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For the degree of Master of Science

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POPULATION GENETIC ANALYSIS OF THE BLACK BLOW FLY *PHORMIA REGINA* (MEIGEN) (DIPTERA: CALLIPHORIDAE)

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John W. Whale

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of

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I would like to dedicate this work to,

My Parents Christine and Tony,

My Aunt and Godmother, Joan and Jill,

And to my Partner Jonathan

Without the continuous love and support from each of you, I would not have been able to take this huge step in my life and have the strength to be who I am today. Thank you from the bottom of my heart.

The aim of life is self-development. To realize one's nature perfectly – that is what each of us is here for.

- Oscar Wilde

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LIST OF ABBREVIATIONS

ADD	Accumulated Degree Days
ADH	Accumulated Degree Hours
AFLP	Amplified Fragment Length Polymorphism
bp	Base Pairs
COI	Cytochrome Oxidase I Gene
COII	Cytochrome Oxidase II Gene
cyt*b*	Cytochrome *b* Gene
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
Eclosion	Emergence of an adult fly from a pupa
HWE	Hardy-Weinberg Equilibrium
IBD	Identity By Descent
ISSR	Inter-Simple Sequence Repeat
LHT	Life History Trait
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Locus
RAPD	Randomly Amplified Polymorphic DNA
SNP	Single Nucleotide Polymorphism
TBE	Tris-Borate-EDTA

ABSTRACT

Whale, John W. M.S., Purdue University, May 2015. Population Genetic Analysis of the Black Blow Fly *Phormia regina* (Meigen) (Diptera: Calliphoridae). Major Professor: Christine J. Picard.

The black blow fly, *Phormia regina* (Diptera: Calliphoridae), is a widely abundant fly autochthonous to North America. Like many other Calliphorids, *P. regina* plays a key role in several disciplines particularly in estimating post-mortem intervals (PMI). The aim of this work was to better understand the population genetic structure of this important ecological species using microsatellites from populations collected in the U.S. during 2008 and 2013. Additionally, it sought to determine the effect of limited genetic diversity on a quantitative trait throughout immature development; larval length, a measurement used to estimate specimen age. Observed heterozygosity was lower than expected at five of the six loci and ranged from 0.529-0.880 compared to expected heterozygosity that ranged from 0.512-0.980, this is indicative of either inbreeding or the presence of null alleles. Kinship coefficients indicate that individuals within each sample are not strongly related to one another; values for the wild-caught populations ranged from 0.033-0.171 and a high proportion of the genetic variation (30%) can be found among samples within regions. The population structure of this species does not

correlate well to geography; populations are different to one another resulting from a lack of gene flow irrespective of geographic distance, thus inferring temporal distance plays a greater role on the genetic variation of *P. regina*. Among colonized samples, flies lost much of their genetic diversity, ≥67% of alleles per locus were lost, and population samples became increasingly more related; kinship coefficient values increased from 0.036 for the wild-caught individuals to 0.261 among the F10 specimens. Colonized larvae also became shorter in length following repeated inbreeding events, with the longest recorded specimen in F1 18.75 mm in length while the longest larva measured in F11 was 1.5 mm shorter at 17.25 mm. This could have major implications in forensic entomology, as the largest specimen is often assumed to be the oldest on the corpse and is subsequently used to estimate a postmortem interval. The reduction in length ultimately resulted in a greater proportion of individuals of a similar length; the range of data became reduced. Consequently, the major reduction in genetic diversity indicates that the loss in the spread of length distributions of the larvae may have a genetic influence or control. Therefore, this data highlights the importance when undertaking either genetic or development studies, particularly of blow flies such as *Phormia regina,* that collections of specimens and populations take place not only from more than one geographic location, but more importantly from more than one temporal event.

1. INTRODUCTION

1.1. Entomology

Insects are among the most speciose and diverse Classes within the animal kingdom. Such diversity has enabled insects to inhabit virtually every ecosystem on Earth. There are many species which have adapted to localized ecozones while others inhabit a wider distribution, the latter may be due to natural expansions or via human introductions.

Due to their abundance, insects typically underpin the sustainability of the ecosystem in which they inhabit, either as pollinators of plants and economically essential crops, manufacturers of materials and/or foods such as silk and honey, or as a food source themselves for many other arthropods and vertebrates, and as such may be considered ecologically indispensable.

Their distribution means they regularly interact and inhabit near or within human civilizations and may be utilized for several different applications such as indicators of water quality (Resh & Unzicker, 1975), biological (pest) control of other invertebrates (for review see: Caltagirone (1981)), production of food and materials (Rinderer, Collins, & Tucker, 1985), and forensic (Catts & Goff, 1992).

This latter application of forensic entomology utilizes insects and other arthropods in legal investigations (Byrd & Castner, 2009; Catts & Goff, 1992). One of its major subdivisions within forensic entomology is medico-legal entomology; which pertains to the role of insects have in death investigations and is often used to estimate a postmortem interval (PMI) (Anderson, 2000; Byrd & Castner, 2009; Catts & Goff, 1992). Forensically, insects are the most accurate indicators of a later PMI estimation as a result of the lack of information that can be obtained by a pathologist following the cessation of rigor mortis up to 72 hours after death (Catts, 1990; Kashyap & Pillay, 1989).

1.2. The Calliphoridae

This family of flies consists of more than 1,000 species distributed worldwide. They are attracted to carrion, and in some cases, vegetative material or waste food products (Byrd & Castner, 2009). Behavioral variation can be observed among the species of this group; many are attracted to and prefer carrion, while species such as *Cochliomyia hominivorax* (Coquerel) and the genus *Protocalliphora* are ectoparasitic. Calliphorids are also passive pollinators of plants which emit carrion-like odors (Stensmyr et al., 2002). They are often the first to detect and colonize carrion, and are often encountered in death investigations (Byrd & Castner, 2009). This is a by-product of their primary ecological role: the efficient removal and breakdown of organic matter recycling

valuable nutrients back into the ecosystem. This family includes the genera *Lucilia* (green bottle flies), *Phormia* (the black blow fly), *Protophormia* (northern flies), *Calliphora* (blue bottle flies), *Chrysomya* (Old World flies) and *Cochliomyia* (screwworm flies).

1.2.1. *Phormia regina*

Phormia regina (Meigen), commonly known as the black blow fly, is distributed across the holarctic region of the world (Byrd & Allen, 2001; Byrd & Castner, 2009). Considered a cold-tolerant species, its distribution across the United States differs throughout the year; during the spring, summer and autumn *P. regina* is common throughout the northern United States (Brundage, Bros, & Honda, 2011), and is prominent across the southern U.S. during winter (Byrd & Castner, 2009). Females primarily deposit their eggs on carrion, however this species is also known to be a secondary myiasis producer (Byrd & Allen, 2001; Byrd & Castner, 2009).

