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Evaluation of Wing Morphometrics for the Identification of the Blowfly Species *Chrysomya albiceps* (Wiedemann, 1819) (Diptera: Calliphoridae) over 9 Years

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ABSTRACT

Geometric morphometrics is a method that analyses the shape of morphological structures. It has been effectively used in the past to examine differences in wing shape among species, populations, and sexes of various Diptera families. The objective of these studies was the simple but reliable identification of numerous Diptera species of applied interest, either for designing effective control plans or accurately estimating the minimum *Post-Mortem* Interval in forensic cases. However, there is a paucity of studies evaluating potential differences in wing shape over time across generations. Significant differences in wing shape over time could affect the models developed for various species and populations, requiring periodic revision. We selected the species *Chrysomya albiceps* (Wiedemann, 1819), which is of great importance in forensic sciences and is widely distributed. Using geometric morphometrics, we analysed the differences in wing shape of *C. albiceps* individuals collected in the same area over 9 years. Our results suggest that wing shape does not change significantly over the years, as cross-validation analyses failed to accurately determine the year of the samples. These positive results support the creation of databases and models for different species and populations, as the lack of significant variation over different generations would ensure the reliability of such models in the medium to long term, making geometric morphometrics a robust tool for the identification of different dipteran species.

Keywords: Calliphoridae, forensic entomology, geometric morphometrics, morphometry, variation across years, wing shape.

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INTRODUCTION

There are multiple techniques, based on either morphological or molecular characters, for the identification of Diptera species of applied interest (Wells & Sperling, 2001; Szpila, 2014; Lutz, Williams, Villet, Ekanem, & Szpila, 2018; Park, et al, 2018). However, these techniques can sometimes lead to inconclusive results under certain conditions. For example, some morphological characters can sometimes be difficult to observe or identify, or even be absent, because of sample damage. In addition, identification by molecular methods may not be possible either because of the sample's preservation condition or because complete reference libraries are not available (Sonet, Jordaens, Braet, & Desmyter, 2012). In addition, certain close species may show insufficient genetic differences to perform a reliable molecular identification, or there may be prior misidentifications in some sequences available in the databases (Sonet, et al, 2012). Because of these potential issues, the development of complementary identification tools is greatly needed. An example of a complementary identification tool is geometric morphometrics (Tatsuta, Takahashi, & Sakamaki, 2018).

Geometric morphometrics is a technique that compares the shape of a particular structure between samples. For that purpose, this method removes the differences due to the size, orientation, and position of the samples (Tatsuta, et al, 2018). Geometric morphometrics uses a series of reference points, or landmarks, to compare the shape of the structures. In the case of adult Diptera, the wings are usually the most used structure. Indeed, geometric morphometrics has already been successfully used for several families of Diptera, analysing differences between species (Grzywacz, Ogiela, & Tofilski, 2017; Macedo, 2017; Sontigun, et al, 2017; 2019; Macleod, Hall, & Wardhana, 2018; Szpila, Żmuda, Akbarzadeh, & Tofilski, 2019; Jiménez-Martín, Cabrero, & Martínez-Sánchez, 2020; López-García, Angell, & Martín-Vega, 2020; Szpila, Johnston, Akbarzadeh, Richet, & Tofilski, 2022), sexes (Virginio, Oliveira Vidal, & Suesdek, 2015; Sontigun, et al, 2017; 2019; Macleod, et al, 2018; Szpila, et al, 2019; Jiménez-Martín, et al, 2020; López-García, et al, 2020; Rodrigues-Filho, Prado e Castro, Lopes, da Fonseca, & Rebelo, 2022), populations (Hall, MacLeod, & Wardhana, 2014; Limsopatham, et al, 2018; Macleod, et al, 2018; Szpila, et al, 2019; López-García, et al, 2020), analysing how competition and captive breeding affect wing shape (Macedo, Arantes, Tidon, & Fryxell, 2020) or analysing differences in wing shape over time across generations (Rodrigues-Filho, et al, 2022). In most of those studies, geometric morphometrics allowed the identification of the samples, at least at the species level, with high reliability.

