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Shape up and Ship out: Do Bt-Resistant Corn Earworm Moths, *Helicoverpa zea*, Have Wing Shapes Better Suited to Long Distance Flight? arworm M oths, *Helicoverpa zea* , H ave W ing S hapes B etter S uited to L ong D istance F light?

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Sarah Barclay

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Shape up and Ship out: Do Bt-Resistant Corn Earworm Moths, *Helicoverpa zea*, Have Wing Shapes Better Suited to Long Distance Flight?

Abstract

Evolution of resistance within insects to pest control has frequently resulted in changes to the organism's morphotype, including changes in wing shape. By measuring these changes, it is possible to distinguish resistant from non-resistant populations. Geometric morphometrics (GM) quantifies morphological variation within and among populations, it has been used in previous studies to identify changes in morphotype and distinguish between resistant and non-resistant populations of insects. *Helicoverpa zea* (corn earworm) is one of the most economically damaging pests for crops across the United States of America. Infestations of *H. zea* annually migrate from southern USA to the north, causing damage to almost all agricultural crops in its path. Although corn (*Zea mays*) is the preferred host and thus the most affected by this insect. A common strategy to manage this pest is the use of Bt Corn; genetically modified corn that produces the Bt toxins. Cry proteins (Cry1AF and Cry1B) are the most common to be produced in Bt Corn, but they are considered low dose toxic to *H. zea*. There are suggestions to include Vip3A by gene pyramiding corn as the combination of Vip and Cry proteins are high dose toxic to *H. zea*. Overtime, *H. zea* has evolved resistance to the Cry proteins expressed by Bt Corn making them a no longer effective control mechanism. This thesis the first study to apply the use of GM methods to the species *H. zea*. The aims of this thesis are to investigate the effect that resistance to Bt Corn has on wing morphology of the *H. zea* moth, and to determine if these changes in wing shape can be detected using GM methods. A total of 145 *H. zea* were provided from two locations across the USA, one in South Carolina and another in North Carolina. Moths were from four different treatments of corn; a control containing corn with no Bt toxins (Treatment 1); crops of Bt Corn containing two toxins (Cry1AB and Cry1F) with a structured refuge present (Treatment 2); crops of Bt Corn containing three toxins (Cry1AB, Cry1F, and Vip3A) with a structured refuge present (Treatment 3); crops of seed blended Bt Corn with 80% containing three toxins (Cry1AB, Cry1F, and Vip3A) and 20% not having any toxins (Treatment 5). Left and right forewings of each moth were chemically bleached, and the scales removed. Once wings were slide mounted, photographs were then taken of each wing. Fifteen type I landmarks were identified in the venation pattern of the forewing for the use of GM analyses. Preliminary tests were conducted and determined that results observed were not confounded by measurement error, bilateral asymmetry, allometry, or sexual dimorphism. A Principal Component Analysis (PCA) was conducted to visualise the whole data set, and a Canonical Variate Analysis (CVA) was done to visualise the relationship between wing shape and treatment of corn. Lastly a Discriminant Function Analysis (DFA) was done to determine the ability of wing shape to act as an indicator and biomarker for the different treatments of corn. The results determined that there was significant difference between forewing shape of susceptible and resistant *H. zea* individuals. The CVA identified that wing shape of moths' resistant to Vip3A protein is significantly different from the wing shape of moths' resistant to just the Cry proteins, thereby identifying resistance to the Vip3A protein. Practical field resistance of *H. zea* populations to the Vip3A protein has only been recorded once before, therefore these findings present a serious concern. If resistance to the Vip3A protein can become established within *H. zea* populations, it may inhibit future pest management strategies and pose a serious economic threat to future corn production within the USA. The results of the DFA showed that the forewing shape of *H. zea* can effectively be used as a biomarker, and that GM can be used to monitor the development and spread of resistance to both Cry proteins, and Vip3A proteins. GM methods are much cheaper and requires less expert knowledge than genetic markers, and thus will allow greater access to the monitoring of resistance within the *H. zea* population to a broader range of professionals.

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OF WOLLONGONG
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School of Earth, Atmospheric and Life Sciences

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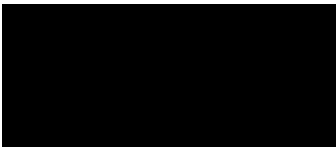
Sarah Barclay

2023

This Thesis is submitted as part of the completion of Honours for Bachelor of Science (Biological Sciences) (Honours) in the School of Earth, Atmospheric and Life Sciences, at University of Wollongong.

Declaration

I, Sarah Barclay, declare that this thesis, submitted as part of the completion of an honours project for a Science (Biological Sciences) (Honours) degree in the School of Earth, Atmospheric and Life Sciences, University of Wollongong. This thesis does not include any material published by another person without due reference within the text. All field and lab work presented was completed by the author, except where acknowledged. This thesis has not been submitted for a degree at any other university.



Sarah Barclay
March 2023

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Evolution of resistance within insects to pest control has frequently resulted in changes to the organism's morphotype, including changes in wing shape. By measuring these changes, it is possible to distinguish resistant from non-resistant populations. Geometric morphometrics (GM) quantifies morphological variation within and among populations, it has been used in previous studies to identify changes in morphotype and distinguish between resistant and non-resistant populations of insects. *Helicoverpa Zea* (corn earworm) is one of the most economically damaging pests for crops across the United States of America. Infestations of *H. zea* annually migrate from southern USA to the north, causing damage to almost all agricultural crops in its path. Although corn (*Zea mays*) is the preferred host and thus the most affected by this insect. A common strategy to manage this pest is the use of Bt Corn; genetically modified corn that produces the Bt toxins. Cry proteins (Cry1AF and Cry1B) are the most common to be produced in Bt Corn, but they are considered low dose toxic to *H. zea*. There are suggestions to include Vip3A by gene pyramiding corn as the combination of Vip and Cry proteins are high dose toxic to *H. zea*. Overtime, *H. zea* has evolved resistance to the Cry proteins expressed by Bt Corn making them a no longer effective control mechanism. This thesis the first study to apply the use of GM methods to the species *H. zea*. The aims of this thesis are to investigate the effect that resistance to Bt Corn has on wing morphology of the *H. zea* moth, and to determine if these changes in wing shape can be detected using GM methods.

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List of Abbreviations

ANOVA: Analysis of Variance

Bt: *Bacillus thuringiensis*

CVA: Canonical Variate Analysis

CV1: First Canonical Variate

CV2: Second Canonical Variate

DFA: Discriminant Function Analysis

df: Degrees of Freedom

GM: Geometric Morphometrics

H. zea: *Helicoverpa zea*

n: Sample size

PCA: Principal Component Analysis

PC: Principal Component

SNP: Single Nucleotide Polymorphism

USA: United States of America

US EPA: United States Environmental Protection Agency

WCR: Western Corn Rootworm

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1. Introduction

1.1 *Helicoverpa zea*: The Corn Earworm Moth

1.1.1 *Helicoverpa zea* as a Polyphagous Pest and its Lifecycle

The Corn Earworm Moth, *Helicoverpa Zea* (Bodie) (bollworm, corn earworm), is native to North and South America (Fitt 1989). It is one of the most polyphagous and economically damaging pests for crops across the agricultural production regions across the United States of America (USA) (Capinera 2000; Jackson et al. 2004; Martin et al. 1976a). *H. zea* has a long history as a pest of cotton and feeds on almost all agricultural crops, although corn (*Zea mays*) is its preferred agricultural host (Lincoln & Isely 2014).

H. zea populations are found throughout the western hemisphere (Cohen et al. 1988) and primarily feed on the fruits of plants. Ovipositing moths are attracted to the flowers and reproductive structures of a variety of cultivated and wild hosts including the ears of corn (Fitt 1989). They can thrive on many host plants and economic crops including artichoke, asparagus, cabbage, cantaloupe, collard, cowpea, cucumber, eggplant, lettuce, lima bean, melon, pea, pepper, potato, pumpkin, spinach, squash, sweet potato, and watermelon (Martin et al. 1976a). Corn and lettuce are considered excellent larval hosts (Harding 1976). Cotton is frequently reported to be injured (Ali & Young 1996; Jackson et al. 2008; King 1994; Lincoln & Isely 2014) but this occurs typically when the more preferred crops have matured.

Corn is one of the most affected crops by *H. zea* infestations, with larvae consuming significant portions of the plant. Oviposition onto the silk of the corn occurs from the silking stage (R1) to the kernel dough (R4) stage of the corn's development (Archer & Bynum 1994). It is during the larval stage of the life cycle where the main damage is done to the agricultural crop with estimated yield losses ranging from 1.5 to 2.5% (McMillan et al. 1976; Wiseman & Morrison 1981). The last two instars (fifth and sixth) of larval development account for over 90% of their total grain consumption (Downes & Mahon 2012).

Once *H. zea* larvae reach maturity, they exit the ear of corn and drop onto the ground before burrowing into the soil so they can pupate and emerge as adult moths (Archer & Bynum 1994). Pupates are typically a mahogany-brown colour and take an estimated thirteen days to emerge as an adult. Adults are variable in colour as well, having a wingspan of 32-45mm. They have an average lifespan of fifteen days, although they have been shown to survive up to thirty days in optimal

conditions. *H. zea* are nocturnal and throughout the day they typically hide in vegetation and are occasionally seen feeding on nectar. The forewings of adults are a yellowish-brown colour and often have a small dark spot centrally. There is a broad dark transverse band distally across the wing, but the margin of the wing is not darkened (Figure 1.1). The hind wings on the other hand are a creamy white colour proximally and are a dark brown to black colour distally (Figure 1.1).

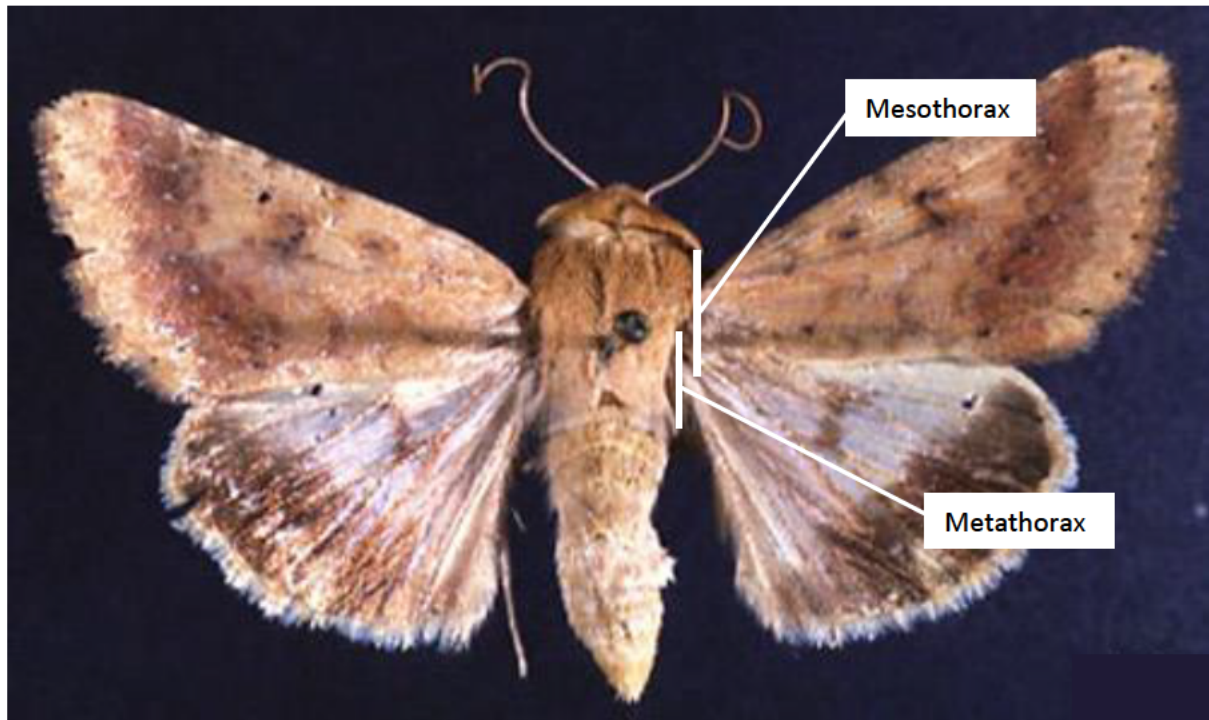


Figure 1.1) Adult *Helicoverpa zea* with forewing and hindwings visible. Mesothorax and metathorax labelled with a set of wing attached to each. Image modified from Capinera (2000).

1.1.2 Distribution of *H. zea*

H. zea are found throughout North America except for northern Canada and Alaska as they typically do not survive well in colder climates due to adverse weather and the collapse of emergence tunnels. Populations from the subtropical region of North America, and progeny produced from overwintering insects from northern locations, undergo an annual migration. They follow northward patterns of host-plant availability and maturity throughout the growing season (Westbrook et al. 1997), thus creating progressive infestations in economic crops (Lingren et al. 1994). They travel north and arrive on crops anytime from mid-July through to September (Sandstrom et al. 2007) (Figure 1.2). *H. zea* infestations are more damaging in areas where they successfully overwinter, because when migrating north they typically arrive too late to inflict economic levels of damage. Due to their highly dispersive nature, immigration allows them to spread into the northern states and

southern Canada, with records showing they have been found as far as 40 degrees north latitude (Morey et al. 2012). Recently, new research has suggested a reverse migration may also occur (Pair et al. 1987; Pietrantonio et al. 2014), where populations migrate from the northern states to the overwintering sites in Texas and Louisiana in the autumn months (Gould et al. 2002).



Figure 1.2) Map of United States of America describing the immigration of *Helicoverpa zea* populations from the southern states to the northern states. First generations appear in the southernmost part between February and March and continue to rise throughout the year. Image from Sandstrom & Changnon (2007).

In order to migrate, *H. zea* rely on the use of convective cells to lift off the canopy layer (Sandstrom & Changnon 2007). Convection cells describe when warm air is pushed upward by the buoyant forces of colder denser air. Then as the warm air cools it sinks and pushes warmer air up, creating a cell cycle. *H. zea* are absorbed by these cells and lifted out of the canopy where they are carried on wind currents at both surface and lower levels of the atmosphere (Sandstrom & Changnon 2007). Consequently, *H. zea* infestations can only be carried to south-to-north direction within the northern

hemisphere (Welshans 2004). The infestations leave these convection cells when they are confronted with cold fronts. Where stormy or rainy weather brings the insects down into a drop zone (Welshans 2004). Crops within these drop zones are under serious risk of infestations.

The severity of infestations varies from year to year and have the ability to change suddenly and unexpectedly based on environmental and ecological factors. Climatology has a very significant impact on migration patterns and intensity. Wind currents control how far infestations can travel, with stronger winds carrying them for longer (Sandstrom & Changnon 2007). Storms and rain caused by frontal boundaries dictate where insects will fall, and which crops are at high risk of infestation (Welshans 2004). Winter temperature play a large factor in the range of infestations, with overwintering often acting as a limiting factor to *H. zea* populations' spread north (Westbrook et al. 1997). Regional moth activity and climatology can be used to provide knowledge about impending immigrations, with sites such as www.insectforecast.com being able to predict these migrations using meteorological forecasting (Sandstrom 2016). Population movements at a landscape level are often dictated by *H. zea* seeking nectar or mates (Lingren et al. 1993). Typically, availability of hosts influences these landscape-level movements as they impact the amount of both nectar and mates present locally (Fitt 1989). Due to the increase in agricultural crops, this no longer acts as a limiting factor for migration which is instead mostly controlled by climatology. During periods of adverse conditions, especially winter, *H. zea* undergo a diapause for up to 20 months (Phillips & Newsom 1966). It is through this evolutionary advantage that migrations are able to survive unfavourable climates and can wait for more beneficial conditions to undergo migration.

1.1.3 Damage Inflicted by *H. zea* and Management Strategies for Infestations

H. zea is one of the costliest crop pests in North America and is the most common aboveground pest of corn in south-eastern USA (Luttrell & Jackson 2012; Niu et al. 2021). Due to its polyphagous nature, it attacks a wide variety of valuable crops. It typically eats the portion of the crop that is harvested and therefore profitable, e.g. blossoms, buds, and fruits. Economic loss in corn crops caused by *H. zea* infestations is caused by a direct weight loss through seeds being partially or wholly eaten (Luttrell & Jackson 2012). The decrease in quality as a result in high levels of chewed grains also causes economic loss. Feeding allows for the enhancement of plant pathogenic fungi within the corn which contributes to economic damage (Capinera 2000).

Chemical management strategies are often used to reduce *H. zea* infestations. Insecticides are usually applied to the leaves of the corn via a liquid medium, with extra focus on any at-risk ears of corn. Typically, the insecticides that are used to treat infestations are a combination of an ovicide (egg killer) and synthetic pyrethroid. A pyrethroid is typically used as it has residual activity over a few days and thus will continue to act over a longer period than other insecticidal options. Application is typically done every 2 to 6 days. Although, in areas with frequent infestations such as Florida, some growers apply the insecticide as frequently as daily. In southern areas of USA, populations of *H. zea* often develop on weed hosts first before dispersing over to crops. Treatments of these weeds via mowing, herbicides and insecticides have been shown to protect the local fields in northern areas. It is common to avoid infestation by harvesting the crops at an earlier time; before the migration of populations have reached these northern areas. This prevents the decrease in yield that is likely to occur once they are there.

There are biological controls that are also often used to combat these pests. The use of *Steinernatidae* nematodes has provided promising results, when applied to the silk of corn it is able to suppress larvae (Purcell et al. 1992). Subsurface soil applications of these nematodes have also shown to control moth populations, feeding on them when the larvae drop into the soil to pupate (Cabanillas & Raulston 1996). These nematodes are available commercially and have application for both at-home garden production and larger commercial production. Besides nematodes, there is also commercially available *Bacillus thuringiensis* (Bt) soil bacterium that is typically mixed with vegetable oil which has been recorded to provide from 80% to 90% control. Another biological option is the use of *Trichogramma* species which are egg parasitoids. It shows a parasitism rate of 40-80% and results in fruit damage levels of only 3% (Oatman & Platner 1971). When using this management method, the host crop seems to have a heavy influence in the parasitism rate, with tomato crops determined to be the most suitable host crop (Martin et al. 1976b).

There are varieties of corn plants that are commercially available that are recognised as being resistant to *H. zea*. These crops have greater husk tightness and ear length which prevents larvae's ability to access the ear kernels. These crops are not completely able to resist *H. zea* infestations although show promising results when used as a component of integrated pest management. Some crops of corn have been genetically engineered to produce naturally occurring toxins that possess insecticidal properties. The most effective and popular is the Bt toxin which has been genetically engineered into a wide variety of crops including corn. This Bt Corn reduces the damage inflicted by *H. zea* and reduces the survival of earworm larvae and damage inflicted onto the corn (Storer et al. 2001; Burkness et al. 2001).

1.1.4 Landscape Movement Patterns

Understanding the flight patterns and migratory nature of the pest species *H. zea* provides crucial information about their distribution. It highlights which geographic areas may be at risk of an invasion and allows for the creation of an effective management plan. For this pest, mass migrations can occur over long distances in a single night (Wolf et al. 1990). Investigations into the pollen found on migrating *H. zea* moths provided evidence that these migrations are occurring over hundreds of kilometres (Westbrook et al. 1997). When migrating, these insects use powered flight to disperse within the boundary layer (Dudley 2000), reaching altitudes of up to 900m (Beerwinkle et al. 1994). They then rely on upward convection and prevailing winds to reduce expenditure during migration, which is gliding flight (Gibo & Pallett 1979).

In most insects, including *H. zea*, forewings generate the main power for the flight compared to hindwings that possess weaker muscles. Hindwings are typically less essential for flight, instead they play a much larger role in the evasive nature and full-speed aerial manoeuvring that is seen in moths (Jantzen & Eisner 2008). These high-speed aerial manoeuvres have been developed in flying insects as a defence mechanism against the predators including bats, birds, and larger insects. (Howland 1974).

Despite the important roles that hindwings play within insects, including *H. zea*, it is the forewings that dictate the ability to fly longer distances for migration purposes. Many studies have found that wing shape is very important for the migratory movement and dispersal strategy of insect species (Kadoić Balaško et al. 2021; Lemic et al. 2014; Mikac et al. 2013; Mikac et al. 2016; Pajač Živković et al. 2019). According to Voss and Ferro (1990), there are three different types of flight with different characteristics: short distance flight, diapause flight, and long distance flight. Long distance is used in migratory flight and is the most important for the dispersal of the species and the colonisation of new areas (DeVries 2010). It is common in insects for the demands of long-distance flight to be an important selective force on the physical characteristics of the species. In birds, the shape and length of wings is able to determine how fast it can travel (Tittler et al. 2009), and its propensity to disperse (Dawideit et al. 2009). Long-distance migrants typically have pointed wings that are longer and thinner (Mönkkönen 1995; Lockwood et al. 1998), whilst rounded wings offer increased manoeuvrability (Dawideit et al. 2009).

Different types of wing morphotypes have been studied to determine the dispersal capabilities of flying insects (Denno et al; Guerra 2011; Sanzana et al. 2013). In insects, elongated wings are better suited to flying with air currents and longer distance dispersal (e.g. migration), while broad wings are more efficient for flapping low-level flights (Altizer & Davis 2010; DeVries et al. 2010). A greater wing

size with more elongated wings, and a lower ratio between body mass and wing area have been shown to allow for longer distance flying (Dingle et al. 1981; Calmaestra & Moreno 2001). A study conducted by Altizer and Davis (2010) compared two different populations of monarch butterflies. They determined that the populations that underwent migratory patterns had longer and larger wings to support them on this long-distance flight, compared to their non-migratory counterparts. In general, longer forewings may create more lift and increase the efficiency of a moth's flight allowing the moth to fly longer distances. This is because the longer wings may have a larger surface area, which produces more lift and allow the moth to fly more easily and efficiently. The decreased weight associated with the thinning of moth wings makes it easier for adult moths to take off and fly. Longer and slender wings are also much faster and more energetically efficient in flight (Mönkkönen 1995). By minimising the induced power requirements, this wing shape allows for flight to be maintained for longer distances.

Long distance/migratory flying is important for the dispersal of the species and colonisation of new areas. Wing morphology is a critical element of an insect's dispersal capacity (DeVries et al. 2010). Understanding which morphotype the superior flier and disperser is has implications for the ways in which this pest will be managed with integrated resistance strategies. This is because the superior flier and disperser has the best chance of spreading its alleles long distance and thus the associated genotype will spread over a larger distance in a shorter amount of time. Pointier and longer wings allow for this rapid colonisation of a large area and hence the population at the front of this expansion wave would compose of individuals that possess these traits in wings (Phillips et al. 2010; Shine et al. 2011). Because wing morphology is heritable in insects (Desender 1989), progeny of these colonisers would display increasing wing length and pointedness. This was seen in the wing shape and frequency of wing shape types of cricket populations that were at the forefront of range expansion (Simons & Thomas, 2004).

