GEOMETRIC MORPHOMETRIC ANALYSIS OF THIRD INSTAR LARVAE OF COMMON BLOW FLY (DIPTERA: CALLIPHORIDAE) GENERA FOR FORENSIC IDENTIFICATION

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Entomology

West Lafayette, Indiana

August 2019

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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To my parents Marilene and Krikor,

and my siblings Caroline and Krikor Jr.

I could not have done this without all

of the love and support you have given me.

Thank you for always being there for me!

ACKNOWLEDGMENTS

I'd like to start by thanking my advisor, Dr. Trevor Stamper for opening his lab for me to do research in. Along with providing me with lots of ideas and freedom to explore a project that interested me, Trevor also provided most of the financial support for my project. Trevor was also the one who convinced me that graduate school was the step in the right direction for me and it is fair to say that I might not have started grad school at all if it weren't for him. Thus, without Trevor I would not be submitting this thesis. Thank you!

I was also blessed with an extremely helpful committee made up of Dr. Chris Oseto and Dr. Erik Otarola-Castillo. Although each were selected for a specific role that they brought to my project, both provided ample guidance in various different parts of the project. Chris shared his knowledge of insect identification and preservation with me which was crucial for the work described in chapter two. And without Erik's knowledge of statistics and geometric morphometrics, chapter three would not exist. People always told me that committee meetings were scary, but I had a very different experience. I always came out of my committee meetings feeling better than I did going into them. And for all of your support, I thank you!

I would like to extend a special thanks to Dr. Lauren Weidner, who inspired me to work with insect identification. Lauren joined our lab as a post doc with a passion for research that she shared and passed on to me. After hours of trying to ID my first set of flies, Lauren came by and told me they were mostly wrong. But she didn't leave it there, instead she spent hours in the lab with me showing me each feature and each hair that was used in the identification. For the countless hours we spent in the lab (and the fun trips to the cactus to clear our heads), I thank you!

My lab mate, Kelie Yoho, also deserves a special thank you. Along with providing me with load of encouragement and mental support, Kelie also provided physical support by helping me

(and sometimes covering me) with the heavy teaching load that we both had. There were moments when we both thought we were crazy for getting into this research (usually around 6am out in the field at -4°C collecting data from pig carcasses), but having Kelie by my side made those times fly by. Thank you for all of your moral support!

There were several undergrads who helped in the lab as well: Julia Betz, Samantha Hittson, and Nat Hone. Their worked ranged from rearing and maintain our fly colonies to testing out the clearing and mounting protocol described in chapter two. Thank you for all the time you put into helping me in the lab.

I would also like to thank my suppliers of flies. This project would not have been possible without the help of Dr. Bob Kimsey and Dr. Krystal Hans who generously supplied specimens from their respective locations. They answered my call for help and were happy to collaborate on this research. In terms of supplying specimens, I would also like to thank Jarrett Hiatt whose work back in 2015 left many extra specimens behind that I was able to incorporate into my project.

This research was funded in part by the Forensic Sciences Foundation Acorn Research Grant Program for 2018-19 (Federal ID Number: 237050691). I thank them for finding value in the research and selecting me for their investment.

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ABSTRACT

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Degree Received: August 2019
Title: Geometric Morphometric Analysis of Third Instar Larvae of Common Blow Fly (Diptera) Genera.
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In terms of forensic entomology, one area that is scrutinized most is the estimation of a minimum post mortem interval (mPMI) based on insects that are present at a crime scene. The identification of the insects found at the scene if the first step to calculate a mPMI. However, currently there are no methods that can present the courts with accurate statistical error rates in identification, because the current methods are reliant on an expert's use of a morphological key to identify the specimen, and this identification method does not produce a confidence value. This project aimed to test a method of identification using geometric morphometrics that can produce confidence intervals to provide to the courtrooms.

Before any identification could start, a standard preservation protocol was developed to ensure that all diagnostic features are preserved, and specimens can be identified in the same way. A clearing method was designed to clear specimens within 24hrs using potassium hydroxide, so they can be dissected and mounted the next day. The dissection of the specimens was a simple six-step procedure to split the mouth hooks, the cuticle and the posterior spiracle. This procedure ensures that all diagnostic features are preserved on a microscope slide.

With all of the features preserved, the microscope slide is photographed for storage and an investigator can perform geometric morphometrics to identify the insect. This study tested the application of geometric morphometrics to distinguish between three genera of Calliphoridae (*Calliphora*, *Lucilia*, *Phormia*), from three locations in the US (Delaware, Indiana, California).

Results showed significant (p-value: <0.05) variation in shape among all genera. When genera were tested for shape differences based on location, these variations were also significant (p-value: <0.05). The implication of these results is that enough shape difference exists to distinguish between these genera and to distinguish between populations.

CHAPTER 1. INTRODUCTION TO THESIS

Background

The succession and life stages of insects found in a death investigation provides critical information regarding minimum time since death, and whether or not the body died at that scene or was relocated. Blow flies (Diptera: Calliphoridae) are some of the first colonizers of corpses, arriving within minutes of the death and laying eggs within the first hour [Anderson & Vanlaerhoven 1996, Anderson 2000]. Once collected, some immature blow flies are preserved, and others reared to adults for species identification [Byrd & Castner 2009]. Rearing flies to adulthood is both time and resource intensive for a forensic entomology lab. For each sample collected from the scene, animal meat is needed as a food source, cups and cages for rearing flies, pins to mount adults, and cabinets to store the specimens [Byrd & Castner 2009]. Along with the physical expense for those items, time must also be considered as an expense since rearing a blow fly from egg to adult can take over two weeks [Hill *et al*. 1947].

Aside from all the costs associated with the current methods, there are currently no all-inclusive larval keys, accounting for all species, to perform the identifications. Larval morphological characters, sufficient for species identification, have been studied for some species of calliphorids. Many of these described features are located either in the cephaloskeleton or near the posterior spiracles of the third instar larvae [Knipling 1939, Erzinclioglu 1987, Liu & Greenberg 1989, Szpila 2009]. But due to the lack of all-inclusiveness in these keys, larvae are still reared to adulthood for identification. There is a critical need to develop a rapid and reliable procedure to identify larval specimens collected from decedents at crime scenes, which produces quantifiable data that can be analyzed for error rates by producing confidence intervals in the form

of likelihood ratios [National Research Council 2009, Bernstein and Jackson 2003] and can reduce the cost and time it takes to rear and identify specimens.

Insect identification

Traditionally, insect identification relies on specific characters that have been determined by a Taxonomist. Taxonomists build dichotomous keys to guide insect classification. Entomologists work from classification of morphological traits that group insects into certain orders, families, genera, and even into species level of identification [Peterson *et al*. 1981]. These characters are sequentially checked during an identification to group individuals with the same traits and separate those that appear different. Each check in the sequence of the key is known as a node. On some occasions these traits are easy to observe and can be classified as present or absent [Cutter and Dahlem 2004]. However, some cases rely on an arbitrary description of traits such as color and relative size (non-metric) [Whitworth 2010]. This type of identification requires that the person identifying the specimens either be trained by an expert or be in the possession of a set of specimens that have been pre-identified by such experts. However, these keys don't always account for user error on issues such as a dispute of color (e.g. – is the trait orange, brown, or tan?). To overcome these issues, some keys include multiple features at the same node [Whitworth 2006]. And although this can provide some insight, it makes the user assume that because the second feature is identifiable, they can ignore the first feature that was difficult to differ. A few keys also include the size or distance of one feature compared to another [Szpila 2010]. However, using distances and ratios creates more room for human error. The user now has to either physically measure a distance or make an ambiguous determination that a feature is larger than another.

Error

In order to reduce the potential for human error there needs to be a change in the way insects are being identified. At each node of the key there is opportunity for error. These errors can potentially lead to a misidentification of the specimen. Errors in insect identification can have wide ranging consequences. Some errors result in trivial problems, such as losing a point or two on an assignment in a classroom setting, should any item be misidentified. But other errors, in the forensic context, have much more dire consequences, such as the misidentification of insects leading to a conviction or release of a suspect from prison. Individuals perform calibrations using the keys on a reference collection of insect, prepared by alpha taxonomists, to ensure the keys are working correctly. At any point when a feature on the key is unclear, one can reference the collection to see what that feature looks like on a physical fly.

Verification of identifications by a partner for cases involving the courts is another option to reduce errors. However, this is more time consuming, creates room for disagreements, and still leaves the court without a statistical rate for error. The next logical step would be a switch away from traditional taxonomy to a geometric morphometric approach, where all the sizes and ratios are not being determined by an individual but rather by a machine that can be calibrated.

Geometric Morphometrics

Geometric morphometrics might offer a solution to the issues discussed above. Geometric morphometrics, or shape analysis, differs from traditional morphometrics in that it retains all of the geometric information of the item in question by recording the data on a coordinate system, versus traditional morphometrics which records linear measurements, ratios, and angles [Rohlf 2015]. To apply this to insects, we find and photograph features on the insect that have diverse landmarks. We then analyze the shape variation of the features for different insects, to discern if

there are quantifiable differences. Following that step, we can select those features and landmarks for any unknown specimen and compare to our data of the known insects.

This type of analysis has been done successfully with wing venation for adult flies [Slice 2007]. By superimposing all of the wings in a set, the shape variation of each landmarks can be measured. The Procrustes superimposition method (fully described in chapter three) allows us to freely scale, rotate, and translate the images and line them up to reduce the impact that size has on shape [Rohlf 2015]. Once the landmarks are selected and saved as coordinates, they can be rotated, scaled or translated to line up, without affecting any latter analysis. If this method of identifying shape can be applied to larval identification, it can reduce the overall time requirement of insect identification, as well as provide the user with quantitative species identity data and most importantly confidence and likelihood intervals. If different species have statistically quantifiable different shapes, then a database of standard morphometric data can be built to include all species. Once such a database is built, the user would input the landmarks of the unknown specimen and compare the shape variation to the database, which would then return the probability that the specimen is a certain species.

Forensic Application

In forensic entomology, there are four central pieces of information needed to establish an accurate time of colonization based on insect development: specimen identification, specimen size/stage, development data, and temperature data (Figure 1). Acquiring these four factors can start the process of calculating a minimum post mortem interval [Higley and Haskell 2001, Grennard 2012]. This project focused on specimen identification and size/stage. With those two items a forensic entomologist can start their work of calculating how long those insects have been feeding on the carcass. Current collection methods include adult and larvae collection from the

carcass [Haskell *et al*. 2001, Gennard 2012], then preservation of half of the collected larvae to establish the stage they were found in and rearing the other half to adulthood to identify the species in order to provide accurate development data for that specific species. As mentioned above, this method is extremely time consuming and relies heavily on the assumption that the specimens collected are representative of the whole population of insects on that carcass. In order to increase the efficiency of insect identification, this thesis is proposing the use of geometric morphometrics as a solution to identify the larvae found on the scene without the need to rear them to adults or to use larval keys.

Using the standard method described in chapter two, an investigator would collect the larvae from the scene and an entomologist would kill, clear, dissect, and mount the larval specimens on slides where they can be photographed for geometric morphometric analysis. My method presents a standard way to preserve specimens on a slide where the diagnostic features can be observed. All supplemental information about the specimen (e.g. – location of collection, size in mm, case number, and who collected it) can either be transcribed on the slide label itself or referenced based on the storage system.

In chapter three I evaluate mouth hook for shape differences between the left and right hook, and shape variations due to genera and location, using statistical software MorphoJ [Klingenberg 2011]. The statistical analysis to test for directional asymmetry was done by performing a Procrustes ANOVA. Patterns of shape variation were determined based on a principal component analysis of shape variation between all specimens. The analysis to group specimens based on predetermined identification for each genera and location was done by performing a canonical variates analysis. These tests were performed based on previous studies comparing shape variation of fly wings [Klingenberg and McIntyre 1998].

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Figures and Tables

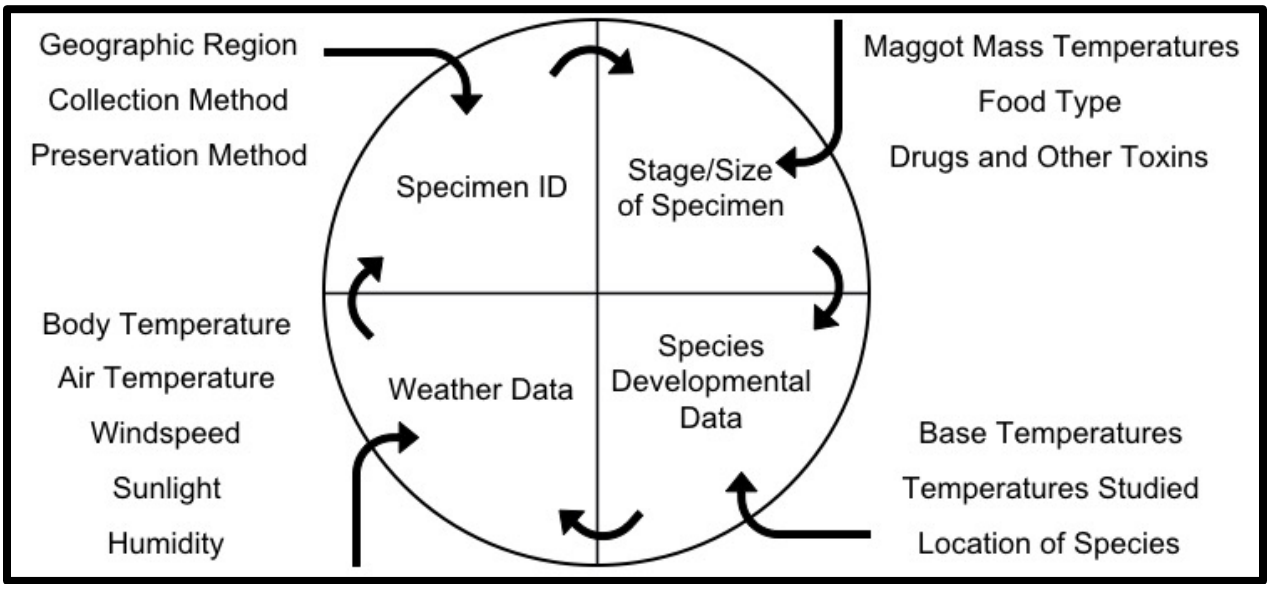

Figure 1: A visual representation of the main factors used in estimating a post mortem interval and external factors that might affect the main ones. Based on the literature of Haskell *et al*. (2001), Higley and Haskell (2001) and Grennard (2012).

CHAPTER 2. TECHNICAL NOTE: A TECHNIQUE TO MOUNT SARCOPHAGID AND CALLIPHORID (DIPTERA) LARVAE FOR FORENSIC IDENTIFICATION USING GEOMETRIC MORPHOMETRICS

Introduction

Current larval keys [e.g. – Knipling 1939, Wells *et al*. 1999, Szpila 2010] are not all-inclusive for U.S. species of forensically important flies and can be difficult to maneuver, while offering no statistical support in for the decisions. These downsides indicate that there is a need for an improved method of larval identification. One technique that offers statistical support is geometric morphometrics. The use of geometric morphometrics in entomology was reviewed by Tatsuta *et al*. [2017]. Although published research on the shape analysis of wings and genitalia of flies are plentiful [Grzywacz *et al*. 2017, Hall *et al*. 2014, Sontigun *et al*. 2017], the use of geometric morphometrics using the acephalic cephalopharyngeal skeleton of muscomorpha larvae is limited [Nunez and Liria 2016]. The cephalopharyngeal skeleton is commonly used in larval identification [Szpila 2010]. We believe this is due to no established protocol for mounting these delicate structures, despite the forensic entomology literature being replete with illustrative examples demonstrating clear species-level differences [Knipling 1939, Szpila 2010, Velazquez *et al*. 2010]. Here we establish a protocol for the clearing and mounting of sarcophagid and calliphorid larval mouthparts for subsequent use in two-dimensional geometric analysis.

In geometric morphometrics, there is a crucial need for "good looking" specimens because data is collected from pictures or scans of the specimen [Slice 2005]. Good looking specimens are defined as specimens with no physical damage or visual obstructions that would make the analysis of shape impossible. Broken or bent specimens alter shape, corrupting the data.

Uniqueness of the data should stem from the underlying shape of the larval mouth hooks—it should not change based on collection and preparation techniques. Several papers detail procedures for clearing and/or mounting specimens for preservation for various types of analyses [Hazeltine 1962, Wilkey 1962, Schauff 2001, Sukontason *et al*. 2004, Niederegger *et al*. 2011, Barbosa *et al*. 2014]. Differences in methods described in the these papers have differing 1) temperatures, 2) type of solutions, and 3) concentration adjustments of the clearing solutions. All of these adjustments can impact quality and structure of the cleared specimen. Geometric morphometrics requires specimens to remain rigid to prevent distortion and displacement when placed on a microscope slide, yet the specimen must also be somewhat malleable to prevent cracking and feature loss while preparing the slide. Szpila [2009 - Workshop] describes how to cut and spread the larval calliphorid insect for identification. We expand and modify those procedures to include the splitting of the mouth hooks on the dorsal side in order to spread (butterfly) the mouth hooks apart. Our process utilizes easily obtainable materials and ensures all relevant features are preserved when mounted on a slide.

