

Short Report: Evaluation of Chemical Preparation on Insect Wing Shape for Geometric Morphometrics

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Abstract. Geometric morphometrics is an approach that has been increasingly applied in studies with insects. A limiting factor of this technique is that some mosquitoes have wings with dark spots or many scales, which jeopardizes the visualization of landmarks for morphometric analysis. Recently, in some studies, chemical treatment (staining) of the wings was used to improve the viewing of landmarks. In this study, we evaluated whether this method causes deformation of the wing veins and tested whether it facilitates the visualization of the most problematic landmarks. In addition, we tested whether mechanical removal of the scales was sufficient for this purpose. The results showed that the physical and chemical treatments are equally effective in improving visualization of the landmarks. The chemical method did not cause deformation of the wing. Thus, some of these treatments should be performed before beginning geometric morphometric analysis to avoid erroneous landmark digitizing.

In recent years, an increasing number of studies using geometric morphometrics in mosquitoes has been used in population-based studies,^{1–5} differentiation between species,^{6–8} or correlation with genetic data that have already been obtained.⁹ This technique allows for multivariate statistical analysis of biological structures; in other words, it simultaneously considers several characteristics of the body structure complex. In dipterans, the target structures of this analysis are the wings, because they have a two-dimensional structure; also, the veins cross to form points that are ideal for a morphometric comparison.

However, some species of mosquitoes have wings possessing dark spots, such as the genus *Anopheles*, or containing many scales, which jeopardize visualization of the landmarks for morphometric analysis. Usually, the specimens collected for these studies are stored in liquid (70% alcohol) or under dry (silica gel or naphthalene) conditions. Traditional methods for dry mounting insect wings in Canada balsam can damage this delicate structure, because after a certain time, the wings become fragile.

For taxonomic studies in general, Carter and others¹⁰ and Lutz¹¹ realized the advantages of phenol in the dehydration and clarification of the insect body, which are required for perfect visualization of the body structures. This method was used on specimens of Diptera (Ceratopogonidae and Chironomidae) with little or no damage to delicate structures, such as wings. For geometric morphometric analysis, recent studies^{4,8} have used a chemical treatment method to clarify, stain, and dehydrate the wing for viewing of the landmarks.

Another method that is widely used to improve the visualization of the landmarks is mechanical removal of the scales using a fine brush. However, the wings of males are weaker than the wings of females, and this method may cause damage to the structure, which affects landmark visualization. Thus, the aims of this work were to verify whether chemical treatment of the wings causes deformation of the wing veins and damage to the geometric morphometrics and determine whether it facilitates visualization of problematic landmarks. In addition, whether mechanical removal of scales alone was sufficient to improve landmark visualization was examined.

We applied three treatments to the wings as follows. The right wings were removed from 30 females of the same species of *Anopheles*, mounted on a slide/coverlip with Canada balsam (Figure 1), and photographed (treatment A). After image acquisition, the wings were dismantled using xylol, the scales were mechanically removed using a soft paintbrush, and the wings were remounted and photographed again (treatment B). Finally, the wings were dismantled, chemically treated, remounted, and photographed (treatment C). Thus, we obtained 30 photosets, and each set consisted of three images of the same wing from treatments A, B, and C.

Chemical treatment involved soaking the wings in a 10% potassium hydroxide (KOH) for 12 hours at room temperature. KOH was removed by washing the wings in 20% acetic acid. The wings were stained with acid fuchsin for 60 minutes and dehydrated in an ethanol series (80–98%). KOH clarified the entire wing, including the scales. The landmarks were not completely visible at this point, because the wing veins became transparent; therefore, it was necessary to dye the wings with fuchsin. Acetic acid baths were necessary to remove excess fuchsin and KOH from the wings.

Wing images were captured using a Leica DFC320 Digital Camera coupled to a Leica S6 Microscope with 40× magnification. Eighteen wing landmarks (LM) for each wing were digitized (Figure 1) using the TpsDig V.1.40 software,¹² and the positional coordinate images were plotted into a Cartesian plane for geometric descriptions. The TpsRelw 1.36 software¹³ was used to plot the landmarks and compare the variables.

REPRODUCIBILITY

A photoset was randomly selected from 30 photosets to test the reproducibility of landmark digitizing. The landmarks of the three images were digitized one time by 100 human operators. The human operators were not previously familiar with wing digitizing and were between 20 and 55 years of age. The digitization order of the photos was A, B, and C. We tested the null hypothesis that the digitizing errors of the operators were equally distributed in the three treatments.