1.2 Bt Corn

1.2.1 Development of Bt Corn

Corn is one of the most important crops worldwide. Over two hundred million hectares are planted, and a subsequent 1150 million tons of corn are harvested every year across the globe (Food and Agriculture Organisation of the United Nations, 2022). Corn has a variety of pests that target its stalks, ears, roots, or leaves including a variety of Lepidopteran larvae (caterpillars) and Coleopteran larvae (beetle grubs). The development of strategies to prevent these pests have been vital throughout corn's agricultural history to protect the crops and reduce economical damage. These strategies typically include cultural, biological, or chemical (insecticide). These strategies can be used together and are referred to as Integrated Pest Management (IPM) to minimise environmental impact (Hellmich et al. 2008).

Some corn crops are genetically modified to use the insecticidal proteins typically produced by the soil bacteria Bt to deter a wide variety of pests. (Figure 1.3) The gene producing the Bt toxin, as well as the promoter sequence, and the marker gene (which allows identification for which plants have been genetically modified) are all transposed from the bacterium into the corn genome. This is what allows the crop to produce the Bt toxin. Bt insecticides are considered "natural insecticides" due to their unusual property of being toxic to only a small range of related insects. This is unlike conventional insecticides which typically have the potential to harm a variety of non-target organisms. Bt's targeted nature comes from its need for specific pH levels, enzymes and midgut receptors to activate. This exact combination of requirements is not found within most insects, only those that are targeted.



Figure 1.3) First column of corn are Bt Corn whilst the second column are non-Bt (Porter 2016).

Bt was first discovered in 1901 by a Japanese biologist Shigetane Ishiwatarias (Milner 1994). In 1956 it was found that the main insecticidal activity against lepidopteran insects was due to the production of certain proteins within Bt (Milner 1994). The Bt protein selectively targets Lepidoptera larvae, which includes several moth species that are pests to the corn, and other non-target butterfly species larvae including the Monarch Butterfly (*Danaus plexippus*). Its main target is the larvae of the European corn borer (*Ostrinia nubilalis*) also a member of the Lepidopteran family, and feeds primarily on the stalks and ears of the corn. To kill the larvae of target species, part of the plant containing the Bt protein must be ingested. With a combination of specific pH levels, enzymes, and midgut receptors, Bt activates and binds to the gut wall, stopping the insect from eating. Within the span of a few hours' pores form within the insect's intestine causing the insects normal gut bacteria to escape into the body cavity where it ultimately dies of septicaemia (Federici 2002).

After approval from the United States European Protection Agency (US EPA), genetically engineered Bt crops, including Bt Corn, were first introduced to the US in 2003 (Gassmann 2021). Bt crops became popular quickly within the USA due to its convenience and simplicity via its innate pest resistance. Functionally, this meant the grower would be handling fewer chemical insecticides which could cause negative health and environmental impacts (Pilcher et al. 2002). Grower's use of Bt Corn as pest protection was also likely encouraged due to the higher yields produced from reduced injury by insects (Gómez-Barbero et al. 2008). Another reason was that there was also a decrease in infections by moulds, caused by insect damage providing the site of infection to the plant (Dowd 2000). Prior to the use of Bt, corn's main form of pest protection was the use of insecticidal agents.

Due to Bt Corn's popularity amongst growers the use of insecticides on corn has had an estimated cumulative decrease of 35% (29.9 million kg) between 1996 and 2008 (Brookes & Barfoot 2010). Today, use has become widespread with over one hundred million hectares of Bt crops being grown worldwide by 2010 (James 2017). By 2020 approximately 79% of all corn and cotton planted within the USA expressed one or more Bt Protein (United States Department of Agriculture-Economic Research Service 2020).

The use of these Bt crops as a pest management strategy has resulted in; reduced use of insecticides, reduced impact on non-target invertebrates, widespread suppression of some insect pests whilst protecting farm yield, and allowing for farm-level cost savings (Cattaneo 2006). This has allowed an increase in profitability whilst reducing environmental costs (Lu et al. 2012). Overall, it has resulted in operational, environmental, and ecological benefits. Bt crops have not eliminated the need for insecticides with a variety of pests showing resistance to the Bt crops. It is a combination of pest management strategies that work together to control pests (Jackson 2008).

1.2.2 Evolution of resistance in *H. zea* populations to Bt Corn

H. zea naturally has low susceptibility to the Cryptochrome Circadian Regulator 1 (Cry1AB and Cry1F) proteins that are typically produced in genetically modified Bt Corn. Control of this moth with the use of Bt Corn is less effective when compared to its ability to control other popular corn pests from order Lepidoptera including the European Corn Borer (Jackson et al. 2004). Consequently, Bt is not considered high dose to *H. zea* (Sivasupramaniam et al. 2014). 'High dose' is defined by the US EPA scientific advisory panel as twenty-five times the protein concentration necessary to kill susceptible larvae. Bt crops are still effective against *H. zea* despite not being 'high dose'. Larval development is significantly delayed when exposed to Bt toxins, with different larval instars have differing levels of susceptibility with younger instars are often the more susceptible (Ali & Young 1996). Larvae developing in Bt Corn are delayed for an average of one week when compared to non-Bt Corn counterparts (Bilbo et al. 2019).

Increase in adoption of Bt crops throughout the USA has led to an increase in evolutionary selection pressure. This promotes the development and consequential spread of field resistance to the Bt proteins (Gould 1998). *H. zea* is known to develop insecticide-resistant populations (Sparks 1981). Practical resistance is described as the field-evolved resistance that reduces pesticide efficacy to a level that has consequences for pest control (Tabashnik et al. 2014). This insect has had three cases of field-evolved practical resistance to Bt crops being detected from 1996 to 2005, and nineteen

cases being detected in 2018 (Tabashnik & Carriere 2017). Bt Corn is not effective against this new population of resistant moths (Bilbo et al. 2019; Gassmann 2021; Tabashnik & Carriere 2017; Yang et al. 2019). Multiple generations of this moth are likely to be exposed to a wide variety of Bt crops. This provides suitable conditions for a fast evolution of resistance to Bt proteins, resulting in the spreading of this resistance.

One strategy to combat this is the refuge strategy, which is growing genetically engineered pest-resistant crops near toxin-free refuges. This promotes survival of susceptible pests and encourages heterozygous progeny between the susceptible and the resistant pests. Decreases in refuge size from 50% to 20% within the southern USA could also contribute to the fast and widespread resistance of Bt Corn (Yang et al. 2014). A decrease in refuges would subsequently lead to a decrease in susceptible moths not exposed to the Bt proteins.

Farmers often use a mixture of non-Bt Corn and Bt Corn seeds to incorporate a refuge into their agricultural practices. The mixing of Bt and non-Bt Corn seeds does not provide refuge for ear eating insects like the corn earworm moth. Instead, the use of seed blend allows for corn-cross pollination; the non-Bt Corn can be pollinated by the Bt Corn and will still produce Bt toxins in at least some portion of the kernels (Chilcutt & Tabashnik 2004). Ear-feeding insects, such as *H. zea* may be exposed to sub-lethal concentrations of toxins present within the kernels, which can accelerate the evolutionary process (Caprio et al. 2016; Vyavhare et al. 2021; Yang et al. 2014). Furthermore, cross-pollination and larval movement in seed-mixed plantings would also encourage the spread of resistance (Brévault et al. 2013) as it will result in the death of susceptible moths and the survival of heterozygotes thus increasing the dominance associated with resistance genes.

The concentrations of the Bt proteins throughout the Bt Corn fluctuate naturally throughout the plant, varying by tissue type, age and environmental condition (Dutton et al. 2004). By the larvae being exposed to an uneven distribution of the toxin, there can be an increase in the dominance of resistant genes within the moth community. This provides the opportunity for behavioural responses to develop (Hoy 1998). This has been observed, with larvae feed along channels of the kernels to reach tissues of the ear that contain less toxins, reducing mortality rate significantly (Dulmage et al. 1978). Hence, evolution of resistance is being influenced via the development of aversion as a behavioural response (Caprio et al. 2016; Hoy 1998).

Evolution of Bt resistance in *H. zea* has become a key threat for the efficacy of Bt Corn crops. There is concern about how this may affect future pest management strategies. This new strain of Bt Corn resistant moths provide a new challenge for pest management strategies. As they are a polyphagous pest that Bt toxins are not naturally high dose towards, resistant *H. zea* populations are

exceptionally hard to control. There is fear that this developed resistance will hinder possible future pest management strategies, such as pyramided Bt crops (Brévault et al. 2013).

Gene pyramiding is described as transgenic plants that express two or more dissimilar Bt proteins. It is believed that they will be more effective as there are a variety of Bt proteins available to kill pests if one were to fail (Zhao et al. 2003). Proteins that are adopted in Bt Corn typically include Cry1AB and Cry1F proteins. There are recommendations to include the Vip3A (Vip3Aa20) protein as promising results have been observed (Burkness et al. 2010; US EPA 2009; Yang et al., 2019). Adding the Vip3A to the Bt crops hopes to cover a wider array of pests. A study conducted by the US EPA has shown that corn producing the Vip3A protein without any other supporting proteins, does not produce a high enough dosage to manage the corn earworm (US EPA 2009). This reinforces that Vip3A requires contribution to a pre-existing pyramided gene to provide the efficiency needed to combat *H. zea*. When Bt crops are pyramided with Vip3A along with Cry proteins, they are considered near high dose for *H. zea* (Burkness et al. 2010). The use of pyramiding is not effective in delaying resistance when the insect is already resistant to one or more toxins expressed in the pyramid (Brévault et al. 2013). Hence, there is concern that the efficacy of further pyramided Bt Corn's will be reduced and that overtime a resistance to Vip3A may be obtained because of this (Brévault et al. 2013; Tabashnik & Carriere 2017). Predictions suggest that *H. zea* will follow a similar pattern of resistance evolution for Vip3A as the Cry toxins (Gassmann & Reisig 2023). Recent studies have reported that *H. zea* is evolving resistance to this toxin (Dively et al. 2021; Yang et al. 2020; Yang et al. 2021a, 2021b), although only one case of practical resistance has been reported so far (Pezzini 2022)

The continued development of resistance within these populations is a dynamic process. To combat this development, the evolutionary changes within these populations require ongoing monitoring. This will guarantee that new circumstances and knowledge is available ensures that management recommendations are still valid. To ensure that the damage done by pests remains contained, early detection of possible resistant populations to Bt Corn is needed and strategies to combat these populations need to be present. Monitoring resistance within populations will also provide a deeper understanding of what evolutionary changes *H. zea* are undergoing and how these changes are making them a superior pest.

1.2.3 Phenotypic Changes Within Insects as a Result of Developing Resistance to Pest Management Strategies

By analysing the body shape of a target species, it is possible to determine whether the physical traits observed have changed according to prevailing habitat characteristics. Phenotypic plasticity is defined as a change in the expression of the phenotype due to a change in the genotype under the influence of various environmental factors (Bradshaw 1965; Schlichting 1986). Phenotypic plasticity has been shown to have serious implications for evolution (Murren et al. 2005; Schlichting 2004). High plasticity of a species is the cause of the formation of different phenotypes that are able to help populations to resist changing environmental factors and to establish and maintain a population in a given area (DeWitt et al. 2004; Helmuth et al. 2005; Van Kleunen & Fisher 2005). Metric properties of insects (including the wing shape) are one of the first physical characteristics to change in an organism in response to environmental stressors (Levine & Oloumi-Sadeghi 1996; Bouyer et al. 2007). Flight studies involving winged animals (birds, bats, and insects) have demonstrated that selection pressure caused by, migration distance, sexual selection, foraging strategies, and geographic clines have the ability to change the shape of wings (e.g. medfly, *Ceratitis capitata*: Lemic et al. 2020). It can be expected that phenotypic changes will be observed in response to the addition of pest control measures as this presents a stressful environment for insects.

Geometric morphometric (GM) methods have been used to study genetic variability and plasticity of different insect species (Benítez et al. 2014a; Kadoić Balaško et al. 2022; Lemic et al. 2014; Mikac et al. 2013; Mikac et al. 2019; Pajač Živković et al. 2019) over the last several years. Mikac et al. (2013) was the first to investigate the wing shape and size differences in the invasive pest; Western Corn Rootworm (WCR) using GM methods. They compared the wing shape of resistance versus susceptible populations. Resistance was tested on individuals resistant to either crop rotation or the use of Bt Corn. Crop rotation involves annually rotating the cultivation of corn with another crop (usually soybean) to disrupt the lifecycle of the beetle. They would lay eggs in the soil of corn crops, only for the eggs to hatch when the field is growing soybean; a crop in which the larvae cannot survive on. Rotation resistance is a behavioural adaptation that is believed to have evolved in response to the high selective pressure imposed by rotation. Population of rotation resistance beetles still lay eggs in corn fields, but also disperse into the nearby non-corn crops (e.g., soybean) for oviposition (Levine et al. 2002). Thus, the eggs laid in the soybean crops will survive if the succeeding crop is corn (Pierce & Gray 2006). As resistant females recognise soybeans as ovipositional sites, they lay eggs into the soil during the 'soybean year' from which larvae hatch the following season to attack the rotated corn crop. The results of Mikac et al. (2013) showed wing

shape and size was significantly different for susceptible individuals versus individuals resistant to rotated corn. Longer and broader wings were present among resistant individuals, although the results revealed that resistance was an only better than average predictor of wing shape. This difference in morphology is likely a result of resistant females having different flight requirements than susceptible females (Onstad et al. 2005). Susceptible females undergo short-distance flight within the same crop for oviposition, whilst resistant females undergo movement between soybean and corn fields for oviposition. Overall, these results provided evidence that resistant individuals differ in both hindwing size and shape compared with susceptible individuals.

Following this study, Lemic et al. (2014) and Benítez et al. (2014a) conducted studies that showed GM could be used as a tool to examine differences in wing shape that have been influenced by the environment. They revealed that the wing shape of the Western Corn Rootworm changed in conjunction with major soil type classifications in Croatia. Lemic et al (2014) investigated the presence of sexual dimorphism within WCR by comparing the hindwing shape and size in population sampled across the USA Corn belt and Europe. The results showed high levels of sex wing shape dimorphism with females having more elongated wings in both USA and Europe populations. Two years later, Mikac et al. (2016) confirmed that GM processes were useful to identify invasion processes for the WCR, in this case the multiple introductions of WCR into Europe. They highlighted that GM methods could be used as a special monitoring tool for this pest species and proposed that it could be used for others. More recently, Mikac et al. (2019) extended the use of GM tools on the hindwing to examine possible differences among rotation resistant, Bt-resistant, and non-resistant (or susceptible) populations in the U.S. Their results showed that morphological differences exist in the hindwing shape of both rotation- and Bt-corn resistant populations versus susceptible populations (Mikac et al. 2019). These changes in WCR wing morphology appear to be a result of developing resistant to Bt Corn and/or crop rotation. In general, the hindwings of susceptible WCR were significantly elongated in shape and narrower in width (Mikac et al. 2019). This study established wing shape and size as a population marker that can detect differences between wild-type and resistant variants of insects (Mikac et al. 2019).

Pajač Živković et al. (2019) took this further and was the first to demonstrate significant differences in wing shape in lepidopterans in relation to resistance. Codling Moth (*Cydia pomonella*) is a significant apple pest in Croatia and Europe and has developed resistance to a variety of chemical-based insecticides. Analysis of the moth's forewings showed a reliable pattern of differences in forewing shape related to orchard control practice type. The results showed that the forewings of moths that were controlled through maintaining high levels of functional biodiversity (susceptible) had a significantly more elongated and narrower wing shape compared to moths that were treated

with chemical insecticides (resistant). Pajač Živković et al. also conducted Finite Element Method (FEM) study that was able to investigate flight performance by modelling various wing attributes (shape, size, vein membrane and thickness) in relation to environmental variables such as wind. The results of the FEM indicated that the wing/vein patterns of resistant had weaker structural integrity compared to other populations. Pajač Živković et al. thus hypothesised that intense selection pressure exerted by decades of pesticide use on the species has altered the structural integrity of its wings, overall making them less efficient at dispersal. This study provided evidence that resistance can influence wing shape in insects across multiple insect taxa (Mikac et al. 2019; Pajač Živković et al. 2019).

A study conducted by Kadoić Balaško et al. (2022) furthered Pajač Živković et al. research on the Codling Moth by using both genetic and GM tools to explore the difference in forewing shape of moths resistant and susceptible to chemical insecticides. The results revealed a significant difference in forewing shape between susceptible and resistant individuals, with susceptible individuals having a more elongated and narrower wing shape. These results corresponded to what was found with Pajač Živković et al. (2019). Interestingly these results were only observed via the use of GM techniques, the genetic tools were not able to isolate the resistant and susceptible population. This is because metric traits, including wing shape and size, are the first morphological trait to change under the influence of environmental and genetic factors (Bouyer et al. 2007; Levine & Oloumi-Sadeghi 1996). Hence, the phenotype adapts at a much faster rate than the genotype. The results produced by Kadoić Balaško et al. (2022) provided evidence that GM techniques is a more reliable monitoring tool of the resistance of pests compared to genetic tools, as they can identify resistance present within morphotypes before it is established within the genome.

1.3 Geometric Morphometrics as a Tool for Investigating Bt Corn Resistant Morphological Changes in Insects

1.3.1 What is Geometric Morphometrics (GM)

Morphometrics is the branch of biology which focuses on the characterising of organism form and the quantifying of morphological variation (Bookstein 1991). GM quantify morphological variation within and among populations and species. It can distinguish different phenotypic populations, revealing invasive adaptations that have arisen due to environmental factors (Bookstein 1985; Klingenberg 2010). Despite morphological variation being very visual in nature, it must be assessed in a rigorous and quantitative manner to test theories and create data for use in mathematical methods. Morphology usually associates tables and lists of numbers. Morphometrics allows us to statistically measure shape whilst maintaining its visual integrity and provides the reader with the resources needed to imagine the shape of the measured object. It allows for the visualisation and interpretation of the results of the statistical analyses.

Morphometrics has been used in science for several centuries. An early example is Albrecht Durer who in 1528 used morphometrics to assess the shape transformation of the human head. More recently, morphometric approaches were used in the 1960s and 1970s as part of the numerical taxonomy movement. During this time, quantitative methods were used to identify and clarify organisms (Sneath & Sokal 1973). Over the last two decades further progress has been made (Rohlf & Marcus 1993). The term 'geometric morphometric' was first coined by Corti in 1993. Since then, the field has continued to grow substantially. GM methods are currently being used in a rapidly growing number of studies, ranging from examination of sperm cells (Firman & Simmons 2009) to measuring of dinosaur skulls (Brusatte et al. 2012). In more recent years has been used more frequently in the fields of ecology and genetic developmental biology. It is a dynamic discipline, with new developments often focusing on the expansion on existing methods and addressing specific biological problems rather than the development of completely new frameworks (Klingenberg 2010).

In GM, size refers to the spatial extent of an organism or part (i.e., magnitude/dimensions). Similarly, shape is defined as "all the geometric information that remains when location, scale and rotational effects are filtered out from an object" (Kendall 1977). The combination of these words is typically referred to as 'form' (Goodall 1991).

GM typically deduces data about shape from the use of landmarks. Landmarks are defined as discrete anatomical loci that are recognisable as the same loci in all specimens in the study (Zelditch 2004). With the development of geometric morphometrics, a criterion for choosing landmarks have developed and include:

- Homology: Each landmark is measured in the exact same spot in all specimens.
- Consistency of Relative Position: Ensuring that landmarks do not alter their topological positions relative to other landmarks.
- Adequate Coverage of Form: Morphology should be adequately covered by choice of landmarks, avoiding as much sparse coverage as possible.
- Repeatability: Can be found repeatedly and reliably across all specimens
- Coplanarity of Landmarks: Associated with analysis of 3-dimensional organisms in 2-dimensions where specimens must be orientated under the camera under one plane.

Within landmarks, there are three distinct type of landmarks that have been defined as Type 1, Type 2, and Type 3 (Bookstein 1991). Within these Types, 1 is the most preferred, followed by Type 2, and Type 3 should be avoided at all costs. Definitions are as follows:

Type 1) Discrete juxtaposition of tissues.

Type 2) Local minima and/or maxima of a curvature, such as a bulge or tip of a structure.

Type 3) Extremes of curvatures or points further along (or away) from structure.

Traditional approaches to morphometric analysis usually involve the application of multivariate statistical procedures involving the collection of distances, angles or distance ratios. There are many problems associated with these methods (Bookstein 1985). The most significant is that the analysis is limited to a set of distances, ratios and angles. These frequently fail to capture the complete spatial arrangement of the landmarks they are trying to describe. As more landmarks are included within a study, more of these lengthy measurements are needed, to the point of impracticality. Instead, these landmarks are represented in the concise format of cartesian coordinates which encode all the information needed. When distances are measured via landmarks, then schemes like triangulations are helpful to achieve good coverage (Figure 1.4) (e.g. Rao and Suryawanshi 1998). Triangulations divide the configuration into triangles and identify a set of distances that do not intersect (except at the vertices of the triangles) (Figure 1.4). They provide a set of distance measurements that avoids redundancy. The coordinates are recorded on a defined axis, where their values reflect the unique location, orientation, and size of each specimen. There have been multiple methods presented over the years to standardise these differences, undesirable properties of the specimen. The one used in

recent scientific communities is referred to as the Procrustes Method. This method is based on the least-squares estimation of translation, rotation, and scaling-parameters that optimally align each set of landmark coordinates for each specimen (Gower 1975; Rohlf 1990).

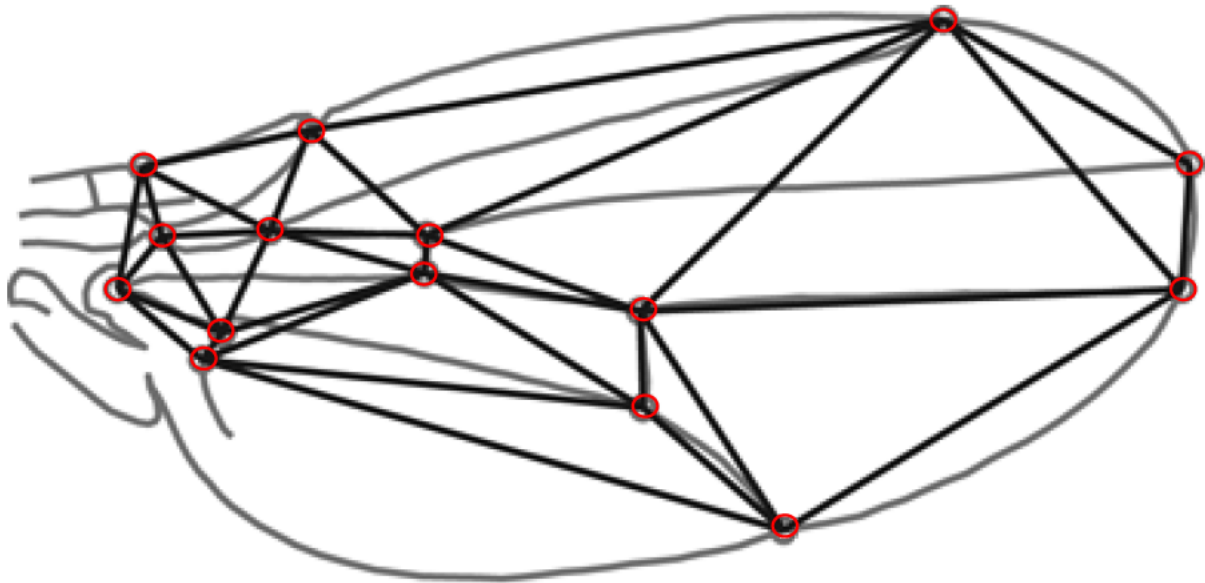


Figure 1.4) Landmarks (red dots) on a 2-dimensional representation of a *Drosophila* wing, with triangulations deduced from these landmarks represented by the black lines. Image modified from Klingenberg (2009).