The aim of many of those geometric morphometric studies was to enable a simple but reliable identification of Diptera species of agri-food, medical, veterinary, or forensic importance. A proper identification is of great importance either to design appropriate control plans or to provide accurate estimations of the minimum *Post-Mortem* Interval (minPMI) (Amendt, et al, 2007). Some authors have observed that the differences in wing shape are big enough to reliably distinguish between populations of the same species (Hall, et al, 2014; Limsopatham, et al, 2018; Macleod, et al, 2018; Szpila, et al, 2019; López-García, et al, 2020). These results suggest that the development of databases for the identification of the main species in each geographical area is greatly needed.

Despite the large number of studies analysing the wide variety of factors mentioned above, the effect of time across generations on wing shape has only been recently addressed by Rodrigues-Filho et al (2022). In this study, no differences in wing shape were found between samples from different years. However, the samples used in this study came from four different populations and non-consecutive years. As a result, further studies are needed to independently analyse the effect of time across generations on the shape of the wing. Significant differences in wing shape over time could have an impact on the accuracy of the models developed for each species and population, requiring updating them periodically to enable reliable identifications.

Chrysomya albiceps (Wiedemann, 1819) (Diptera: Calliphoridae) is a highly relevant species in forensic investigations, due to its frequent occurrence of colonizing cadavers (Grassberger, Friedrich, & Reiter, 2003; Vanin, et al, 2009), including indoor scenarios (Bugelli, et al, 2015; Martín-Vega, Martín Nieto, Cifrián, Baz, & Díaz-Aranda, 2017). In addition, the larvae of this species have the particularity of being facultative predators of the larvae of other Calliphoridae that colonize carrion, like *Lucilia sericata* (Meigen, 1826), which may alter the estimation of minPMI in forensic cases (Grassberger, et al, 2003). Moreover, *C. albiceps* is a widely distributed species, occurring in most of Europe (Makovetskaya & Verves, 2018), the tropical and subtropical regions of Africa and Asia, and Central and South America (Baumgartner & Greenberg, 1984; Grassberger, et al, 2003). It is a thermophilic species whose distribution seems to be expanding to more temperate regions (Grassberger, et al, 2003; Makovetskaya & Verves, 2018). In central Spain, it is a common species during the summer and autumn seasons. It is the most abundant sarcosaprophagous Diptera species during the summer months (June-August), both in wild and urban habitats (Martín-Vega & Baz, 2013; Martín-Vega, et al, 2017).

This study analyses the differences in wing shape of the blow fly *Chrysomya albiceps* over 9 years, using geometric morphometrics. We hypothesize that these differences are not significant enough to require constant updating of databases, thereby maintaining the viability of geometric morphometrics as a reliable tool for dipteran identification. Our objective is to evaluate the extent of these differences and confirm the stability of identification databases over time.

MATERIALS AND METHODS

Wild specimens used for this study were collected at the Scientific-Technological Campus of the University of Alcalá, in the city of Alcalá de Henares, Community of Madrid, Spain (3°20’5,45”W, 40°30’24,846”N). It is a semi-urban environment characterised by a predominance of herbaceous vegetation. The specimens were captured using a squid-baited pitfall trap, successfully used in previous studies for collecting necrophagous insects (Baz, Cifrián, Díaz-Aranda, & Martín-Vega, 2007;

Martín-Vega & Baz, 2013). None of the specimens were captive-bred. The trap was working continuously from 2005 to 2013, both years included. The trap was active only during the first half of each month to minimise the potential effect of the continued capture of specimens on the same population in the area. For this research, we selected the samples captured only during September each year, as this was the month in which the greatest number of specimens were collected. Thirty specimens from each year were randomly selected and identified using a key (Szpila, 2014). Of these 30 individuals per year, 20 were females and 10 males. This difference is because the number of collected males was much lower than the number of collected females. There is often a bias towards more females being caught in carrion-baited traps (Martín-Vega & Baz, 2012). However, to make sure that the same trend was observed with the males, we thought it might be interesting to do a small test with 10 specimens from each year (except for 2011, for which there was not enough material available). A total of 260 specimens of *C. albiceps* were used for this research.