Materials and Methods

We developed this method using samples from two different families of forensically important flies: first on 3rd instars of *Sarcophaga bullata* (Parker, 1916) that were obtained from colony, and second on 3rd instars of *Phormia regina* Meigen (1826). The Sarcophagidae larvae were selected for the first trial as they are much larger in size than blow fly larvae.

Specimen Preparation

Both species were maintained on a diet consisting of sugar (Dominos, Sugar-Pure Cane Granulated) and water, *ad libitum,* in a 30.5cm x 30.5cm x 30.5cm colony box (Bioquip,

Collapsible Cages 1450B). Beef liver (purchased from the Purdue Butcher Block) was used as a medium for oviposition and as a food source for resulting larvae. Third instars with empty crops (wandering stage) were parboiled for one minute and preserved in ~80% ethanol until being cleared. Specimens were prepared for clearing by making a 2-3mm longitudinal incision on its ventral side between abdominal segments III through V (aIII-aV; Figure 1).

Clearing

Prepared specimens were placed in a flask with a 15% potassium hydroxide (KOH) solution for 24 hours at room temperature (~23°C). To ensure saturation, the flask was agitated for approximately 30 seconds, every 3-4 hours during the first 8 hours. After 24 hours the entire cephaloskeleton was visible from the outside of the specimens; any remaining opaque gut content or fatty tissue was removed during dissection (Figure 2). The larvae were then removed from the KOH solution and rinsed in a bath of 100% ethanol, before being placed in ~90% ethanol for storage until dissection. Although the dissection can be done without clearing the specimen, it is much more labor intensive as the dissector must scrape away the fatty tissues surrounding to the mouth hooks.

Dissection

Once cleared, specimens were prepared for slide-mounting, which requires dissection. To dissect the specimen:

1) Extend the initial incision on the ventral side toward the posterior end through segment aVII and towards the anterior end through thoracic segment II (tI; Cuts 2 & 3 in Figure 3).
2) Using a sharp scalpel, disconnect the posterior end just past the seventh abdominal segment (Cut 4 in Figure 3).

3) Disconnect the anterior end between the first and second thoracic segment (tI & tII), with caution to not slice through the cephaloskeleton. (Cut 5 in Figure 3).

4) Remove the cephaloskeleton using scalpels and micro forceps (Bioquip, Micro Dissection Kit 4761) to separate the connective tissue.

5) Cut the cephaloskeleton along the dorsal side to allow the specimen to be butterflied and spread on the slide (Figure 4). The cephaloskeleton will be fragile and should be handled gently to avoid damaging important lateral segments.

6) Both the cephaloskeleton and the larval exoskeleton are butterflied and mounted onto the same slide using a PVA mounting medium (Bioquip, PVA Mounting Medium 6371A) and a cover slip (Bioquip, Square Cover Slips 6341B). As the mounting medium dries, add weight to the cover slip to keep the parts spread apart. (Figure 5).

Results and Discussion

This technique was developed from the need to have a standard protocol to prepare specimens for analysis using geometric morphometrics. Specimens processed using this method are butterflied neatly on the slide and maintain the original features of interest. Following the clearing and mounting procedure outlined above will result in a specimen mounted on a slide with minimal loss of two-dimensional diagnostic features. Although the repeatability depends on the skill of the dissector, the geometric morphometric tools available are able to ignore things such as rotation, translation, and scaling issues [Slice 2007]. This means that as long as both sides of the cephalopharyngeal skeleton are preserved without damage on the slide, the investigator can perform various types of statistical analysis on them (e.g. – generalized Procrustes analysis, principal components analysis). We recommend that anyone trying to use shape analysis on these parts, analyze the hooks separately from the rest of the cephalopharyngeal skeleton. Since mouth

hooks and the rest of the skeleton are not connected by a solid structure, but rather by tissue that dissolves during clearing, mouth hook and pharyngeal sclerite do not maintain the same relative configuration during the clearing and mounting process and thus should not be analyzed together. This standard protocol for preparing specimens, allows multiple questions to be asked of the same specimen. Even though we are working on specimen identification for forensic entomology, geometric morphometrics is able to provide phylogenetic [Polly *et al*. 2013], evolution [Brusatte *et al*. 2012], and feeding behavior [Meloro 2011, Meloro *et al*. 2015] answers to questions as well. Once the specimen is preserved as described in this study, all that is needed for analysis is an image of the mounted specimen with a ruler for scale. Using this standard protocol also simplifies the ability to share data with collaborators, since each collaborator can prepare their own specimens from their regions and the share the images with anyone else.

Even if geometric morphometrics is not the end goal, the clearing technique provides a procedure that aides larval specimens identification. The potassium hydroxide solution clears the specimen's cuticular layer and disintegrates the fatty tissue that normally obstructs the view. Once the specimen is more transparent, the important diagnostic features (e.g. – mouth hooks, anterior and posterior spiracles) can be viewed without taking the specimen apart. (Figure 6). The dissection process we describe is delicate, and results varied between our first samples and those produced after practicing the procedure (Figures 7-8). We recommend users learn on disposable specimens before beginning the work with research-grade or casework samples.

This protocol was developed to provide a standard for scientists utilizing geometric morphometrics on larval Diptera. Shape analysis is conducted on images, so any deviation between specimens limits the possibility for comparison to other specimens. Larvae preserved with this technique could be useful across labs as well; images can be uploaded to a GenBank-like database

[Benson *et al*. 2017] for anyone to access and use to increase confidence in their identification. Although this method is destructive to the individual pieces of the specimen, key features used for identification are preserved. Notably, the internal contents of the dissected larvae can also be stored and could be useful for molecular identification. Further research is needed for this to be the verified.

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Figures and Tables

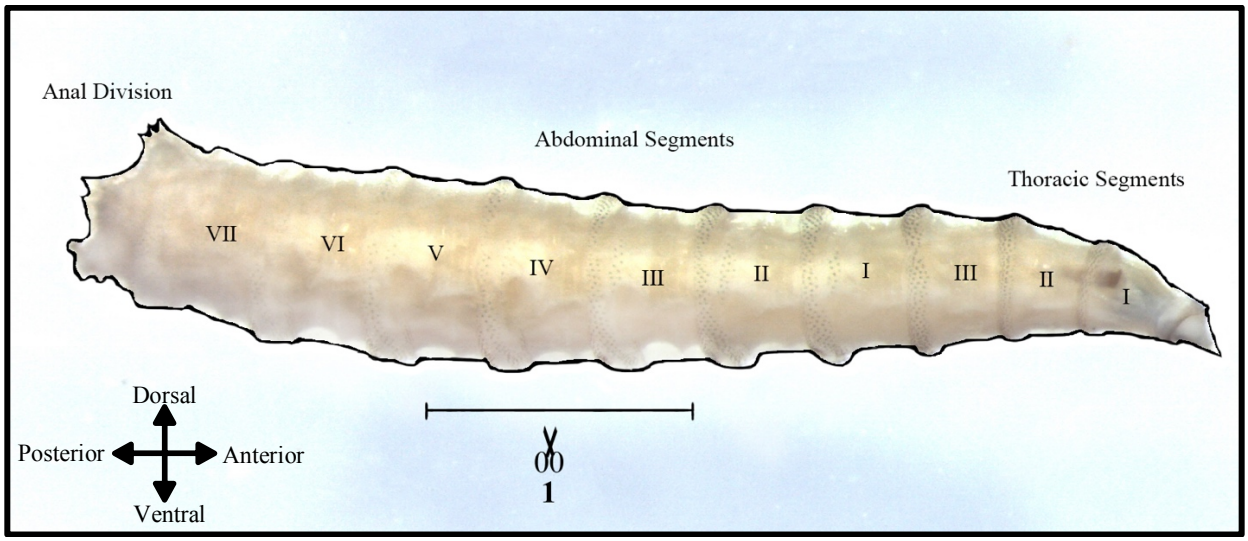

Figure 2: An example of a Dipteran larva showing the location for Cut 1.

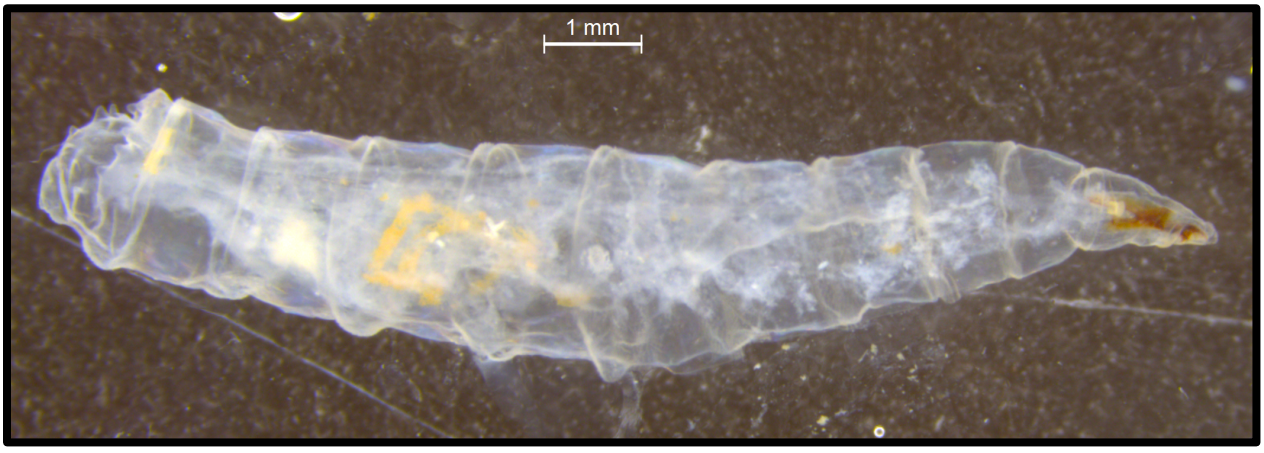

Figure 3: Third instar *Phormia regina* larva, cleared using this protocol.

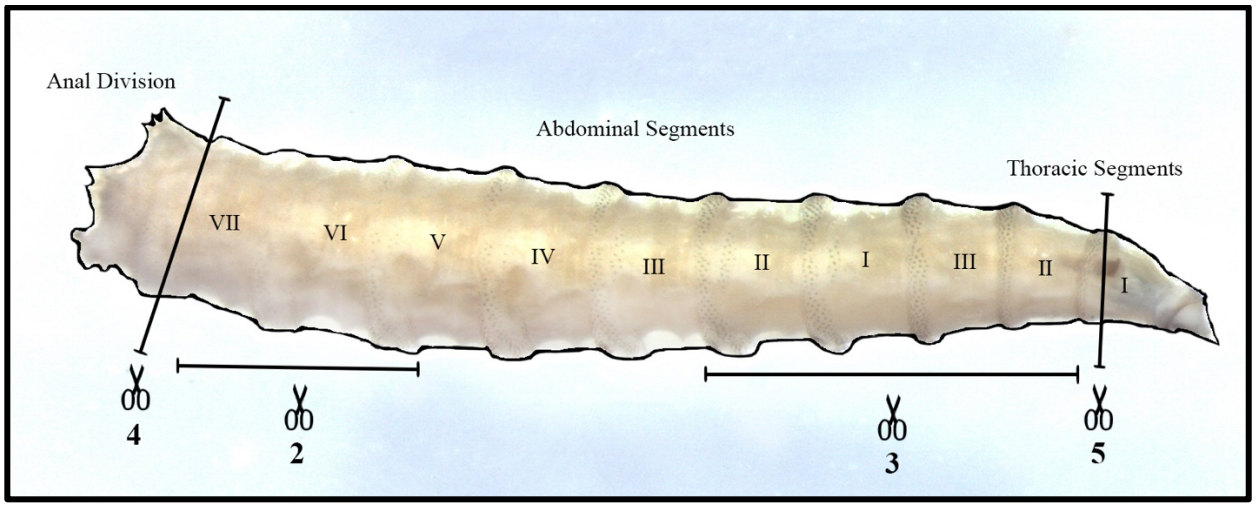

Figure 4: An example of a Dipteran larva showing the locations for Cut 2-5.

Figure 5: Butterflied cephaloskeleton of a 3rd instar *P. regina* larva on a slide with a scale.

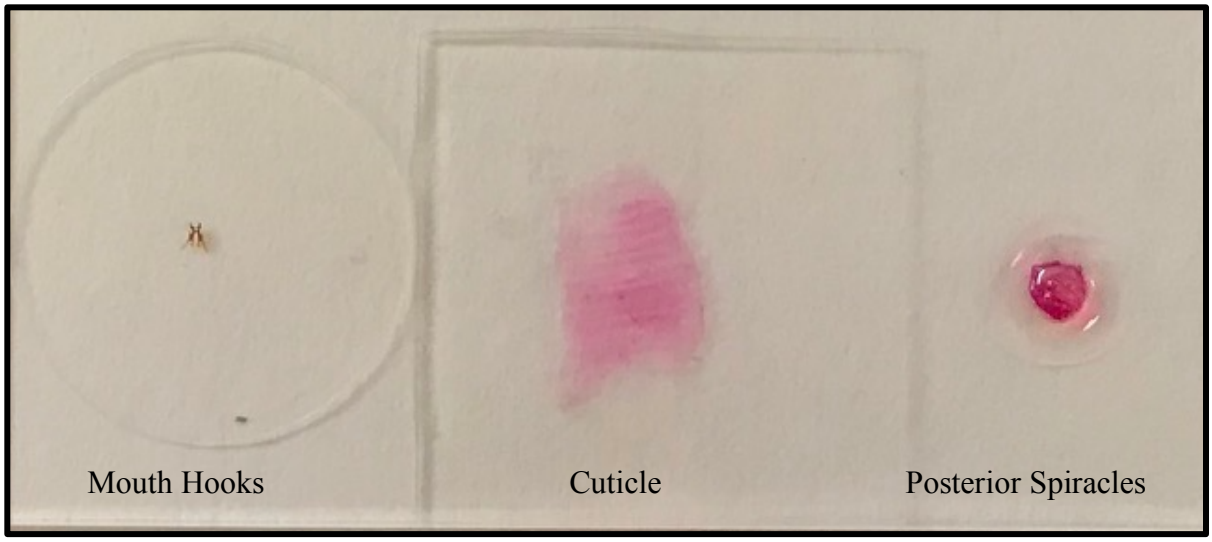

Figure 6: An image of a slide with the mouth hooks, the cuticle and the posterior spiracle preserved. An example of how all pieces are preserved on the same slide.

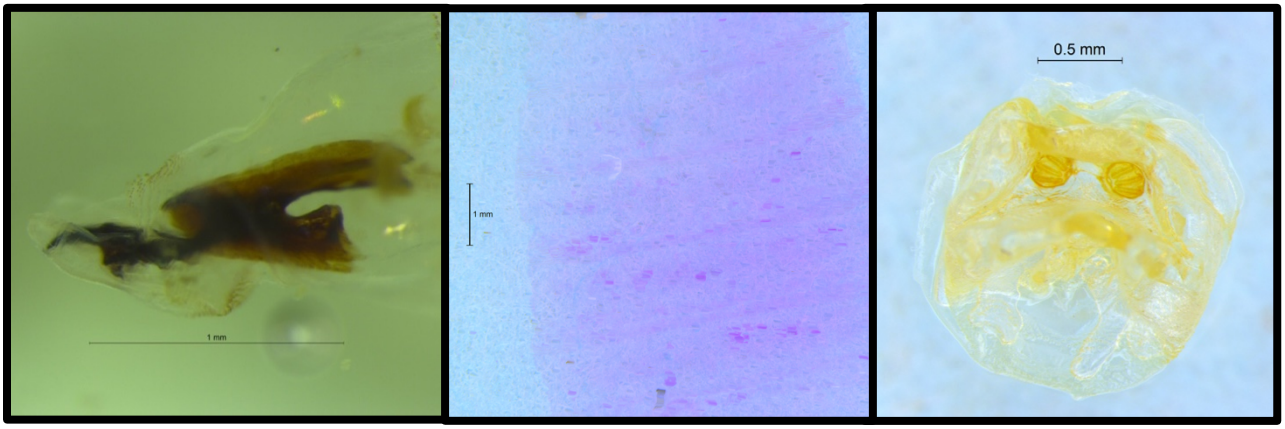

Figure 7:An image of the mouth hooks, lateral spines (stained with Lignin Pink), and the posterior spiracles after the clearing process.