1.3. Carrion Detection and Blow Fly Development

1.3.1. Blow Fly Development

Blow flies progress through four life stages: egg, larva, pupa and adult (Figure 1.1). Blow flies are poikilothermic (Fraenkel & Herford, 1940) and as such, their development is positively correlated with temperature resulting in a known, predictable life cycle. Development is affected by both the ambient temperature and by the increased temperatures generated from a large larval mass (Charabidze, Bourel, & Gosset, 2011; Slone & Gruner, 2007).

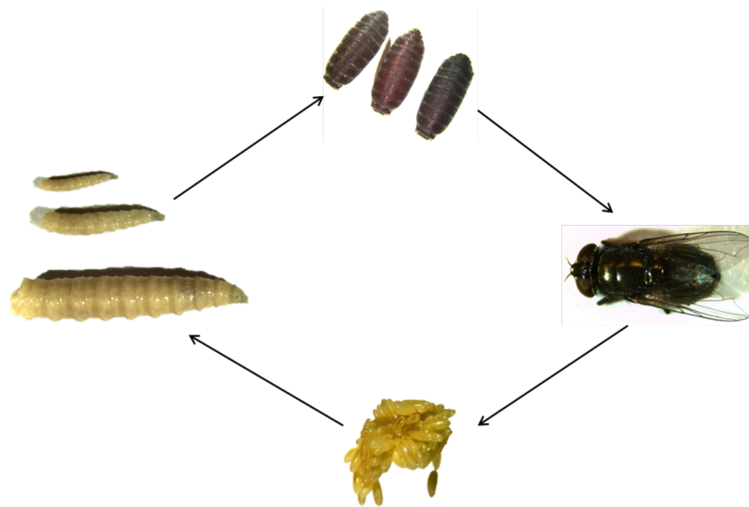

Figure 1.1: Typical life cycle of a blow fly.

Once basic nutritional needs have been met, an adult female will actively seek a source of protein, this may be in the form of carrion or some alternative. The maturation of her ovaries depends on this protein meal. Dethier (1961) observed that female blow flies require both a carbohydrate and protein source for survival;

females fed exclusively protein died within four days, while carbohydrate-only fed females resulted in prolonged survival. However, her eggs failed to mature. Once fully gravid, a female will begin to search for a suitable resource for oviposition. Eggs are typically deposited in or around natural orifices or wounds (Singh, Venketasan, Aggarwal, & Raj, 2014), and a blow fly can potentially locate carrion within minutes of its death (Anderson & VanLaerhoven, 1996; Erzinclioglu, 1983). Females tend to oviposit together at the same location on carrion, it is thought this prevents desiccation (Norris, 1965). It has been observed that a single female can oviposit as many as 200 eggs (Charabidze et al., 2011). Following incubation, a larva will hatch from the egg and begin feeding on the soft tissues of the carrion and will proceed through two larval molts. Once in the third instar, larvae feed edaciously until they meet their nutritional developmental threshold and enter the post-feeding stage (also known as the wandering stage, as the larvae leave the resources in search of a pupation substrate). Following this migration away from carrion, the larvae begin pupariation and enter the longest phase of immature development. It is throughout this stage that the larvae will metamorphose into adults. When metamorphosis is complete, eclosion occurs and the cycle begins anew.

1.3.2. Development Variation

Used by forensic entomologists to estimate a minimum postmortem interval (PMI_{MIN}), blow fly development is complex due to a number of variables which can directly affect it; such as temperature, species, light, humidity and its underlying genetics. For example, at lower temperatures, blow fly development is slow, while its rate increases as temperature increases until a maximum rate is achieved (Figure 1.2). Beyond this maximum, development rates decrease rapidly as the temperatures experienced become intolerable and are near the lethal temperatures for a given species (Higley & Peterson, 1994).

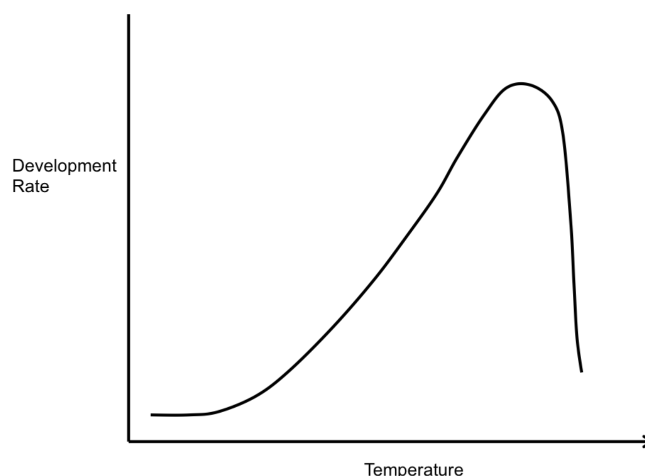

Figure 1.2: Insect temperature-development curve. The simplified curve highlights the relationship between development and temperature; development rate increases with temperature until an upper temperature limit in reached, at this point, development rate slows (Higley & Peterson, 1994).

Development differs between different blow fly species. Kamal (1958) studied the growth rates of thirteen different Calliphoridae and Sarcophagidae species at a number of different temperatures. The study found that development differed

across species at a constant temperature and humidity; total immature development of *P. regina* took 11 days, *Cynomyopsis* (=*Cynomya*) *cadaverina* (Robineau-Desvoidy) took 18 days and *Calliphora vomitoria* (Linnaeus) 23 days.

Aside from temperature, the amount of light exposure throughout development has been shown to affect development. Development of *P. regina* under a 24-hour light treatment resulted in delayed development and also greater variability in overall development time when compared to a 12-hour cyclic light-dark treatment (Nabity, Higley, & Heng-Moss, 2007). As a result, one might consider that seasonal day lengths have an impact on blow fly development, and that the longer summer days may retard development, however this may be offset by typically warmer ambient temperatures.