The methodology was similar to that used in other wing morphometrics studies (Grzywacz, et al, 2017; Sontigun, et al, 2017; 2019; Szpila, et al, 2019). We removed the right wing of each specimen. We placed a few drops of DMHF medium on a slide, placed the stretched wing on top, and covered it with a coverslip. All wings were placed with the same orientation. We left the samples drying for a few hours and then we photographed them using a Leica EZ4D stereo microscope with a built-in camera and the Leica Application Suite V. 2.1.0 software. Once photographed, we digitised each wing and placed the reference points used, using the TpsUtil V. 1.76 and TpsDig2 V. 2.31 software (http://life.bio.sunysb.edu/morph/), respectively. We placed 19 landmarks by sample, which corresponded to the intersections between the main wing veins and between the wing veins and the wing margin (Fig. 1). These or similar landmarks have been used previously in other wing morphometry studies performed on calliphorids (Hall, et al, 2014; Sontigun, et al, 2017; Limsopatham, et al, 2018; Jiménez-Martín, et al, 2020). All reference points were always placed in the same order and by the same person.

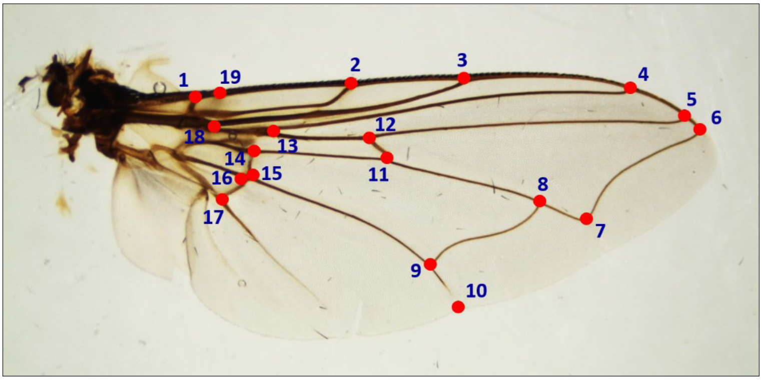

Figure 1. Wing of *Chrysomya albiceps* showing the landmarks, and the placement order, used for this research.

Once all landmarks were digitised, we performed a series of statistical analyses using PAST V. 3.21 (Hammer, Harper, & Ryan, 2001). First, we performed a

Procrustes analysis to remove the differences in the data due to sample size, orientation and position, comparing uniquely the shape. Then, we performed a principal component analysis (PCA) to observe the variation in wing shape between years, and a canonical variant analysis (CVA) to determine the characteristics that maximise these differences, which facilitates the separation of the groups. We also obtained the Mahalanobis distances between years, using MorphoJ V. 1.06d software (Klingenberg, 2011). Finally, we performed a linear discriminant analysis (LDA) and a leave-one-out cross-validation analysis to determine the ability to identify the year of origin of the samples using geometric morphometrics. The cross-validation test removes one specimen from the sample and performs an LDA with the remaining specimens. Subsequently, it proceeds to identify the extracted specimen using the model developed with the rest of the specimens. The same process is performed with all the specimens in the sample. Although it is a process that requires more computing power, it is a stricter analysis, providing more reliable results. All these analyses were performed directly on the values obtained after the Procrustes analysis.