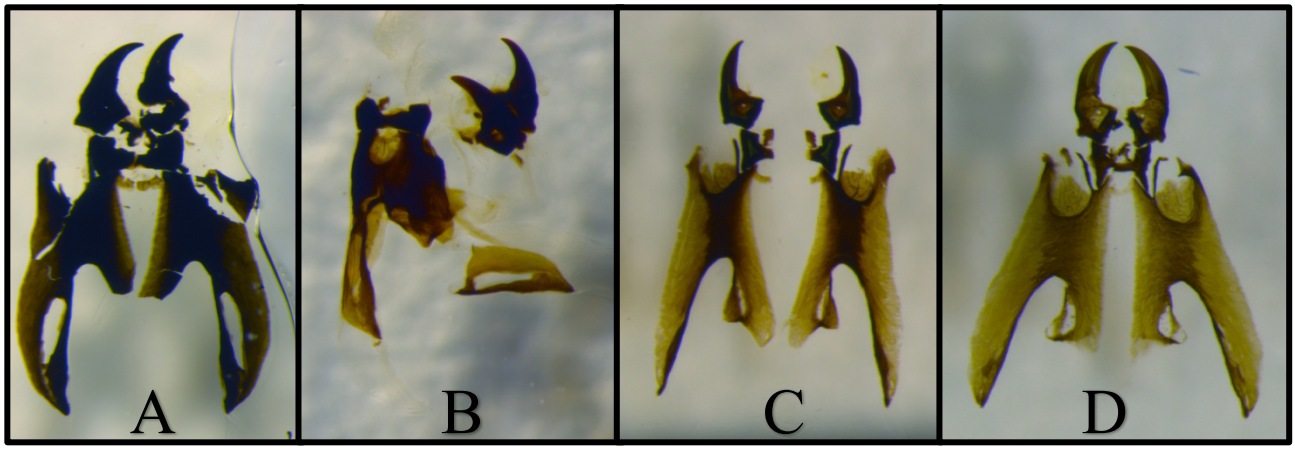

Figure 8: A and B are examples of *S. bullata* samples that were damaged during dissection that cannot be used for analysis. C and D are examples of more recent dissections: C is a *L. sericata* and D is a *P. regina*. C and D are good examples of what kind of dissection is needed for analysis.

CHAPTER 3. GEOMETRIC MORPHOMETRICS ANALYSIS OF THIRD INSTAR LARVAE OF BLOW FLIES (DIPTERA: CALLIPHORIDAE) FOR FORENSIC IDENTIFICATION

Introduction

Background

The current problems with identification of insects for forensic investigation were discussed in chapter one. To summarize, there are three main issues that forensic entomologists face: current morphological keys are not all inclusive, processing is time and resource consuming, and lacks confidence intervals for each identification. Geometric morphometrics was discussed as a means to overcome these issues. This chapter focuses on how geometric morphometrics can be applied to forensic entomology.

Geometric Morphometrics

In the simplest of terms, geometric morphometrics is shape analysis [Rohlf and Marcus 1993]. Morphometric software detects shape variations between a selected set of landmarks, even those that microanalysis with the human eye cannot discern. Studies on shape analysis have many potential applications, such as: comparing evolutionary shape changes [Wiley *et al*. 2005], wing venation in flies for identification [Grzywacz *et al*. 2017, Hall *et al*. 2014, Sontigun *et al*. 2019], and even skull shape in humans to determine if there is any variation [Maass and Friedling 2019]. Even though my study uses the mouth hooks of dipteran larvae, there may be other larval features that can also distinguish between specimens, such as the distribution of spines on the exoskeleton or the shape of posterior spiracle. The application of statistical shape analysis to different fields are endless. For example, shape variation can be applied to tooth/mandible of pig carcasses to

distinguish wild boars versus domesticated pigs [Owen *et al*. 2014]. Geometric morphometrics has been used to differentiate species of flies based on the different shapes of wing venation [Slice 2007, Sontigun *et al*. 2017, Grzywacz *et al*. 2017] and larval cephaloskeleton [Nuñez and Liria 2016]. However, the study by Nuñez and Liria [2016] of the cephaloskeleton was performed on different species, and using a different method of preparation, so the results cannot be compared to this study. As mentioned in the previous chapter, some keys have already incorporated morphometric criteria such as the ratio of feature size or distances between features [Szpila 2010, Wells *et al*. 1999]. However, those keys are prone to human error, due to the ambiguity of the features. Using geometric morphometrics, the only decision left to humans is where to place landmarks. The rest of the comparison and analysis is done by comparison of coordinates of landmarks without operator prejudice. Tests vary depending on what the user wants to see from the data: if analyzing bilateral asymmetry, Procrustes ANOVA is appropriate [Klingenberg 1998]; determining which specific feature has the most shape variation requires a principal components analysis [Rohlf 1993B]; while testing how an unknown sample compares in shape to a set of pre-identified samples utilizes canonical variates analysis [Klingenberg and Monteiro 2005]. Software transforms the landmarks into a coordinate system and calculates variation from the average shape of the feature [Klingenberg 2013]. For the majority of geometric analyses the software works from an average of size or shape, and gives results showing how the size/shape of specimens fluctuates from those averages [Klingenberg 2013].

Once landmarks are selected, Procrustes Superimposition orients and aligns the specimens to the same position and scale [Rohlf and Slice 1990]. Procrustes Superimposition ensures that any variation analyzed is due to difference in shape of the specimen, instead of differences due to specimen orientation on the slide or specimen size [Zelditch 2012]. This Procrustes method

accounts for mirror imaging, scaling, superimposition, and rotation differences in each specimen, since those are not caused by differences in shape [Klingenberg 2015]. For the purpose of shape comparison, size is seen as a variable to be standardized and the best way to accomplish this is to scale all the specimens as if all were the same size [Slice 2005]. Scaling is important to view shape variation within a group, since some specimens might be larger than others within the same species. This means that size is not directly a factor of shape and can be ignored, as specimens are all rescaled using a centroid size [Slice 2005]. Centroid size is calculated for each specimen as a marker of size from the square root of the sum of squared distances from a set of landmarks to the centroid [Stegmann and Gomez 2002]. Principal component analysis (PCA) is used to show the individual components of shape variation attributed to each of the measured components [Rohlf 1993]. PCA is used in geometric morphometrics to determine the direction of shape changes based on each principal component, as eigenvectors. Each eigenvector has its own eigenvalue, as a result for each principal component [Pavlicev *et al.* 2009]. Canonical variates analysis is used to find the best combination of distinguishing features between known groups [Zelditch 2012]. This method alone should not be used to classify individual specimens, unless previous identification has already grouped specimens under analysis [Klingenberg and Monteiro 2005]. Much like a discriminate function, canonical variates analysis in MorphoJ [Klingenberg 2011] performs pairwise analysis of the given groups, providing statistical significance between the shape comparison of each of the groups tested against each other. The aforementioned tests are useful for determining shape variation between sets of specimens but before testing for any shape variation, one first needs to account for symmetrical components.

Symmetrical Components

Symmetry can be found on many different objects in nature [Slice 2005]. There are two types of symmetry discussed in morphometrics: object symmetry and matching symmetry [Klingenberg 2002]. The symmetrical component that splits the human face down the middle displays object symmetry. Object symmetry is used when an object can be split down the middle and the landmarks on one side line up to the landmarks on the other side of the specimen [Klingenberg 2002]. Matching symmetry is symmetry between two parts that look and act the same way although might not be analyzed together [Klingenberg 2002]. An example of matching symmetry is wing venation. Wings are connected through the body but they are truly independent from each other and can be analyzed by themselves or compared to each other. Geometric morphometric research done on wing venation of flies should start with the comparison of the left and right wings to each other to test for directional asymmetry [Klingenberg 2015]. Directional asymmetry is a feature of bilateral symmetry which shows variation that is present, and always towards a specific side. An example of directional asymmetry is when all left wings have two landmarks closer that connect at a shorter distance than the right wing. If there is directional asymmetry, then all the groups being studied should be compared on both sides. However, if there is no significant shape variation between the sides, then one side can be selected to study the shape variation among different classification of flies.

For studies of asymmetry, a two-factor ANOVA (Procrustes ANOVA) is recommended to calculate the shape variation between the coordinates of the specimens superimposed by the Procrustes method [Rohlf and Slice 1990]. The main effect between specimens tests the level of individual shape variation (e.g. some mouth hooks are sharper than others) [Klingenberg 2015]. The main effect from the sides tests for directional asymmetry (e.g. – the right hooks tend to be

thinner than the left hook) [Klingenberg 2015]. The interaction of the individual and side test for levels of fluctuating asymmetry [Klingenberg 2015]. In order to test shape variation based on classification or due to asymmetry, a broad number of specimens need to be compared to ensure that any variation isn't due to a local change in morphology. There are many factors that might influence shape, such as: sex, geography, local food, and inter-vs-intra specific variation.

This project could not test every factor that might influence shape variation, so I chose to focus on testing the shape variation between the sides of the mouth hooks and the variation based on the different genera and the different locations available. I hypothesize that there is no difference in shape between the left and right mouth hooks, based on the symmetrical larval development of the cephaloskeleton. I also hypothesize that there will be significant variance in shape between the different genera tested, since they display other morphological features that are different as well. Due to the use of morphological keys for the same species throughout North America, I hypothesize that location differences will not influence any significant shape variation. Given these hypotheses, the next steps are to establish which genera to use and from which location, then select which landmarks should be used.

Specimen and Location Selection

Given the potential for variation between single specimens it is important to perform these types of analysis on a set of individuals specimens representing each group. I chose three different genera in the blow fly (Diptera: Calliphoridae) family: *Calliphora* Robineau-Desvoidy (1830), *Lucilia* Robineau-Desvoidy (1830), and *Phormia* Robineau-Desvoidy (1830). These genera were selected because they represent commonly found flies in forensic entomology in North America [Byrd and Castner 2009]. Therefore, should this method work with them, then this trial can be expanded to include different genera and even try to go down to species level classification. An

outgroup of thirty *Sarcophaga bullata* (Parker, 1916) were selected, since they are from a different family of Diptera but are still forensically important [Byrd and Castner 2009]. To overcome potential geographic variation, three locations which contained the aforementioned fly populations were chosen to represent a sample across the United States: Dover, DE; West Lafayette, IN; and Davis, CA. These three locations are similar in latitude and vary from the east coast to the west coast, which provide a central and two longitudinal extremes in North America. With the specimens selected, the next step is to determine which landmarks to use.

Landmark Selection

There are three types of landmarks that have been designated by Bookstein [1997]. Type 1 landmarks are discrete juxtapositions of tissues and require the meeting of three different structures [Bookstein 1997]. Type 2 landmarks are designated as maximum curvatures or other local morphogenetic processes, which can be the tips of claws and teeth or tips of bony processes where muscle attachments occurred [Bookstein 1997]. Type 3 landmarks are defined as extremal points, or points that have at least one deficient coordinate, which account for the widest diameters or bottom of any concavity [Bookstein 1997]. In addition to the original three types of landmarks, Bookstein [1997] discussed a fourth type: semi- (or pseudo) landmarks. These landmarks are not based on biological features but based on mathematical spacing or locations between landmarks.

Landmark selection is very important due to the fact that the landmarks must include points that are present in and representative of every specimen analyzed. While traditional taxonomy would rely heavily on the presence or absence of features, geometric morphometrics relies on features that are present in every specimen [Zelditch 2012]. Any feature that sticks out and makes one specimen easier to identify can only be included if all other specimens also have that feature. This means that autapomorphies should be avoided when working with landmark morphometrics,

due to the fact they would create a different number of landmarks between a specimens that did display a feature versus one that did not. Slice [2007] explains that research using semi landmarks on shape outlines is rare because of the lack of software available to work from only semi landmarks. Due to this dilemma and how soft and malleable the pharyngeal sclerite became after the clearing process, this project focused on the shape variation between the mouth hooks themselves versus the whole chephalopharyngeal skeleton.

Selection of the data (images and landmarks) to be studied should be well established before beginning data analysis, since it is hard to re-digitize all the landmarks on the specimens after the analysis has started. My project tests three things: 1) asymmetry between left and right mouth hooks, 2) patterns of variation based on centroid size, and 3) patterns of variation based on shape differences.

Methods

Specimen Collection and Preparation

I collected larvae from West Lafayette, IN using chicken baited traps or decomposing pig carcasses. Once collected the larvae were reared to adulthood until they were identified, then split into independent colony boxes by species. Colonies were maintained according to chapter 2, with beef liver used for oviposition. Once the flies oviposited on the beef liver, eggs were removed from the colony box and raised in a smaller container until they reached the wandering phase of the third instar. At that point the larvae were parboiled in sub boiling water, then preserved in <80% ethanol. The outgroup chosen for comparison were *Sarcophaga bullata* (Parker, 1916), which are kept in colony at Purdue University. Table 1 shows how many of each specimens were able to be used for analysis, since some were either damaged or not enough were available from the collection.

Larvae received from Davis, CA were collected by Robert Kimsey's Lab, reared in colony, and preserved in the same way and shipped to West Lafayette for the next steps. And larvae from Dover, DE were handled in the same procedure by Krystal Hans' Lab, with the exception of *Phormia regina* and *Lucilia sericata* which were not reared in colony, but collected and preserved from the field and identified once received in West Lafayette, using Szpila's [2010] larval key.

All larvae were then prepared according to the specimen preparation protocol established in chapter two. Following the clearing, the mouth hooks were dissected and preserved as described in the dissection protocol of chapter two, with the only modification that the rest of the larval body was preserved in ethanol for future molecular identification.

Photography

Images were acquired on a Leica DMC 2900 z-stacking microscope (Leica Microsystems™; Wetzlar, Germany) with a 3.1-megapixel camera to ensure the images were completely in focus. A 5mm micro ruler (TDI International Inc.; Tucson, Arizona) was placed on the slide to provide a scale for the image. The Leica Application Suite software (https://www.leica-microsystems.com/products/microscope-software/p/leica-application-suite/) also provided an extra scale on the image as well as an image label. The files were saved as high-resolution Tagged Image File Format (TIFF; extension: .tif) with lossless compression of the files.

Landmark Selection

Landmarks were digitized on both the right and the left mouth hook of most of the specimens, with some specimens having to be excluded due to a missing or damaged mouth hook (Table 1). Figure 9 shows the selected landmarks for the right mouth hook and those landmarks

were mirrored for the left hooks. Different types of landmarks were selected based on the features present on all of the mouth hooks (Table 2).

Software Usage

Once the images were acquired on the microscope, the files were saved as a single TPS file, so the landmark software could read it. I used tpsUTIL32 [Rohlf 2004] to create an image path so all images could be accessed by the digitization program. The tpsDIG2w32 [Rohlf 2001] software was used to digitize the landmarks onto the images. I selected where to add each landmark and ensured that all images had the same number of landmarks, because the number of landmarks must be equal on all sample for analysis. For each image digitized on tpsDIG2w32 [Rohlf 2001] I also selected two points on a 5mm ruler that was photographed with each specimen, so the software had a scale for the measurements.

MorphoJ [Klingenberg 2011] was used for all analysis due to the variety of tests the software offers. Once all of the landmark coordinates were uploaded on to MorphoJ, I put in all the classifiers (location, genus and species) for each specimen. The software also needed to know whether the data is two dimensional or three dimensional, and whether there is a symmetrical component to it.

Statistical Analysis

Through the preliminaries menu in MorphoJ, a Procrustes Superimposition was performed which aligned the data by principal axis and provided three data matrices: raw coordinates, centroid size calculations for all specimens, and Procrustes coordinates.

Procrustes ANOVA were performed to compare between the samples. A Procrustes ANOVA is similar to a two-factor ANOVA which calculates the shape variation between the

Procrustes coordinates of the specimens [Rohlf and Slice 1990]. For the analysis of asymmetry, the individual specimens were selected as the random effect and the side of the mouth hook for each of those specimens as the fixed effect. The random effect between specimens tested for the level of individual shape variation. The main effect from the sides tested for directional asymmetry. Fluctuating asymmetry was tested as the interaction between the random effect and the main effect for side. The other Procrustes ANOVA did not have a fixed effect for size because the left and right mouth hook data was averaged for each specimen. Procrustes ANOVAs were also used to test the effect on shape by Genus and then Location, with the residuals showing any change of shape that was not accounted for by those effects.

Various principal components analysis (PCA) were performed to test the components of shape that displayed the greatest variance for the tested groups. This type of analysis was performed on all classifications from the dataset (every genus, every location and the group as a whole). A PCA returned an eigenvalue for each principal component and a scatterplot of PC scores where shape variance among the group can be compared in space.