1.3.2 GM as a Method for Describing Evolutionary Change in Resistant Pest Populations

Resistant populations of pests need to be constantly monitored to suppress the further spread of resistance. To keep management recommendations as recent as possible, monitoring is essential, especially considering changing conditions or new data gained (Bosch et al. 2018). Suppressing and preventing the further spread of pests is a prerequisite for the successful and sustainable production of crops. There is a need for new control tools and new approaches to pest management. One such being the use of Integrated Pest Management (IPM). IPM is imperative to suppress resistant populations. One component of this is understanding the population dynamics and dispersal patterns of the pest (Blommers 1994). To ensure the earliest level of detection and to understand dispersal patterns, novel methods like the use of GM should be used (Kadoić Balaško et al. 2022; Mikac et al. 2016).

Knowledge of pest's invasion pathways and dispersal routes is especially important for the accurate application of control measures. By analysing the shape and size of wings, it is possible to reveal the invasive adaptation of the resistant pests' traits to different environmental influences. GM offers a powerful method for studying intraspecific variations or ecotypes, such as resistant versus susceptible populations of the same species. It has been shown to act as a useful bio-monitoring tool (Hood 2000). One of the most important characteristics for invasive species is phenotypic plasticity as it has many evolutionary implications (Murren et al. 2005; Schlichting 2004). Because of high plasticity, wings within pest species are seen to evolve at fast rates, making GM an ideal technique to detect and monitor population variation and resistant variants in the field (Bouyer et al. 2007; Benítez et al. 2014a). GM can be used as a monitoring technique to detect resistant insect populations to provide crucial information needed for effective integrated pest management strategies (Lemic et al. 2016; Mikac et al. 2019; Pajač Živković et al. 2019).

GM can reveal differences in forewing shape and size amongst populations of pest insects and can act as a phenotypic biomarker. Integration of different techniques to understand the plasticity and variation of wing shape and size is vital to understanding how they adapt to new environments. From this, strategic planning can occur ahead of possible new invasions (Lemic et al. 2015). GM is a monitoring tool for monitoring inter and intraspecific variation in the shape change of the pest.

Morphology can retain useful info about genetic structure (Camara et al. 2006; Garnier et al. 2005; Henriques et al. 2020; Ortego et al. 2011). Understanding morphological traits of pests can provide additional information about underlying population genetics. Often genetics and GM tools are used together when attempting to identify differing populations (Garnier et al. 2005; Camara et al. 2006). Studies that use genetics are thorough and provide useful info about the genetic data of a population. Although, they are often expensive and impractical (Roush & Daly, 1990). GM on the other hand is inexpensive and easily accessible. This contributes to its use for widespread monitoring and hence early detection of resistance populations (Camara et al. 2006; Garnier et al. 2005; Henriques et al. 2020; Kadoić Balaško et al. 2022; Mikac et al. 2013; Mikac et al. 2016). Over the last 20 years GM has been used to study the genetic variability of different insect species (Benítez et al. 2018; Camara et al. 2006; Lemic et al. 2016; Lemic et al. 2020; Lemic et al. 2021; Pajač Živković et al. 2019).

Due to the emerging nature of resistance in pest populations, genetic tools have failed previously in identifying resistant populations that GM have been able to isolate (Lemic et al. 2014; Benítez et al. 2014b). Kadoić Balaško et al (2021) recently used both single nucleotide polymorphism (SNPs) and GM to detect resistant variants in beetles, detecting three genetic clusters that were only revealed

via the use of GM and not through SNPs. Similarly, Benítez et al. (2014a) used GM to explore the directional asymmetry of shape variation of the wings of the WCR. Their research showed genetic variation that genetic surveys conducted on the same species of insects had not detected previously. This is because metric traits, including wing shape and size, are the first morphological trait to change under the influence of environmental and genetic factors (Bouyer et al. 2007; Levine & Oloumi-Sadeghi 1996). Hence, the phenotype adapts at a much faster rate than the genotype. Methods that explore these phenotypic changes can reveal population resistance at a faster rate than methods focused on genetics (Bouyer et al. 2007). Alleles within areas that have become invaded by resistant pests have not had enough time to become fixed within the population to act as a biomarker for monitoring purposes (Lemic et al. 2016). Changes in an organism's genotype takes much longer to manifest than in its phenotype (Bouyer et al. 2007) thus making GM a much more useful tool than genetics for detecting changes in populations in the short term. GM methods are also relatively simple, easy to apply, require minimum financial investment, expert guidance, or equipment (Hood 2000). GM is a reliable, accurate, and cost-effective technique for detecting population changes associated with pests.

Over the last two decades, GM has been used to study the genetic variability within a variety of different insect species (Benítez et al. 2014b; Camara et al. 2006; Kadoić Balaško et al. 2021; Kadoić Balaško et al. 2022; Lemic et al. 2014; Lemic et al. 2020; Lemic et al. 2021; Mikac et al. 2013; Mikac et al. 2016; Mikac et al. 2019; Pajač Živković et al. 2019). GM has been used to detect other pests but not *H. zea*.

TSETSE FLY

The Human African trypanosomiasis (HAT) is a serious condition that attacks the nervous system of humans, causing extreme lethargy and death. In West Africa, HAT is mainly transmitted by the Tsetse fly species (*Glossina palpalis gambiensis*). A study was conducted by Camara et al. (2006) on three populations in Guinea to determine if the tsetse population from the Loos islands were isolated from two other populations off the mainland mangrove area. Population genetics and GM models was used. The GM analyses were based on 11 landmarks defined by vein intersection, from the forewings of 64 flies. Eighteen partial warps that corresponded to the landmarks, and the first eleven relative warps (principal components of the warps) were calculated due to the small sample size. A Multivariate regression of the partial warps on size was conducted on the total sample and separately on each sex, with permutation tests run for 1 000 rounds. This was done to estimate any residual allometry present within the dataset. Bilateral asymmetry and size asymmetry were tested

on a subsample of the individuals, with the use of a Procrustes ANOVA. Overall, both microsatellite and wing geometry data concluded that the Loos island population was separate from those of the mangrove areas on the mainland.

MEDFLY

Medfly (*Ceratitis capitata*) is one of the most economically important fruit flies. It is an extremely polyphagous pest that has been reported in five different continents across a variety of climates (Gilstrap & Hart 1987) and has been shown to have phenotypic changes as a response to change in environmental factors.

A study conducted in Croatia by Lemic et al. (2020), compared populations of Medfly found in mandarins collected from the island of Brac and the coastal location of Dubrovnik. Climate data and soil samples at the two locations were also collected and used to determine if they had any influence on wing shape. GM analyses were conducted on the wings of Medflies using 14 landmarks identified within the venation pattern, to estimate patterns of sexual shape dimorphism and the influence of environmental factors. Differences in environmental factors from both locations were identified using an Analysis of Variance (ANOVA). A Principal Component Analysis (PCA) was conducted to analyse the shape variation between the different sexes and populations. Allometry was also measured by conducting a multivariate regression of shape (Procrustes coordinate) on centroid size. A two blocks partial least squares analyses (PLS) was done to investigate the influence of environmental conditions between island and coastal geography on wing shape, by using 13 different environmental variables extracted from the climate and soil data collected. Lastly a Canonical Variate Analysis (CVA) was conducted to find the shape characteristics that best distinguished the groups of specimens. The results determined that even though mean wing shape comparisons revealed differences between populations, site-specific population differentiation was not strongly supported by wing traits. The results also showed that environmental effects had a non-significant effect on wing shape. When investigating wing shape, a significant difference was found between the mean shape of male and female flies. The females had more elongated wings that were better for long-distance flying and migration. This made sense as males typically establish mating territories and attract females, thus suggesting females are the main sex to undergo migration and there are no reason for males to undergo long movement. The theory highlights how sex-specific dispersal requirements is a key factor in influencing the wing shape of Medflies. This research provides key insight into the biological adaptation of medfly in newly invaded habitats, a significant process that ensures invasion success.

Further research was conducted on the Medfly with Lemic et al. (2021) exploring population variability based on wing shape of three host plants. A method that has been implemented in order to control the spread of this pest is the Sterile Insect Technique (SIT). SIT is a species-specific control method that can be used for many Diptera, Lepidoptera, and Coleoptera species. SIT works by releasing a large number of sterile males into the wild, that then compete with wild males and disrupt the reproduction of wild populations (Bjeliš et al. 2014). GM methods were used to determine what influence the host plant may have on the invasiveness and adaptability of the pest. A total of 386 individuals (i.e. 772 wings) were analysed using GM in Croatia. The flies came from infested fruits of three different host plants (peach, fig, and mandarin fruits) that were collected from within the fields. Each of these fields had different agroecological growing areas and different pest management practices. As part of the GM analysis, fourteen landmarks were used, based on the venation pattern on the forewing. A PCA was conducted to distinguish between sexes and populations. A Multivariate Regression tested the presence of allometry and analysed whether there was an effect of size on wing shape. Finally, a CVA was performed as a discriminant analysis to highlight differences between both the host and population groups. These GM analyses revealed that all medfly populations exhibited sexual dimorphism in wing shape, with females having more elongated and larger wings compared to males. The plant host type from which the medfly developed, influenced wing shape with flies from mandarin fruit differing largely from flies from peaches or figs. Both females and males of mandarin individuals manifested longer wings. This is a result of localised phenotypic plasticity within the population, which may act as a prerequisite for the high level of invasiveness seen within this species. Considerable variability was also detected between the sterile and wild populations of medflies. GM analysis determined that individuals from sterile population have narrower and smaller wings compared to individuals from the wild. The overall size of flies plays a key role in the mating rituals of this species; hence this result suggests that sterile individuals are overall less 'attractive' to females than the wild counterparts. This poses a risk to the use of SIT as a method for pest control. Lastly, high phenotypic plasticity was observed between the different study sites, and thus agroecological conditions, with a low but clearly detected variability present. This indicates genotypic stability but high phenotypic plasticity, which can be considered a prerequisite for medfly invasiveness and its ability to disperse into new areas.

WESTERN CORN ROOTWORM (WCR)

The Western Corn Rootworm (WCR) (*Diabrotica virgifera*) is the most serious pest of corn in the USA and recently Europe. Crop rotation remains the most effective control tactic against this beetle, although the use of the Bt toxin has also shown positive results. Crop rotation acts to break the lifecycle of these beetles as eggs laid in fields would hatch when a different crop is planted that the larvae are unable to survive on such as soybean. Resistance to both rotation and Bt Corn has been detected within populations, posing a serious problem for future pest management.

An analysis conducted on the WCR explored the effects of sexual dimorphism and developed resistance to crop rotation, through GM analysis on hindwings (Mikac et al. 2013). GM analyses was conducted on thirteen type I landmarks present within the beetles' hind wing venation from 223 specimens from 9 locations in the US Corn belt. Allometry was tested by multivariate regression, with Procrustes coordinate data as the independent variable and centroid size as an indicator of hind wing size. Sexual dimorphism was tested between males and females individually between the susceptible and resistant populations. Discriminant Function Analysis (DFA) was conducted on Procrustes transformed data with males and females representing two categories that were tested for significance using Hotelling's T^2 distribution. For between sex and site comparison, wing centroid size was analysed using an ANOVA. Wing shape differences were analysed using DFA, and the reliability of the DFA results were assessed by cross-validation. The results suggested that allometry did not influence wing shape. It also detected that there was no significant difference of wing size detected between the sexes, although they had significantly different shapes with the females being more elongated than the males. This corresponded to their sex-specific dispersal behaviour where females mate and then undergo long-distance flying before laying eggs, whilst males typically have no reason to undergo long-distance flying. Wing shape and size was significantly different between susceptible and resistant individuals. The results showed that resistant individuals had longer and broader wings compared to their susceptible counterparts. Although, cross-validation of these results revealed that resistance was only a better than average predictor of shape in males and females. This study provided preliminary evidence that wing shape and sizes were different in resistant and susceptible populations.

Lemic et al. (2014) further explored sexual dimorphism and allometry in the WCR across populations from Europe and USA Corn Belt. 686 adults were collected by hand from four locations in Europe and five from the USA Corn Belt. The hind wing shape of these adults were analysed by GM techniques using fourteen landmarks that were based on the venation pattern of the wing. A CVA was conducted to test for the sexual dimorphism associated with location, i.e. on an intercontinental

scale. A Multivariate statistical method with 10 000 iterations was used to find the shape characters that best distinguished among groups of specimens. These results were reported using Mahalanobis and Procrustes distance and accompanying P-Value. A Procrustes ANOVA explored sexual dimorphism was associated with shape and size differences across all locations. Lastly a Multivariate Regression of shape, pooled within sex based on centroid size tested for the presence of static allometry. These results depicted high levels of sex-based hindwing shape dimorphism, with females having more elongated wings than males. This pattern was observed across all locations but was stronger and more obvious in locations from the USA Corn Belt. This observed sexual dimorphism in wing shape are likely to be explained by the sex-specific requirements of the species.

GM analyses was conducted on the hind wings of WCR to test wing development and function (Benítez et al. 2014b). Morphological integration refers to the connection between or among an organism's morphological traits (Klingenberg et al. 2001; Olson & Miller 1958), whilst modularity refers to morphological traits that are internally coherent by tight integration among parts which are relatively independent of others (Klingenberg 2004; Klingenberg 2013; Wagner 1996). Previous research suggests that anterior and posterior wing compartments of hind wings could be considered separate modules/developmental units (Pezzoli et al. 1997). By correlating the left to right asymmetries in the anterior and posterior parts of the wing it is possible to determine if the same level of disturbance has affected both, thus reflecting the existence of the pattern of integration. GM analysis was used to test the pattern of morphological integration and modularity on the beetle's hindwing shape. A total of 334 individuals were collected from corn plants in Croatia, and fourteen type I landmarks were defined in the venation pattern of the hindwing. Right wings were mirrored, and all images were superimposed via the Procrustes method to produce averages and contrasts of the original and reflected configurations. This produced the symmetric variation and asymmetric component of shape which distinguished the left to right variation among individuals and analysed the shape exhibiting bilateral asymmetry. Following this, a PCA was conducted so that the individual variation and fluctuating asymmetric could be calculated. Allometry was a serious concern for this research so a multivariate regression of shape on centroid size was conducted, with the dataset pooled by sex and location of the symmetric and the asymmetric component. Percentage of the total shape covariation between the anterior and posterior wing modules was visualised using a Partial Least Squares analysis. Lastly a RV coefficient was measured to calculate the strength of association between the posterior and anterior parts of the wing. The RV values were as different as would be expected by random partitions of the wing, thus the results rejected the null hypothesis and suggested the hindwings act as a singular integrated structure. The wing shape showed internal integration which could enable flexibility and consequently enhance flight manoeuvrability.

Mikac et al. (2016) undertook a large-scale study over Europe to investigate the hind-wing morphology of the WCR. They hypothesised that differences in wing shape would correlate to invasion processes previously established using genetic markers. Over 560 specimens were collected from Croatia, Italy, Hungary, Serbia and Austria, and GM analyses was conducted on 14 landmarks identified in the venation patterns of the hindwings. A Procrustes ANOVA calculated the impact that measurement error had on the data collected. A PCA was done to visualise hind-wing shape variation related to invasion processes. Both Procrustes and Mahalanobis values were measured to determine the magnitude of shape variation present between countries and invasion processes. Finally, a multivariate regression analysis of wing shape on centroid size was conducted to detect any allometric effects that size may have on shape. The results determined that changes within the wing shape were related to identifiable invasion processes, i.e. introduction events into Europe. Both the PCA and the two distance measures separated population from Italy and those from the first known introduced populations in Europe, corroborating what had been identified through genetic studies. Differences in wing shape also suggest an East to West direction of spread which had also been highlighted in previous studies. This study confirmed that GM can be used to identify invasion processes within the WCR species and recommended its use for future detection of possible invasion occurrences.

In Mikac et al (2019), the morphology of WCR hindwings were analysed to determine if there was a significant difference between populations of resistant and non-resistant beetles. Resistant populations included individuals that were resistant to both Bt Corn and/or rotation crop methods. WCR were collected from Iowa, Indiana, and Illinois. GM analyses was conducted on the 358 individuals, based on fourteen type I landmarks identified in the venation pattern of their hindwings. A preliminary test using 30 specimens was conducted by using a Procrustes ANOVA to test for measurement error within the data. A PCA was also performed to visualise the hind-wing shape variation related to the development of resistance. A CVA was performed to compare the morphological relationship among resistant and non-resistant populations. A Procrustes ANOVA was conducted to determine if there was a significant difference between the hind wing shapes of different beetle populations. Lastly a Multivariate Regression of shape versus centroid size was done to determine if size had an allometric effect on the shape of the forewings. These tests showed the presence of morphological differences that existed in hind-wing shape of both rotation- and Bt Corn- resistant beetles compared to their non-resistant counterparts. Resistant hindwings were shown to be shorter and length and greater in width, suggesting a greater propensity for short-distant movement. Whilst the susceptible hind-wings had longer and more-narrow wings, suggesting they were better at long-distance dispersal and sustained flight at higher altitudes. It was

hypothesised that susceptible beetles would consequently be able to spread their genes more efficiently than their resistant counterparts; useful information that should be incorporated when preparing an Integrated Pest Management strategy for this pest.

Another study was conducted on American populations of WCR (Kadoić Balaško et al. 2021). Individuals were analysed using both genetic and morphometric analysis to determine if there were differences between resistant and non-resistant populations. The GM results confirmed the genetic results, with different genetic populations showing similar wing shape.

CODLING MOTH

The Codling Moth (*Cydia pomonella*) is a significant apple pest throughout Croatia and Europe, and has developed resistance to 22 different chemical insecticides that were used to suppress it. Thus the development of resistance poses a serious problem in apple production and in future pest management of the Codling Moth (CM).

A study conducted in Croatia in 2019 by Pajač Živković et al. collected 294 CM individuals from three different types of apple orchards. The first type of apple orchard that moths were collected from was an integrated pest management (IPM) orchard that relied on; pest and disease monitoring systems, and threshold-based applications of a variety of pesticides that were sprayed approximately ten to fifteen times during the growing season. The second was an ecological orchard (ecological) that did not treat with chemical agent but instead controlled pests through maintaining high levels of functional biodiversity, i.e. beneficial insect assemblages. The last were laboratory reared orchard (lab) that had no exposure to any form of pest deterrent and reared non-resistant moths. Basic GM procedures were conducted based on 18 landmarks within the venation pattern on the moths' forewings. Procrustes ANOVA was first done to measure measurement error, and allometry was accounted for by conducting a multivariate regression of shape (Procrustes coordinate) on size (centroid size). A PCA was conducted for visualisation of the entire dataset. A Procrustes ANOVA with permutation procedures was performed to assess differences in wing shape amongst groups. A CVA with cross-validations was run to explore morphological shape variables that were maximised between resistant and non-resistant group variance, relative to within-group variance. Finite Element Method (FEM) was also used to measure the level of wing deformation versus wing speed. It investigated the flight performance of the moths by modelling various wing attributes in relation to environmental variables such as wind. The combination GM and FEM methods allowed for a comparison between wing shape differences based on resistance, and flight

efficiency. This method followed work done by Mikac et al. (2019). The results showed that forewing shape differences were detected, and a reliable pattern of differences were established that related forewing shape to orchard control practice type. This was the first study conducted to demonstrate evidence of distinct lepidopteran wing shape differences related to resistance. It suggested that these results may be caused by intense selection pressure causing the phenotype of the moth to change as a result. The results indicated that moths from IPM orchards, had wing shape more suitable to withstand higher windspeeds. Moths from the ecological population, on the other hand, had wings that were most suitable for flying. Presumably, they were the best dispersers and were most likely to spread their alleles through the landscape. Despite these observable changes, no net difference on capability of flight was observed.

Kadoić Balaško et al. (2022) also explored the impact that different management systems had on the forewing shape of the Codling moth (*Cydia pomonella*). Populations were collected from three different type of apple orchards; organic, integrated pest management (IPM), and lab-reared. Both genetic and GM methods were used. GM was conducted on 18 landmarks within the venation pattern of the forewings. A PCA was done to visualise forewing shape variations, and a Procrustes ANOVA was done to detect statistical differences between orchards. The presence for allometry was also tested using a multivariate regression of shape versus centroid size. GM was highly sensitive and showed three distinct groups. It differentiated IPM from organic clearly and showed that organic and lab-reared orchards were remarkably similar. They both produced moths with more elongated and narrow wings which are more aerodynamic and have positive implications for long-distance/migration movement. They hypothesised this would provide better dispersal and thus these susceptible moths were likely responsible for the expansion of the population. Overall, they concluded that the use of pesticides has altered the structural integrity of wings, making them less efficient.

1.4 Hypothesis and Aims of Thesis

1.4.1 Specific Aims

This thesis will:

1. Investigate the effect that resistance to the crop Bt Corn has on the wing morphology (shape and size) of the *H. zea* moth.
2. Determine if these changes in wing shape can be detected via the use of geometric morphometrics (GM).

1.4.2 Hypotheses

1. There will be a difference in wing shape and size between *H. zea* moths that are from different treatments of Bt corn including non-Bt Corn, 2-toxin Bt Corn, 3-toxin Bt Corn, and 3-toxin Bt Corn seed blend.
2. Differences in wing shape can act as an indicator for which treatment of Bt Corn, *H. zea* individuals were collected from.

2. Methods

2.1 Sample Sites and Specimen Collection

H. zea pupae were collected at two sites across USA during the 2020 corn growing season. The first site was: (1) Clemson University Pee Dee Research and Education Centre, in Florence South Carolina; and the second site (2) North Carolina State University Vernon James Research, in Plymouth North Carolina (Figure 2.1).



Figure 2.1) Map of United States of America with states outlined. Location of the first site (1) Clemson University Pee Dee Research and Education Centre, in Florence South Carolina (34.288770928° N, -79.7380787674° W), and second site (2) North Carolina State University Vernon James Research, in Plymouth North Carolina (35.7796° N, 78.6382° W).

To increase the likelihood of *H. zea* infestations in these areas, the collection of specimens was repeated. One early in the corn growing season (early) and again during a second planted crop during the same season (late). At each location, there were five treatments (Table 2.1) from a complete randomised block design with four blocks per trial. Each treatment was located at least 200m away from other fields to avoid pollen contamination (Yang et al. 2014). Fertiliser and

herbicide were applied according to guidelines recommended by the respective Cooperative Extension Service in each state.

Table 2.1) The different treatments of corn (*Zea mays*) from which *H. zea* were collected from. n = 145. Treatments consisted of Bt corn expressing either 2-toxins or 3-toxins, which either contained structured non-Bt refuges or were part of a seed blend. Seed blends consist of the mixing of two different types of seeds containing different amounts of Bt toxins. Structured refuges are patches of corn crops that consist of non-Bt Corn.

