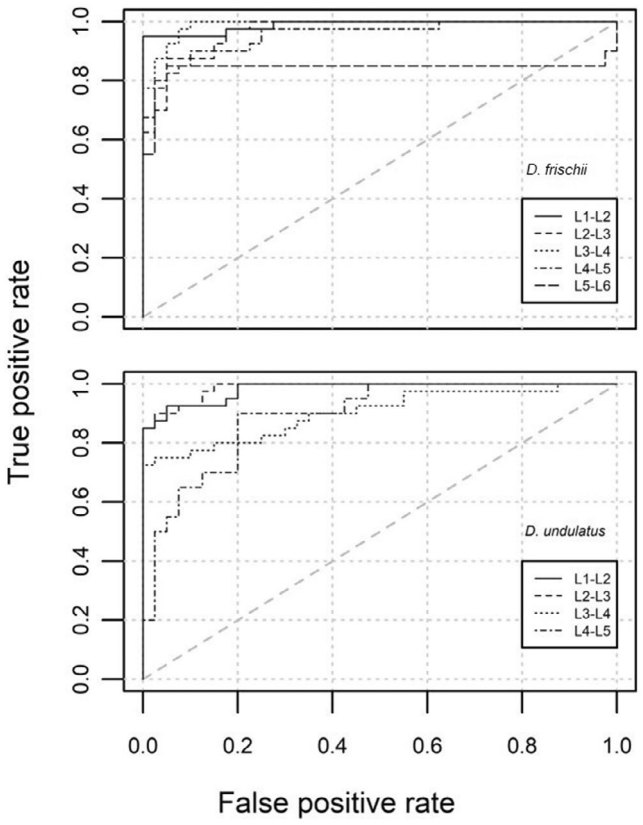

FIGURE 2 Receiver operating characteristics curves for two contiguous larval instars in *Dermestes frischii* and *Dermestes undulatus*. For both species, the area under the curve is high with slightly higher sensitivity for *D. frischii*.

TABLE 2 Diagnostic performance of morphological measurements for discrimination of contiguous larval instars in *Dermestes frischii* and *Dermestes undulatus*.

	AUC	Sensitivity	Specificity	FNR	FPR
D. frischii					
L1-L2	0.99 ± 0.02	0.950	1.000	0.050	0
L2-L3	0.97 ± 0.02	0.950	0.950	0.050	0.050
L3-L4	0.98 ± 0.03	0.975	0.975	0.025	0.025
L4-L5	0.97 ± 0.03	0.900	1.000	0.100	0
L5-L6	0.84 ± 0.02	0.850	0.975	0.150	0.025
D. undulatus					
L1-L2	0.98 ± 0.03	0.925	1.000	0.075	0
L2-L3	0.99 ± 0.02	0.950	1.000	0.050	0
L3-L4	0.89 ± 0.07	0.750	1.000	0.250	0
L4-L5	0.87 ± 0.10	0.800	0.900	0.200	0.100

Abbreviations: AUC, area under the curve; FNR, false-negative ratio; FPR, false-positive ratio.

Table 1 shows the first discriminant functions for larval instar classification in *D. frischii* and *D. undulatus*. In *D. frischii*, the discriminant function is primarily influenced by the width measurement, whereas in *D. undulatus*, the length measurement plays a more significant role; these factors are shown in Figure 1, where it is clear how measurement can isolate different larval stages with great accuracy.

Table 2 displays the diagnostic performance of morphological measurements for discriminating between contiguous larval instars in both species. The results showed excellent performance, the latter with high AUC values (ranging from 0.84 to 0.99) and sensitivity and specificity values close to one. These results demonstrate that the morphometric approach is reliable in discriminating between adjacent instars of both *D. frischii* and *D. undulatus*.

To confirm the findings described in Table 2, Figure 2 displays the receiver operating characteristics (ROC) curves of contiguous larval stages. It is evident that for both *D. frischii* and *D. undulatus*, with slightly lower values, the curves align closely with the true-positive rate, particularly for the first larval stages.

The approach described in this research is less invasive compared to traditional methods such as histological examination, which requires the destruction of specimens for microscopic observations: this method is costless and faster compared to histological and genetic examinations and is optimal for analyzing groups of specimens rather than individuals.

5 | CONCLUSION

The results obtained from this trial show how measuring length, width, and thickness can be very effective in determining the instar to which the larvae of *D. frischii* and *D. undulatus* belong.

In particular, the effectiveness of the measurements is better regarding larvae of *D. frischii* and implies a great ability to be able to discriminate the different larval stages only through the measurements of the three dimensions and therefore a faster analysis of the samples.

For *D. undulatus*, the specificity values are 1 for almost all instars, even if the sensitivity values are lower than for *D. frischii*. This means that even in this case measurements of the dimensions of the larvae can be used to define the stage of the samples with a notable accuracy.

The trial results demonstrate the effectiveness of morphometric analysis in enabling forensic entomologists to study large quantities of Dermestid larvae quickly. This approach also provides valuable insights into the larval age of the specimens, which further helps in improving the accuracy of the minPMI period. Additionally, this technique ensures that there is no loss of any specimen that can be utilized for any future counter-analysis, thus maximizing the efficiency of the process.

Future studies are needed to expand this approach to include other Dermestidae species to test its validity under different environmental conditions.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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