Kaneshrajah and Turner (2004) studied the effects of different feeding substrates on the development of *Ca. vicina* and observed a delay of up to two days on pork liver when compared to other pork tissues (brain, kidney, lung and heart muscle), perhaps due to the nutritional content of liver. They also noted a reduction in pupal size on cohorts reared on heart and brain in comparison to the other substrates. This has serious implications for estimating PMI_{MIN} and determining specimen or larval age if correlated to data where larvae were reared on a substrate that stimulates a faster growth rate (Kaneshrajah & Turner, 2004). Similarly, Day and Wallman (2006) and Thyssen *et al.* (2014) observed slower development with larvae reaching maximum lengths later and undersized pupae

on sheep and cattle liver, respectively, when compared to the alternative tissues (sheep meat and brain in Day & Wallman, and cattle muscle, tongue, stomach and chicken heart in Thyssen *et al.*). However, unlike the study by Kaneshrajah and Turner (2004), larvae reared on sheep brain tissue did not produce undersized pupae (Day & Wallman, 2006). Meanwhile, *Co. macellaria* showed no difference in larval growth or rate between horse and pig muscle (Boatright & Tomberlin, 2010). Slower development on liver tissue may be due to its biological role of detoxification, therefore if high levels of, or a number of different toxins are present, larvae may require a greater nutritional meal to counteract this (Thyssen et al., 2014). Additionally, larvae can synthesize lipids from proteins that are stored and utilized later for energy particularly throughout metamorphosis, therefore substrates with less fat content (such as liver) coerce larvae to direct energy away from growth into this process (Day & Wallman, 2006). The variation observed between these studies may be due to a number of different factors, such as the use of different species, different substrate (and their subsequent origins) and therefore a variable nutritional content or perhaps even an underlying genetic effect of the flies used from different geographic sources.

Duration of development of the same blow fly species, in theory, should be very similar from one study to the next; however this has been shown to not be the case. A number of studies of *P. regina* from across the United States and Canada have exhibited variation in development rate between them (Anderson, 2000; Byrd & Allen, 2001; Greenberg, 1991; Kamal, 1958; Nabity, Higley, &

Heng-Moss, 2006; Nunez-Vazquez, Tomberlin, Cantu-Sifuentes, & Garcia-Martinez, 2013) making it difficult to determine which is the most reliable dataset. Conversely, Cyr (1993) found no significant difference in development duration between *P. regina* populations collected from Indiana, Louisiana, Texas and Washington. Although slightly different temperatures were used in these studies, blow fly development can be standardized by converting these rates into physiological time units known as accumulated degree hours (ADH) or accumulated degree days (ADD). These are measurements of the thermal units required for the development and growth of the insect based on the temperatures experienced per hour or per day, respectively. The variation between studies is thought to either be attributable to variation in experimental and lab practices, in genetic variation, or both (Tarone & Foran, 2006). For instance, a significant difference in minimum development time and pupal size was observed among three different populations of another blow fly species, *Lu. sericata*, from California, West Virginia and Michigan (Tarone, Picard, Spiegelman, & Foran, 2011). The population from California developed quickest at 20 °C, slowest at 33.5 °C but produced the largest individuals on both occasions. In another study, significant differences were observed in pupal size and development time between three populations of *Co. macellaria* from three distinct ecoregions in Texas (Owings, Spiegelman, Tarone, & Tomberlin, 2014). Should this developmental variation not be considered, estimations of PMI_{MIN} may be miscalculated, particularly if the blow fly in question does not conform to available and/or reference data (Tarone et al., 2011). These variations between samples of

the same species indicate potential genetic differences specific to each region or location. These divergences in observed phenotypes may be controlled by a number of genetic processes, such as selection, to alter allele frequencies and genotypes within in each sample and drive divergence further from the original population from which they derive.

1.4. Molecular Analyses and Population Genetics

Population genetics is defined as the analysis of allele frequency changes within a population or groups of populations (Hamilton, 2009). Allele frequencies can be affected by a number of processes such as gene flow (the transfer of alleles into or from another population); selection, genetic drift (the random sampling of alleles); inbreeding or other events which constrict and reduce the overall genetic variation; and meiotic recombination. Population genetics and the study of alleles within populations can assist in answering questions of population structure, diversification and evolutionary relationships.

A population may be defined as the total group of individuals of the same species which inhabit the same geographic location or ecosystem. The individuals within this area are able to breed with one another or with individuals from another group from another area to produce fertile offspring (Hartl & Clark, 2007). A population sample is a small group of individuals from this population; they

should be representative of the variation present within the population enabling adequate and accurate data accumulation for conclusions to be drawn. The island model as described by Wright (1951) states that migration (or gene flow) into the population is equal to the migration leaving it between all populations. A more appropriate population model for blow flies may be the stepping stone model as described by Kimura (1953) which describes that a population from a larger area experience isolation by distance and that gene flow occurs mostly between nearby or adjacent sub-populations due to their more proximal distance than between populations much further away (Hamilton, 2009; Kimura & Weiss, 1964). For this thesis, the stepping stone model is assumed.

1.4.1. Selection and Population Differentiation

The basic assumption of selection is that alleles which improve fitness are or become more prominent and deleterious alleles are lost. However, deleterious alleles can be common throughout a population. There are three extensions to the selection theory which can explain this; non-Mendellian inheritance, non-independence between alleles as a result of linkage, and non-independence between the same alleles from different genomes (Hurst, 2009). Positive selection is the increase in frequency of an allele which benefits or improves the fitness of an organism until it reaches fixation within a population (Hurst, 2009). Meanwhile, negative selection removes deleterious alleles from the population,

maintaining the species' optimum fitness, which would otherwise be compromised if retained. The emergence of deleterious alleles within the genome is part of the neutral theory.

The neutral theory is based on genetic drift, in that allele frequencies have an equal ability to increase or decrease by chance (Hamilton, 2009; Hurst, 2009) rather than by selection. This is a stochastic process that is affected by the size of the population, isolation of breeding groups and by the geographic expanse in which these populations live (Hamilton, 2009). The majority of mutations that occur are considered to be neutral rather than beneficial or detrimental, and as such are subsequently assumed to have no effect on an individual's fitness (Kimura, 1968). These selection pressures may promote divergence between (isolated) populations, which may result in prezygotic (behavioral) or postzygotic (genetic) variations (White, 2001). The analysis of these alleles and variants can be detected via experimental evolution studies which use population genetic theory with life history traits and detect phenotypic-fitness trade-offs resulting from the organism's plasticity to new or stressful conditions or environments. To better understand how population differentiation occurs as a result of isolation, we can perform a series of experiments (known as experimental evolution experiments) in order to create ≥2 separate lineages which derive from a common starting population.