Treatment	Bt Toxins Present	Descriptions	<i>H. zea</i> collected (n)
1	Control (No toxins)	Crop of non-Bt Corn	40
2	Cry1Ab + Cry1F	Crop of Bt Corn containing two toxins with a structured refuge present	35
3	Cry1Ab + Cry1F + Vip3A	Crop of Bt Corn containing three toxins with a structured refuge present	35
5	Cry1Ab + Cry1F + Vip3A	Crop of seed blended Bt Corn with 80% containing three toxins and 20% not having any toxins (non-Bt Corn)	35

Two modified hibernation boxes were placed in each field treatment (Phillips & Barber 1929). These boxes allowed pre-pupae *H. zea* to drop from ears of corn and bury themselves within the soil that was within the box. Such boxes ensure that pupae from a specific treatment type were exclusively sampled. For seed blend treatments hibernation boxes were placed on the ears of Bt and non-Bt Corn treatments. Measurements were taken of the pupae and again when the adults were allowed to emerge, these included weight, depth of burying, and sex. Individuals were numbered from 001 onwards. Sex was determined via examination of abdominal apex (Wu & Wang 2012).

Within the first 24 hours after eclosion, the flight performance of moths (i.e. adults) was measured using a flight mill study (Attisano et al. 2015). Following this, the wings of the moths flown on the flight mill were dissected, stored dry and sent to the University of Wollongong to be used in this thesis. The specimens were stored at -20°C until they were prepared, and slide mounted for the geometric analyses described hereinafter.

2.2 Wing Preparation

Wings of individual moths were prepared for slide mounting by initially removing their scales. This was done by: soaking wings in a solution of (10%) bleach for three minutes, followed by an ethanol (70% v/v) solution for another three minutes, and a final rinse in a distilled bath (Pajač Živković et al. 2019). When necessary, the chemically treated wings were lightly brushed with a fine paint brush to ensure scale removal and clear exposure of vein anatomy of the wing. Left and right forewings of each specimen were then slide mounted on glass slides (75mmx26mm), using coverslips (22mm x 22mm), and the fixing agent Euparal (Australian Entomological Supplies, Melbourne) based on methods outlined by Upton and Mantle (2010).

2.3 Wing Landmark Acquisition

Venation pattern of *H. zea* was drawn and divided into the Subcosta (S), Radius (R), Medial (M), Anterior Cubitus (CuA), and Anal (A) veins. They were labelled with formal entomological terminology (CSIRO: Division of Entomology 1970) (Figure 2.2). From this venation pattern, fifteen “type 1 landmarks” were identified for GM analysis (Bookstein 1991) (Figure 2.3). When choosing landmarks, the number of landmarks were limited by the number of recognisable homologous features that exist on the structure (Bookstein 1991). The choice of landmarks was of crucial importance for all subsequent analysis. They were located precisely and unambiguously on each specimen (Klingenberg 2004). Landmarks that were duplicated or absent in some specimens would have posed difficult problems and were avoided. For many biological forms, there are landmarks on both the top and bottom of the specimen. By using the venation patterns within the wings this eliminated that issue as venation is visible on both the top and bottom of wings. Hence only one side of the wing needed to be assessed: in this case the top.

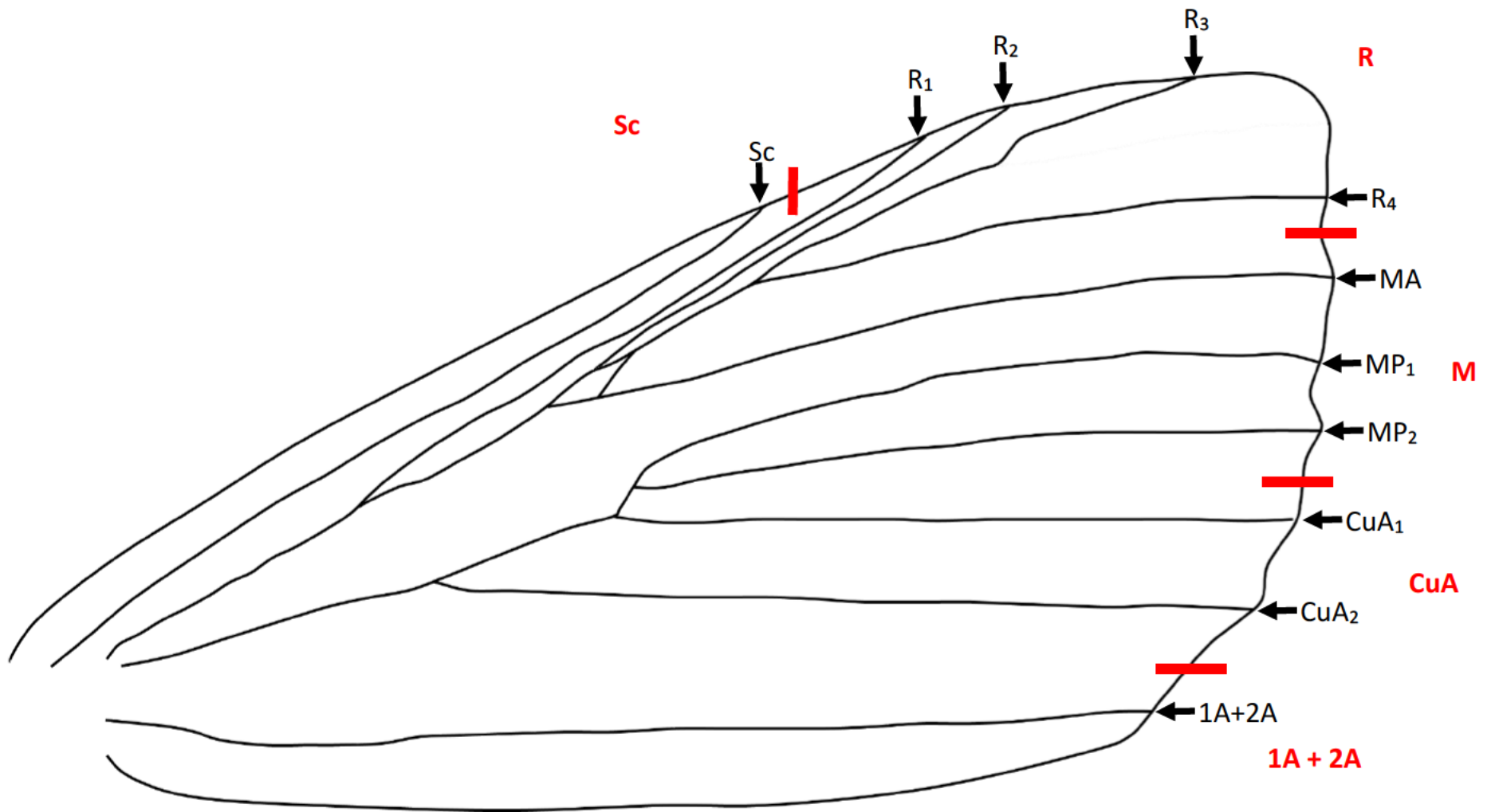


Figure 2.2) Venation pattern of the forewing of *Helicoverpa zea*, with veins divided into Subcosta (Sc), Radius (R), Medius (M), Cubitus Anterior (CuA), and Anal (A) sections by red lines. Each vein is labelled according to standard terminology (CSIRO: Division of Entomology 1970).

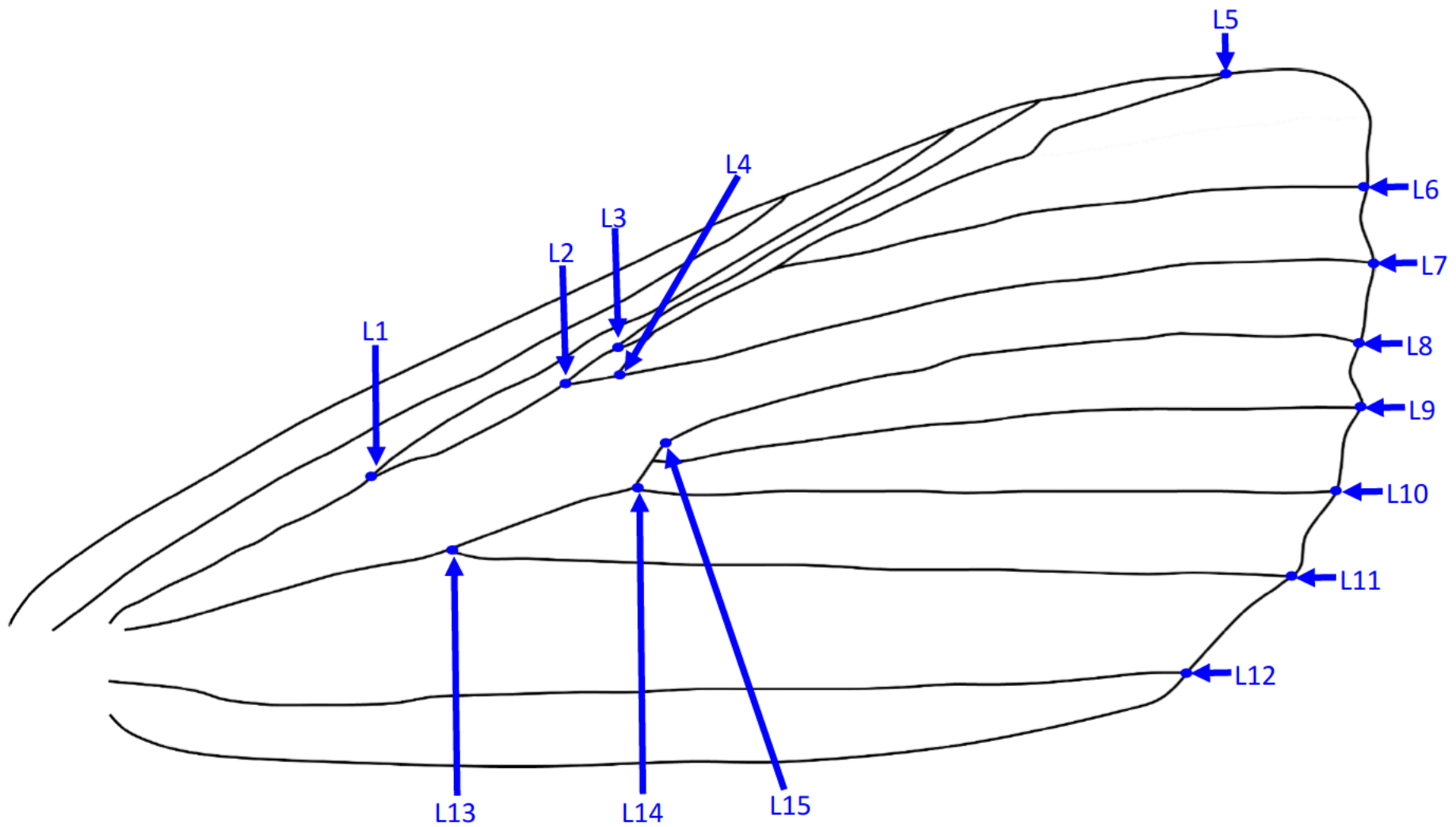


Figure 2.3) Venation pattern of a *Helicoverpa zea* forewing, with the 15 type 1 landmarks used in this study.

The 15 landmarks identified and used (Cañas-Hoyos et al. 2016; Nagoshi & Allan 2020) were: LM1: intersection of origin of Radial R_1 Vein from the Primary Radial Vein; LM2: second division on Posterior Radial Vein; LM3: intersection of Radial R_4 and Radial R_5 Vein; LM4: intersection of origin of Medial Anterior MA Vein from Radial Vein; LM5: termination of Radial R_4 Vein at the distal wing border; LM6: termination of Radial R_5 Vein at the distal wing border; LM7: termination of Media Anterior MA Vein at the distal wing border; LM8: termination of Medial Posterior MP_1 Vein at the distal wing border; LM9: termination of Medial Posterior MP_2 Vein at the distal wing border; LM10: termination of Cubitus Anterior CuA_1 Vein at the distal wing border; LM11: termination of Cubitus Anterior CuA_2 Vein at the distal wing border; LM12: termination of combined Anal $A1+A2$ Vein at the distal wing border; LM13: branching of Cubitus Anterior CuA_2 Vein from Primary Cubitus Anterior Vein; LM14: branching of Cubitus Anterior CuA_2 Vein from Primary Cubitus Anterior Vein; and LM15: intersection of origin of Media Posterior R_4 Vein from Primary Cubitus Posterior Vein.

When measuring specimens, it was important to analyse possible human induced error in the dataset. Differences in experiences and possible subconscious biases could result in differences in measurements (Lee 1982). Different observers may have used slightly different criteria when locating landmarks and thus could cause differences among how observers locate landmarks (Robinson et al. 2002). To minimise the risk of this happening, a single observer landmarked all the specimen photographs for this study. As the observer became more familiar with the specimens, they may have adjusted to accommodate for the whole range of variation. Also, as the observer gained more experience with landmarking the measurement variability was likely to decrease (Yezerinac et al. 1992). To address this, a single observer randomised the sequence with specimens coming from different groups, whilst concealing the identity of specimens to avoid possible subconscious biases.

Slide mounted wings were photographed using a Nikon D 7500 camera (20.9M Pixel) on a trinocular mount with a Micro Nikkor 40mL lens. For downstream landmarking it was recommended that an image resolution of 1-3 million pixels is needed (Klingenberg 2013), thus the resolution of 20.9 million that this camera provided was more than adequate. The camera was placed so that each specimen occupied approximately the same area in the viewing field, to minimise distortion. Damaged and folded wings caused by the slide-mounting process were discarded and excluded from further analysis. These photographs were saved in a NEF file, before being converted into a TIFF file using Nikon Transfer 2 (v2.15). The software program tpsUtil v1.82 was used to convert files from TIFF to TPS format. tpsDig v2.32 (Rohlf 2005) was then used to landmark, record the scale of and digitise every specimen. This produced cartesian coordinates (x,y) that included the complete

information of all the pairwise distances between the landmarks and all angles simultaneously. The landmark data was saved as a TPS file and was imported into MorphoJ v1.07a (Klingenberg 2011) for GM statistical analysis.

2.4 Preliminary Data Analysis

A subsample of the *H. zea* samples (n = 30) was selected for preliminary tests to determine the effects of asymmetry, allometry, and possible sexual dimorphism on the shape of *H. zea* forewings. Out of the 30 moths used for preliminary analysis, individuals were selected that represent an even distribution of all populations, genders, and treatments of Bt Corn (Table 2.2)

Table 2.2) The gender, population and treatment of Bt Corn of the 30 *Helicoverpa zea* individuals used in the preliminary analysis, with accompanying totals.

Treatment of Bt Corn	Population						Total:
	North Carolina Early		South Carolina Early		South Carolina Late		
	Males	Females	Males	Females	Males	Females	
1	1	2	1	1	1	1	7
2	1	1	2	2	1	1	8
3	2	1	1	1	2	1	8
5	1	1	1	1	1	2	7
Total:	5	5	5	5	5	5	30

To compare shapes, unnecessary information such as position, orientation and scale were removed, this was done by rescaling the configurations to a standard size, shifting them to a standard position and rotating them to a standard orientation (Goodall 1991)(Figure 2.4). After this, the difference between landmark configurations that remained was exclusively caused by differences in shape. The process of Procrustes superimposition does this by dividing each landmark coordinate by the centroid size resulting in all wings having a centroid size of 1.0. Centroid size is the summed squared distances of each landmark from the centroid (Klingenberg 2013). The centroid is the centre of all the specimen landmarks and is defined as where the x and y coordinates of all landmarks are averaged. Centroid size is a measure of the amount of dispersion of landmarks around the centroid and thus a larger number is representative of a wing bigger in size. After the centroid size of all wings were converted to 1.0, averages of x and y coordinates of all landmarks for every wing were averaged so that the centroid coordinates lie on (0,0). Finally, all wings were rotated to produce an overall best fit which is determined by the smallest sum of squared distances between

corresponding landmarks (Bookstein 1991). This process produces a new set of coordinates, and the difference between these coordinates is called the Procrustes distance and is one of the main measures of shape difference used in GM (Goodall 1991).

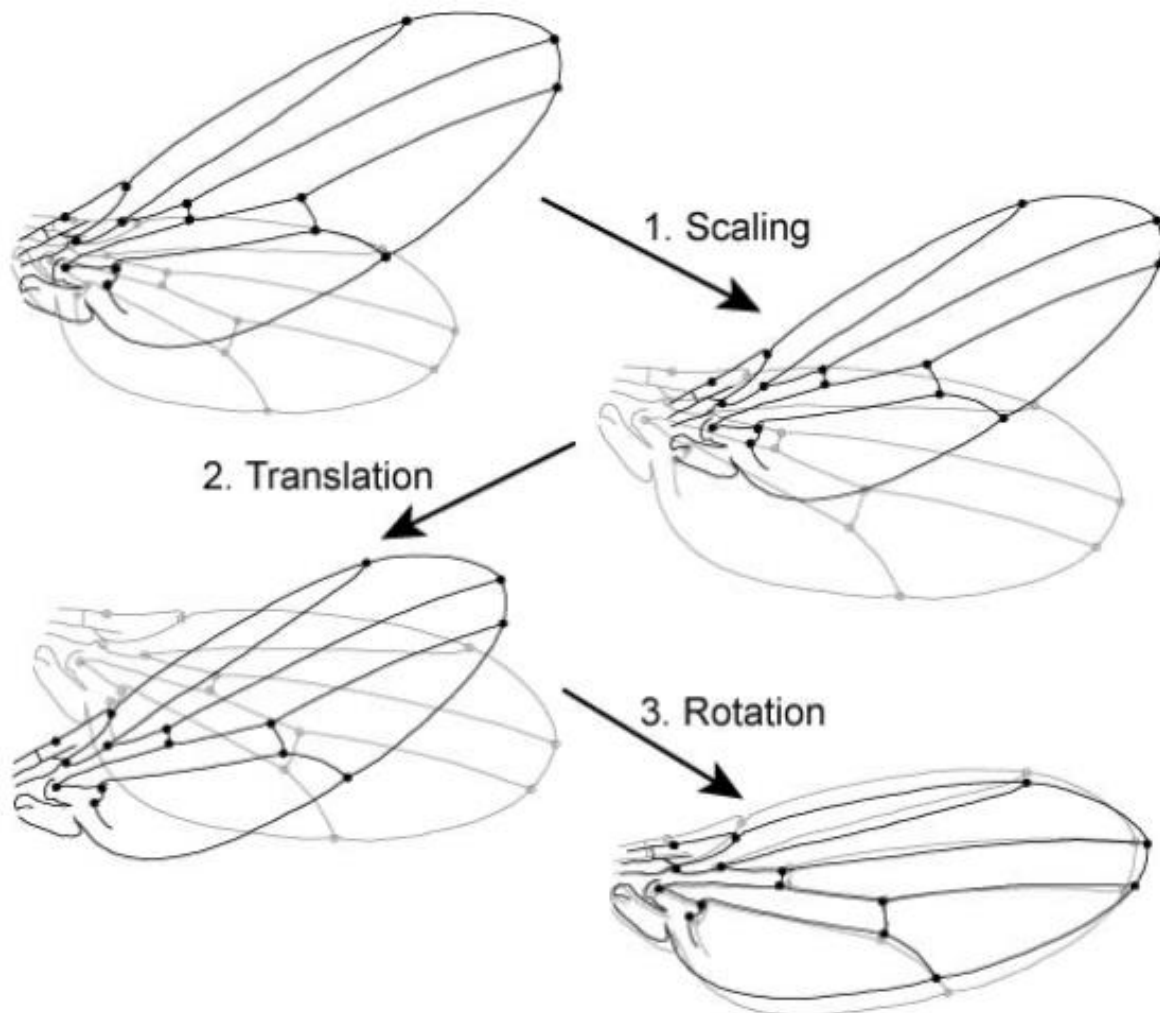


Figure 2.4) Summary of Procrustes Superimposition. All components of variation other than shape are eliminated; first by scaling to the same size, then translating to the same location of centroids, and then rotating to an overall best fit of corresponding landmarks. Image from Klingenberg (2009).

A variety of preliminary tests were conducted on the Procrustes transformed data to test for the presence of any confounding factors. These factors may have impacted the observed relationship between forewing shape and Bt Corn treatment such as: measurement error, bilateral asymmetry, allometry, and sexual dimorphism (Figure 2.5).

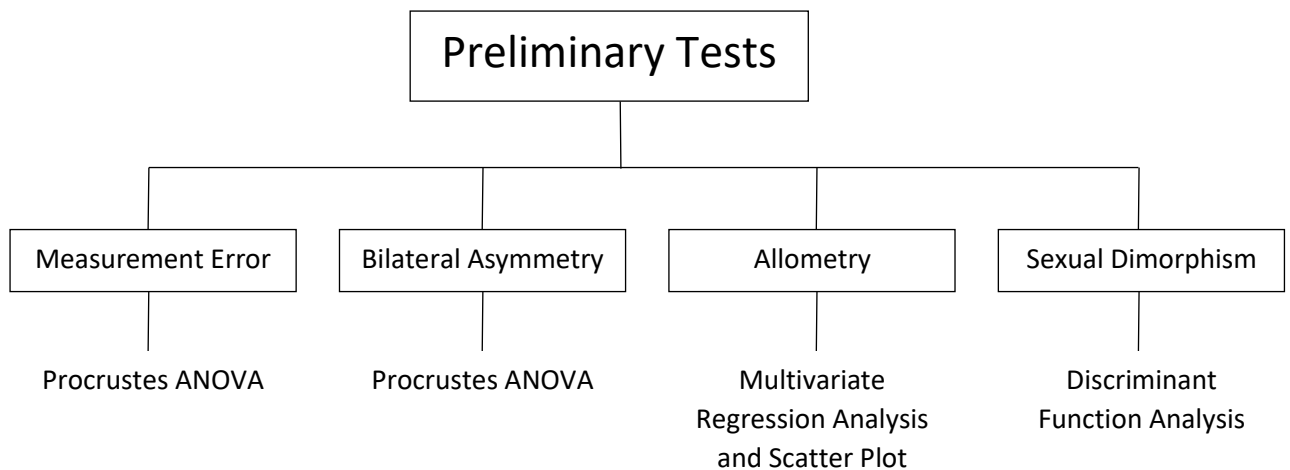


Figure 2.5) Flow diagram of preliminary data analyses to test for confounding factors that may affect downstream geometric morphometric analyses.

2.4.1 Measurement Error

Whilst measuring measurement error, it was important to acknowledge that practical procedures associated with the experimental process could have allowed for the introduction of errors. Errors could have been introduced into the dataset via the distortion of the specimen during the slide preparation and mounting, photographing or during the landmarking of specimens. Measurement error is always undesirable but can be tested for. It must be monitored and minimised so that it does not interfere with the subtle biological shape changes being investigated in this study (Arnqvist & Martensson 1998). Some systematic errors irreversibly change the value of the measurement to no longer represent a biologically accurate measurement (Lee 1982). This posed a threat to the reliability of the results.

In this thesis, measurement error was calculated to determine if error-reducing strategies needed to be implemented throughout the slide preparation and mounting process. To determine the systematic measurement error associated with the landmarking and photographing of the slide mounted specimens, each specimen was photographed twice, and each wing photograph was landmarked twice. This resulted in a total of four sets of cartesian coordinates for each specimen. A Procrustes ANOVA was applied to this data to compare the measurement error to the biological effects of asymmetry (Klingenberg & McIntyre 1998). Fluctuating Asymmetry was chosen to compare measurement error with, as it is typically the subtlest biological affect that can be measured and thus be compared with. If the measurement error was calculated to be similar to, or

larger than fluctuating asymmetry, that would represent a significant amount of measurement error present within the data.