Much like the PCA, various canonical variates analysis (CVA) were performed on every classification of the dataset. First the specimens were grouped based on the locations and tested for variance among the genera from those locations. Then the specimens were grouped based on the genera and analyzed for variation based on the locations they came from. The dataset as a whole was also tested using a CVA.

Results

General

Two groups of specimens were selected for a preliminary study to test symmetrical variation between left and right mouth hooks. The preliminary study resulted in significant directional asymmetry between the left and right mouth hooks (p-value: <0.0001), which meant that specimens in the main study would be digitized and analyzed on both sides independently to investigate directional asymmetry.

Once both mouth hooks from every specimen was superimposed, a Procrustes distance was calculated using the square root of the sum of the squared differences between the position of the landmarks between two configurations that have been optimally superimposed based on the centroid size [Bookstein 1989]. The Procrustes fit results in an image of all landmarks observed superimposed on each other with an average calculated for each landmark (Figure 10A). Superimposition also produces a lollipop graph for each specimen with an average Procrustes coordinates for each land mark as plotted as the top and the deviation unique for each specimen as the stick (Figure 10B). The variation of each individual specimen can also be viewed as a wireframe graph, showing the averaged landmarks in light blue and the shape variation unique to each specimen in dark blue (Figure 10C).

Analysis of Asymmetry

Centroid size of Left vs. Right Mouth Hooks

Table 3 show the results of the centroid size comparison for all samples. The only effect that were significant for centroid sizes was the individual specimen component which accounts for natural differences in sizes among the specimens (p-value: <0.0001). Although there was some

slight variation in centroid size between the left (0.4444mm) and the right mouth hook (0.4432mm), the results were not significant (p-value: 0.3006). The results also show there was no significance in the directional or fluctuating asymmetry attributed based on the centroid sizes of the specimens.

Procrustes ANOVA

The results of the Procrustes ANOVA for shape, show that the largest variation was attributed to the difference in the left and right mouth hooks for each specimen (Table 4). Results were significant in both the main effects for shape variation based on the individual (p-value: <0.0001) and based on the side of the mouth hook (p-value: <0.0001). No significance was found for the interaction of the individual by side (p-value: 0.9951).

Shape Differences between Left vs. Right Mouth Hooks

A principal component analysis showed that the first three principal components accounted for 55.06% of the total variance between specimens (PC1 = 28.79%, PC2 = 13.88% and PC3 = 12.39%; Figure 11). The shape variation attributed to PC1 appears to stem from the wider base, where the tooth starts to protrude from the main hook, between landmarks 2 and 6 (Figure 12A). PC2 appears to account for the wider area of the tooth itself, between landmarks 7 and 8 (Figure 12B). And PC3 accounts to a sharper curve on the distal end of the mouth hook, shifting the location of landmark 1 (Figure 12C).

The full dataset was then analyzed using a canonical variates analysis where the left and right side were given as two separate groups. The results found a Procrustes distance of 0.0176 between the left and right mouth hooks, which was significant (p-value: <0.001). Since there were only two sides, there was only one canonical variate which attributed to all of the shape differences between the groups (Figure 13). Although there is some overlap left and right mouth hooks shape

differences attributed to CV1, they are mostly grouped together on the frequency chart (Figure 14).

Patterns of Variation Due to Size

Analysis of centroid Size

After averaging the dataset by specimen, to compensate for the directional component of variation, I performed two more Procrustes ANOVAs changing the individual effect between: genera and location. Results show that there is a significant different between centroid size and shape at all levels: genera and location (Table 5). The average centroid size for each genera is shown in Table 6.

Patterns of Variation Due to Shape

Shape Variation Split by Location

Variation due to shape was analyzed individually for each location. For specimens from California, principal components analysis showed that there was a clear difference in shape between *Lucilia* and *Phormia* (Figure 15). The first two principal components accounted for 56.08% of the total variance (PC1 = 35.64% and PC2 = 20.44%). A canonical variates analysis grouped by genera from California found a Procrustes distance of 0.0876 between the two genera, which was significant (p-value: <0.001). Since there were only two genera, there was only one canonical variate which attributed to all of the shape differences between the groups (Figure 16).

The principal components analysis for Delaware showed that the first and second principal components accounted for 61.95% of the total variance of shape (PC1 = 48.13% and PC2 = 13.82%). When plotted on a PC scores plot, the shape variation attributed to PC1 and PC2 was very useful in separating out *Calliphora* from the other two genera (Figure 17). *Lucilia* and

Phormia had some overlap. Canonical variates analysis for genera from Delaware showed Procrustes distances between all three genera were significant (Table 7). Much like the principal components analysis, canonical variates analysis scatterplot of CV1 and CV2 showed that the genus *Calliphora* was separate while some overlap was present between *Lucilia* and *Phormia* (Figure 18).

Principal components analysis for Indiana showed that the first and second principal components accounted for 47.63% of the total variance of shape (PC1 = 26.95% and PC2 = 20.67%). Since neither of the first two principal components accounted for a large majority of the shape variance, it was not surprising that the scatterplot between them showed a lot of overlap in shape variation between the three genera (Figure 19). Canonical variates analysis showed that Procrustes distances between all three genera were significant (Table 8). Even though principal components analysis showed lots of overlap, the canonical variates analysis scatterplot of CV1 and CV2 showed no overlap between any of the three genera for Indiana (Figure 20).

Shape Variation Split by Genus

The variation due to shape was also analyzed individually for each genus, to observe any variation found based on location of specimen. For the *Calliphora* specimens, principal components analysis showed that there was a clear difference in shape between specimens from Indiana and specimens from Delaware, with barely any overlap between the locations (Figure 21). The first two principal components accounted for 64.55% of the total variance (PC1 = 51.47% and PC2 = 13.08%). Canonical variates analysis with the grouping based on location found a Procrustes distance of 0.1155 between the Indiana and Delaware specimens of *Calliphora*, which was significant (p-value: <0.001). Since there were only two locations to compare, there was only one canonical variate which attributed to all of the shape differences between the groups (Figure

22). The variation found between *Calliphora* from Indiana and Delaware could also stem from the fact that two different species of *Calliphora* were used for each of those locations.

The principal components analysis for all *Lucilia* specimens showed that the first and second principal components accounted for 54.59% of the total variance of shape (PC1 = 31.45% and PC2 = 23.14%). When plotted on a PC scores plot, the shape variation attributed to PC1 and PC2 was able to separate the three locations into individual groups with little overlap (Figure 23). The canonical variates analysis with the locations set as groups showed Procrustes distances between all three genera were significant (Table 9). Canonical variates analysis scatterplot of CV1 and CV2 showed that *Lucilia* specimens from all three locations could be separated based on their shape profiles, with minor overlap between California and Indiana (Figure 24).

The principal components analysis for all *Phormia* specimens showed that the first and second principal components accounted for 44.77% of the total variance of shape (PC1 = 26.47% and PC2 = 18.30%). The scatterplot of PC scores showed a lot of overlap, which is expected since neither of the first two principal components accounted for a large percentage of shape variance (Figure 25). The canonical variates analysis showed Procrustes distances for the *Phormia* specimens were only significant for specimens from Delaware compared to Indiana (p-value: <0.0001) and Delaware compared to California (p-value: <0.05; Table 10). The canonical variates analysis also showed almost no overlap for the *Phormia* specimens from any of the locations (Figure 26).

Shape Variation for Genera based on Full Dataset

A principal component analysis showed that the first three principal components accounted for 60.27% of the variance (PC1 = 32.91%, PC2 = 15.56% and PC3 = 11.80%; Figure 27). The

scatterplot from PC1 and PC2 show the amount of variation between the genera tested based on the first and second principal components, which account for 48.47% of the total variation (Figure 28). There are large amounts of variation within each genus, and it fluctuates in both directions of PC1 and PC2.

Figure 29 shows the variation attributed to each of the first three principal components. The shape variation attributed to PC1 appears to stem from the wider base, where the tooth starts to protrude from the main hook, between landmarks 2 and 6. PC2 appears to account for the wider area of the tooth itself, between landmarks 7 and 8. And PC3 accounts to a sharper curve on the distal end of the mouth hook, shifting the location of landmark 1.

The results at the genus level for the full dataset were displayed in a scatterplot of on the first two canonical variates (CV1 and CV2), since they accounted for about 91.91% of the total variation (CV1=60.81% and CV2=31.10%). The four genera each appear to be split into their own grouping with the closest being *Lucilia* and *Phormia* which showed some overlap, and the farthest being *Sarcophaga* which had no overlap with any other genus (Figure 30). Table 11 shows the statistical significance of pairwise differences in the mean shapes of the specimens, using Procrustes distances.

Discussion

I hypothesized that there was no difference in shape between the left and right mouth hooks, based on the symmetrical larval development of the cephaloskeleton. However, the analysis of asymmetry found that there is a significant difference (p-value: <0.0001) in shape based on the side of the mouth hook (Table 4). The analysis failed to find any significance between the sizes of the left or right side (Table 3). This established that when analyzing shape in the future, both mouth hooks should be analyzed to avoid missing any variation due to directional asymmetry. With that

in mind, I then averaged each specimen by its left and right hook to continue the analysis of shape variation.

Analysis of variation due to size showed that there was significant difference between centroid sizes of all classifications based on genus and location (Tables 5 & 6). Although there was a significant difference in centroid size, we cannot use size as a means for identification due to the large size range found in each classification set. In general, specimens from one genus will tend to fall within the same size range but when dealing with unknown specimens we cannot know for sure if the specimen being tested wasn't an abnormally larger specimen for that genus.

I also hypothesized that there would be significant variance in shape between the different genera tested, since they display other morphological features that are different as well. The principal components analysis performed on samples split by locations, found that there is a significant variance in shape between genera from each location (Figures 15, 17 & 19). Although the two genera from California and the three genera from Delaware showed a significant difference in the way shapes changed within each location, the two genera from Indiana had considerable overlap. However, the number of *Phormia* specimens from California (n=11) and the number of samples of *Calliphora* (n=12) and *Phormia* (n=18) from Indiana were relatively low and thus cannot be used to draw conclusions from the set. Once specimens were defined by the genera for the canonical variates analysis, the test found specific changes in shape due to the morphology of each genera (p-values: <0.05; Figures 16, 18 & 20). The implications of these results are that even though the way that shape changes varies among specimens from each genus, when the genus is known for each specimen, it will be able to calculate average shape changes for each classification and in turn determine what genus an unknown specimen from each location would be classified to.

Similar results were found when the tests were switched. Here, I hypothesized that location differences will not influence significant shape variation since the same morphological keys to these species are used throughout North America. The analysis testing shape differences at location when split by genera, also found that there was some overlap in the way shape changed among specimens from the same genus (Figures 21, 23 & 25). These results can be inferred as changes due to location. However, once data was organized by location, canonical variates analysis was able to distinguish between the sets based on shape variation due to the location (Figures 22, 24 & 26). The implication of a difference in shape based on locality is that in the future we might be able to determine not only specimen identification but also where a specimen came from. However, at present data might not be strong enough to draw conclusions, due to the limited number of samples of *Phormia* from California (n=11) and Indiana (n=18).

Analysis of the full dataset showed similar results to the independent studies. There was significant variation based on the principal components found (Figures 28 & 29). Once the genera were selected as identifiers, canonical variates analysis was able to distinguish between all of them, although there was some overlap between *Lucilia* and *Phormia* specimens (Figure 30). Although all four genera were distinguishable based on their shape variation, there are limitations to the conclusions we can draw due to the small number of specimens available for certain genera or certain locations.

It is important to follow an established protocol as described in chapter two for specimen preparation so that data can be compared between collections. As Nuñez and Liria [2016] analyzed different species than those analyzed for this project, comparison between these two is not possible since critical analytical steps were different. Geometric morphometrics requires preparing specimens and labeling landmarks in the same manner across all compared specimens. Nuñez and

Liria [2016] differed from the current study in both the preparation and the choice of landmarks. Due to the lack of a common protocol, the two studies differed enough that they could not be compared.

I believe the next step is to increase dataset size to include more specimens from additional different locations in North America. An increase in sample size should also be accompanied by an increase in species diversity within each genus in order to test this method for species identification. Communication between collaborators is key for the increase in diversity of specimens, to ensure that everyone is following the same protocols established. If species identification becomes possible using geometric morphometrics, this could revolutionize the field of forensic entomology.

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Figures and Tables

Table 1: Total number of specimens collected and used for this study, from each location.

Location	Species	Specimens Collected	Specimens used for Analysis
Davis, California	*Phormia regina*	20	11
	Lucilia sericata	30	29
	Calliphora vicina	--	--
Dover, Delaware	*Phormia regina*	31	31
	Lucilia sericata	31	30
	Calliphora vicina	30	29
West Lafayette, Indiana	*Phormia regina*	25	18
	Lucilia sericata	27	25
	Calliphora vomitoria	13	12
Lab Grown (Carolina.com)	*Sarcophaga bullata*	30	26
Total Specimens		237	211

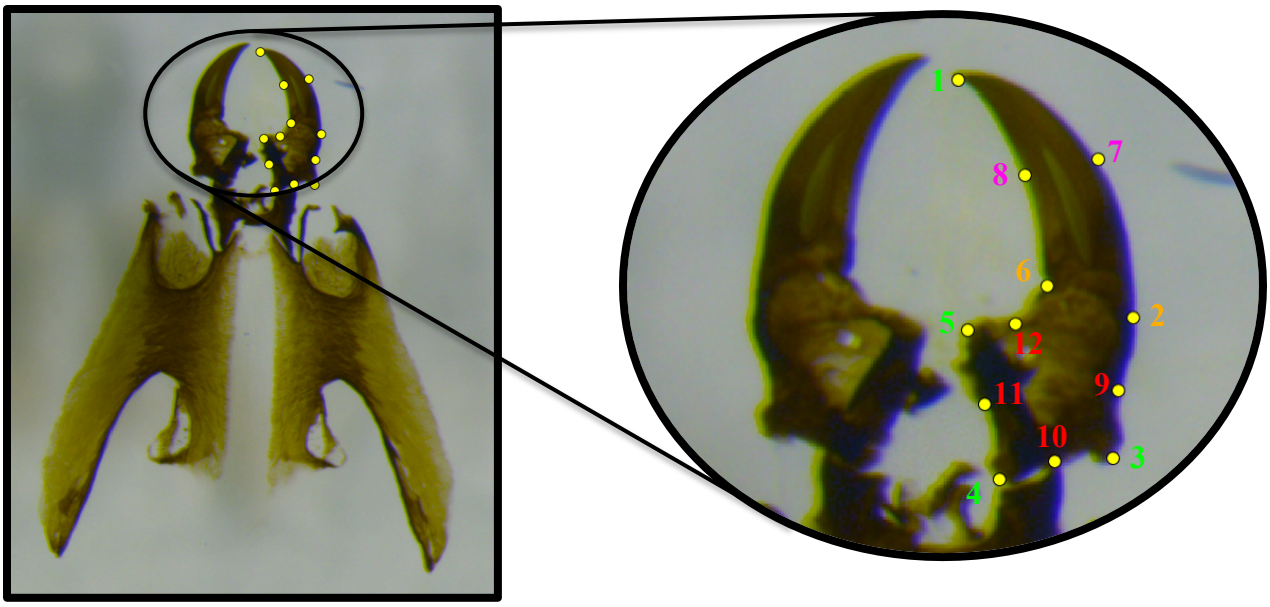

Figure 9: Landmarks selection on larval mouth hooks for geometric morphometric analysis. Type 1 in orange, type 2 in green, type 3 in pink and semi-landmarks in red. Types of landmarks based on Bookstein (1997).

Table 2: Description of landmarks on larval mouth hooks for geometric morphometric analysis. Types of landmarks based on Bookstein (1997).