REPEATABILITY

Two landmarks were selected for this test: a difficult-to-visualize landmark (LM2) and a conspicuous landmark

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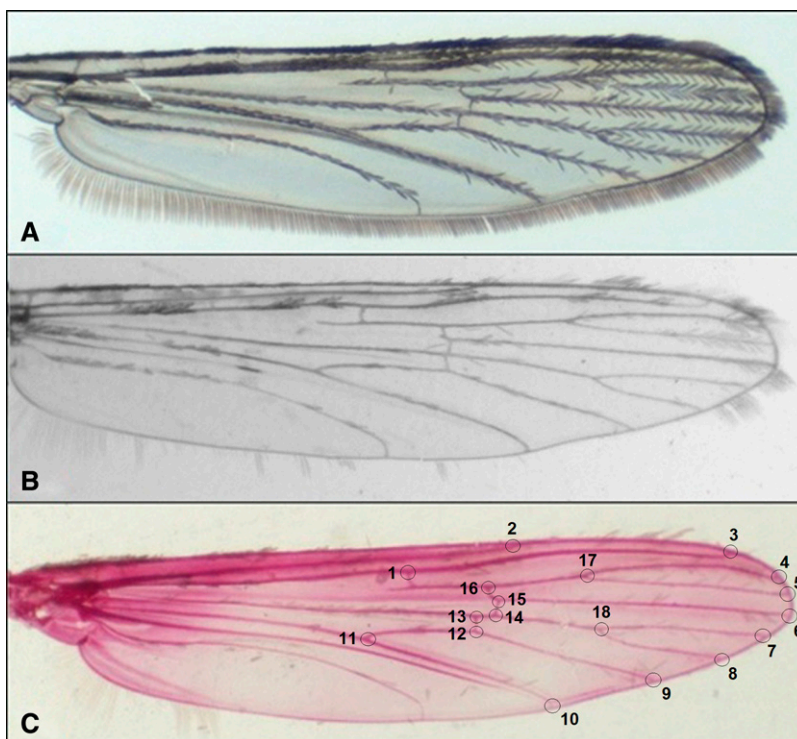


FIGURE 1. The wing of *Anopheles* photographed under three different treatments: (A) no treatment, (B) physical treatment, and (C) physical and chemical treatments.

(LM6). Only operator C.L. (familiar with the morphometrics) digitized the two selected LMs in all 3 photosets. We tested whether the repeatability of an experienced operator was equal in the three treatments.

Statistical tests were based on the Automotive Industry Action Group Measurement Systems Analysis Guideline Manual^{14,15} for comparison between methods. We used the software Statistica 7.0,¹⁶ MiniTab 16.2.3,¹⁷ and Microsoft Excel 2010¹⁸ for statistical analysis and the generation of the graphs.

Physical and chemical treatments helped to visualize the anatomic LMs of the wings of *Anopheles*. We concluded that the chitin of the wing veins was not deformed by the reagents used. Table 1 summarizes the main differences of the treatments, and the highest percentage of losses (damage that precluded analysis caused by the tweezers or brush or occurring during the assembly of the slides with balsam) occurred using the physical treatment. Removal of the scales with the brush resulted in a significantly higher number of losses among all treatments, because it is a more aggressive treatment compared with the chemical treatment. Surprisingly, we observed that KOH provided increased resistance to

the wing, because manipulation with tweezers resulted in almost no damage.

In general, the reproducibility scores of A were lower than the reproducibility scores of B and C. We noted that the most inconspicuous LMs (over 30% error) were located on densely scaled wing locations. The visibility of LMs was improved by treatments B and C, which showed significant increases in the accuracy of the data. Note that the inconspicuous LMs appear only in wings with treatment A, and the accuracy of the digitization process was low (Figure 2).

Treatments B and C did not differ with respect to the reproducibility rates of each LM (analysis of variance [ANOVA]

TABLE 1
Comparison of three treatments

Treatments	Percent of losses	Repeatability (same operator)	Reproducibility (various operators)	Problematic LMs*
A (none)	2	85.6 to 94.2	14.7 to 91.6	LM1, LM2, LM11, LM17, and LM18
B (physical)	30	92.0 to 98.4	90.1 to 97.8	-
C (chemical)	6	92.2 to 99.5	90.5 to 98.5	-

*We considered the LMs with error scores higher than 30% to be problematic (they were observed only during reproducibility analyses).

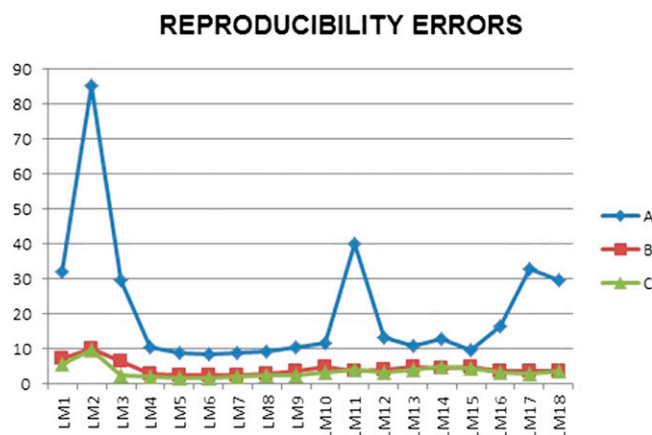


FIGURE 2. Reproducibility errors after the three treatments (A, no treatment; B, physical treatment; C, physical and chemical treatments).