Since the pilot study determined that the measurement error in the early stages of this study was negligible, further methods of reducing error such as replication in the actual data collection phase was not required.

2.4.2 Bilateral Asymmetry: Asymmetry between Left and Right Wings

Bilateral asymmetry was tested to determine if there were significant differences between left and right wings. A Procrustes ANOVA was used to test for asymmetry both wing shape and centroid size (Palmer & Strobeck 1986).

2.4.3 Allometry

Allometry refers to the association between size and shape (Mosimann 1970). Changes of mechanical loads often result in scaling relationships between various physiological and biomechanical factors. These factors need to be taken in consideration when comparing these results. To test for the presence of allometry within the *H. zea* samples, a multivariate regression was conducted on shape versus size (Loy et al. 1996; Monteiro 1999; Drake & Klingenberg 2008). Centroid size was used to describe size whilst Procrustes coordinate data described shape. A permutation test with 10 000 iterations was also conducted. Permutations assess the null hypothesis that two or more populations have identical distributions for the variables of interest; this is done via computer simulation that randomly exchange observations between the samples of specimens drawn from different populations (Good 2013; Pitman 1937).

These results were expressed visually in a scatter plot which had the regression score as the independent variable and centroid size as the dependent. The regression score chosen for representation in the scatter plot was the score that had the most influential impact on the independent variable, i.e. centroid size (Mosimann 1970). Allometry was tested to ensure that size did not have any influence on changes in shape, and that differences detected because of the treatments of Bt Corn were not confounded by size.

Ontogenetic variation is also of concern. It describes how the development of an individual organism from the earliest stage to maturity will have significant effects on shape. A solution to avoid

morphological changes caused by ontogenetic growth was to focus either on the aspects of the form that do not change with growth, or to compare specimens that were all in the same stage of growth. In this case, all moths had emerged as adults and within 24 hours were placed through the flight mill for three complete days. This ensured that they were all extremely close in development and age and ontogenetic variation would not have been influential in shape change.

2.4.4 Sexual Dimorphism

Sexual dimorphism was measured to determine differences in wing shape between males and females. To test for the presence of sexual dimorphism, left and right-wing shapes for each specimen within the subsample were averaged to produce a new dataset. This was done to ensure that the effects of bilateral asymmetry and sexual dimorphism was not confounded. A DFA was conducted on this dataset with 10 000 permutations run. The DFA was used to find the features of shape that distinguish between the distinct groups, in this case between males and females.

Distinguishing groups is more difficult in directions where there is a large amount of within-group variation. Thus, DFA transforms the whole space so that the within-group variation is the same in all directions. This transformation was done by multiplication of the inverse within-group variation. The differences between the means of males and females in the transformed space produced the discriminant function that distinguished the sexes. As part of the DFA, the Procrustes distance between the two-sample means was determined. A Parametric P-value test was calculated, and a Permutation P-value for Procrustes distance and Hotelling's T^2 was computed. Hotelling's T^2 is a multivariate counterpart to the univariate T^2 test, that tests for the significance difference between the means of two groups (Bookstein 1996).

The different treatment of corn (Table 2.1) was one classifier for this analysis, and sex was the other. In total this created 28 possible pairs of groups that could be analysed. Although, pairs that confounded sex effects and treatment effects were ignored, resulting in a total of 17 pairs of groups being analysed. The difference in Procrustes and Mahalanobis distance between the means of the two groups were also measured as part of the DFA. Mahalanobis distance is the measure of relative distance that indicates how well two groups are separated (Mardia et al. 1979). It is the multivariate equivalent to the measurement of the distance of a univariate value from a sample mean in units of SD.

2.5 Geometric Morphometric Analysis

In GM, sample size should ideally be greater than the number of variables included in the analysis (Klingenberg & McIntyre 1998). For two-dimensional (2D) data the number of specimens should ideally be greater than twice the number of landmarks. A total of 145 moths, left and right wings were prepared for use in GM methods (Table 2.1).

A variety of different statistical analyses were conducted on the entire dataset (n=145) to determine; whether there was a relationship between *H. zea* forewing shape and different treatments of Bt Corn, and whether forewing shape in *H. zea* was an indicator of which treatment the moths came from (Figure 2.6)

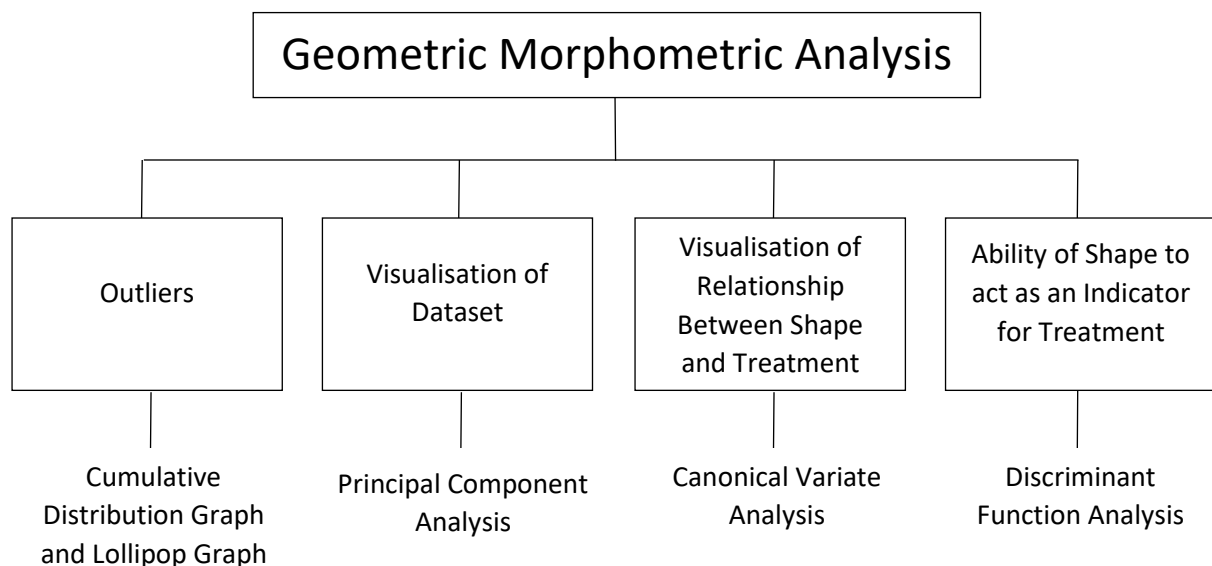


Figure 2.6) Flow diagram of the geometric morphometric analysis and the accompanying statistics undertaken. 'Shape' refers to the forewing shape of *Helicoverpa zea* samples, and 'Treatment' refers to the different treatments of Bt Corn from which *H. zea* were collected.

2.5.1 Identifying Outliers

To identify outliers within the study, a cumulative distribution graph was created. It compared the cumulative distribution function of the data, with the normal distribution expected for a multivariate analysis. Cumulative frequency was on the y-axis and Mahalanobis distance squared was on the x-axis.

A potential outlier was identified (Specimen SE3nf122). A lollipop graph was produced to compare the shape changes in the specimen to the average. This determined that there was no reason for this specimen to not be used in this study and hence no outliers were excluded.

2.5.2 Visualisation of Complete Dataset through Principal Component Analysis

Firstly, a PCA was conducted on the entire data set to visualise and identify any treatment-based groupings or associations that were present. PCA is the most common technique used to explore patterns of variation within data points (Jolliffe 2002). It allows for the examination of the variation in the dataset overall. It shows which shape changes are associated with the most variation, thus allowing for the most variable or most constant shape features to be identified. It is used in this study as an exploratory analysis to provide a visualisation of the overall variation.

PCA transforms the original variables of the analysis into a new set of variables which are referred to as Principal Components (PC) which are not correlated with each other and successively amount for the maximum amount of possible variation (Figure 2.7). It recentres the variables so that the average is the origin for the new coordinate system, which is aligned to the main axes of variation in the data. As it is not possible to visualise all the variations in the many dimensions of all the PCs, plots are constructed using pairs of PCs that show as much variation as possible.

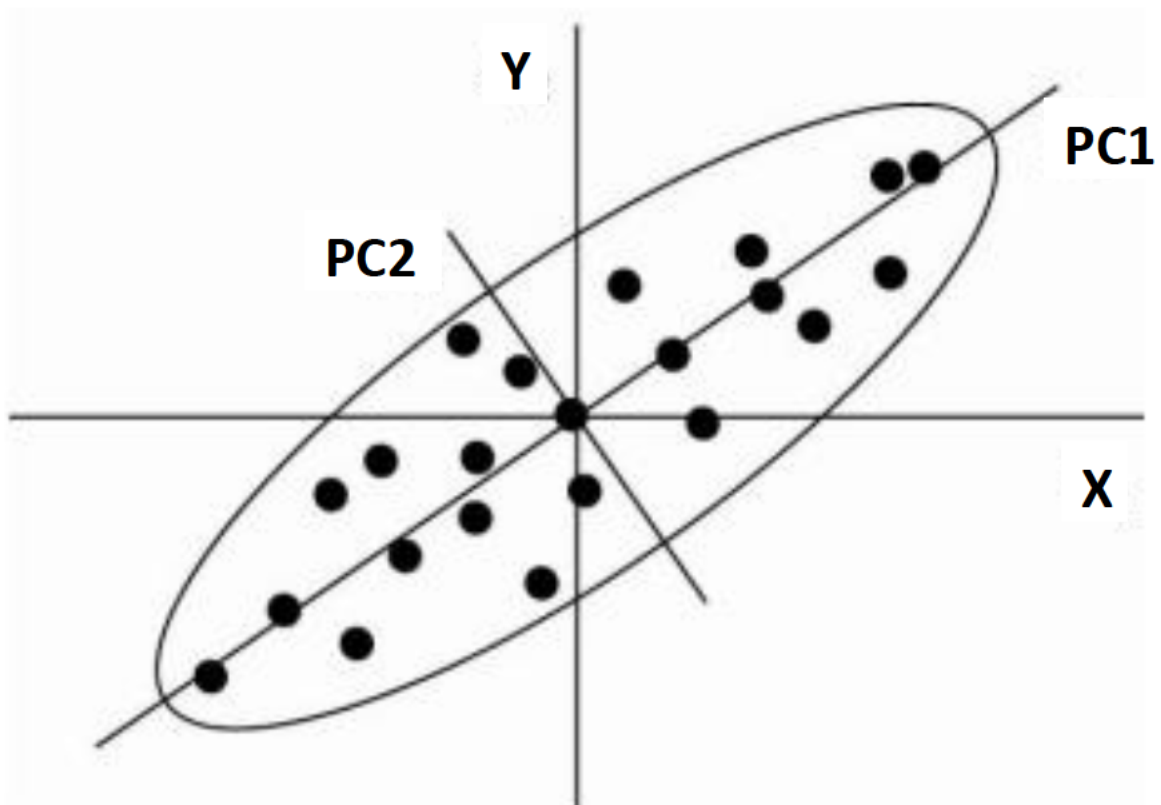


Figure 2.7) Example of the first (PC1) and second (PC2) Principal Component being determined via the process of Principal Component Analysis (PCA). PC1 is the direction in the plane that accounts for the most variance, whilst PC2 is the direction with the least variances. Image from Klingenberg (2009).

There are some limitations to using PCA as an exploratory graphical data analysis. There is no guarantee that a PCA will show group differences. However, gaps between groups that are seen in a PC plot are real. Typically, leaving out the last four PCs of shape data that has undergone Procrustes This is because the first three to five PCs typically cover a significant percentage of variation, with a cumulative variation typically well over 90%. Superimposition has no effect on the results as the PC do not contain any variation, MophoJ does this automatically and is not shown. Another benefit of this is that the first few PCs are usually less affected by measurement error than the subsequent ones (Lougheed et al. 1991).

2.5.3 Visualisation of Differences in Wing Shape According to Different Treatments of Bt Corn through Canonical Variate Analysis

A CVA (Albrecht 1980) was conducted on the complete data set to examine and summarise the variation in wing shape among the different treatments. It presents this variation in as few dimensions as possible. CVA is a combination of both DFA and PCA, and like PCA, produces a new set of variables; the canonical variables (CVs). The space of CVs is a transformed version of ordinal data space whereby the distances are now Mahalanobis distances. CVs successively account for the maximum among-group variance relative to within-group variance. The computation of the CVs can be considered geometrically as a rescaling and rotation of the coordinate system in two steps. First scaling of the multivariate space that makes within group variation equal in all directions. Then, secondly, aligning the CVS with the major axes of variation among groups. The CVs are then used to produce a scatter plot that visualises the variation among the different treatments. For this analysis, CVA instead of PCA was used. PCA does not consider any structures such as group memberships of observations in the data set, but instead displays the general pattern of variation. PCA is therefore a useful tool for general inspection of the data but for the purpose of distinguishing difference in wing shape in correspondence to the different treatments, CVA is used.

2.5.4 Determine if Wing Shape Can Act as an Indicator for Different Treatments of Bt Corn through Discriminant Function Analysis

A DFA was conducted to determine if wing shape could be used to identify which treatment of Bt Corn it was collected from. Pairwise analysis of treatments was undertaken along with 10 000 permutations to distinguish between the different treatments. They determined if there was a discriminating function present that would allow moths from unknown treatments to be characterised into certain treatments with high certainty. The difference in Procrustes and Mahalanobis distance between the means of the two groups was measured again as part of the DFA. A Parametric P-value test was also calculated, as well as the Permutation P-value for Procrustes distance and Hotelling's T^2 .

2.6 Different Visualisations of Results

Transformation grids have been used as an effective way to visualise changes in shapes since their invention in 1961 (Thompson 1961). Transformation grids describe a rectangular grid superimposed on a drawing of a starting form before distorting to match the drawing of the final form (Klingenberg 2013) (Figure 2.8). The “lollipops” in the graph indicate the relative shifts of the landmarks; with the starting form being represented by the dot and the bold blue lines representing the changes to the target shape (Figure 2.8).

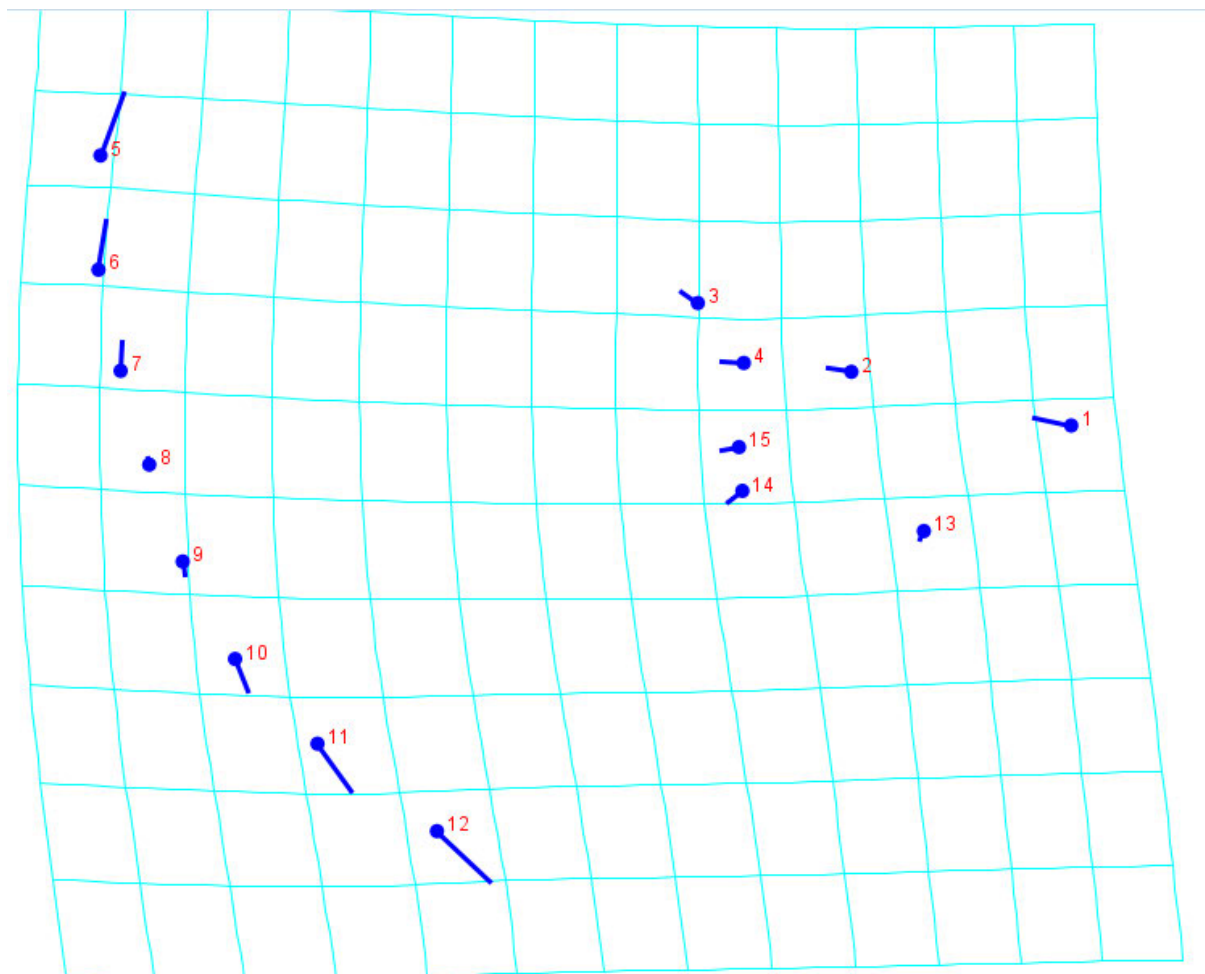


Figure 2.8) Lollipop graph with transformation grid. The dot of the lollipop represents the mean wing shape, whilst the stick represents changes that occurred. Graph was produced by MorphoJ v1.07a (Klingenberg 2011).

There are disadvantages associated with the interpretation of this kind of graph. The shifts in landmarks should not be interpreted independently but in the context of all the landmarks together (Klingenberg 2013). The changes in the shape can look different depending on the directions of the grid lines and the orientation of the specimen. Also, these grids can be hard to understand and often

lead the casual reader to be confused or to misunderstand some of the subtler aspects of the shape changes (Klingenberg 2013).

To address these challenges, new methods to visually present these changes in shape have been developed. These include warped outline drawings, whereby an outline of a form with corresponding landmarks is aligned to the average landmarks to create an average outline (Figure 2.9). Then changes in these landmarks, results in a shift in the outline resulting in a 'warped outline'. It should be noted that all the information in these diagrams come from the changes in landmark positions and not the actual outlines, but the changes in shape will be consistent despite changes in orientation.

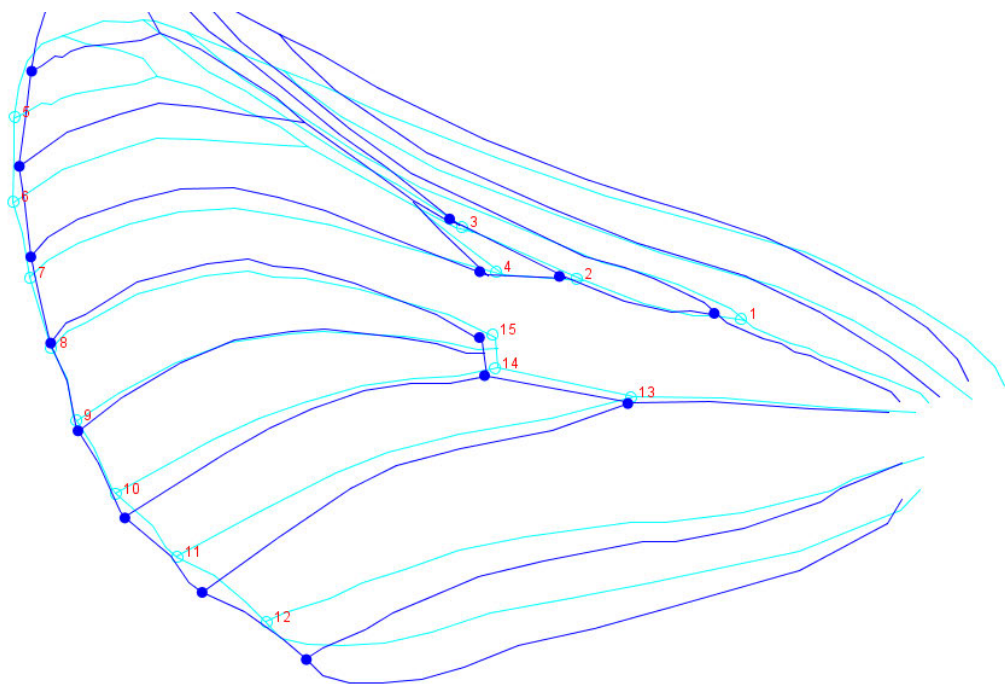


Figure 2.9) Transformed outline graph showing changes that may occur in the forewing of individual *H. zea*. The light blue outline is representative of the average wing shape whilst the dark blue outline represents the change in shape. Graph was produced by MorphoJ v1.07a (Klingenberg 2011).

Transformed outline graphs were chosen to visualise the results of the above analyses as they are much easier to interpret and for a casual reader to understand.

3. Results

3.1 Preliminary Tests for Asymmetry and Allometry

3.1.1 Measurement Error

The shape change associated with photographing error and landmarking error were both significant (Table 3.1). To determine if the measurement error observed within the data was significant enough to influence results, it was compared to the biological effect of fluctuating asymmetry. The mean square associated with fluctuating asymmetry was 5.74 times larger than the mean square associated with photographing error, and 9.62 times larger than the mean square associated with landmarking error (Table 3.1). Error was subsequently deemed negligible and would not interfere with results about shape changes caused by other biological effects.

Table 3.1) A Procrustes Analysis of Variance conducted on the forewings of a subsample (n = 30) of *Helicoverpa zea* moths, exploring the effects that Measurement Error and Asymmetry have on the shape of forewings. Individual refers to the individual forewing specimen, Side refers whether it is a left or right wing, Individual*Side is a measure of any fluctuating asymmetry present, photographing error refers to the measurement error calculated from the different photographs used of the same specimen, and landmarking error refers to the measurement error calculated from the different landmarking done on the same photograph.

Effect	Mean Square (MS)	Degrees of Freedom (df)	F ratio	P-value
Individual	0.0002401687	754	9.43	<.0001
Side	0.0000335672	26	1.32	0.1344
Individual*Side	0.0000254799	754	5.74	<.0001
Photographing Error	0.0000044406	1560	1.68	<.0001
Landmarking Error	0.0000026486	3120		

The change in centroid size associated with fluctuating asymmetry, photographing error, and landmarking error all had an F-ratio of 1 (Table 3.2). An F-ratio of 1 means that no matter what significance level was produced, all variances of centroid size are equal. Hence, photographing error and landmarking error did not have a significant effect on the centroid size of forewings.