In equilibrium, allele frequencies remain constant within a population from one generation to another, in accordance with the Hardy-Weinberg Equilibrium (HWE) model. This ideal is based on populations randomly mating (panmictic), no genetic drift, alleles are unaffected by selection or bias to gender, a large population size, negligible migration and no mutations (Hamilton, 2009; Hartl & Clark, 2007). Hierarchical population structures within a species occur as a result from a deviation of these assumptions, particularly unequal allele frequencies due to restricted gene flow. Populations that do not conform to HWE ideals, often have at least one evolutionary process acting upon them to drive allele frequencies, often increasing heterozygosity due to migration or gene flow, or drift. Migrations are somewhat limited by geography; females in one location are more likely to mate with a male from the same or nearby location rather than those from afar. It has been observed that populations of a species which inhabit a relatively small geographic region exhibit divergence from one another (Haber et al., 2012; Wallman & Adams, 1997). This is usually due to population isolation caused by rugged topography or island groups, which despite the short geographic distance, prevent gene flow from occurring. Ikeda *et al.* (2012) noted that some beetle species from Japan’s southern island Honshu are not found on Hokkaido, and vice versa despite the distance of 12 miles between these islands. These populations are unlikely to move away from a region where their desirable food source is common. This, compounded with their inability to migrate long distances due to their flightless nature, over a short period of time, has promoted the diversification of these species. Such migrations would almost certainly mean

travelling away from an ecosystem they are well suited and adapted for, and potentially through those they are unfamiliar with and to which they have a lower tolerance.

Life history traits (LHTs) are an explanation of diversity, and account for how selection pressures enable adaptation in organisms to optimize their survival within their environment (Kingsolver & Huey, 2008). These traits may be dependent on genetic variation upon which selection drives adaptation. Among insects, there are three LHT rules: bigger is better, hotter is smaller, and hotter is better (Kingsolver & Huey, 2008). The first rule, suggests that larger individuals possess greater fitness than smaller individuals within a population by providing greater opportunity for survival, fecundity (ability to produce large numbers of offspring) and mating success (Kingsolver & Huey, 2008; Kingsolver & Pfennig, 2004). Positive selection pressures favor these larger individuals within a natural population (Kingsolver & Huey, 2008). However, larger individuals require greater energy and nutritional demands that will lengthen development and increase the risk of predation during vulnerable life stages. Therefore a trade-off for this may be that intermediate-sized individuals may provide the optimal balance between size and development. The second rule, hotter is smaller, proposes that higher temperatures result in smaller individuals (Kingsolver & Huey, 2008). The trade off in size at higher temperatures maximizes fitness with insects such as *Drosophila subobscura* inhabiting cooler environments tend to have larger body sizes (Gilchrist, Huey, Balanya, Pascual, & Serra, 2004). Similarly, greater

altitudes have also shown to promote larger sized individuals among both *D. subobscura* and *N. americanus* (R. J. Smith et al., 2000). However, phenotypic plasticity may promote fragility with this rule; *Caenorhabditis elegans* (Maupas) can possess a SNP within the *tra-3* gene which eliminates this rule (Kammenga et al., 2007), while a study found that a Californian population of *Lu. sericata* generated larger individuals regardless of the temperature throughout development (Tarone et al., 2011). The final rule, hotter is better, suggests that species with higher optimal temperatures possess greater fitness (Kingsolver & Huey, 2008). This may in fact be biologically logical, and benefit enzymatic kinetics and other cellular processes; however, greater nutritional requirements may be needed for this. *Drosophila melanogaster* has been observed to have increased metabolism in response to cold temperatures (Lee Jr, Damodaran, Yi, & Lorigan, 2006) as part of a mechanism known as rapid cold hardening (RCH). This process has also been observed in *Eurosta solidaginis* (Fitch) and *Sarcophaga bullata* (Parker) (Teets, Yi, Lee Jr, & Denlinger, 2013). Warm adapted insects possess shorter development and generation times than cold adapted insects (Kingsolver & Huey, 2008). There is however, an upper limit; as mentioned previously, too hot becomes unfavorable to key traits such as development.

Quantitative Trait Locus (QTL) mapping is a statistical method in which phenotypic data (LHTs such as behavior, morphology or development) is associated with genotypic data in order to determine the genetic basis in

variation of complex traits and characteristics (Seaton, Haley, Knott, Kearsey, & Visscher, 2002). This analysis requires at least two lineages that differ genetically in both the phenotype of interest and genetic markers which enable the differentiation between them (Fedoroff, 2012). Its aim is to determine whether phenotypic variation is caused by a collection of genes with little effect or few genes of large effect (Miles & Wayne, 2008; Weber, Peterson, & Hoekstra, 2013). Changes in quantitative traits between populations are characterized by genotype variations which directly affect the phenotype, this may be driven by phenotypic plasticity that promotes the population's adaptability to its changing environment (Kopp & Matuszewski, 2014). In order to detect these variants, they must exist within the parental strains, therefore a large sample size of diverse individuals would be appropriate in order to cover this variation. This method has been utilized identifying SNPs and alleles indicative of burrow design among two species of *Peromyscus* mice (Weber et al., 2013). Similar experiments have determined the genetic role underlying body size variation (Turner, Stewart, Fields, Rice, & Tarone, 2011), development rate variation (Burke et al., 2010), and the ability to adapt to novel environments (Orozco-TerWengel et al., 2012) in *Drosophila*. Burke *et al.* (2010) selected for accelerated development in *D. melanogaster* over 600 generations, observing a ~20% decrease in total development time compared to the original populations. The development phenotype has an important application within forensic entomology for blow flies. If the phenotype of development time in blow flies could be increased or decreased as a result of genetic inheritance as seen in *D. melanogaster*, this

could lead to both over- and under-estimations of a postmortem interval in death investigations. Better understanding of the genetic role behind development would subsequently improve the accuracy of PMI estimation.

1.4.2. Population Analyses of the Calliphoridae

Several types of molecular markers have been developed and used in order to determine variation within and among species. Both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) have been utilized in molecular studies of Calliphorids; the former have, however, primarily focused in species identification. Due it its low non-coding content and unilateral mode of inheritance (Lightowlers, Chinnery, Turnbull, & Howell, 1997), mtDNA sequences are often conserved in order to retain its functionality. Mutations within coding regions are more likely to occur in the third codon position, the position of least impact on amino acid determination. Nuclear DNA undergoes recombination, and as such, contains more variable regions than mtDNA. An effect on the increased variation present is genetic drift; the random fluctuations in allele frequencies within a population or a group of populations over time or generations due to chance alone (Charlesworth, 2009; Holsinger & Weir, 2009; White, 2001). The effect of drift may be enhanced by two population events; bottlenecks and founder effects. A bottleneck is an event in which the population size decreases significantly, resulting in the reduction of genetic variation available in the remaining members

of the group, therefore increasing the effect of drift during recovery. A founder effect is the formation of a new population group from a relatively small number of breeding individuals who have migrated or become separated from a much larger group. This new population, whose genetic variation will be limited (in that it covers only a portion of what was contained in the larger population), begin to exhibit new differences from this original population, the differentiation occurs as a result of the restricted genetic content available. Table 1.1 highlights the different population studies undertaken using various molecular markers.