Landmark Number	Landmark type	Landmark Description
1	2	The sharpest curvature of the apical mouth hook
2	1	Dorsal histological point of connection between the tooth and the main body of the mouth hook
3	2	Dorsal posterior apodeme of mouth hook
4	2	Ventral posterior apodeme of mouth hook
5	2	Ventral anterior apodeme of mouth hook
6	1	Ventral histological point of connection between the tooth and the main body of the mouth hook
7	3	Extremal point on the dorsal side of the hook
8	3	Extremal point on the ventral side of the hook
9	semi	Equidistant point between landmarks 2 and 3
10	semi	Equidistant point between landmarks 3 and 4
11	semi	Equidistant point between landmarks 4 and 5
12	semi	Equidistant point between landmarks 5 and 6

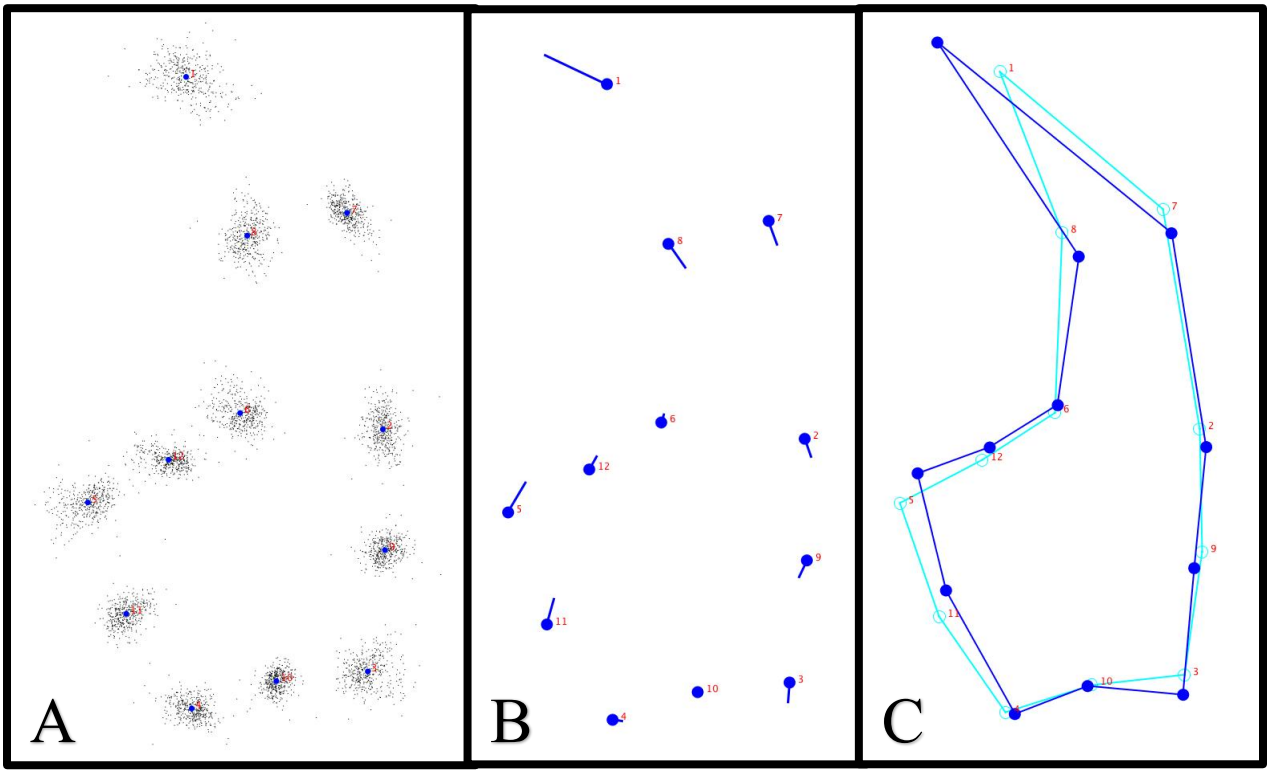

Figure 10: Different types of plots from a Procrustes superimposition of the full dataset displaying: (A) the Procrustes superimposition of all specimens with the mean landmark positions plotted in blue, (B) a lollipop graph showing unique shape variation of a single specimen, and (C) a wireframe graph showing the unique shape variation of a single specimen.

Table 3: Procrustes ANOVA results of centroid size comparison for all specimens.

Centroid Size - Individual					
Effect	Sum of Squares	Mean Squares	Degrees of Freedom	*F* statistics	*P*-values
Individual	0.965584	0.004620	209	30.29	<0.0001
Side	0.000164	0.000164	1	1.08	0.3006
Individual x Side	0.015940	0.000076	209	0.08	1.0000
Residual	0.003607	0.001804	2		

Table 4: Procrustes ANOVA results for shape variation for all specimens

Shape, Procrustes ANOVA:							
Effect	Sum of Squares	Mean Squares	Degrees of Freedom	*F* statistics	Parametric *P*-values	Pilai's trace	Parametric *P*-values
Individual	2.63553915	0.0006305118	4180	3.72	<0.0001	13.53	<0.0001
Side	0.03252164	0.0016260821	20	9.53	<0.0001	0.64	<0.0001
Individual x Side	0.70772923	0.0001693132	4180	0.60	0.9951		
Residual	0.01135234	0.0002838086	40				

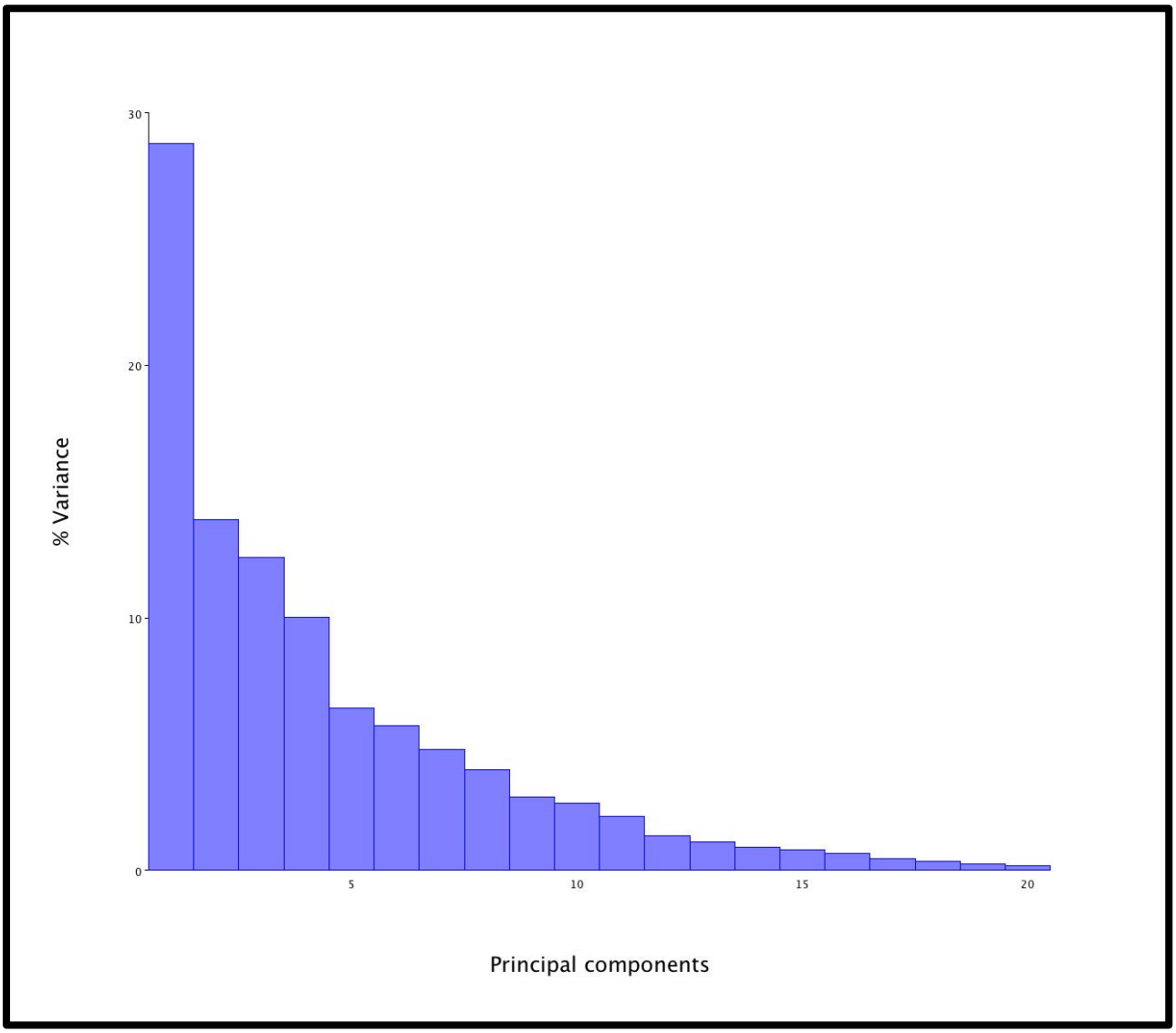

Figure 11: PCA Eigenvalues based on shape difference for all specimens. Each specimen landmark data for both left hook and a right hook, separately.

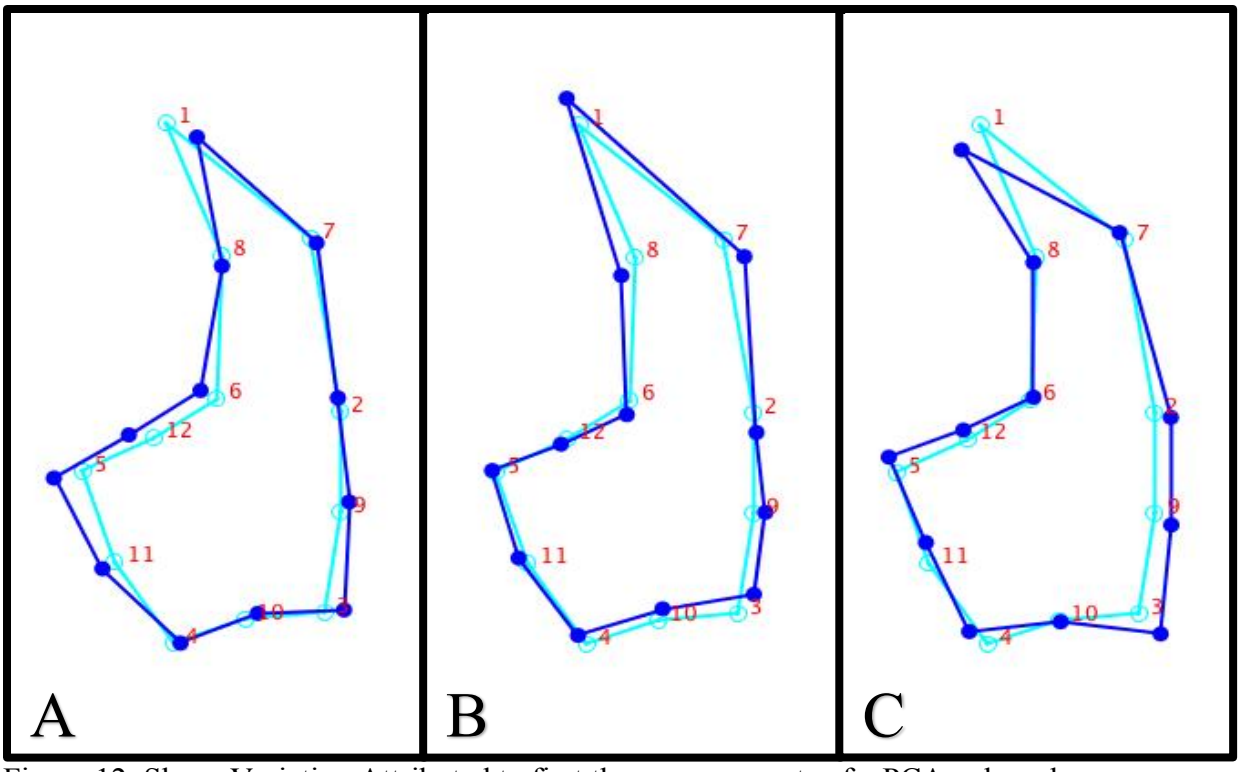

Figure 12: Shape Variation Attributed to first three components of a PCA, when shape was compared between all specimens, left and right hooks separately. (A) PC1, (B) PC2, and (C) PC3. Mean is displayed in light blue with the variation in dark blue.

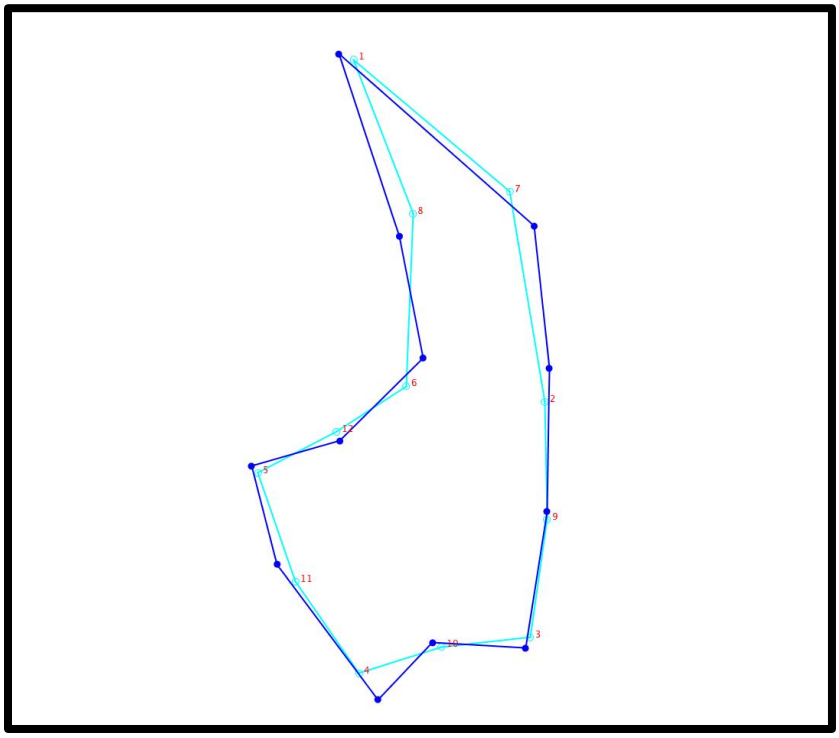

Figure 13: Shape differences based on side of mouth hook, from a canonical variates analysis separating left and right hooks. Mean is displayed in light blue with the variation in dark blue.

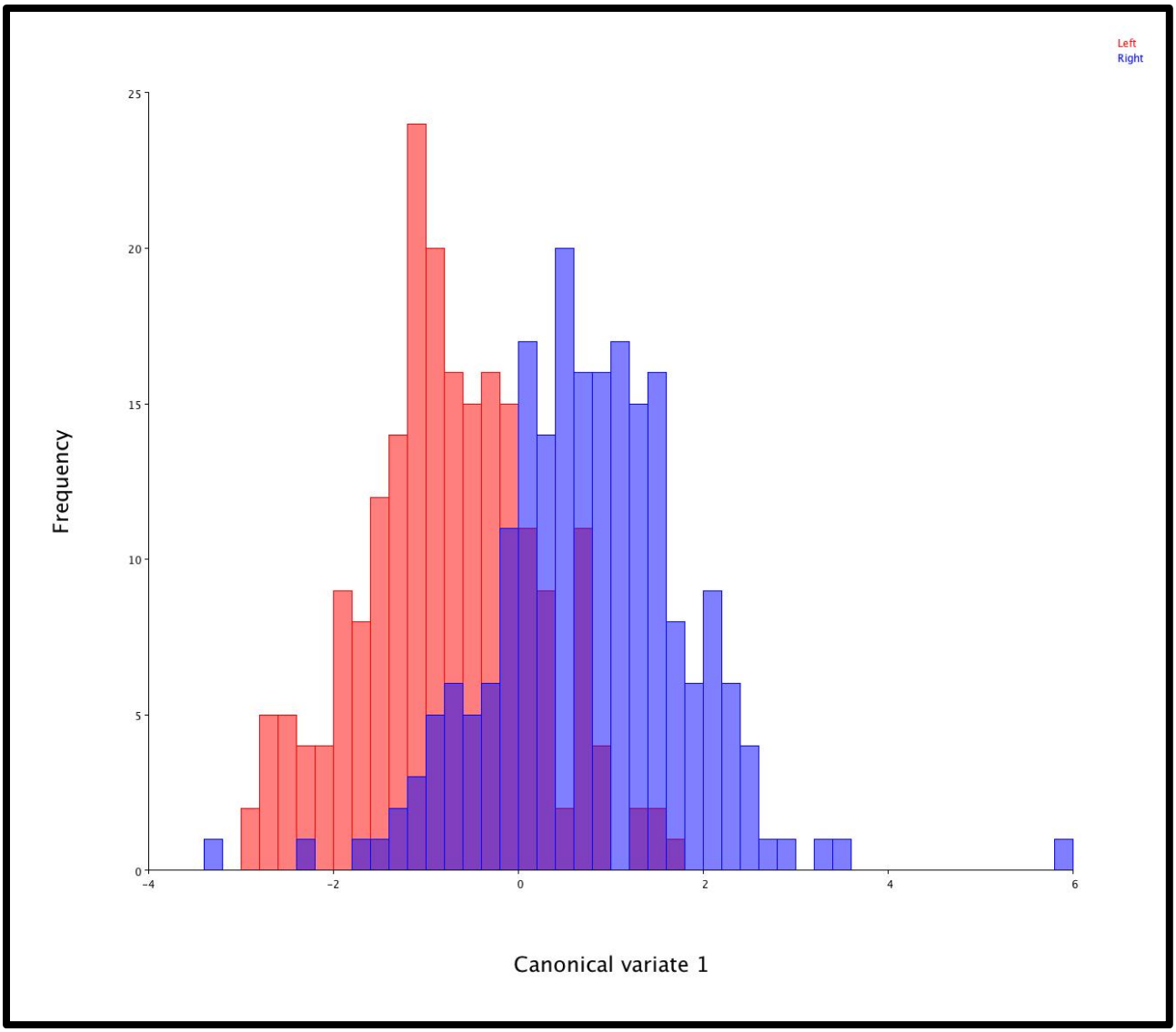

Figure 14: Canonical variates analysis frequency scores for CV1 grouping based on left (red) and right (blue) mouth hooks.