Table 3.2) A Procrustes Analysis of Variance conducted on the forewings of a subsample (n = 30) of *Helicoverpa zea* moths, exploring the effects that Measurement Error and Asymmetry have on the size of forewings. Individual refers to the individual forewing specimen, Side refers to whether it is a left or right wing, Individual*Side is a measure of any fluctuating asymmetry present, photographing error refers to the measurement error calculated from the different photographs used of the same specimen, and landmarking error refers to the measurement error calculated from the different landmarking done on the same photograph.

Effect	Mean Square (MS)	Degrees of Freedom (df)	F ratio	P-value
Individual	46956.374625	29	1.00	0.4997
Side	46937.164158	1	1.00	0.3256
Individual*Side	46942.990760	29	1.00	0.4851
Photographing Error	46940.747849	60	1.00	0.4901
Landmarking Error	46940.811411	120		

3.1.2 Bilateral Asymmetry: Asymmetry Between Left and Right Wings

The shape changes associated with the individuals' left and right forewings was not significant (Table 3.1). Thus, no shape difference was detected between an individual's left and right wings and no bilateral asymmetry was observed.

The centroid size changes associated with the individuals' left and right wing was not significant but had an F-ratio of 1 (Table 3.2). Again, since the F-ratio was 1.0, no matter what significance level was produced, all variances of centroid size were equal. Hence, bilateral asymmetry does not have a significant effect on the centroid size of forewings.

It can be concluded that both the shape and size of left and right forewings were not consistently different from each other and thus there was no detection of bilateral asymmetry within the wing shape of this population. Since no bilateral asymmetry was detected, left or right wings of specimens were used indiscriminately.

3.1.3 Allometry

The 10 000 iterations of permutation testing produced a non-significant P-value ($P = 0.246$) suggesting that size and shape were not associated. Therefore, the effects of allometry did not need to be considered when interpreting results.

Within the scatter plot (Figure 3.1) there was a distinct trend in which the regression score had a weaker effect on specimens with small centroid size, compared to specimens that have a larger centroid size. This was indicative of a positive relationship between the two variables. The regression score only ranges from -0.030 to 0.030 (Figure 3.1), an extremely small range that remained close to zero. Units in the centroid size axis range from 1.30 to 1.80 (Figure 3.1), having a range of only 0.50. Since the landmarks in this study were measured in units of 0.1mm the centroid size shares the same units and therefore the wings in this subsample only vary by 0.05mm.

The amount of shape change predicted by the regression ($S_{\text{Predict}} = 0.00108501$) was extremely smaller than the total amount of shape change observed ($S_{\text{Total}} = 0.05026753$), thus the regression only predicted 2.1585% of the total shape change observed could be attributed to the effects of centroid size.

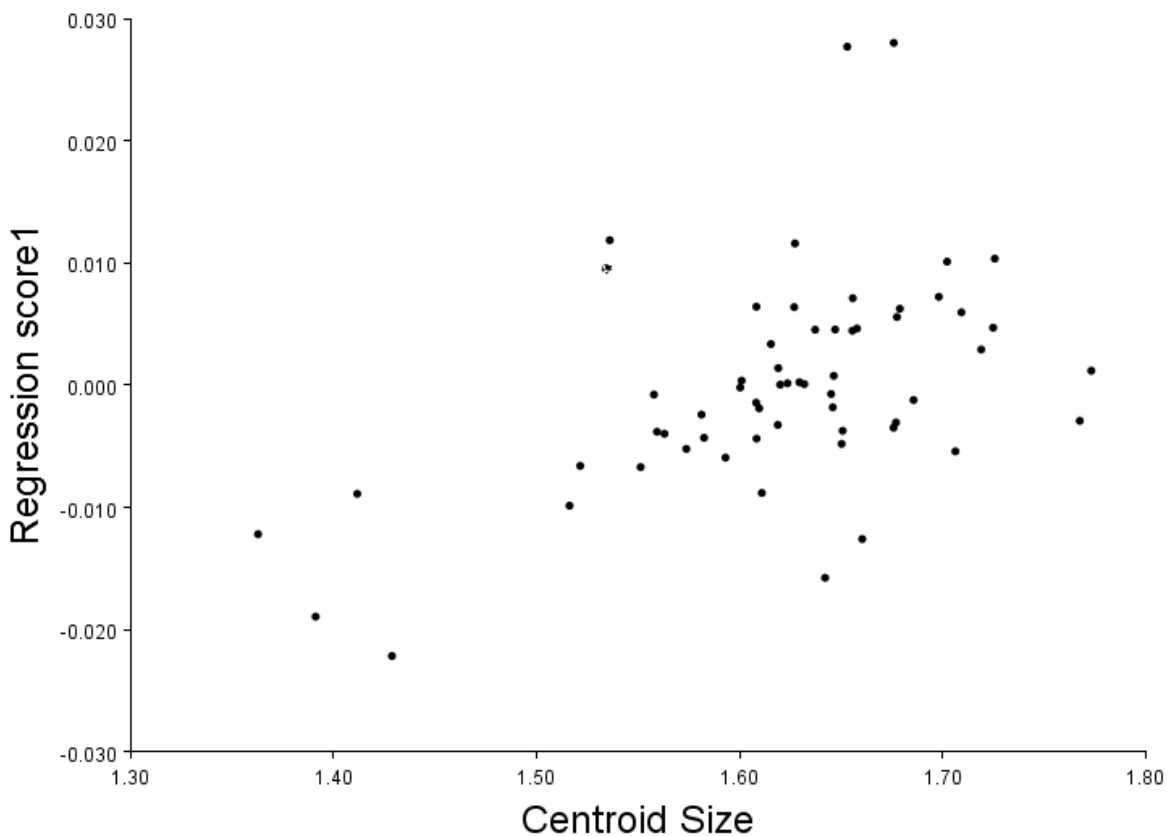


Figure 3.1) Scatter plot depicting any potential allometry within the shape of the forewings of a subsample (n = 30) of *Helicoverpa zea*. Regression Score's act as the independent variable and centroid size as the dependent. Graph was produced by MorphoJ v1.07a (Klingenberg 2011).

3.1.4 Sexual Dimorphism

Overall, there was no apparent sexual dimorphism detected within or amongst treatments. All P-values for parametric and permutation tests were non-significant; except for the permutation test that measured the Procrustes distance between the males and females for treatment 3 (Table 3.3). The parametric p-value and Hotelling's T^2 p-value were both non-significant for this treatment (Table 3.3). The Procrustes distance measured how similar the overall variation within the wing morphology of males and females was, whilst Hotelling's T^2 measured how similar the mean wing morphology of both males and females were. Thus, these results suggested that both males and females from treatment 3 had similar variation in wing shape morphology but significantly different mean wing shapes. Hence, it was determined that the males and females in treatment 3 did not show signs of sexual dimorphism.

The overall variation in wing shapes between males and females, within or amongst treatments, was small as both the Procrustes and Mahalanobis distance was relatively small between all pairs. Procrustes distance ranged from 0.01227311 when comparing the males and females from Treatment 1, to 0.03013030 when comparing males and females from Treatment 5 (Table 3.3). Mahalanobis distance for all pairs was quite small as well, ranging from the females in Treatment 2 and Treatment 5 being 0.6842 standard deviations from each other to the males and females from Treatment 3 being 4.0157 standard deviations away from each other (Table 3.3).

Overall, sexual dimorphism was not detected in the DFA and hence female and male specimens were used indiscriminately for further analyses.

Table 3.3) Discriminant Function Analysis conducted on a subsample (n = 30) of male and female *Helicoverpa zea* forewings that were raised on the following treatments of Corn (*Zea mays*) crops in North and South Carolina: 1) Structured Refuge with non-Bt Corn 2) Structured refuge with 2-toxin 3) Structured refuge with Bt 3-toxin and 5) Seed blend consisting of 80% Bt 3-toxin and 20% non-Bt Corn.

Groups Being Compared; Females (F) or Males (M) from a Treatment (1, 2, 3, 5)		Difference Between Means		P-Vales for Parametric Tests	P-Values for Permutation Tests	
Group 1	Group 2	Procrustes distance	Mahalanobis distance		Procrustes distance	Hotelling's T ²
F1	F2	0.01322411	1.0829	0.9671	0.9780	0.9887
F1	F3	0.01481721	1.2010	0.9995	0.6837	0.9399
F1	F5	0.02048901	1.3779	0.9342	0.7655	0.3135
F1	M1	0.01227311	0.8493	0.9936	0.9639	0.9315
F2	F3	0.02129030	1.6214	0.9955	0.6236	0.7949
F2	F5	0.01984993	0.6842	0.9002	1.0000	0.6695
F2	M2	0.02776112	1.3574	0.8630	0.0664	0.1449
F3	F5	0.02301792	2.6331	0.9570	0.4742	0.3674
F3	M3	0.02272611	4.0157	0.8675	0.0170	0.1657
F5	M5	0.03013030	1.2453	0.7505	0.6630	0.6630
M1	M2	0.02044178	1.1132	0.9574	0.4845	0.2057
M1	M3	0.02351703	1.7933	0.9719	0.0789	0.0815
M1	M5	0.02269134	0.8643	0.9495	0.8023	0.6030
M2	M3	0.02317743	2.1048	0.9490	0.0579	0.0584
M2	M5	0.02668156	1.0371	0.9224	0.3695	0.5379
M3	M5	0.02003051	1.2690	0.9916	0.4911	0.8176

3.2 Geometric Morphometric Analysis

3.2.1 Identifying Outliers

The Cumulative Distribution curve is an S-shape, with the specimens that have shapes further away from the average on the top to the right whilst the observations that are closer to the average are on the bottom to the left (Figure 3.2). The difference between the normal distribution (blue) and the data distribution (red) line was small, with only slight fluctuations. The red line had slightly less cumulative frequency than its blue line counterpart towards the bottom and middle of the curve, but at the top the red had more cumulative frequency (Figure 3.2). This means there were slightly less observations that were close to the mean and slightly more observations that were further from the mean within the data than when compared to a normal distribution.

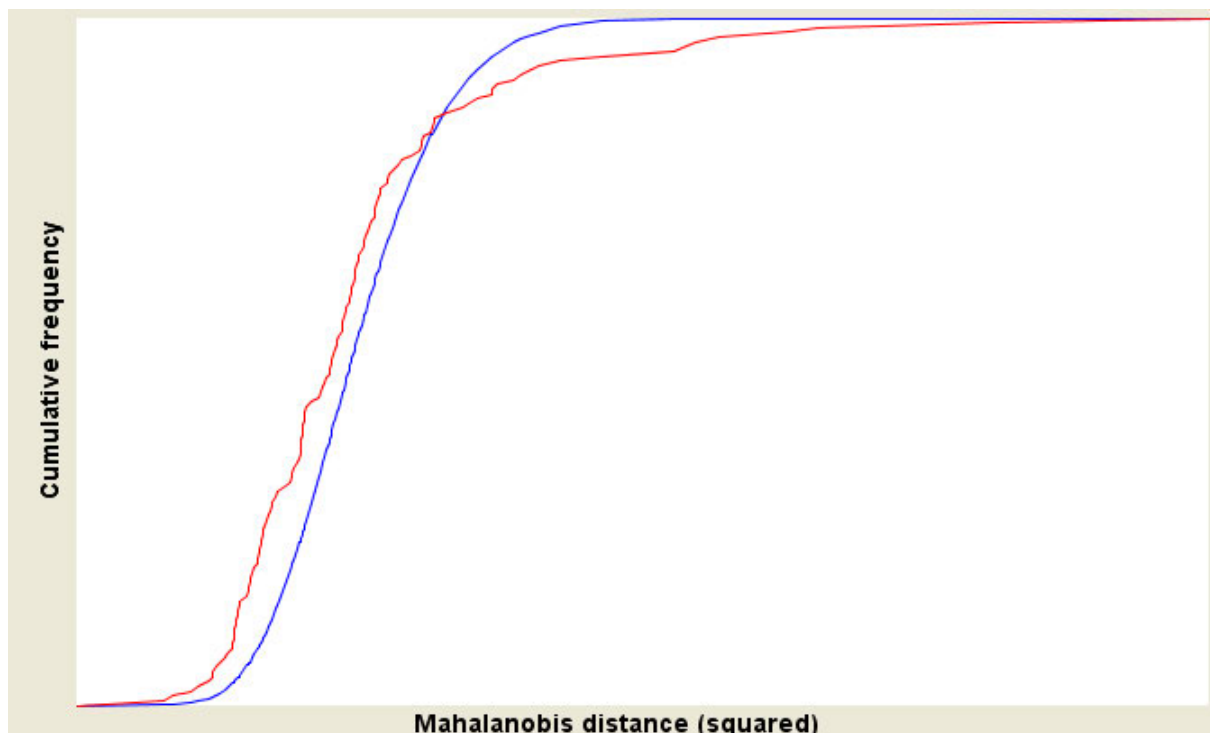


Figure 3.2) Cumulative Distribution Curve conducted on a sample ($n = 145$) of *Helicoverpa zea* forewings. The red line represents the cumulative distribution function for the data and the blue representing the normal distribution expected from a multivariate analysis. Squared Mahalanobis distance for each observation is ordered from smallest to largest on the x-axis and, Cumulative Frequency is represented on the y-axis. Graph was produced by MorphoJ v1.07a (Klingenberg 2011).

The largest Mahalanobis squared distance observed was from the specimen SE3nf122 which had a distance of 104.36, meaning that this specimen was 10.22 standard deviations away from the mean. The rest of the specimens had a Mahalanobis distance that ranged from 85.25 to 7.98, meaning that the specimens ranged from 9.23 to 2.82 standard deviations away from the average.

A lollipop graph visualised the difference from the average position of landmarks to the landmarks of specimen SE3nf122 (Figure 3.3). This kind of graph was chosen as it was not the overall shape changes of the wing that needed to be explored, instead, the changes of each individual landmark were of more interest.

The lollipop graph indicated there was nothing obviously wrong with the wing. There were not any landmarks that appeared out of order or in an obviously wrong/highly improbable positions (Figure 3.3). There were no consistent movements across all landmarks as if half of the wing has been slightly pushed one way or rotated (Figure 3.3). Overall this graph suggests that the specimen SE3nf122 was not an outlier and hence it was included in all future analyses.

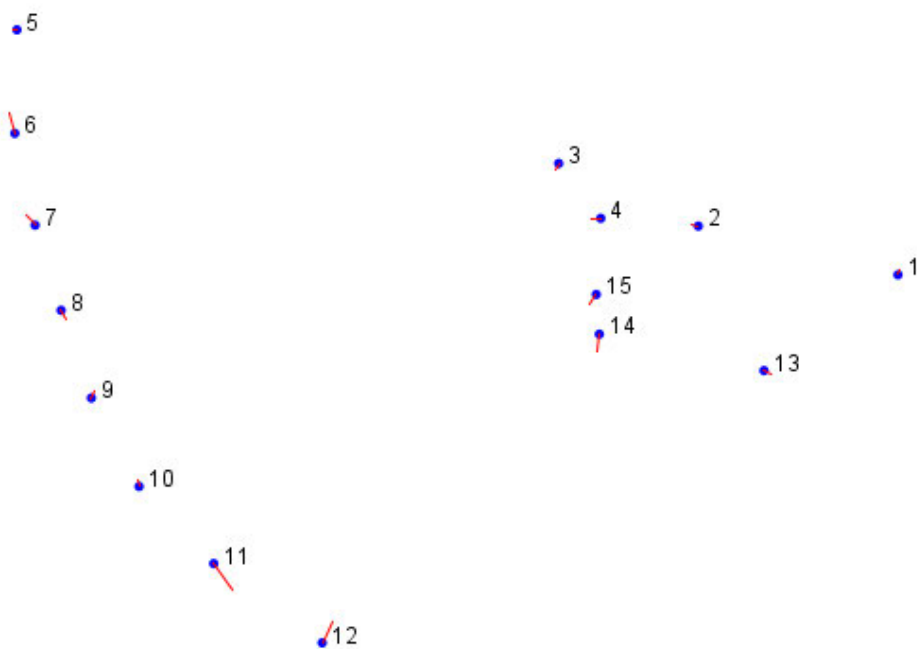


Figure 3.3) Lollipop graph (scale of 0.1) showing the differences in forewing shape of *Helicoverpa zea* specimen SE3nf122 compared to the average forewing shape of the entire sample (n=145) of *Helicoverpa zea* studied. The blue dot of the lollipop represents the mean wing shape, whilst the red stick represents the specimen. Landmarks are labelled 1 to 15 in black text next to landmark. Graph produced by MorphoJ v1.07a (Klingenberg 2011).

3.2.2 Visualisation of Complete Dataset through Principal Component Analysis

The shape change observable in the first principal component (PC1), generated because of a PCA, shows a widening and stunting of the wing (Figure 3.4). It contributed to 30.367% of the variance observed in shape changes. Whilst Landmark 8 remained in the centre of the distal edge of the forewing, all other landmarks spread away from the centre (Figure 3.4). The landmarks at the very top (Landmark 5) and bottom (Landmark 12) of the wing moving the furthest away (Figure 3.4). It was also observable that most of the landmarks from the inner venation of the wing move towards the distal edge of the wing, suggesting that the wing will become more contracted on the horizontal axis and overall shorter in length (Figure 3.4). Landmarks 1, 2, 3, and 4 all appear to move distally in an upwards direction, most likely due to the heightening of the top of the distal edge; this pattern is also seen with landmarks 13, 14, and 15, moving distally in a downwards direction due to the lowering of the bottom of the distal edge (Figure 3.4).

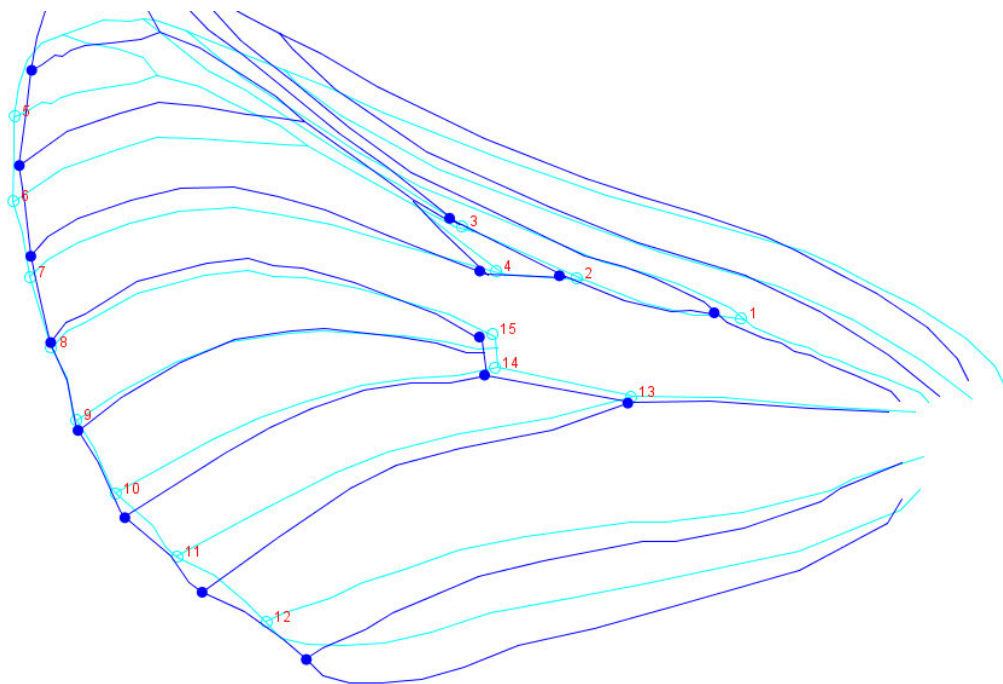


Figure 3.4) Transformed Outline graph with a scale of 0.1 showing the effects that PC1 has on the shape of the forewing. The PC has produced via a Principal Component Analysis conducted on the forewings of a sample (n=145) of *Helicoverpa zea* forewings. The light blue outline is representative of the average wing shape whilst the dark blue outline represents the change in shape inflicted by PC1. Landmarks are represented by dark blue dots. Landmarks are labelled 1 to 15 with red text adjacent to corresponding dot. Graph was produced by MorphoJ v1.07a (Klingenberg 2011).

The second Principal Component (PC2) produced by the PCA, provided 14.763% of the total shape change in the forewings. It described an elongation of the wing (Figure 3.5). The distal edge on the other hand did have a noticeable difference. There was a slight shortening on the top of the distal edge and the landmarks at the bottom moved slightly more proximal to the body, but overall kept a similar shape (Figure 3.5). The landmarks in the middle appear to be elongated with larger spaces between them. Landmark 2 remained in line with the mean shape. Landmarks distal to Landmark 2 (3, 4, 14, and 15) move even more distally, and landmarks proximal (1, and 13) move more proximal. This results in an elongation of the centre part of the wing and of the wing overall (Figure 3.5).

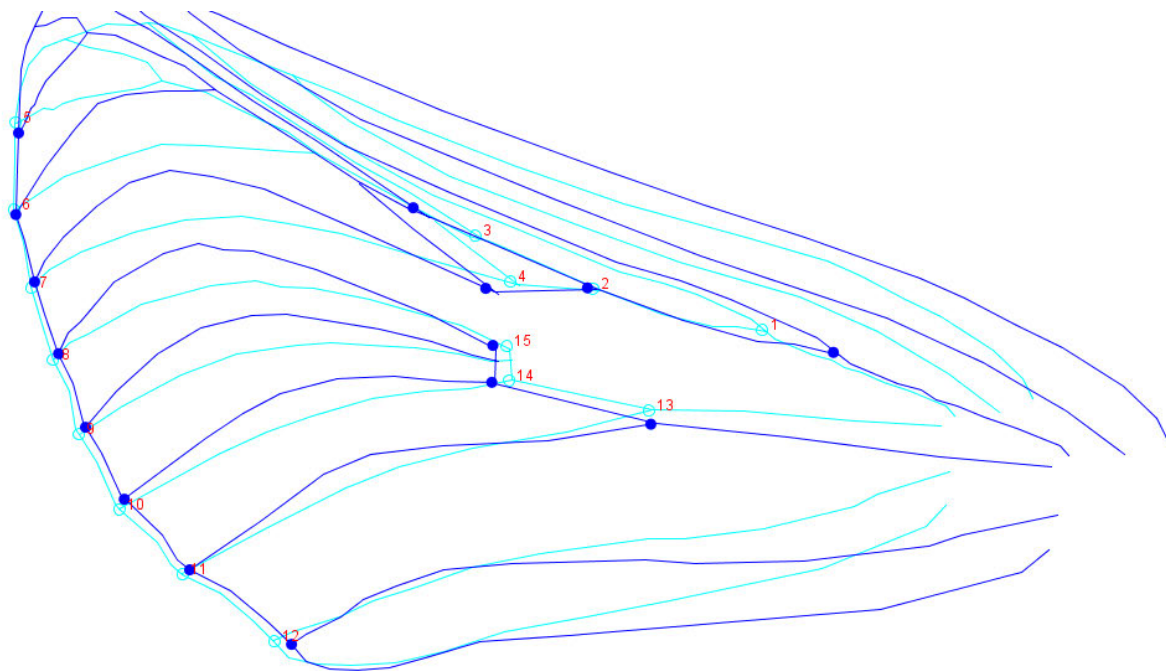


Figure 3.5) Transformed Outline graph with a scale of 0.1, showing the effects that PC2 has on the shape of the forewing. The PC has produced via a Principal Component Analysis conducted on the forewings of a sample (n=145) of *Helicoverpa zea* forewings. The light blue outline is representative of the average wing shape whilst the dark blue outline represents the change in shape inflicted by PC2. Landmarks are represented by dark blue dots. Landmarks are labelled 1 to 15 with red text adjacent to corresponding dot. Graph was produced by MorphoJ v1.07a (Klingenberg 2011).