Table 1.1: Comparison of Calliphorid population genetic studies: molecular markers used and whether population sub-structure was detected.

Species	Population Location	Molecular Marker Used	Population Structure Identified?	Article
Ca. hilli hilli	Australia	Allozyme	Yes	(Wallman & Adams, 1997)
Ch. bezziana	Africa/Asia	mtDNA	Yes	(M. J. Hall, Edge, Testa, Adams, & Ready, 2001)
	Asia	mtDNA, nDNA	Yes	(Ready et al., 2009)
Ch. megacephala	United States	AFLP	No	(Bao & Wells, 2014)
Co. hominivorax	Uruguay	Microsatellites, mtDNA	Yes, limited	(Torres, Lyra, Fresia, & De Azeredo-Espin, 2007)
	Jamaica, Mexico	AFLP	Yes	(Alamalakala, Skoda, & Foster, 2009)
	Jamaica, Trinidad	Microsatellites	Yes	(Griffiths, Evans, & Stevens, 2009)
	Caribbean	Microsatellites	Yes	(Torres & De Azeredo-Espin, 2009)
	Caribbean, South America	mtDNA	Yes	(Lyra, Klaczko, & Azeredo-Espin, 2009)
	Central America	AFLP	Yes	(Skoda, Figarola, Pornkulwat, & Foster, 2013)
Lu. cuprina	New Zealand	Allozyme	No	(Gleeson & Heath, 1997)
	New Zealand	mtDNA	No	(Gleeson & Sarre, 1997)
Lu. sericata	UK	RAPD	Yes	(Stevens & Wall, 1995)
	UK	RAPD, mtDNA	No	(Stevens & Wall, 1997)
	Australia	mtDNA	Yes, potentially	(Harvey, Dadour, & Gaudieri, 2003)
	Australia, southern Africa	mtDNA	No	(Harvey, Mansell, Villet, & Dadour, 2003)
	United States	AFLP	Yes	(Picard & Wells, 2010)
	Texas, USA	Microsatellites	Yes	(Archambeault, 2012)
P. regina	United States	AFLP	No	(Picard & Wells, 2009)
	Canada	Microsatellites	No	(Farncombe, Beresford, & Kyle, 2014)

Allozyme markers are an early but indirect method in detecting variation in that the variants present affect the amino acid sequence and thus electrophoretic mobility. These can be detected using native gel electrophoresis which separate

these proteins by both charge and size (Schlotterer, 2004). Gleeson & Heath (1997) identified moderate gene flow among samples of *Lu. cuprina* (Wiedemann) in New Zealand which suggested a high degree of migration. Pairwise estimates of gene flow (using Wright's F-statistic) were calculated between populations; the highest value (10.58, values >2.5 are considered high) was observed between populations collected from Palmerston North and Flockhouse which are 30 miles apart from one another, while the lowest value of 0.20, what detected between the Palmerston North and Hastings populations, a distance of 120 miles. They also noted that geographic barriers, such as the Ruahine and Tararua ranges, did not prevent the observed gene flow, and that migrations may potentially occur by hitchhiking on vehicles or fly-stricken sheep as well as by flight. However, population differentiation was found to exist between mainland and island populations of at least three blow fly species in Australia; *Calliphora hilli hilli* (Patton), *Ca. stygia* (Fabricus), and *Ca. albifrontalis* (Malloch). Despite the ~8.5 mile distance between Kangaroo Island and the mainland, this barrier prevents migration and gene flow, which subsequently promoted bottleneck events on the island (Wallman & Adams, 1997).

RAPD-PCR (randomly amplified polymorphic DNA – polymerase chain reaction) is a method of detecting variation between individuals, populations and species by randomly amplifying regions of DNA using one or two short, simple primers (Williams, Kubelik, Livak, Rafalski, & Tingey, 1990). Differentiation between groups is accounted for by the number of bands generated and their sizes. This

can be an unreliable method, as results can be difficult to reproduce (Schlotterer, 2004). RAPD markers have been used to determine intraspecific genetic variation among *Lu. sericata* samples from southern England (Stevens & Wall, 1995). Closely related individuals could be distinguished, while populations were determined to be free mixing with no specific barriers inhibiting their movement. Skoda *et al.* (2013) initially used these markers to differentiate between the screwworm flies *Co. hominivorax* and *Co. macellaria* but also found intraspecific variation among *Co. hominivorax* populations from Central America. Populations from a single country were not found to form a single monophyletic cluster; four Mexican populations were grouped together, while two additional populations from Mexico grouped with populations from Jamaica and Panama thus inferring population substructure among the Mexican populations. This may have arisen due to differentiation as a result of distance between populations from the two clusters or via gene flow from adjacent non-Mexican populations into the two Mexican populations that are separated from the others (CECH, LH (both Mexican populations), CR92 (Costa Rica), J98 (Jamaica) and P95 (Panama).

The AFLP technique is both robust and reliable which combines RFLP analysis with PCR (Vos et al., 1995). Picard and Wells (2009, 2010) used AFLP markers to determine the population structure of *P. regina* and *Lu. sericata* collected from across the United States and found that different populations were equally divergent genetically irrespective of the geographic sampling location. Flies from a single collection event were also found to consist of more closely related

individuals than would be expected in a random sample. They also noted that when a location had been revisited multiple times, the individuals collected were genetically distinct from the prior collection event, indicating geographic distance alone does not play a significant role in the genetic variation observed. Similarly, Bao and Wells (2014) observed little geographic structure and lower genetic diversity using AFLP among the oriental latrine fly, *Ch. megacephala* from Florida in comparison to other native North American flies. Data from the 15 distinct populations collected also exhibited a higher-than-random relatedness (Bao & Wells, 2014), with much of the variation observed occurring within each population sample. AFLP markers have also been used to differentiate between *Co. hominivorax* and *Co. macellaria* with several loci appearing to be species-specific. Among *Co. hominivorax*, 13 loci were sufficient to differentiate between 10 different populations from Central America. Populations from the four countries sampled (Costa Rica, Jamaica, Mexico and Panama) formed four individual clades, highlighting population substructure among this species in Central America and the Caribbean (Alamalakala et al., 2009).