Table 5: Centroid size comparison for all specimens (averaged by left and right mouth hooks)

Centroid Size – Genera					
Effect	Sum of Squares	Mean Squares	Degrees of Freedom	*F* statistics	*P*-values
Individual – Genus	0.175366	0.058455	3	39.17	<0.0001
Residual	0.307390	0.001492	206		
Centroid Size - Location					
Effect	Sum of Squares	Mean Squares	Degrees of Freedom	*F* statistics	*P*-values
Individual – Location	0.274295	0.091432	3	90.35	<0.0001
Residual	0.208461	0.001012	206		

Table 6: Average centroid sizes (in mm)

Average Centroid Size (in mm)					
	All Locations	Delaware	Indiana	California	Lab Grown
All Genera	0.4438	0.4105	0.4758	0.4321	0.5094
Calliphora	0.4293	0.3895	0.5256	N/A	N/A
Lucilia	0.421	0.4	0.4511	0.4168	N/A
Phormia	0.4572	0.4403	0.4769	0.4727	N/A
Sarcophaga	0.5094	N/A	N/A	N/A	0.5094

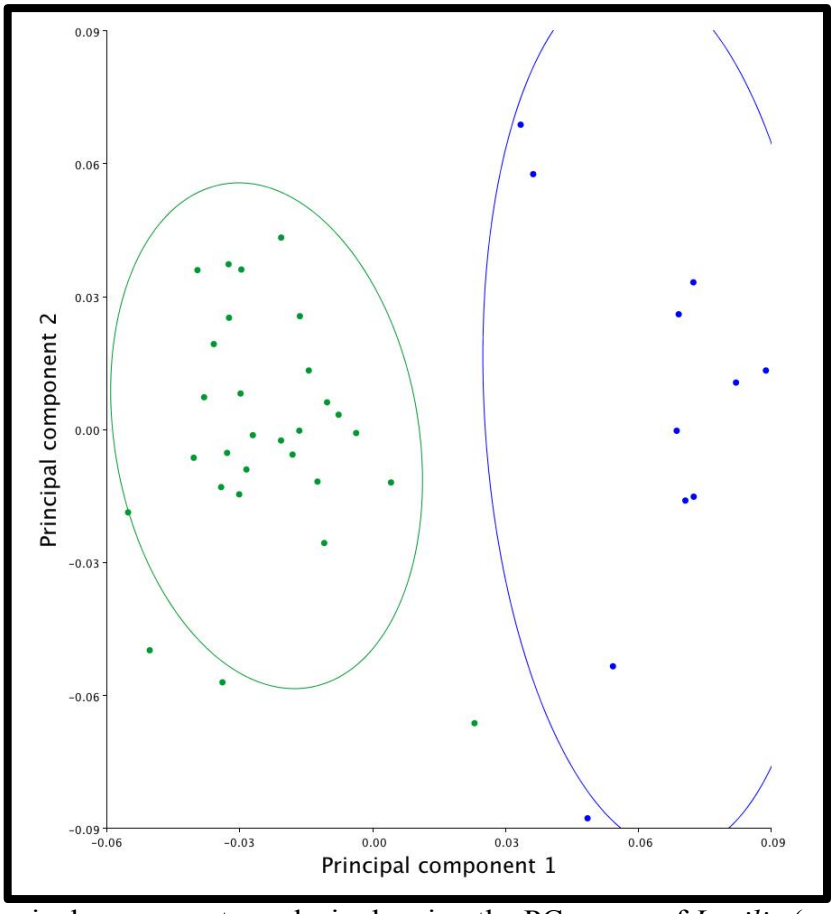

Figure 15: A principal components analysis showing the PC scores of *Lucilia* (green) and *Phormia* (blue) from California. 90% confidence ellipses plotted for each genus. Plotted on the axis of PC1 and PC2.

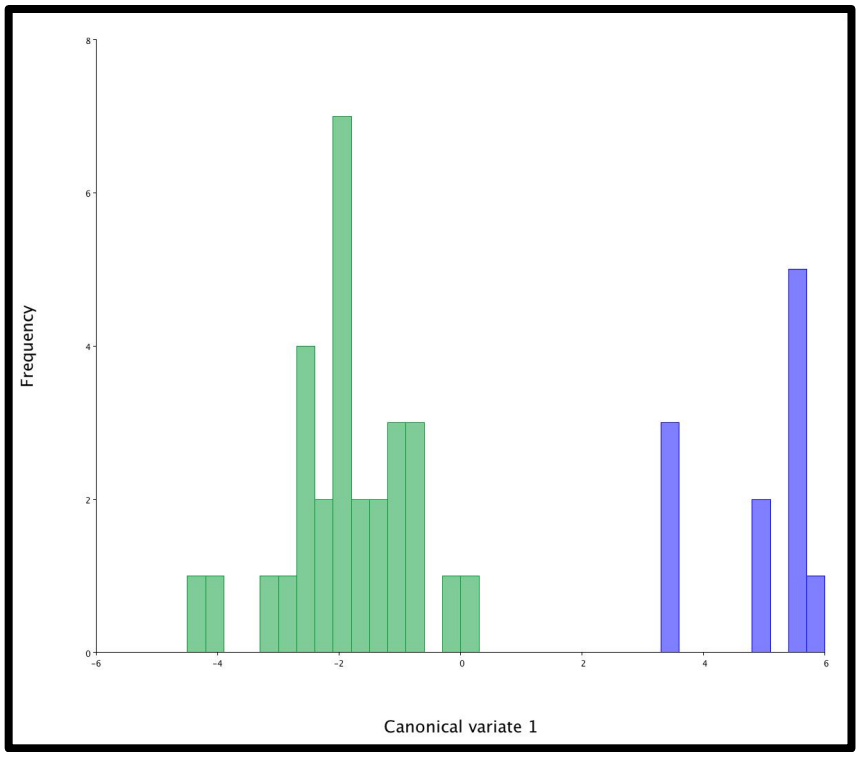

Figure 16: A canonical variates analysis showing the frequency of grouping of *Lucilia* (green) and *Phormia* (blue) from California based on canonical variate 1.

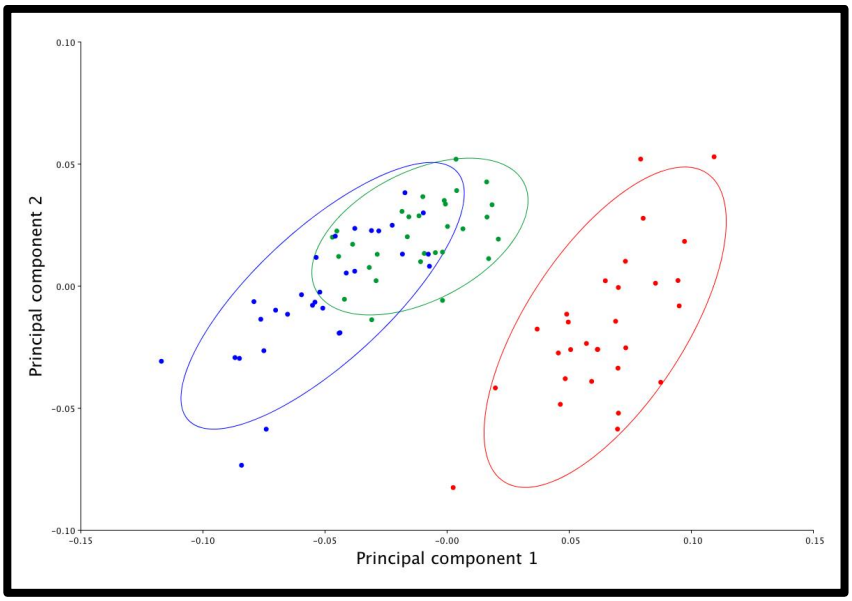

Figure 17: A principal components analysis showing the PC scores of *Calliphora* (red), *Lucilia* (green) and *Phormia* (blue) from Delaware. 90% confidence ellipses plotted for each genus. Plotted on the axis of PC1 and PC2.

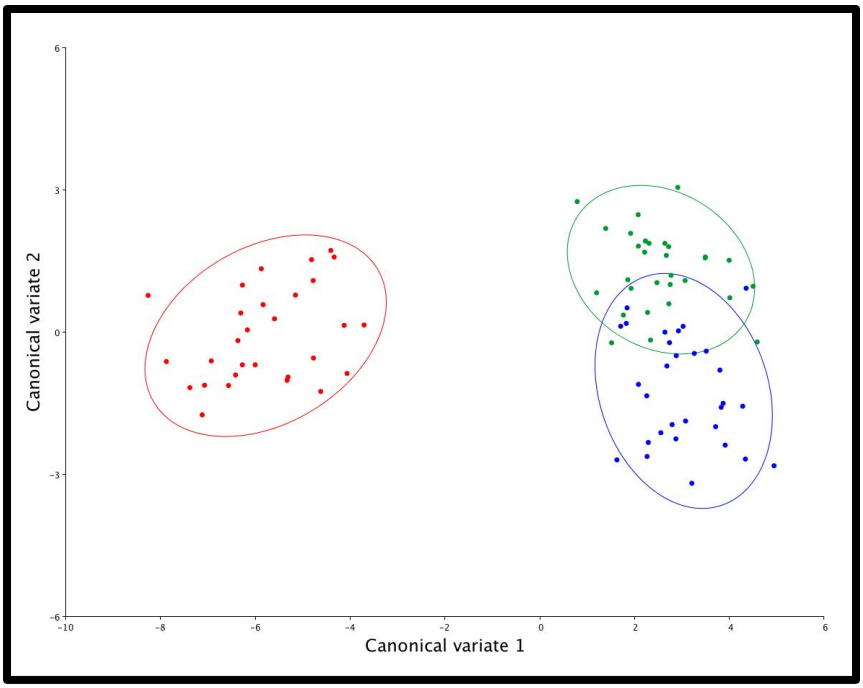

Figure 18: A canonical variates analysis showing the frequency of grouping *Calliphora* (red), *Lucilia* (green) and *Phormia* (blue) from Delaware. 90% confidence ellipses plotted for each genus. Plotted on the axis of CV1 and CV2.

Table 7: Pairwise comparison of Procrustes distances between different genera from Delaware.

Procrustes distances among groups		
	Calliphora	Lucilia
Lucilia	0.0862***	
Phormia	0.1166***	0.0501***
*** denotes a p-value <0.0001		

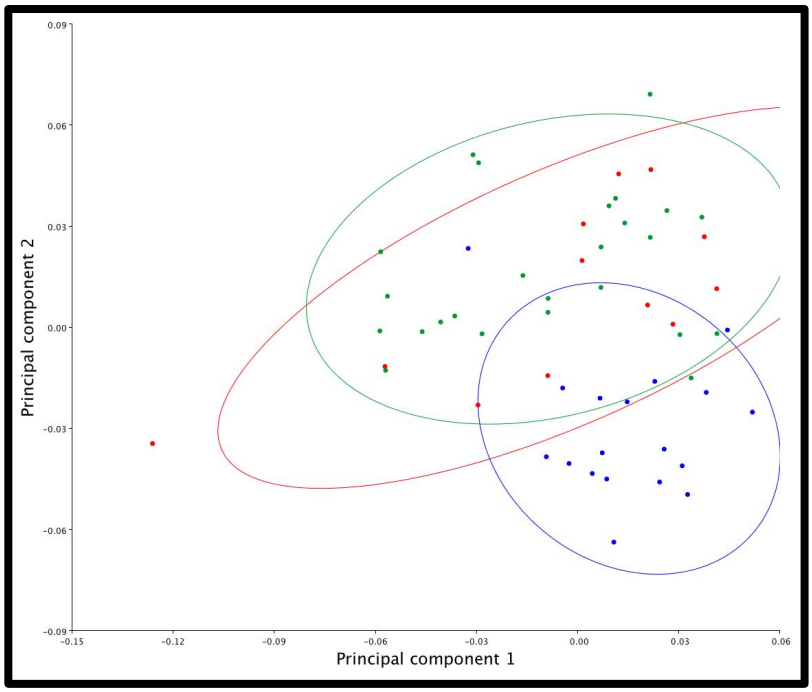

Figure 19: A principal components analysis showing the PC scores of *Calliphora* (red), *Lucilia* (green), and *Phormia* (blue) from Indiana. 90% confidence ellipses plotted for each genus. Plotted on the axis of PC1 and PC2.

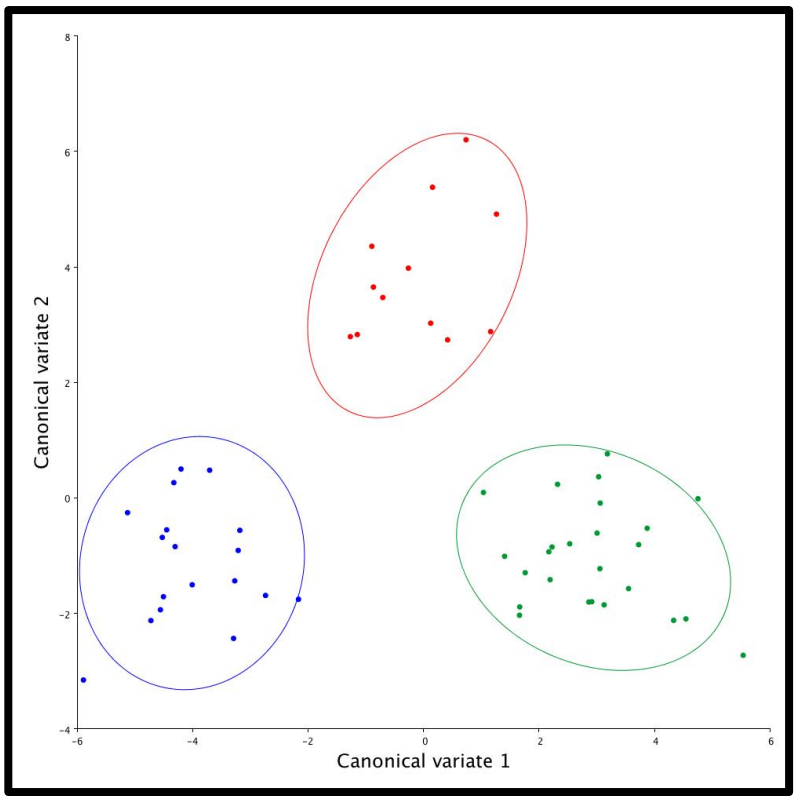

Figure 20: A canonical variates analysis showing the frequency of grouping *Calliphora* (red), *Lucilia* (green), and *Phormia* (blue) from Indiana. 90% confidence ellipses plotted for each genus. Plotted on the axis of CV1 and CV2.

Table 8: Pairwise comparison of Procrustes distances between different genera from Indiana.