Cumulatively PC1 and PC2 contributed almost half (45.130%) of the variation in the shape change within the wings. The PC1 appeared to have a range of 1.2 centring around 0, from -0.06 to 0.06 (Figure 3.6). PC2 had a slightly smaller range of 0.08 also centring around 0, ranging from -0.04 to 0.04 (Figure 3.6). When comparing the distribution of the different treatments according to their different colours, visually there were no distinct clusters with many of the confidence ellipses overlapping and circling the main large cluster in the centre (Figure 3.6).

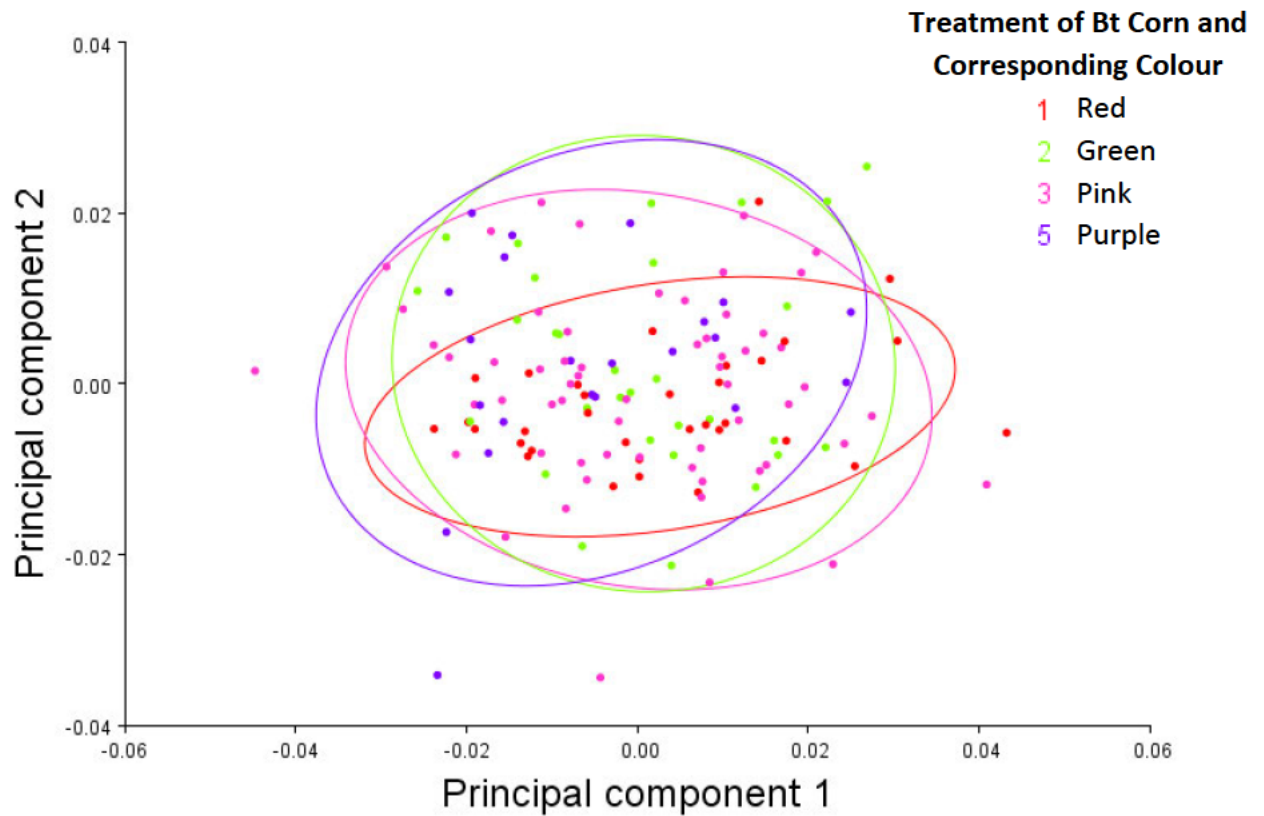


Figure 3.6) A Principal Component Analysis conducted on the forewings of a sample ($n = 145$) of *Helicoverpa zea*, with PC1 acting as the independent variable and PC2 as the dependent. Points are colour co-ordinated dependent on which treatment of corn the moths were extracted from. Colour and treatments of corn are as follows: 1) Structured Refuge with non-Bt Corn 2) Structured refuge with 2-toxin 3) Structured refuge with Bt 3-toxin and 5) Seed blend consisting of 80% Bt 3-toxin and 20% non-Bt Corn. Confidence ellipses of 95% encircle the treatments in their corresponding colours. Graph was produced by MorphoJ v1.07a (Klingenberg 2011).

3.2.3 Visualisation of Differences in Wing Shape According to Different Treatments of Bt Corn through Canonical Variate Analysis

The CVA attributed 49.301% of the variation of shape change between species relative to within species, to the first Canonical Variate (CV1). The shape change associated with the CV1 were mainly associated with the sharper angling of the distal edge of the forewing (Figure 3.7). Landmarks 5 and 6 are pushed more distally whilst Landmarks 10, 11 and 12 are pushed more proximally, overall making the distal edge of the wing have a sharper angle (Figure 3.7). Within the centre of the wings, Landmarks 2, 3, and 4 are moved slightly more proximal suggesting that the top of the wing has undergone an elongation (Figure 3.7). Landmarks 13 and 14 on the other hand have moved more downwards and distal, suggesting that the bottom of the wing has contracted (Figure 3.7).

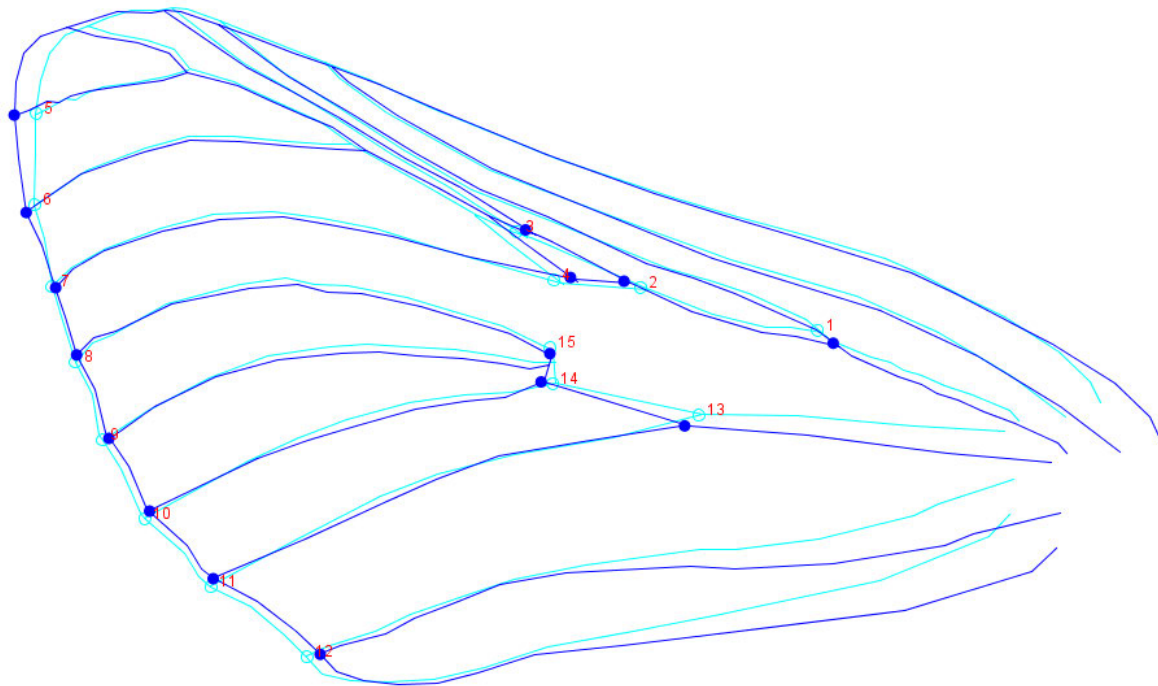


Figure 3.7) Transformed Outline graph with a scale of 10.0 showing the effects that Canonical Variate 1 (CV1) has on the shape of the forewing. The CV was produced via a Principal Component Analysis conducted on the forewings of a sample (n=145) of *Helicoverpa zea* forewings. The light blue outline is representative of the average wing shape whilst the dark blue outline represents the change in shape inflicted by CV1. Landmarks are represented by dark blue dots. Landmarks are labelled 1 to 15 with red text adjacent to corresponding dot. Image was produced by MorphoJ v1.07a (Klingenberg 2011).

The CVA attributed 29.692% of the total shape change to the second canonical variate (CV2), together with CV1 dictating 78.993% of the total shape change. The shape change associated with the CV2 was mainly associated with the narrowing and elongation of the wing. The landmarks on the upper distal edge of the wing (Landmarks 5, 6, and 7) moved slightly down and distal compared to the average wing (Figure 3.8). This created a narrowing and elongation of the upper part of the distal edge (Figure 3.8). The landmarks on the lower distal edge (Landmarks 10, 11, and 12) moved upwards and proximal, overall creating a thinning and slightly contracting effect on the lower distal edge of the wing (Figure 3.8). Some of the landmarks in the inner part of the wing moved closer to the centre of the horizontal plane; with landmarks in the upper middle wing (Landmarks 1, 2, 3, and 4) moved slightly downwards, and a landmark in the lower part of the wing (Landmark 14) moving slightly upward (Figure 3.8). This is most likely a result of the narrowing of the distal edge of the wing. Most landmarks in the middle of the wing (Landmarks 3, 4, 14, and 15) move distally, towards the distal edge of the wing (Figure 3.8). This was likely due to the elongation shape change. The landmarks in the centre of the wing that are more proximal (Landmarks 1, 2 and 13) appear to move more proximal, creating more elongation within the inner part of the wing (Figure 3.8).

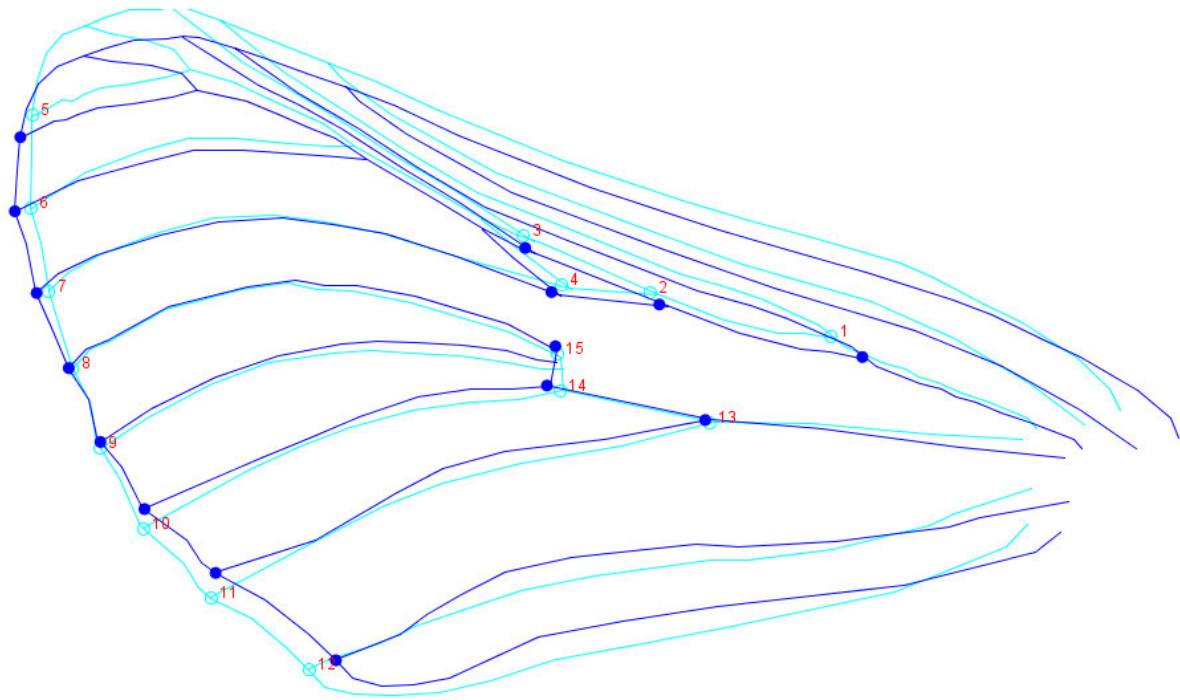


Figure 3.8) Transformed Outline graph with a scale of 10.0 showing the effects that Canonical Variate 2 (CV2) has on the shape of the forewing. The CV was produced via a Principal Component Analysis conducted on the forewings of a sample (n=145) of *Helicoverpa zea* forewings. The light blue outline is representative of the average wing shape whilst the dark blue outline represents the change in shape inflicted by CV1. Landmarks are represented by dark blue dots. Landmarks are labelled 1 to 15 with red text adjacent to corresponding dot. Image was produced by MorphoJ v1.07a (Klingenberg 2011).

The CVA determined that the Procrustes distance for each treatment was significantly different to the next, except for treatments 2 and 5 (Table 3.4). The difference of the means between each treatment had very small differences in Procrustes distance, ranging from treatments 1 and 3 that had a difference of 0.0072, and treatments 1 and 5 that had a difference of 0.0128 (Table 3.4).

Table 3.4) Procrustes distance and accompanying P-values produced by a Canonical Variate Analysis conducted on a sample (n=145) of *Helicoverpa zea* forewings, comparing the different treatments of Bt Corn that the moths were collected from. The CVA compared the following treatments: 1) Structured Refuge with non-Bt Corn 2) Structured refuge with 2-toxin 3) Structured refuge with Bt 3-toxin and 5) Seed blend consisting of 80% Bt 3-toxin and 20% non-Bt Corn

	1	2	3
2	0.0101 (P = 0.0345)		
3	0.0072 (P = 0.0164)	0.0091 (P = 0.0465)	
5	0.0128 (P = 0.0107)	0.0088 (P = 0.2763)	0.0103 (P = 0.0400)

The Mahalanobis distance measured between all treatments were statistically significant, except again for the distance recorded between treatments 2 and 5 (Table 3.5). The CVA determined that the difference between the means of the different treatments also had an extremely small Mahalanobis distance and were approximately 1.72 to 2.02 standard deviations apart (Table 3.5).

Table 3.5) Mahalanobis distance and accompanying permutation P-values produced by a Canonical Variate Analysis conducted on a sample (n=145) of *Helicoverpa zea* forewings, comparing the different treatments of Bt Corn that the moths were collected from. The CVA compared the following treatments: 1) Structured Refuge with non-Bt Corn 2) Structured refuge with 2-toxin 3) Structured refuge with Bt 3-toxin and 5) Seed blend consisting of 80% Bt 3-toxin and 20% non-Bt Corn

	1	2	3
2	1.7623 (P = 0.0007)		
3	1.7297 (P < 0.0001)	1.7874 (P < 0.0001)	
5	1.8461 (P = 0.0017)	1.6954 (P = 0.0777)	2.0281 (P < 0.0001)

Within the CVA scatterplot, there was separation between the four distinct treatments, as observed by the confidence ellipses that encircle the treatments (Figure 3.9). Overall, this suggested that there were distinct differences in wing shape according to different treatments. Treatment 1 can be seen having more specimens that have a negative CV2 effect, with some samples reaching as far down as -3.0 and only achieving a high of approx. 1.25 (Figure 3.9). Treatment 3 on the other hand has an extremely negative CV1 effect, with a sample reaching as far down as -4 and others reaching a max of approx. 1 (Figure 3.9). Treatments 5 and 2 have extreme overlapping, with treatment 5 sitting

almost entirely inside of treatment 2, except for one specimen that lies just outside its confidence ellipse (Figure 3.9). Treatment 5 has a smaller confidence ellipse, and therefore less variation among specimens, than all other treatments. Both treatment 2 and treatment 5 are characterized by their positive CV1 and CV2 values. The CV1 of treatment 2 ranges from approx. -1 to approx. 2.25, and CV2 ranges from -2.5 to 3.5 (Figure 3.9).

Overall, the CVA suggested that there were significant differences between all treatments, except for specimens collected from treatment 2 and treatment 5 which instead had severe overlap in their forewing morphology.

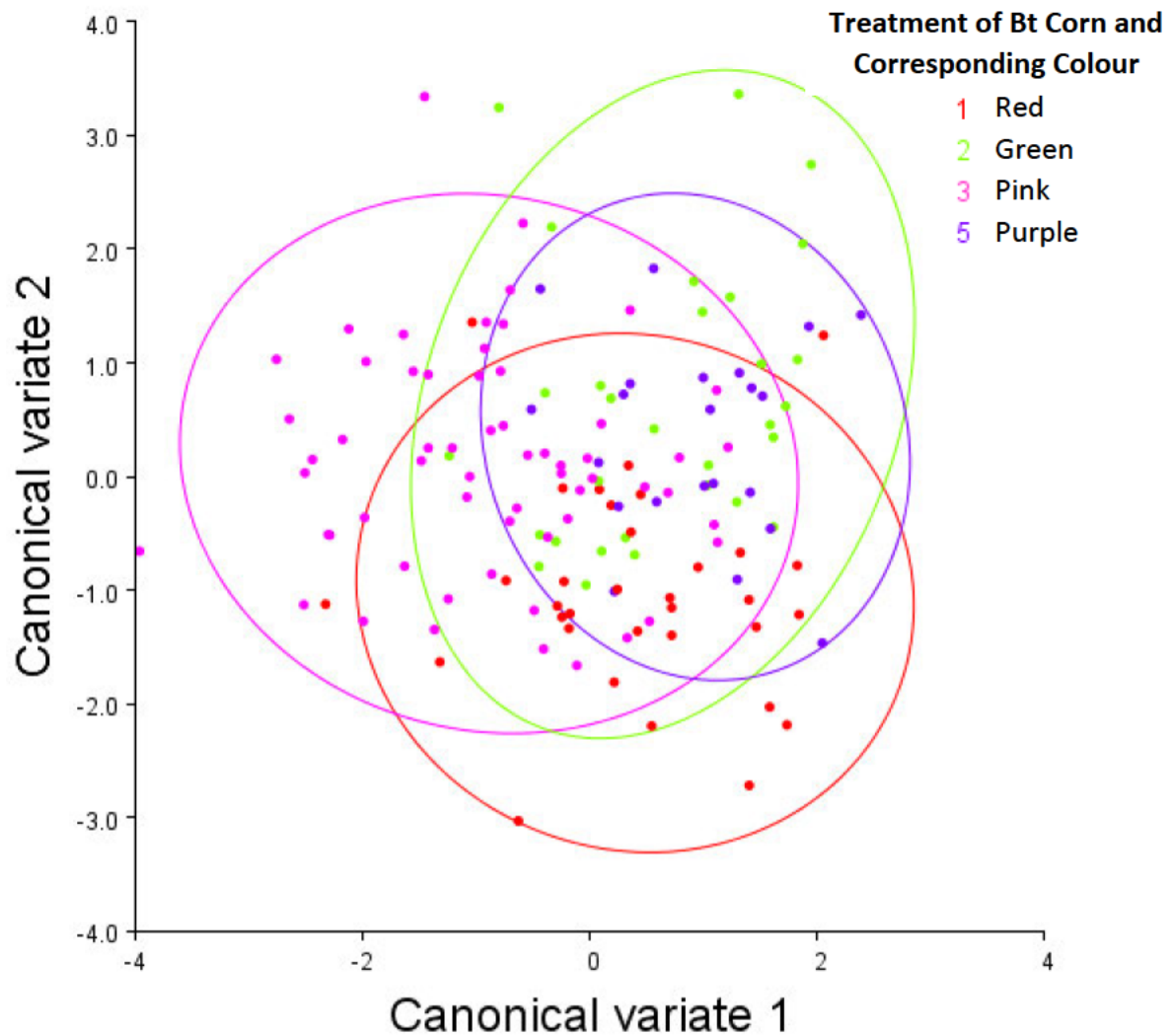


Figure 3.9) A Canonical Variate Analysis conducted on the forewings of a sample (n = 145) of *Helicoverpa zea*, with CV1 acting as the independent variable and CV2 as the dependent. Points are colour co-ordinated dependent on which treatment of corn the moths were extracted from, treatments of corn are as follows: 1) Structured Refuge with non-Bt Corn 2) Structured refuge with 2-toxin 3) Structured refuge with Bt 3-toxin and 5) Seed blend consisting of 80% Bt 3-toxin and 20% non-Bt Corn. Confidence ellipses of 95% encircle each treatment. Graph was produced by MorphoJ v1.07a (Klingenberg 2011).

3.2.4 Determine if Wing Shape Can Act as an Indicator for Different Treatments of Bt Corn through Discriminant Function Analysis

The DFA determined that all treatments had significantly different wing shapes, except for treatments 2 and 5. Treatments 2 and 5 had non-significant p-values for both permutation and the parametric test (Table 3.6). This suggested that moths from treatments 2 and 5 had extremely similar wing shape that were indistinguishable from each other. All other p-values were statistically significant except for the parametric p-value between treatments 1 and 2 (Table 3.6).

For treatment 1 and 2, both permutation p-values were significant, it was only the parametric p-value that was non-significant. Parametric tests are conducted under the assumption that normal distribution is present within the population. Instead, the permutation tests considers the possibility of non-normal distribution, thus presenting more reliable outcomes in this situation. This implies that normal distribution is not present within this population, and that the results of the permutation tests should be prioritised over the parametric tests. Overall, the wing shape between treatments (except for 2 and 5) were distinguishable from one another and could act as an indicator of which treatment an individual had come from.

Table 3.6) Discriminant Function Analysis (DFA) conducted on a sample (n = 145) of *Helicoverpa zea* forewings, comparing the different treatments of Bt Corn that the moths were collected from. The DFA compared the following treatments: 1) Structured Refuge with non-Bt Corn 2) Structured refuge with 2-toxin 3) Structured refuge with Bt 3-toxin and 5) Seed blend consisting of 80% Bt 3-toxin and 20% non-Bt Corn.

Treatments Being Compared		Difference Between Means		P-Vales for Parametric Tests	P-Values for Permutation Tests	
		Procrustes distance	Mahalanobis distance		Procrustes distance	Hotelling's T ²
1	2	0.01007489	2.1748	0.0726	0.0308	0.0476
1	3	0.00718788	1.7056	0.0387	0.0162	0.0360
1	5	0.01282804	3.0127	0.0116	0.0123	0.0125
2	3	0.00906083	1.8824	0.0161	0.0500	0.0131
2	5	0.00877917	2.2464	0.3024	0.2652	0.3087
3	5	0.01026691	2.1956	0.0127	0.0427	0.0112

4. Discussion

This study is the first to use GM analysis to detect Bt-resistance within populations of *H. zea*. Further, this study is the first to describe patterns of resistance spread when information on population genetics is not available. This is a new species which this method is being applied to and highlights the effectiveness of GM analysis as a biomarker. Both the PCA and the DFA demonstrated that forewing's shape can be reliably used to identify patterns related to the development of resistance within populations of *H. zea*. The data generated in this thesis provides the second recorded identification of practical resistance to the Vip3A protein in field populations of *H. zea* (first: Pezzini 2022) and suggests that continuous use of GM as a monitoring tool will allow for continued surveillance on this resistance.