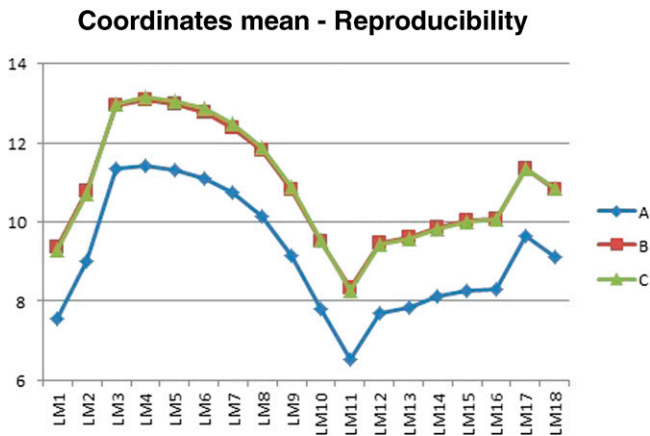


FIGURE 3. Mean value of raw coordinates of each LM scored after the three treatments. Values of Y axis are actual values in pixels divided by 1000.

and post-hoc Tukey–Kramer test, $P > 0.05$). The total reproducibility mean of each treatment is presented in Figure 3, and the physical and chemical treatments showed no significant differences (ANOVA and post-hoc Tukey–Kramer test, $P > 0.01$).

The LMs that were the most problematic are obvious by looking at the scatter plot of the points scored by 100 operators (Figure 4). For example, in treatment A, the position of some LMs (Table 1) in the same wing may be interpreted differently depending on the operator. However, all LMs were observed and digitized with ease after treatments B and C, and the repeatability range was equivalent to the reproducibility (both were below 10%). According to the American Society for Quality Control/Automotive Industry Action Group protocol,¹⁴ a measuring system should be considered acceptable if the total reproducibility/repeatability is less than 10%; these results indicate that even layperson operators were able to locate the anatomical LMs with high accuracy.

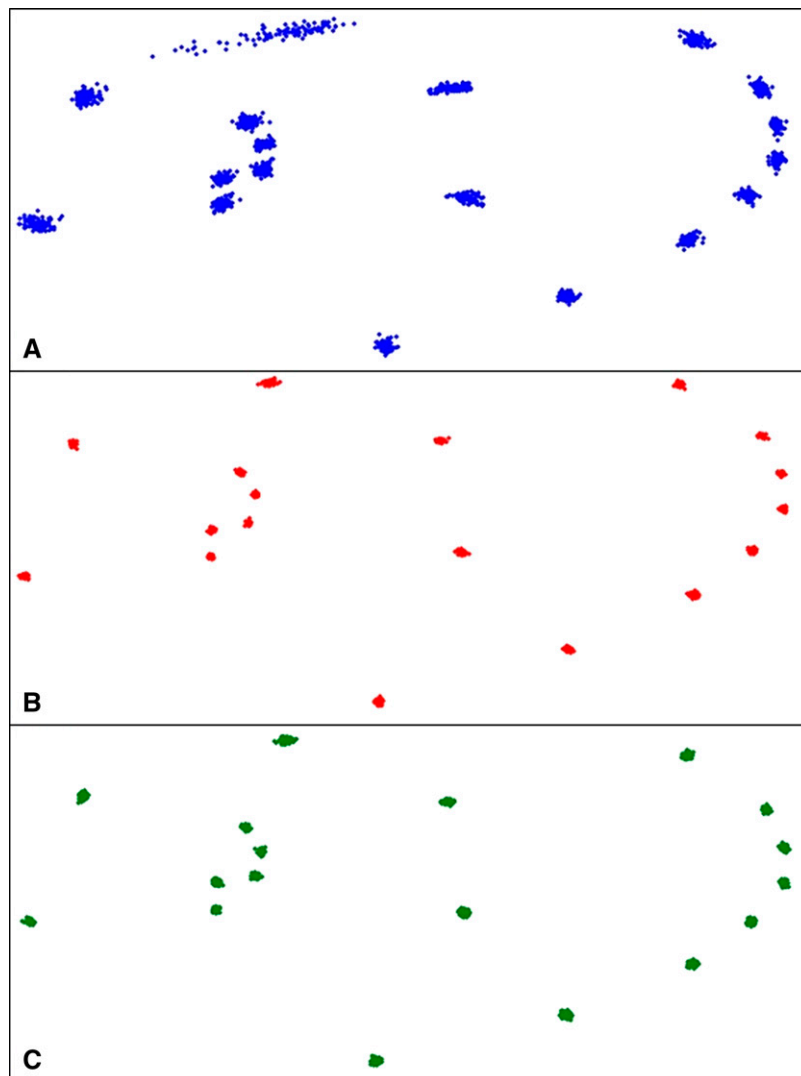


FIGURE 4. Scatter plot of 18 LMs scored by 100 human operators: (A) no treatment, (B) physical treatment, and (C) physical and chemical treatments.

Because no differences in visualization were observed after the physical and chemical treatments of the wings, we concluded that both treatments are equally effective at improving visualization of the LMs. We suggest using some of these methods before initiating geometric morphometric analysis of *Anopheles*, because removal of the scales eliminates errors when marking the LMs on the wing. These methods may facilitate the study of wings from other mosquitoes as well.

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