Inter Simple-Sequence Repeat (ISSR) markers are generated by amplifying regions of DNA between two adjacent microsatellites. The microsatellites themselves are used as the primer binding sites (Reddy, Sarla, & Siddig, 2002) and are not necessarily specific to their target organism. Like RAPD-PCR and AFLP, variation is determined by the presence or absence of fragments of a particular size and requires no *a priori* knowledge of the genome (DeSalle &

Amato, 2004; Schlotterer, 2004). Typically used as a method of molecular identification, variation in profiles have been observed in specimens of *Lu. sericata* and *Ch. megacephala* from different locations in China (Zheng, Hu, Kunnon, & Chen, 2010). For *Lu. sericata*, 4, 6 and 4 different profiles (each pertaining to its own population) were observed at three loci, while 3, 7 and 2 different profiles were observed for *Co. macellaria* at the same loci. These profiles also differed between species. Meanwhile, a total of 60 individuals from four populations of *Ch. megacephala* from Malaysia were split into two groups. One group consisted of individuals from Penang and Selanger both located along the west coast of west Malaysia, although separated by ~350 Km, are connected by a main road which is often used to transport perishables between the two regions, allowing for easier migration (Chong, Chua, & Song, 2014). The second group, consisted of individuals collected from Johor and Pahang (both located on west Malaysia's east coast) and Sabah from east Malaysia. East and west Malaysia are separated by the South China Sea.

The molecular markers and their use described thus far, while they can be used to determine differentiation between populations as has been shown with AFLPs, but they do not have the power to determine deviation from HWE. Microsatellites however, do possess this power. Historically known as variable number of tandem repeats (VNTRs) (Jeffreys, Wilson, & Thein, 1985; Nakamura et al., 1987), they are short repeated sequences of DNA, typically 2-6 nucleotides, that are distributed throughout the genome, are highly polymorphic and co-dominant

(DeSalle & Amato, 2004; Schlotterer, 2004). Microsatellites have been increasingly used to identify and understand the natural genetic variation and population structures of many organisms with mixed results. Population structures have been observed in populations of *Co. hominivorax* from the Caribbean and South America (Griffiths et al., 2009; Torres & De Azeredo-Espin, 2005, 2009) but was not tested for among a Canadian population of *P. regina* consisting of 60 individuals collected from 7 locations (Farncombe et al., 2014).

Like microsatellites, prior genome or sequence knowledge is required for SNP variant analysis. Variation in nucleotide sequence can provide useful insights into historical demographic events such as population expansions, migrations and admixture (Schlotterer, 2004). SNPs are usually bi-allelic, however, hypervariable (HV) regions may possess multi-allelic loci as found within HV-I and II of mammalian mtDNA (Lott, Procaccio, Derbeneva, & Wallace, 2013; Schlotterer, 2004). Within coding DNA, mutations are more likely to occur in the third codon position for an amino acid. These polymorphisms may be detected by a number of methods, such as the use of restriction enzymes or DNA sequencing. The use of restriction enzymes enables the rapid identification of different strains based on the presence or absence of a SNP at a particular locus, but they may also be employed to insert dominant lethal genes as part of a process called release of insects carrying a dominant lethal (RIDL) (Thomas, Donnelly, Wood, & Alphey, 2000) as part of controlling pest species. Studies have particularly focused on myiasis-causing flies due to their economic impact. Knowledge of genetic data

assists in the control of these flies; *Co. hominivorax* has been successfully controlled and its spread into North America restricted using the sterile insect technique (SIT). However, if populations of *Co. hominivorax* begin to diverge, control techniques that have previously proved successful may become ineffective. Analysis of *Co. hominivorax* using PCR-RFLP from 34 populations from 10 countries in Central and South America identified population substructure which resulted from limited gene flow (Lyra et al., 2009). Populations from the Caribbean were highly structured with little variation in comparison to mainland populations, which possessed no clear clustering of populations. This therefore means that the implementation of SIT among the Caribbean islands would be appropriate due to their genetic isolation from the mainland, while mainland populations may prove more difficult to eradicate (Lyra et al., 2009).

Often the use of mtDNA will involve the direct sequencing of gene that has been identified to have discriminatory power between species and populations within a species. Implementation of a DNA sequencing method on mtDNA have included partial and whole gene sequencing i.e. the cytochrome oxidase I (COI) gene in order to detect variation in the form of SNPs. Single nucleotide polymorphisms are particularly useful in QTL mapping, selection and phenotypic analyses. The presence of these markers within a population may be prevalent as a result of selection pressures. Analysis of a short region (<300bp) of the mtDNA COI sequence identified potential population structure of *Lu. sericata*, separating

populations from Queensland and Western Australia (Harvey, Dadour, et al., 2003). However, when a ~1200 bp segment was analyzed, populations from Australia and southern Africa could not be differentiated from one another (Harvey, Mansell, et al., 2003). Among *Lu. cuprina*, no intra-population variation could be detected from populations from New Zealand alone with little variation also found between these populations and those from Australia (Gleeson & Sarre, 1997).

Cytochrome *b* sequence analysis of the Old World screwworm fly, *Ch. bezziana*, found support for two population clusters; the first from sub-Saharan Africa, and the second from the Gulf region and Asia (M. J. Hall et al., 2001). The Asian lineage can also be split into two sub-clades; mainland Asia and the islands of Papua New Guinea. Population substructure of this species seems to have occurred as a result of the significant geographic barriers (separation due to an ocean) between these populations making migrations (and therefore gene flow) between the islands and the mainland difficult (Ready et al., 2009).

Microsatellites are a highly important tool for genetic analyses in that they allow for the accurate testing of population structure and deviations from Hardy-Weinberg equilibrium. They have a benefit over SNPs, particularly those used in studies of the Calliphoridae (using mtDNA) as these polymorphisms which define the various haplotypes, are unilaterally inherited and are not subject to recombination. Meanwhile, the other molecular markers described are unable to

distinguish between a heterozygote or a homozygote genotype, a feature of microsatellites, that make the latter suitable for analyses of population structure. It is also not common practice within these studies to collect specimens separated by large periods of time (months/years), specimens are collected from a location once, and not often revisited.

1.5. Aims of this Study

The focus of this thesis is to determine the genetic diversity of wild and lab-colonized *P. regina* populations, and the correlation between genetic and development rate variation. Herein this study looks to:

I. Isolate, characterize and test polymorphic tetranucleotide microsatellite markers to determine a hierarchical structure among wild populations of *P. regina* collected from across the United States.

II. Determine the effect of reduced genetic variation on *P. regina* development, with particular emphasis on the quantitative measurement of length in the third instar stage, the most variable of the larval stages and a common tool used by forensic entomologists to estimate a postmortem interval.