Procrustes distances among groups		
	Calliphora	*Lucilia*
Lucilia	0.0389*	
Phormia	0.0526***	0.0584***
* denotes a p-value <0.05 *** denotes a p-value <0.0001		

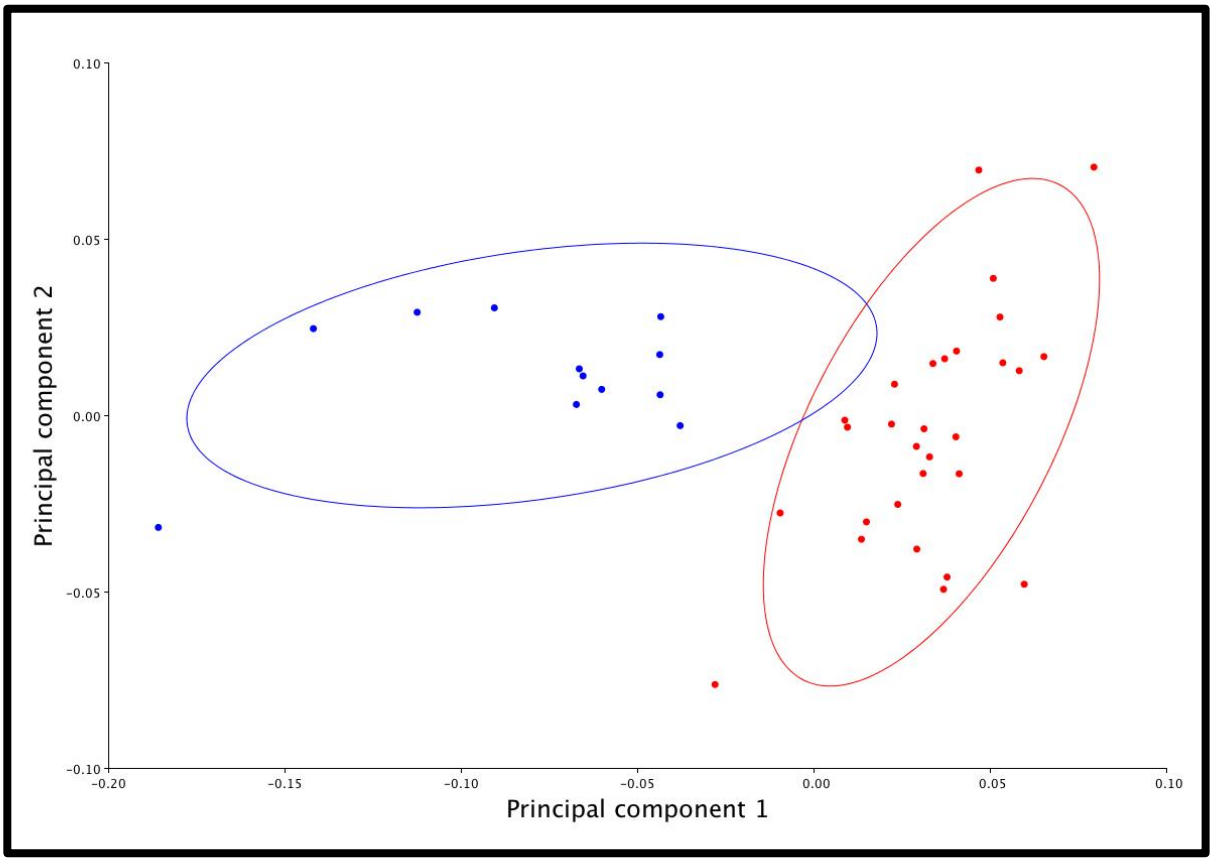

Figure 21: A principal components analysis showing the PC scores of Indiana (blue) and Delaware (red) for *Calliphora*. 90% confidence ellipses plotted for each location. Plotted on the axis of PC1 and PC2.

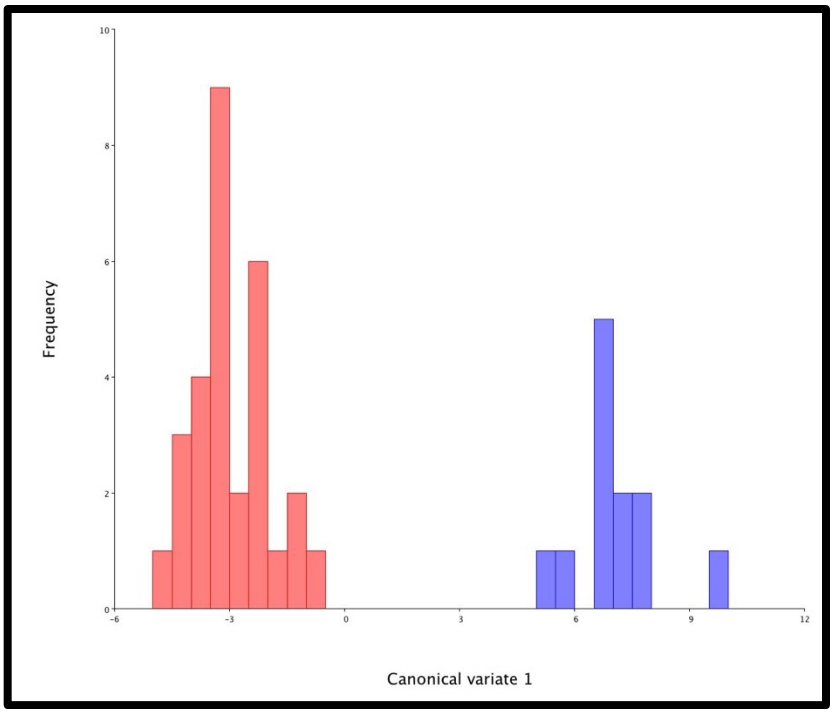

Figure 22: A canonical variates analysis showing the frequency of grouping of Indiana (blue) and Delaware (red) for the genus *Calliphora*. 90% confidence ellipses plotted for each location. Plotted on the axis of CV1 and CV2.

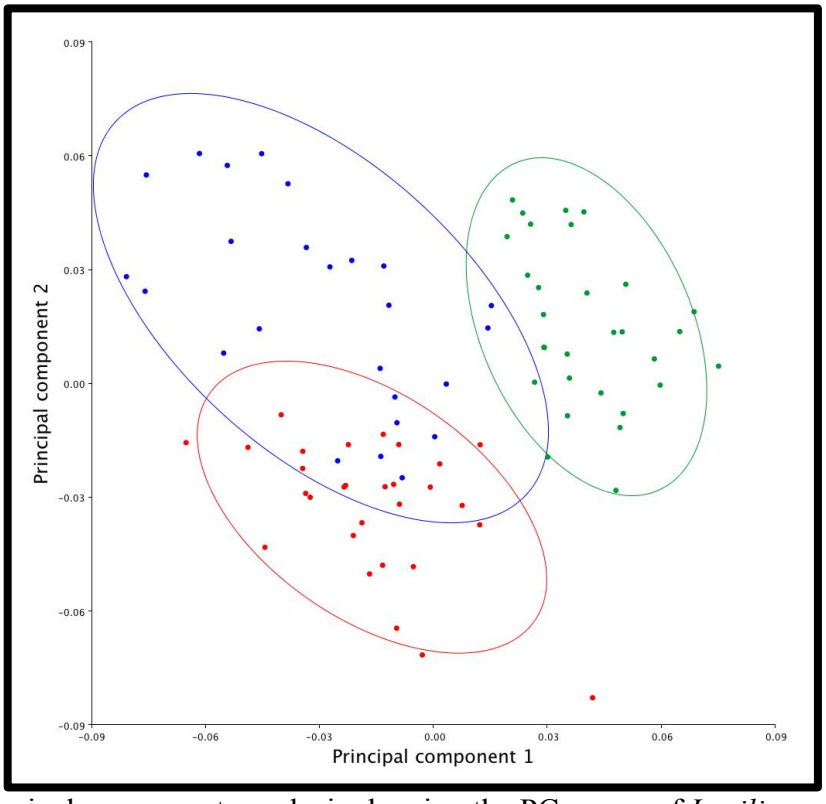

Figure 23: A principal components analysis showing the PC scores of *Lucilia* specimens from California (red), Delaware (green) and Indiana (blue). 90% confidence ellipses plotted for each location. Plotted on the axis of PC1 and PC2.

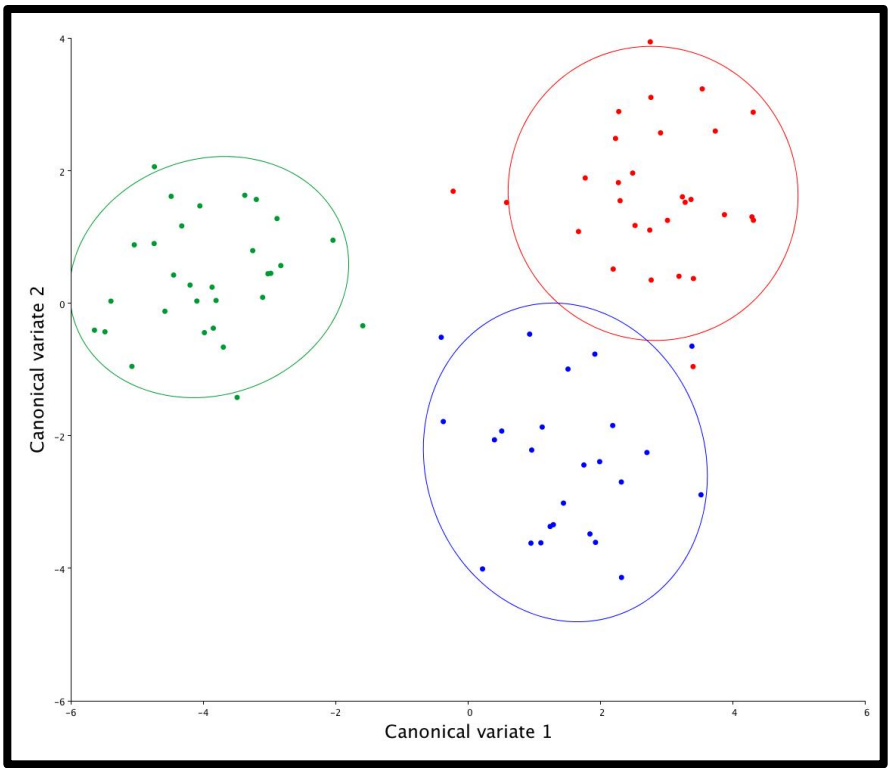

Figure 24: A canonical variates analysis showing the frequency of grouping California (red), Delaware (green) and Indiana (blue) for the genus *Lucilia*. 90% confidence ellipses plotted for each location. Plotted on the axis of CV1 and CV2.

Table 9: Pairwise comparison of Procrustes distances for *Lucilia* specimens grouped by location.

Procrustes distances among groups		
	California	Delaware
Delaware	0.0743***	
Indiana	0.0574***	0.0718***
*** denotes a p-value <0.0001		

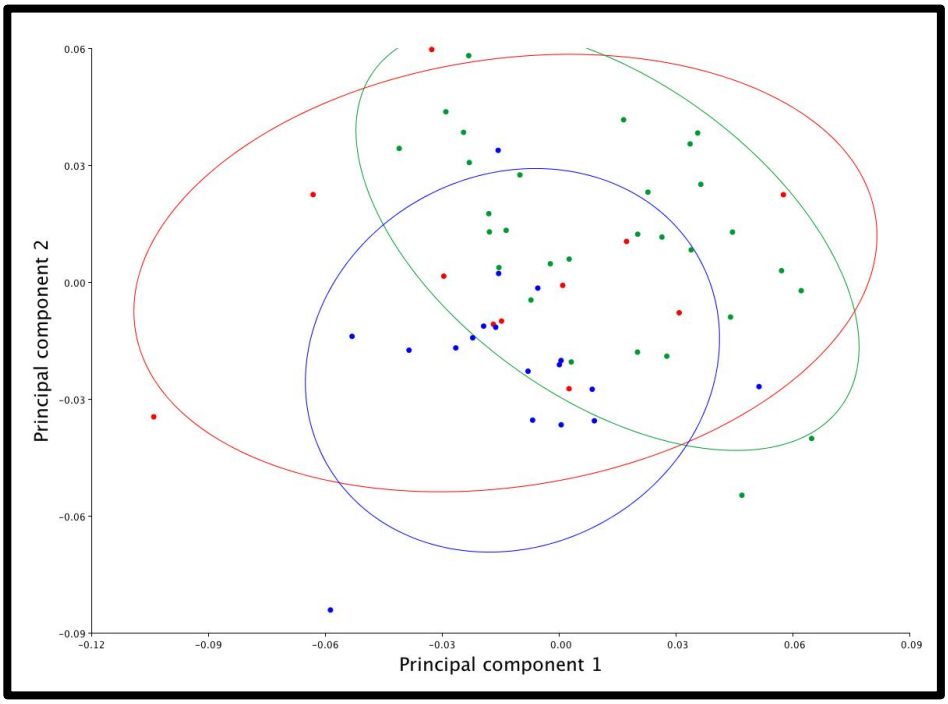

Figure 25: A principal components analysis showing the PC scores of *Phormia* specimens from California (red), Delaware (green) and Indiana (blue). 90% confidence ellipses plotted for each location.

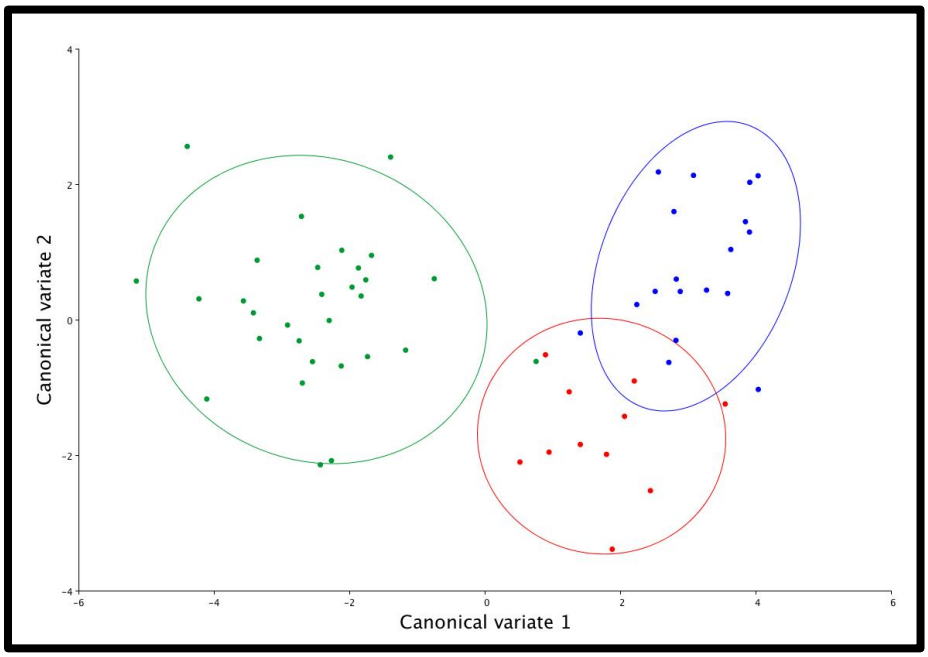

Figure 26: A canonical variates analysis showing the frequency of grouping California (red), Delaware (green) and Indiana (blue) for the genus *Phormia*. Plotted on the axis of CV1 and CV2. 90% confidence ellipses plotted for each location.

Table 10: Pairwise comparison of Procrustes distances for *Phormia* specimens grouped by location.

Procrustes distances among groups		
	California	Delaware
Delaware	0.0353*	
Indiana	0.0297	0.0441***
* denotes a p-value <0.05 *** denotes a p-value <0.0001		

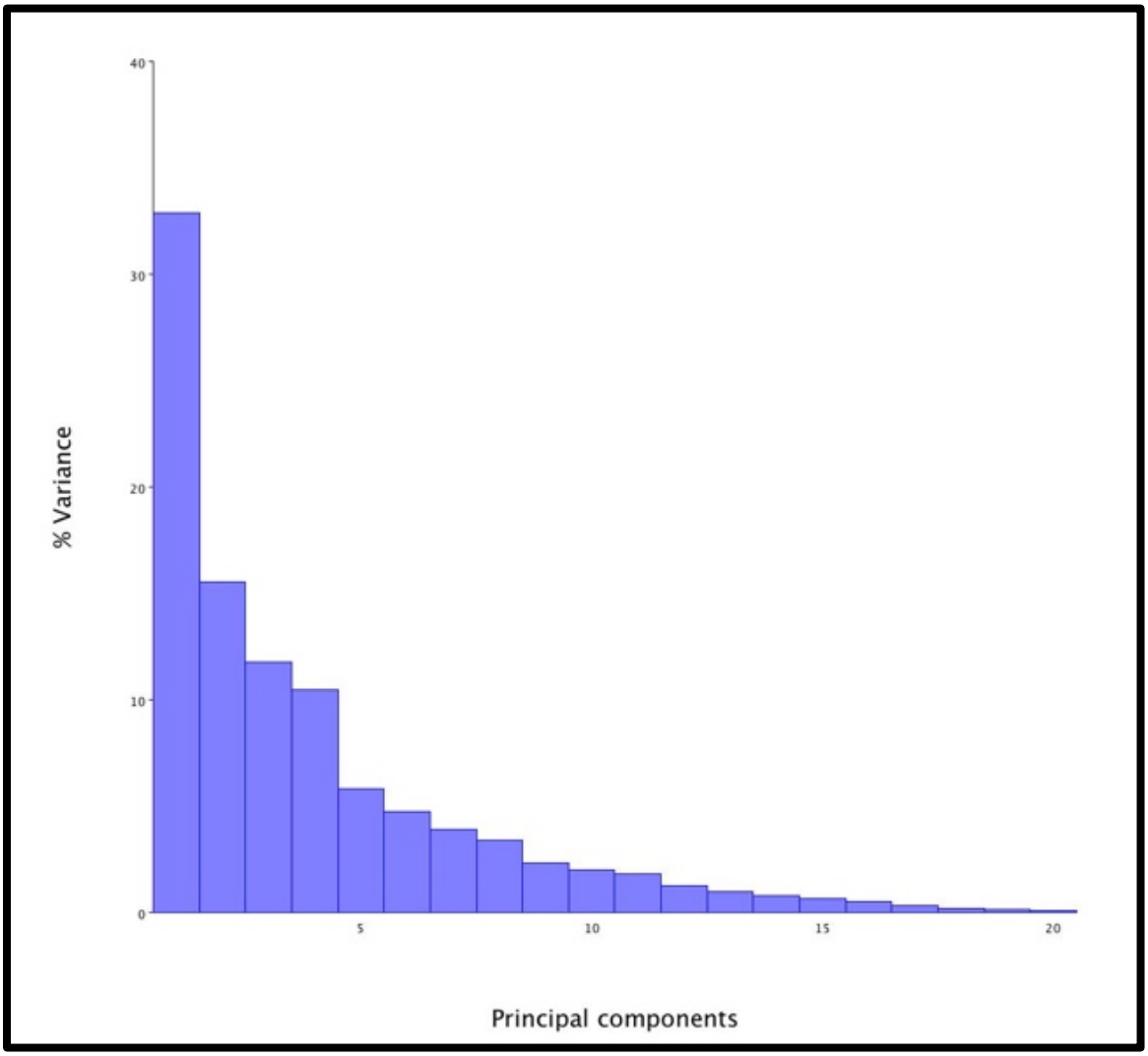

Figure 27: Principal Components Analysis – Eigenvalues accounting for the shape differences between all specimens from all locations.