4.1 Preliminary Tests

The Preliminary tests concluded that bilateral asymmetry, allometry, and sexual dimorphism had no confounding effects on the data analysed within this thesis. The Procrustes ANOVA determined that the wing centroid size among individuals was not significant and hence all wings within the sample were similar in size. The moths that were examined within this study were all adults and of similar stages in maturity. Consequently, it is not unusual that there were no significant differences among the moths. The Procrustes ANOVA did detect significant differences within the shape of wings; consequently, the hypotheses of this study were only able to address the shape of the wing.

The Procrustes ANOVA did not detect any effects of bilateral asymmetry on the shape or size differences between left and right wings. Thus, it determined that bilateral asymmetry of wings was not present within this sample. Bilateral asymmetrical traits have been suggested as an indicator for environmental stress, with asymmetry being expected to increase when stressful conditions disturb normal development of an organism. Despite this, previous studies on stress-asymmetry associations are extremely inconsistent (Bjorksten et al. 2000; Leung et al. 2000). Often, there are contradictory results produced from similar experiments and if significant results are detected, they are small and difficult to interpret (Hoffmann et al. 2005; Leung & Forbes 1996; Palmer & Strobeck 1986). There are many studies where apparent stressful environmental conditions in an organisms' habitat have not resulted in a detectable presence of asymmetry (Bjorksten et al. 2000; Leung & Forbes 1996). With some studies suggesting that shape changes are more common a response to stressful environments, rather than asymmetry (Hoffman et al. 2005). Overall, there is no reliable

evidence for increases in bilateral asymmetry in either shape or size under stressful conditions (Hoffman & Woods 2003).

Since there was no significant difference in centroid size of the wing, the effects of allometry on the dataset would not be detectable as the effect would be the same for all of the wings. Despite this, the multivariate regression analysis and accompanying permutation tests determined there was a non-significant effect of size on the shape of the wings. Overall, allometry was not a concern when interpreting the results of downstream statistical analyses.

The DFA determined that sexual dimorphism was not present within the sample. Sexual dimorphism typically results from evolutionary pressures acting differently on the sexes, and is present within numerous species (Shine 1989). Hernández et al. (2010) explored the effects of altitudinal ranges on the wing shape of in the invasive moth *Tecia solanivora*, revealing wing shape differences between sexes occurred as a result of the stressful conditions (i.e. increased altitude). Another study conducted by Queiroz-Santos et al. (2018) determined that adult *Helicoverpa armigera* exhibited sexual dimorphism in the number of setae on the frenulum and spines on the prothoracic leg. Sexual dimorphism is typically evident in insects with differences related to sex-specific behaviours, which can be evident by variation in wing morphology (Gilchrist 1990; Hernández et al. 2010). Typically, these wing configurations relate to the varying ecological roles and physiological constraints of flight that are particular to each sex. For example, Lemic et al. (2020) investigated sexual dimorphism present within the Medfly (*Ceratitidis capitata*) and determined that females had more aerodynamic and elongated wings compared to males that had shorter and wider wings. Male medflies are known to establish mating territories to attract females for mating (Hendrichs et al. 2002) and hence it is mated females that undergo migratory flights over long distances before oviposition (Churchill-Stanland et al. 1986). Lemic et al. (2020) hypothesised that it is the sex-specific dispersal requirements of this species that has resulted in the sexual dimorphism they recorded. *H. zea* have no distinct differences in observed sexual behaviours with both undergoing localised breeding before partaking in migration characterised by long-distance flight across the US.

4.2 Wing shape differs in differing treatments of Bt Corn: Canonical Variate Analysis

This study compared wing morphology of *H. zea* from different treatments of Bt Corn (Table 2.1). These results provide the first morphological evidence that *H. zea* individuals resistant to Bt Corn differ in wing shape compared to susceptible individuals. It cannot be concluded that the differences observed are purely from the resistance phenotype, due to the number of uncontrollable site-specific variables within this study including weather, soil, etc. Nevertheless, the results for this study do suggest that despite such variation, there is evidence of selection for different wing morphology dependent on the treatment of Bt Corn that moths were sampled from.

In this thesis it was shown that wing shape was significantly different among the four different treatments with the CVA, grouping them into three main wing shapes. The results identify a unique planform of forewing shape within each *H. zea* population from developing different levels of resistance to the widely used pesticidal Bt proteins. Changes in phenotype have been recorded in many different insects, mainly the WCR (Benítez et al. 2014a; Lemic et al. 2014; Mikac et al. 2013; Mikac et al. 2019). These wing shape changes have also been identified in Lepidopterans such as the Codling Moth (Kadoić Balaško et al. 2022; Pajač Živković et al. 2019).

Treatment 1 was representative of *H. zea* specimens collected from fields of non-Bt Corn and were thus considered 'susceptible' individuals (Table 2.1). Susceptible individuals presented a widening and shortening of the wing, with a sharper angling of the distal edge. This shape of wing supports short-distance movement. Susceptible individuals are unable to survive on any Bt Corn crops and hence would have to undergo short-distance movement to different fields before they were able to find a non-Bt Corn crop or a non-Bt Corn refuge. Since approximately 79% of all corn and cotton planted in the USA expressed one or more Bt proteins by 2020 (United States Department of Agriculture-Economic Research Service 2020), high amounts of energy would be demanded for this level of short-distance movement. Hence, it is likely that the need for short-distance movement could act as a selective pressure on this population.

Treatments 2 and 5 represent *H. zea* individuals that were collected from treatments of corn that; had structured refuges in place and contained the 2 toxins Cry1AB and Cry1F (Treatment 2); or contained a seed blend with 20% containing non-Bt Corn and 80% Bt Corn containing the 3 toxins Cry1AB, Cry1F, and Vip3A (Treatment 5) (Table 2.1). These *H. zea* individuals have developed resistance to the most commonly used Cry proteins and are developing resistance to the Vip3A protein when it is diluted using seed blending practices. Seed blending practices are conducted to

efficiently add non-Bt Corn refuges into crops, although this method allows for the cross pollination of the corn resulting in a reduction in the Bt Corn's efficacy. Cross pollination of corn describes the non-Bt Corn being pollinated by Bt Corn, which causes the production of Bt toxins to a significantly lesser extent (Chilcutt & Tabashnik 2004). This allows *H. zea* to be exposed to sub-lethal amounts of the Vip3A protein, thus speeding up the evolutionary process of resistance development in *H. zea* individuals (Caprio et al. 2016; Vyavhare et al. 2021; Yang et al. 2014). The death of moths susceptible to Vip3A and the survival of heterozygotes is likely to result in the increase dominance associated with the resistance gene and will also encourage the spread of resistance throughout populations of *H. zea* (Brévault et al. 2015). Individuals collected from treatments 2 and 5 were considered 'resistant' individuals as they were able to survive Cry1AB and Cry1F proteins, and the Vip3A protein when it is weakened using seed blending practices. The resistant *H. zea* individuals found in this study had a narrowing and elongation of their wings and a sharper distal edge. Elongated wings are better suited to flying with air currents and long-distance dispersal (e.g. migration). This wing shape is typically seen in insects that undergo migratory patterns, as long-distance migrants typically have pointed wings that are longer and thinner (Mönkkönen 1995; Lockwood et al. 1998). The wing shape of resistant *H. zea* individuals is most likely due to the demands of long-distance flying and dispersal. This differs from the wing shape of susceptible populations as the demand for short distance movement is released. Since resistant individuals no longer require short distance movement to find a suitable crop to survive, they evolve to favour migratory movements and dispersal.

Treatment 3 represented *H. zea* individuals that were collected from fields of Bt Corn that contained three toxins (Cry1AB, Cry1F, and Vip3A) and had structured refuges of non-Bt Corn (Table 2.1). When Vip3A is added to pre-existing pyramided Bt Corn they are considered near high dose for *H. zea* (Burkness et al. 2010). Because of this strength, *H. zea* from Treatment 3 were considered 'super-resistant' individuals. In this thesis it was found that the wings of super-resistant *H. zea* individuals had no distinct elongation or shortening. Instead, their wing width fell in between susceptible and resistant *H. zea* wing shapes. Super-resistant individuals had a distinctly more rounded/rectangular shaped wing, with the angle of the distal edge weakened thus making it overall more vertical. Like resistant populations, these *H. zea* no longer have the natural constraint of requiring short-distance movement to find a suitable corn crop to survive in, consequently wings start changing to enable migratory flight and movement. Although, compared to resistant individuals there is no distinct narrowing and elongation of the wing, hence this appears to not be the case.

Wing morphology is one of the most critical elements of an insect's ability to disperse (DeVries et al. 2010), with differences in wing shape impacting on the dispersal or long-distance movement of moths. Finding the wing morphotype of superior fliers and dispersers, as well as the morphotype with specific resistance patterns, is crucial to the success of resistant pest management strategies. The associated genotype of the superior flier/disperser will most likely spread over a larger distance in a shorter period. Even though susceptible and super-resistant individuals are less capable of long flights, they still represent a pool of resistant genes, which means that they can transfer these resistant genes at a continental scale (Lemic et al. 2014). By integrating different techniques to understand the plasticity and variation of wing shape and how they adapt to new environments (i.e. develop resistance), it provides the crucial information needed to coordinate a strategic plan ahead of possible new invasions (Lemic et al. 2015).

4.3 Practical Resistance to Vip3A Developed in the Field, and its Potential Effects on Future *Helicoverpa zea* Management

There are several possibilities as to why this rectangular wing shape has developed within the super-resistant *H. zea*; with one being they are the result of genetic variation associated with the development of resistance to Vip3A. Shape changes are particularly sensitive to environmental stress (Hoffman & Woods 2003). One reason for this is that shape requires a cascade of genes to act throughout development (García-Bellido 1977; García-Bellido & de Celis 1992). Minor perturbations in this cascade (i.e., perturbations caused by the development of resistance to Vip3A protein) can lead to larger changes in wing shape (Emlen & Nijhout 2000). For example, a study conducted on male beetles revealed that individuals expressing long horns typically had disproportionately small wings, suggesting that changes in development that allowed the growth of longer horns also caused the growth of smaller wings (Kawano 1995, 1997). This resource trade-off may be what has caused the rectangular-like shape seen in super-resistant individuals; with wing development being modified because of other genetic changes.

Another reason may be that this rectangular-like shape provides superior structural support and strength to the wing. Previous research on other insects has suggested that development of resistance may cause a decrease in the structural integrity of the wings. A study conducted by Pajač Živković et al. (2019) used the Finite Element Modelling to investigate the morphological changes in

Codling Moth (*Cydia pomonella*) populations caused by developed insecticide resistance. Their study showed that individuals that were exposed to high levels of insecticide treatment (resistant) had higher amount of wing deformation and the lower wind speeds models compared to field-collected susceptible individuals. The results indicated that the wing and venation pattern was weaker in its structural integrity for resistant Codling Moths. It was suggested that the intense selection pressure exerted by widespread use of pesticides had ultimately altered the structural integrity of resistant Codling Moth wings, overall making them less efficient at dispersal. Ultimately, it provided clear evidence that the development of resistance could affect the fitness of the organism itself. This may be occurring within *H. zea* super-resistant populations as well; with widespread use of Bt Corn throughout the USA implementing intense selection pressure on populations and causing weaker structural integrity within the forewings. The development of the rectangular-like wing shape may be a consequence of a decrease in forewing integrity, to maintain the wing's stability during flight. With wing stability compromised, there could potentially be increased injury or damage to the finer parts of the wing, i.e., the tip. By rounding the wing to produce a more rectangular shape, the wing may be less prone to these damages. There is a need to further investigate *H. zea* wing shape and size performance in relation to key environmental dispersal constraints (i.e. wind) to determine how well different wing morphotypes perform and to be able to effectively compare the differing flying abilities according to differing levels of resistance. The combined approach of GM and finite element modelling would provide vital information on wing structure and flight efficiency of wing shape differences based on levels of resistance. This would produce robust data and a deeper understanding on how pest insects change their wing shape and size, and dispersal efficiencies under a resistance scenario.

To suppress *H. zea* populations and mitigate the economic damages inflicted on the corn agricultural business, suggestions to implement the Vip3A protein into gene pyramiding alongside the Cry proteins has been highly recommended (Niu et al. 2021). Previously, promising results have been observed when including Vip3A in Bt Corn (Burkness et al. 2010; US EPA 2009; Yang et al. 2019). Despite these studies, our results show that practical field resistance of *H. zea* to Vip3A has occurred, and that resistant alleles already exist within wild populations. If the frequency of Vip3A resistant alleles builds up within the population unchecked, resistance could potentially become 'fixed' within the population. Once resistance reaches very high levels, strategies to restore susceptibility are unlikely to be effective and the use Vip3A protein as pest management strategy may no longer be successful. The results highlight that further analysis needs to be conducted, as the efficacy of Vip3A as a pest management strategy may have changed and Vip3A is currently less effective on *H. zea* than previously thought. The use of pyramiding is not effective in delaying

resistance when insect is already resistant to one or more toxin expressed in the pyramid (Brévault et al. 2013). Tabashnik & Carriere (2017) predicted that pyramiding Vip3A proteins with the Cry proteins would not be effective due to the pre-existing presence of Cry protein resistance within the population. They hypothesised that overtime a resistance of Vip3A may be obtained because of this. Gassmann & Reisig (2023) also predicted that similar patterns of resistance development for the Cry toxins may also occur for Vip3A. Evolving resistance to Vip3A has been reported in laboratory environments multiple times (Dively et al. 2021; Yang et al. 2020; Yang et al. 2021a, 2021b) although this is only the second time practical resistance has been reported in the field so far (first: Pezzini 2022). This provides serious concern for the control of *H. zea* as a pest and could potentially pose threats to agricultural corn production in future years.

4.4 Determine if Wing Shape Can Act as an Indicator for Different Treatments of Bt Corn through Discriminant Function Analysis

This study was conducted to determine if wing shape can indicate the presence of Bt resistance within populations. Like the CVA, the DFA was able to distinguish among the same three populations: Treatment 1-susceptible, Treatment 2 and 5-resistant, and Treatment 3-super-resistant. The DFA was unable to distinguish individuals from treatments 2 and 5, this was because there was no significant difference in the wing morphology between these two treatments as also shown by CVA.

The results of the DFA confirmed that there are features of the wing morphology that can help distinguish between different levels of resistance. It can be used to identify the group membership of an unknown individual *H. zea* based on wing morphology. The results show that wing morphology can act as a biomarker of not just resistance, but different kinds of resistance. This study provides techniques for the monitoring and management of *H. zea*, and potentially other pests, particularly where genetic markers do not exist for the species in question.

Consistent monitoring of *H. zea* is essential when determining if management recommendations remain relevant or if they need to be revised considering newly acquired knowledge or change in circumstances (Bosch et al. 2018; Yang et al. 2020). This study is the first to show that the use of DFA

on *H. zea* forewing shape can monitor resistance to Bt proteins within populations. By monitoring this resistance, it will provide the reliable information about the extent and intensity of resistance that effective resistant management relies on (IRAC 2020). To inhibit the spread of resistance within the field, it is crucial to detect specific biotypes in a timely manner to apply effective control manner. The DFA conducted in this study has been able to identify three unique biotypes (susceptible, resistant, super-resistant) that each have different genotypes and alleles in relation to varying levels of resistance. By monitoring each biotype as an individual entity rather than monitoring the species, it provides imperative information on resistance development and spread within populations. Monitoring of resistance within populations is essential to proactively prevent it from compromising control; whilst providing reliable data that is essential for successful resistance management. It is only by the monitoring, characterising and predicting the appearance and spread of resistance, that existing chemical tools can be used in a sustainable manner (Abbott 1925; Foster 2016; Liu 2012). Determining the factors that positively or negatively affect, or limit the growth of resistant populations, will facilitate in the development of IPM strategies that aim to slow the spread of resistance. By using GM as a monitoring tool, it will provide vital information on the basic biology of *H. zea*. If used consistently over a longer period, it will generate data on temporal and spatial changes. Overall, consistent monitoring of resistance to Bt proteins within *H. zea* populations would provide a wide array of essential information. Implementing this information into management recommendations will increase the effects of overall results considerably and may mitigate the damage to corn crops on a national and potentially international scale.

Future integrated control strategies should consider incorporating GM techniques into the monitoring and resistance management strategies. Overall implications from the GM work conducted so far suggests that GM can be used to monitor population changes related to the invasion process and the development of resistance (Benítez et al. 2014b; Camara et al. 2006; Kadoić Balaško et al. 2021; Kadoić Balaško et al. 2022; Lemic et al. 2014; Lemic et al. 2020; Lemic et al. 2021; Mikac et al. 2013; Mikac et al. 2016; Mikac et al. 2019; Pajač Živković et al. 2019) . It can be used in lieu of more expensive and time-consuming genetic techniques; it does not require expert equipment or analysis and hence is more accessible whilst providing robust and reliable data. To implement appropriate changes to pest management in response to development of resistant populations, timely detections of such populations are vital. GM has been shown to be a useful biomonitoring tool (Hood 2000) that can potentially act as a better biomarker than genetic markers as they are able to detect resistant populations much sooner. Metric properties (including wing shape and size) are typically the first morphological characters to change as influenced by environmental and genetic factors (Bouyer et al. 2007; Levine & Oloumi-Sadeghi 1996). Because of

this, there are instances where phenotypic changes have been detected in the same populations for which genetic markers show no difference (Benítez et al. 2014b; Kadoić Balaško et al. 2021; Lemic et al. 2014). This is because alleles within resistant populations have not had enough time to change within populations to serve as a reliable biomarker for monitoring. Overall, if the aim of a population biomarker is so that it can be used to conduct biomonitoring, then a marker based on phenotypic differences is more appropriate than a genetic marker (Bouyer et al. 2007; Mikac et al. 2013; Mikac et al. 2016). Use of wing shape and size differences as a biomarker has been repeatedly demonstrated to be effective and reliable (Benítez et al. 2014a, b; Lemic et al. 2014, 2016; Mikac et al. 2016).

4.5 Future Directions

The development of resistance in *H. zea* poses a severe risk to future production of corn and cause significant economic damage to corn production. *H. zea*'s evolution of resistance to Cry proteins is well documented (Bilbo et al. 2019; Gassmann 2021; Tabashnik & Carriere 2017; Yang et al. 2019). Many pest control strategies have been recommended as an attempt to suppress the damage caused by resistant *H. zea* populations. Most of these strategies typically involve the use of the Vip3A protein, as adding VIP3A to gene pyramided Bt Corn is highly effective and makes it high dose against *H. zea* (Burkness et al. 2010; US EPA 2009; Yang et al. 2019). The results shown in this thesis provides evidence that the evolution of resistance to Vip3A proteins has occurred within field populations of *H. zea* already. Evolved resistance to Vip3A has been voiced as a serious concern, with resistance to Vip3A already recorded within laboratory environments (Dively et al. 2021; Yang et al. 2020; Yang et al. 2021a, b). This is only the second paper to detect resistance to Vip3A within field populations of *H. zea*, with the first coming from a paper only published last year (Pezzini 2022). This may cause serious issues for future pest management strategies, as Vip3A becoming ineffective against *H. zea* may result in populations becoming uncontrolled. Infestations may be more severe, causing increasing damage to corn crops and overall causing serious economic damage to the agricultural industry. The results of this study reinforce the need of monitoring the evolution and spread of resistance to both Cry and Vip3A proteins within *H. zea* populations.

This study illustrates that GM methods are able provide this monitoring. By measuring the forewing shape, GM analysis can provide information on whether *H. zea* individuals are resistant to Bt Corn and what kind of Bt proteins they may be resistant too. This information is vital to manage these pests, and is needed by scientists, agricultural workers, and all other people that are damaged by *H. zea* infestations. Genetic markers provide similar information but are very expensive and involve

complicated process that often do not detect changes as early as GM methods do. This is because the genotype typically takes longer to change as a result of developed resistance than the morphotype, i.e. wing shape, and hence methods that measure these changes (GM) would detect changes earlier (Bouyer et al. 2007; Levine & Oloumi-Sadeghi 1996). Since GM methods are both easily accessible and cheap, they allow a wider array of people to monitor the resistance of *H. zea*, hopefully leading to a more complete and robust collection of recordings.

Future studies should concentrate on monitoring the evolution of resistance to the Vip3A protein within *H. zea* populations and the affect this has on the morphotype. They should prioritise determining how prevalent Vip3A resistance is within populations and identifying strategies that may be able to reduce the spread of resistance, e.g. increasing the size and/or frequency of non-Bt refuges, reducing or changing seed blend practices, etc. Identifying the effects that resistance to Vip3A protein has on the morphotype may also provide insight on how to prevent the spread of resistance. The results of this study suggested that the structural integrity of Vip3A-resistant forewings may be weaker, and that the change in wing shape may be a response to reduce wing injury. Further studies should investigate this using the finite element method, modelling various wing attributes in relation to environmental variables. Structural integrity of insect wings has a great effect on their ability to fly, thus investigation into this may also provide information on the true dispersal capabilities of Vip3A-resistant individuals.

The establishment of long-term monitoring of Bt resistance within *H. zea* populations is long overdue and highly necessary. Using GM methods will allow for more complete and robust data to be collected by a wider variety of people, establishing the monitoring of the spread of resistance within *H. zea* populations. Further research should focus on long-term studies, with monitoring of *H. zea* occurring on an annual basis to ensure that all information about resistance is both relevant and recent, and so any new information discovered may be implemented into future pest management strategies. This study provides supporting evidence that GM can be used to monitor resistance within a variety of insects from different taxa (Benítez et al. 2014b; Camara et al. 2006; Kadoić Balaško et al. 2021; Kadoić Balaško et al. 2022; Lemic et al. 2014; Lemic et al. 2020; Lemic et al. 2021; Mikac et al. 2013; Mikac et al. 2016; Mikac et al. 2019; Pajač Živković et al. 2019). Hence, future studies should explore resistance in insect pests using GM methods and further establish its use as a biomonitoring tool.

4.6 Conclusion

To conclude, this thesis confirms that development of resistance to Bt proteins in Bt Corn does significantly alter the forewing shape of *H. zea*, and that these differences in shape can be detected using GM methods. Hence, GM can be used as a monitoring tool to measure the development and spread of resistance to Bt Corn in *H. zea* populations.

The results show that susceptible moths have shorter wings to facilitate short distance movement, whilst resistant moths have longer wings to assist in migration and longer-distance movement. Super-resistant moths have medium-sized, more rounded wings which may be a consequence of either random genetic variation, or resource trade-off during development. These differences in wing shape can be used as a biomarker with the use of GM to identify different levels of resistance within a population of *H. zea*.

This thesis is the second case of practical resistance to Vip3A in field populations of *H. zea* to be recorded and provides supporting evidence that evolved resistance to Vip3A within field *H. zea* populations is a serious threat to future pest management strategies.

Overall, GM can be used as an effective way to monitor the evolution and spread of resistance within *H. zea* populations, including both the resistance to Cry proteins and the resistance to Vip3A.

5. References

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