2. POPULATION GENETIC ANALYSIS OF *PHORMIA REGINA* FROM THE UNITED STATES

2.1. Population Genetics of the Calliphoridae

Phormia regina is a widespread and abundant blow fly species across North America, particularly the United States (Byrd & Allen, 2001; Byrd & Castner, 2009). Knowledge of the genetic structure is limited with a few studies identifying mtDNA gene haplotypes (Boehme, Amendt, & Zehner, 2012; Desmyter & Gosselin, 2009; Jordaens et al., 2013), AFLP markers (Picard & Wells, 2009, 2012) and among some Canadian populations, microsatellites (Farncombe et al., 2014).

Many of the previous population genetics studies among Calliphorids using microsatellites have focused on the ectoparasite *Co. hominivorax*, a veterinary pest. Alternative markers have been utilized to determine the population structure of other blow flies of forensic importance such as mtDNA COI gene (Harvey, Dadour, et al., 2003) and AFLP of *Lu. sericata* and *P. regina* (Picard & Wells, 2009) with varying degrees of success. Harvey et al. (2003) found apparent population differentiation between *Lu. sericata* specimens from Western Australia

and a specimen from Queensland based on a 278bp sequence of the COI gene. Meanwhile, when a ~1200 bp fragment of the same gene was sequenced resolution could not be determined between specimens collected from South Africa, Zimbabwe and Australia (Harvey, Mansell, et al., 2003). The apparent differentiation found by the former study is likely due to the low number of specimens from Queensland, as well as the shorter sequence examined. A global study of 27 Calliphorid species using the same sized fragment also found it difficult to differentiate between samples belonging to a different geographic origin (Harvey, Gaudieri, Villet, & Dadour, 2008). There is ample genetic data on the Calliphoridae using the COI gene, but its primary use has been directed toward species identification. Based on these studies, it appears to be difficult to identify population structure of the Calliphoridae based on mtDNA sequence data, and that the utility of nuclear markers may be appropriate in defining structure in relation to geography.

Microsatellites are common markers in identifying population structures and are suitable for such analyses as they are multi-allelic, co-dominant and can easily detect gene flow. However, their use in blow fly species is not widespread, and becomes increasingly difficult in the absence of a reference genome, which is beneficial in their isolation. Advancements in next generation sequencing (NGS) technologies are now providing an avenue around this.

Microsatellite analysis of *Lu. illustris* and *Lu. sericata* (Florin & Gyllenstrand, 2002), and *Co. hominivorax* (Torres & De Azeredo-Espin, 2005) have observed low levels of genetic variation. They found lower observed heterozygosities than would be expected, at all 11 loci (where data was available) for *Lu. illustris*, which may be due to population subdivision or to the presence of null alleles (Florin & Gyllenstrand, 2002). *Cochliomyia hominivorax* has probably been the most extensively studied blow fly using microsatellites, and differentiation has been detected between populations on Caribbean islands and the South American mainland (Griffiths et al., 2009). Ten populations collected from four Caribbean islands were found to be highly structured resulting from a lack of gene flow limiting population expansion (Torres & De Azeredo-Espin, 2009). It is apparent that the geographic barriers which separate one island from another prevents gene flow between populations allowing for divergence. Island populations are more likely to experience bottleneck events resulting from reduced genetic variation from restricted gene flow (Frankham, 1997). Analysis of 12 polymorphic microsatellite loci identified a population sub-structure of the non-native blow fly *Ch. putoria* within the same region. Eight of these loci possessed at least five alleles (Rodrigues, de Azeredo-Espin, & Torres, 2009). It has been observed in *Lu. mexicana*, *Lu. sericata* and *P. regina* that when a localized population is analyzed, they exhibit a greater relatedness among the individuals (Archambeault, 2012; Picard & Wells, 2009, 2010, 2012). Archambeault (2012) was able to detect population differentiation between 11 different populations of

Lu. mexicana. Analysis of 572 specimens from across Texas split these 11 populations into four genetically distinct groups that were not bound by ecoregion similarity.

Previous population genetic analyses of *P. regina* have found little geographic structure, whether with the use of AFLP markers (Picard & Wells, 2009) or very recently, di- and tetra-nucleotide microsatellites (Farncombe et al., 2014). Both of these studies have identified that *P. regina* populations appear to be panmictic, but individuals within each sample population consist of highly related individuals. This may not be unexpected in that a female may lay up to 300 eggs (Yin & Stoffolano, 1997) which hatch, develop and emerge as adults together. Therefore, as adults, they may be attracted to the same odor cues from a particular carrion source. Picard & Wells (2009) also observed that much of the observed genetic variation (23%) remained within each sample. Among the seven Canadian populations, 8 of the 12 microsatellite loci (9 di-nucleotide and 3 tetra-nucleotide motifs) studied found observed heterozygosity to be lower than expected. Based on these data, it remains to be seen whether this species exhibits geographic structure or that the physical boundaries of North America e.g. Rocky Mountains, Appalachian Range or the Mississippi do not affect this species' ability to migrate and admix.

The aim of this chapter was to investigate the population genetic structure of *P. regina* using microsatellites. Population structures occur as a result of restricted

gene flow between subpopulations leading to differences in allele frequencies. This differs from previous work on this species in that new, novel tetra-nucleotide microsatellite markers developed here have been employed. Since the abundance and distribution of *P. regina* is vast, understanding its population structure bears importance particularly due to its role in death investigations.

2.2. Methods

2.2.1. Collection of *P. regina* Specimens

Previous collections of adult *P. regina* flies were collected from 21 different locations (Table 2.1) from across the contiguous United States in 2008 over a 23 day period as reported in (Picard & Wells, 2012). An additional unreported population from Texas (Smith County, TX, 32.438788, -95.095876) was collected on 22nd May 2008. All specimens were stored in ≥70% ethanol until further use. Two additional populations were collected from Indianapolis, IN, details about these collections are reported in Chapter 3.

Table 2.1: Collection sites for the *P. regina* populations in this study. *denotes samples from these populations were analyzed using AFLP technique in Picard & Wells (2012).