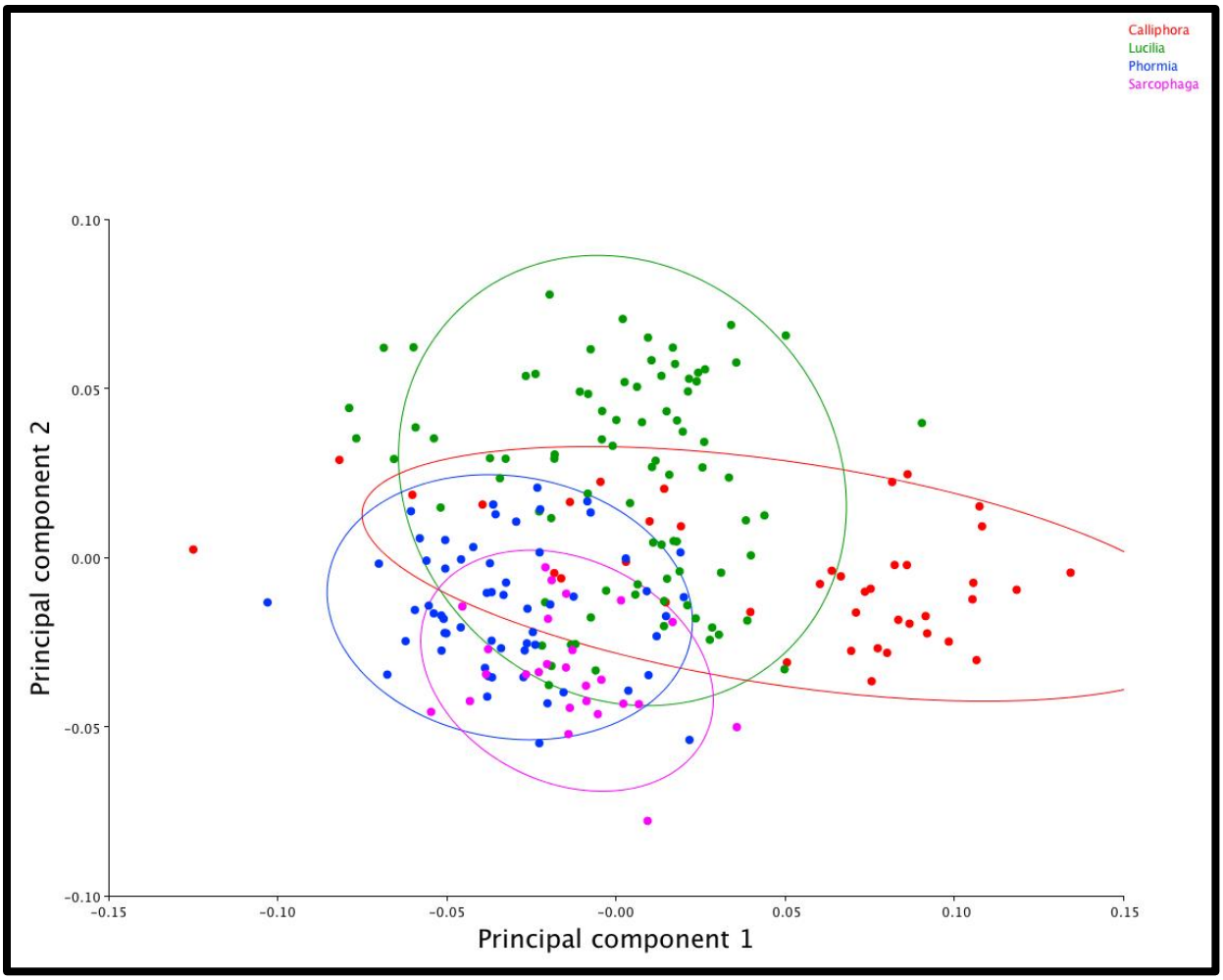

Figure 28: Principal Components Aanlysis - PC Scores Scatterplot showing the shape differences based on PC1 and PC2 for all specimens from all locations. *Calliphora* (red), *Lucilia* (green), *Phormia* (blue), and *Sarcophaga* (pink). 90% confidence ellipses plotted for each genus.

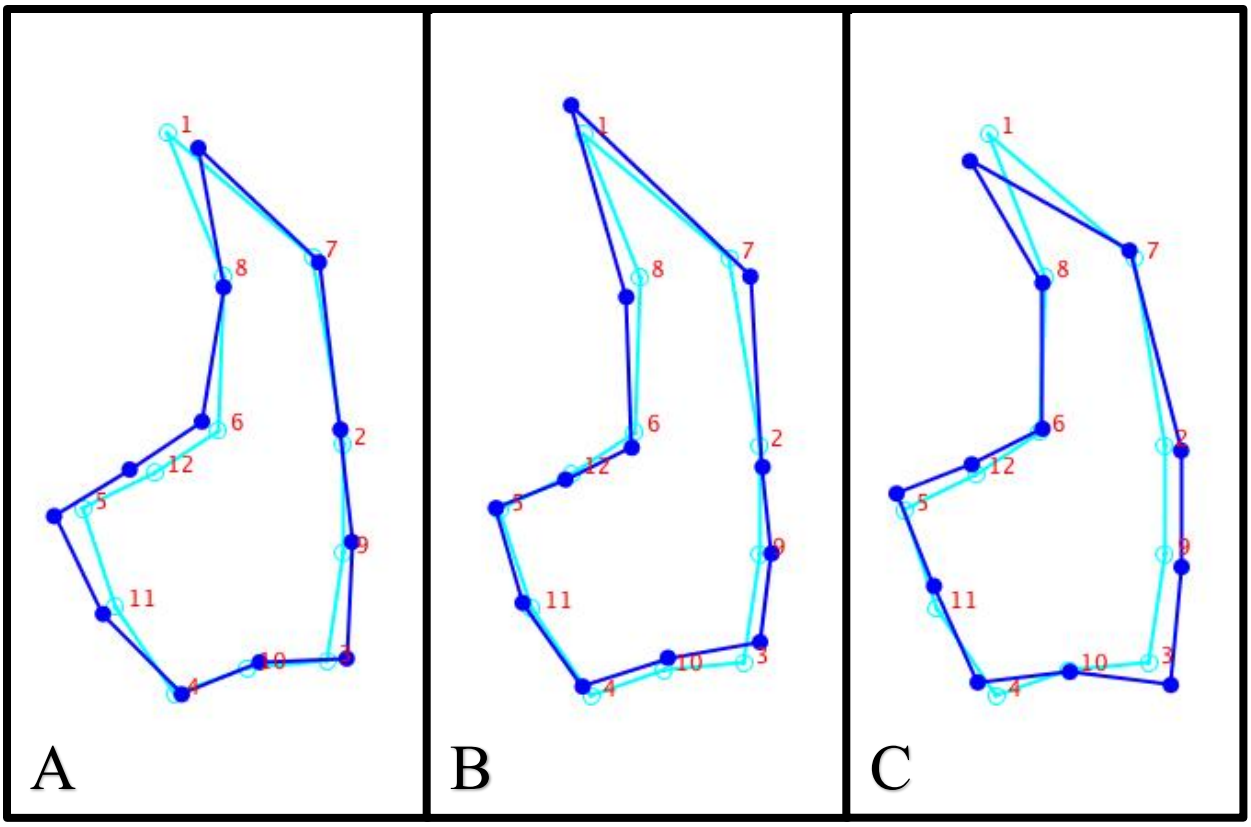

Figure 29: Shape variation attributed to the first three components, for all specimens from all locations. A - PC1, B - PC2 and C - PC3.

Table 11: Pairwise comparison of Procrustes distances between different genera from all locations.

Procrustes distances among groups:			
	Calliphora	*Lucilia*	*Phormia*
Lucilia	0.0637***		
Phormia	0.0888***	0.0513***	
Sarcophaga	0.0839***	0.0781***	0.061***
***p-values <0.0001 with 10000 permutations.			

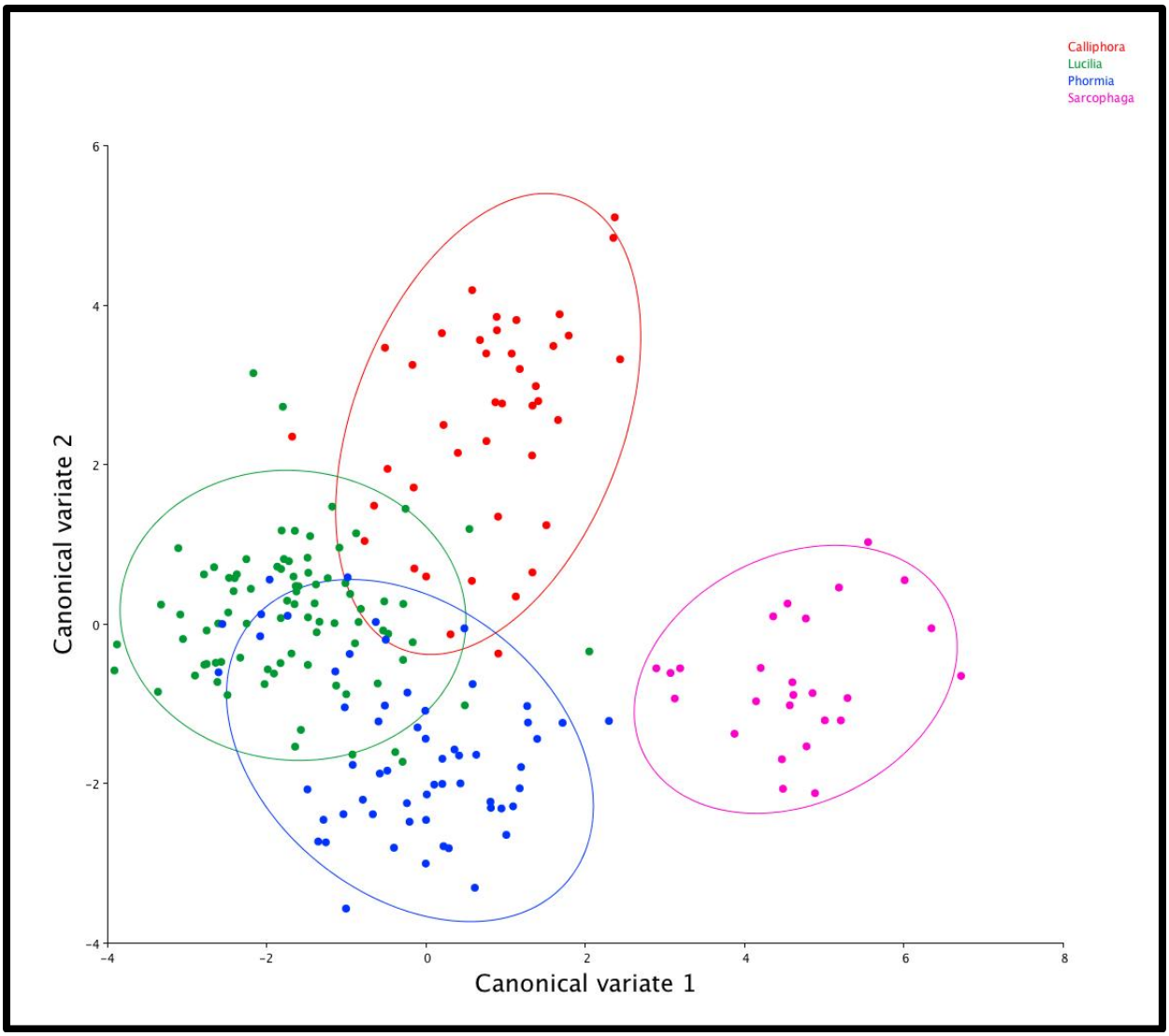

Figure 30: Canonical Variates Analysis for all genera from all locations, plotted on CV1 vs. CV2 axis. *Calliphora* (red), *Lucilia* (green), *Phormia* (blue), and *Sarcophaga* (pink). 90% confidence ellipses plotted for each genus.

CHAPTER 4. THESIS CONCLUSION

Conclusion

Larval mouth hooks have been used previously for insect identification [Liu and Greenberg 1989, Velasquez *et al*. 2010, Szpila 2010], although the majority of identifiable features reside in the pharyngeal cephaloskeleton. This study aimed to test the distinguishability of the tooth portion of the mouth hook. The mouth hooks displayed variation in size and shape between the genera and locations tested. Even though size does not directly influence shape, it is important to note that there was a significant difference between the sizes of the mouth hooks for each genus. This variation in size, however, should not be used to draw conclusion since the size of the larvae may have been different based on feeding habits. Although there was an attempt to standardize collection and preparation of specimens, some wild-caught specimens were used and their access to food might have influenced the size of their mouth hooks. The more important aspect to focus on is shape differences. Due to the significance of shape variation between the two hooks, it is important for investigators to use both sides of the mouth hooks for identification.

In chapter 2 a method of specimen preservation was established to ensure that all specimens being prepared for geometric morphometric analysis can be prepared in the same manner. Having a standard to follow means that regardless of where the analysis is being performed or the kinds of specimens analyzed, all will be treated the same. Standard preparation is key to geometric morphometrics because all landmarks need to be visualized in the same way for analysis.

In chapter 3 a variety of geometric morphometrical tests were performed on the data. Although there was lots of overlap between genera and location groupings there was still significant differences found when the genera and locations were tested by canonical variates analysis.

Using this information, an investigator could first use geometric morphometrics to compare between local genera and then compare to specimens more broadly throughout North America to test for locality of an unknown specimen.

This new method of identification could change the field of forensic entomology as we know it. Using a statistical approach to quantify specimens into genera and species would increase the reliability of the data when presented in a courtroom setting. The results would not rely on an investigator's morphological identification but on statistical analysis with error rates provided for the identification. Additionally, this method can also reduce the overall time it takes for a forensic entomologist to identify all of the specimens collected from a scene as well as the amount of storage space that is used. The investigator collecting the specimens only needs collect enough larvae from the carcass and preserve them all in ethanol, instead of the current methods where half are preserved, and half are reared to adult for identification.

With the standard method for specimen preparation, completed in chapter two, and the determination that there is enough shape variation to classify specimens by mouth hooks, the next step is to expand the reference samples to include all pertinent species with samples from a wide geographic range to build a full reference database. A reference database of standards is needed because, software alone can only analyze whether or not there is shape variation, and in which direction shape variation is moving. In order to be able to group the specimens with known species, a database of standards is needed to perform comparative analysis that then groups specimens based on the standard groups. This is the equivalent of calibration in traditional analyses, allowing the software to know what known specimens look like before comparing to the unknowns.

Collaboration is extremely important for this type of database-building because it would take any one researcher a very long time to set up a database for all the forensically important flies

available in the United States. Having multiple collaborators across the country submitting their sample specimens would speed up the expansion of the reference database. Individuals trying to identify larvae could then download the database and add their unknown specimens to test how the software would classify their specimens in relation to the reference collection. Users would select specific landmarks for analysis and analysis would return a chart with classification standards and the querie samples classified to species (or family, or genus) level designation based on that standard.

Works Cited

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