RESULTS

Principal component analysis (PCA) showed no significant differences between specimens from different years, all being completely overlapping, both in females (Fig. 2A) and males (Fig. 2B). The first two principal component axes accounted for 36.8% of the variation (20.5% and 16.2% on the first and second axes, respectively) in females and 36.1% of the variation (22.9% and 13.3% on the first and second axes, respectively) in males. The canonical variant analysis (CVA) also showed no significant differences between the different years of the study, with all the ellipses overlapping (Fig. 3), although this overlap is somewhat smaller than that observed with the PCA. However, this overlap is still remarkably high, which does not allow the separation of any group unambiguously. The first two axes accounted for 57.4% of the variation (39.4% and 18.1% on the first and second axes, respectively) in females and 56.8% of the variation (40.4% and 16.3% on the first and second axes, respectively) in males. The Mahalanobis distances between different years are shown in Table 1.

The percentage of specimens correctly classified between years for females, calculated by the linear discriminant analysis (Table 2A), was relatively high, with the lowest being in 2010 (60%) and the highest in 2012 (85%). The overall percentage of correctly classified specimens was 70.6%. These percentages decreased drastically with the cross-validation test (Table 2B), with an overall percentage of correctly classified specimens of 14.4%. In this case, the highest percentage was obtained for the 2005 specimens (25%), and the lowest percentage remained for the 2010 specimens (5%). For males, the results were slightly higher using linear discriminant analysis (Table 3A), the lowest being for the year 2012 (70%) and the highest for the years 2006 to 2009 (100%). The overall percentage of correctly classified specimens was 90%. However, these percentages decreased even more than with the females using the cross-validation test (Table 3B), with an overall percentage of correctly classified specimens of 10%. In this case, the highest percentage was obtained for the 2006 and 2012 specimens (20%), and the lowest percentage for the 2007 and 2010 specimens (0%).

Table 1. Mahalanobis distances obtained for females and males between the years of study (lower triangle) and p-value obtained after a permutation test with 1000 rounds (upper triangle). A) Females. B) Male.

A	2005	2006	2007	2008	2009	2010	2011	2012	2013
2005	-	0.0001	0.0257	0.138	0.0002	<0.0001	<0.0001	<0.0001	<0.0001
2006	2.58	-	0.0017	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001
2007	2.19	2.48	-	0.4282	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
2008	2.05	2.59	1.89	-	<0.0001	<0.0001	<0.0001	<0.0001	0.0001
2009	2.62	3.17	2.92	3.00	-	0.7114	0.0336	0.0017	<0.0001
2010	3.21	3.22	2.94	3.20	1.70	-	0.2571	0.0792	0.0002
2011	2.77	3.11	2.69	2.93	2.18	1.94	-	0.0265	<0.0001
2012	3.40	3.49	3.37	3.39	2.46	2.14	2.25	-	0.0057
2013	2.92	2.54	2.80	2.62	2.93	2.58	2.72	2.37	-
B	2005	2006	2007	2008	2009	2010	2011	2012	2013
2005	-	0.0002	0.0002	0.0061	0.002	0.0001	-	0.0022	0.0014
2006	3.71	-	<0.0001	0.0202	<0.0001	<0.0001	-	0.0001	<0.0001
2007	3.47	4.64	-	<0.0001	0.0008	0.3157	-	0.0001	0.0143
2008	3.29	3.11	3.83	-	<0.0001	<0.0001	-	0.0003	0.0005
2009	3.56	4.55	3.36	3.82	-	0.0904	-	0.0005	0.0002
2010	4.15	5.72	2.72	4.42	2.96	-	-	0.0004	0.0217
2012	3.48	4.42	3.86	3.54	3.63	3.90	-	-	<0.0001
2013	3.50	4.23	3.14	3.52	3.61	3.17	-	3.93	-

Table 2. Percentage of specimens correctly classified between the different years of study, for females, obtained using LDA (A) and cross-validation analysis (B).

A	2005	2006	2007	2008	2009	2010	2011	2012	2013	Total	Correctly classified (%)
2005	13	1	1	1	2	0	1	1	0	20	65.0
2006	1	15	2	0	0	2	0	0	0	20	75.0
2007	1	1	14	1	0	1	1	0	1	20	70.0
2008	0	2	2	14	0	1	0	0	1	20	70.0
2009	0	1	0	0	13	2	2	2	0	20	65.0
2010	0	0	2	0	2	12	3	0	1	20	60.0
2011	1	0	1	1	0	2	13	1	1	20	65.0
2012	0	0	0	0	1	1	0	17	1	20	85.0
2013	2	1	0	0	0	0	0	1	16	20	80.0
Total	18	21	22	17	18	21	20	22	21	180	70.6
B	2005	2006	2007	2008	2009	2010	2011	2012	2013	Total	Correctly classified (%)
2005	5	2	3	5	2	0	2	1	0	20	25.0
2006	4	3	4	0	0	3	0	1	5	20	15.0
2007	2	4	3	3	1	2	3	1	1	20	15.0
2008	6	2	4	2	0	1	1	2	2	20	10.0
2009	1	1	0	1	4	5	3	5	0	20	20.0
2010	0	0	3	0	7	1	3	2	4	20	5.0
2011	2	0	1	1	1	5	3	5	2	20	15.0
2012	1	1	0	0	3	4	4	3	4	20	15.0
2013	2	4	2	2	0	3	1	4	2	20	10.0
Total	23	17	20	14	18	24	20	24	20	180	14.4

Table 3. Percentage of specimens correctly classified between the different years of study, for males, obtained using LDA (A) and cross-validation analysis (B).

A	2005	2006	2007	2008	2009	2010	2012	2013	Total	Correctly classified (%)
2005	8	0	0	1	1	0	0	0	10	80.0
2006	0	10	0	0	0	0	0	0	10	100.0
2007	0	0	10	0	0	0	0	0	10	100.0
2008	0	0	0	10	0	0	0	0	10	100.0
2009	0	0	0	0	10	0	0	0	10	100.0
2010	0	0	0	0	1	9	0	0	10	90.0
2012	1	0	1	0	1	0	7	0	10	70.0
2013	0	0	1	0	0	1	0	8	10	80.0
Total	9	10	12	11	13	10	7	8	80	90.0
B	2005	2006	2007	2008	2009	2010	2012	2013	Total	Correctly classified (%)
2005	1	0	2	2	2	0	1	2	10	10.0
2006	3	2	0	2	0	0	3	0	10	20.0
2007	2	0	0	1	2	3	1	1	10	0.0
2008	1	2	1	1	2	0	2	1	10	10.0
2009	1	1	0	1	1	5	0	1	10	10.0
2010	1	0	3	1	3	0	1	1	10	0.0
2012	1	0	2	1	4	0	2	0	10	20.0
2013	1	1	2	3	1	1	0	1	10	10.0
Total	11	6	10	12	15	9	10	7	80	10.0

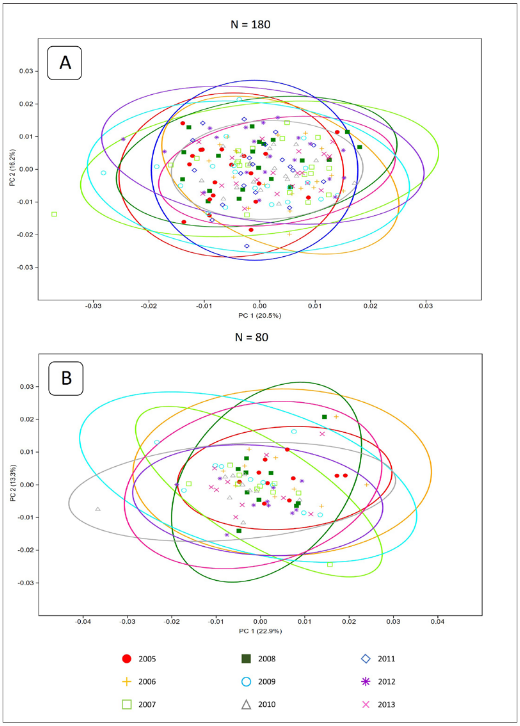

Figure 2. Scatter plot showing variation in wing shape for the different years of study in females (A) and males (B), along the first two principal component axes.

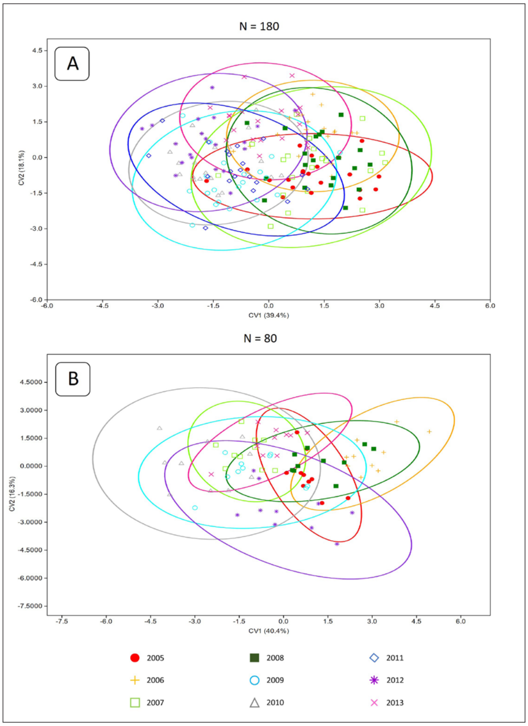

Figure 3. Scatter plot showing variation in wing shape for the different years of study in females (A) and males (B), along the first two canonical variant axes.

DISCUSSION

Our results strongly suggest that there are no significant differences in wing shape over the years. Although the LDA had, in some cases, high success rates (even 100 % for some years), these percentages decreased drastically when the cross-validation test was performed (reaching a maximum of 25 % identification for females in 2005). Overall, the findings of this study are similar to those presented by Rodrigues-Filho et al (2022). Their study shows a strong overlap between specimens from various years, with the Mahalanobis distances resembling those found in our study. However, Rodrigues-Filho et al (2022) did not perform a discriminant analysis or a cross-validation analysis between years, so it is not possible to compare the success of their method to distinguish between years with our own.

The morphological variation in the wings of *Chrysomya albiceps* over almost a decade was not large enough to identify the year of collection of the specimens. We believe that these negative results strongly support the use of geometric morphometrics for the identification of species, sex, or populations of Diptera. If these results are confirmed in the future by similar studies with other species and different families, it could mean a consolidation of geometric morphometrics as a stable and reliable identification tool. Due to the lack of resources, facilities, and/or qualified practitioners, species identification by molecular methods may not be possible (Sonet, et al, 2012). Therefore, the development and consolidation of alternative simple and affordable tools are of paramount importance.

Moreover, other similar studies (Grzywacz, et al, 2017; Macedo, 2017; Sontigun, et al, 2017; 2019; Macleod, et al, 2018; Szpila, et al, 2019; Jiménez-Martín, et al, 2020; López-García, et al, 2020) showed that, with little training and solid baseline data, it is possible to identify species which, because of their size, rarity, or complex morphological characters, might be difficult to determine for practitioners unfamiliar with the taxonomy of these insects. In many cases, moreover, the applied interest of many of these insects (forensic, veterinary-medical, or food) requires a reliable and unambiguous identification (Amendt, et al, 2007). With these results, the development of wing morphometrics databases including representatives from different populations of relevant species and families of Diptera would provide a valuable identification tool to both taxonomists and forensic, medical, veterinary, or pest control practitioners.

In conclusion, our results suggest that blowfly wing shape does not vary significantly over different generations, remaining relatively stable. Although it would be desirable to test this with other species and populations, the lack of temporal variation would be a great advantage when creating different databases and models, as these could continue to be used in the medium to long term without the need for major updates, making geometric morphometrics a simple reliable and robust tool for the identification of different dipteran species.

CREDIT AUTHOR STATEMENT

JL-G, BC, AB, and DM-V conceived the study. JL-G carried out the laboratory work, analysed the data and wrote the manuscript draft. BC, AB and DM-V collected the data and critically revised the manuscript draft. AB and DM-V also supervised the study. All authors gave final approval for publication.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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