Location: City, State	Year Collected	Population Code	Number of Specimens
*Birmingham, AL	2008	AL1	10
*Tuscaloosa, AL	2008	AL2	5
*Clarksburg, CA	2008	CA1	10
*West Haven, CT	2008	CT1	5
*Quincy, FL	2008	FL1	10
*Riggins, ID	2008	ID1	7
*Mountain Home, ID	2008	ID2	10
*Blackfoot, ID	2008	ID3	9
*Boise, ID	2008	ID4	9
Indianapolis, IN	2013	IN1	15
Indianapolis, IN	2013	IN2	10
*Otis, MA	2008	MA1	10
*Mount Airy, NC	2008	NC1	5
*Severance, NY	2008	NY1	5
*North Olmstead, OH	2008	OH1	7
*West Springfield, PA	2008	PA1	10
*Tilford, SD	2008	SD1	10
Smith County, TX	2008	TX1	5
*Pullman, WA	2008	WA1	10
*Tucannon River, WA	2008	WA2	6
*New River Gorge, WV	2008	WV1	8
*Buffalo, WY	2008	WY1	5
*Greybull, WY	2008	WY2	10
*Shell, WY	2008	WY3	4

2.2.2. DNA Extractions

DNA from the samples collected in 2008 was extracted from thoracic tissue, while fly heads were used for flies collected in 2013. Corresponding bodies were retained as voucher specimens and stored at -80 °C. All new extractions were conducted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Fly heads were crushed and ground using a sterile micro-pestle and the remainder of the manufacturer’s protocol was followed and eluted into a final

volume of 100 µl and stored at -20 °C. DNA extracts were quantified (Appendix II) using a Qubit® 2.0 Fluorimeter (Life Technologies, Valencia, CA).

2.2.3. Microsatellite Selection and Primer Design

The program MSATCOMMANDER (Faircloth, 2008) was used to search the draft *P. regina* genome (CJ Picard, unpublished) generated from 100 bp paired end Illumina (San Diego, CA) reads for tetranucleotide microsatellite motifs. A total of 8,346 tetranucleotide motifs were identified and filtered for motifs that contained six or more repeats and were not wholly adenine and thymine. Tetranucleotide motifs were chosen over di- or trinucelotides due to their greater stability. Additional filtering was applied to motifs which would possess primers with similar annealing temperatures, from here primers were designed for 14 preliminary microsatellite loci using Primer3 (Rozen & Skaletsky, 2000), automated within MSATCOMMANDER. Primer sequences were designed based on the criteria of: GC content >30%, final product length of 200-600 bp, primer length of 19-25 bp, and an annealing temperature <2 °C between primer pairs.

2.2.4. Amplification, Fragment Length Analysis and Sequencing

Forward and reverse primers (Integrated DNA Technologies, Coralville, IA) were used to determine the polymorphic nature of the 14 preliminary microsatellite loci. Polymerase chain reaction (PCR) was optimized on a subset of ten wild-caught *P. regina* individuals and subsequently, six loci were selected for further analysis (Table 2.2). For these six loci, forward primers were fluorescently labeled with either 6-FAM, NED, PET or VIC (Life Technologies, Valencia, CA) were used with the appropriate unlabeled reverse primer (Table 2.2). DNA from the 225 *P. regina* specimens from the 24 wild-caught populations (collected in both 2008 and 2013) were amplified by polymerase chain reaction (PCR) using a Veriti 96-well Thermal Cycler (Life Technologies Inc., Carlsbad, CA).

Table 2.2: Primer sequences, motif and fluorescent label for the six loci genotyped.

Locus	Primer Sequence (5'-3')	Tm (°C)	Repeat Motif	Fluorescent Label
L3	3F: TGTATGACTTGTTGTATTCCTTTGC 3R: ACAGTACCGCTATTTAGGCAC	59	ACAT	VIC
L8	8F: ACAATCAGCGCCCATTTCC 8R: GGATCCACTTTGACGGATGG	59	ACAT	6-FAM
L9	9F: ACCACTGTGCAACGTCAAAC 9R: ACGCTGAATTATAGCCGTTCATC	60	ACAT	6-FAM
L12	12F: TGGACTGGGTACTGGTTAGC 12R: AGGCCTACCTCCCAATGAC	59	ACAT	6-FAM
L13	13F: TGAAGTATTCCAGTGTTTCAGCG 13R: ACGAACGCAACGTCTAAGTG	60	ACAT	PET
L14	14F: GTGAATATTTGCAGTTTGGGAGAC 14R: TGTTTAGAGGCTAATCCTTGTCG	59	AGAT	NED

The PCR master mix consisted of: 1x PCR Master Mix (containing 50 units/ml *Taq* DNA polymerase (pH 8.5), 400 µM of each dNTP, 3 mM $MgCl_2$) (Promega

Corp., Madison, WI), 2.5 pmoles each of forward and reverse primer, 1 ng of template DNA and nuclease-free water (when required) to a 20 µl volume. Cycling conditions were an initial denaturation step at 95 °C for 3 minutes, 28 cycles of: 95 °C for 25 seconds, 56 °C for 30 seconds and 72 °C for 30 seconds, followed by a final extension step at 72 °C for 8 minutes. PCRs were multiplexed into two reactions, each containing three primer pairs: the first contained pairs 3, 8 and 13 while the second contained pairs 9, 12 and 14. Each multiplex consisted of differently labeled primers or in the case of primer pairs 9 and 12 (both have 6-FAM tagged primers), where no fragment length overlap occurred.

Multiplexed samples were then genotyped for fragment length at each locus. Using a 96-well plate, 1 µl of amplified product was mixed with 9 µl HiDi™ Formamide and 0.5 µl GeneScan™ 600 Liz® size standard v2.0 (both Life Technologies Inc.) and placed into a 3500 Genetic Analyzer (Life Technologies Inc.) for analysis. Fragment lengths were evaluated using GeneMarker v2.4.0 (SoftGenetics LLC, State College, PA). Where peak height imbalance was observed, peaks <30% the height of the major allele peak were not scored.

Homozygous genotypes at each locus were selected for both forward and reverse sequencing to determine the number of repeating motifs present within each microsatellite. Amplified products from using unlabeled primers were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) as per manufacturer's recommendations. The purified products were then prepared for DNA

sequencing using BigDye® Terminator v3.1 Sequencing Buffer (Life Technologies Inc.). Sequencing reactions were prepared as half reactions of the directed protocol into a final volume of 10 µl. The default BigDye® reaction and cycling conditions were used. Samples were sequenced in both directions.

BigDye XTerminator® purification kit (Life Technologies Inc.) was used to purify the sequencing reactions by removing unincorporated BigDye® terminators and salts. To each reaction, 45 µl SAM™ solution and 10 µl XTerminator™ solution were added. Samples were vortexed gently for 30 minutes, before being centrifuged briefly to ensure the entire sample was at the bottom of the well. The samples were then placed onto the 3500 Genetic Analyzer for analysis. Quality of the DNA sequence was checked using Sequence Scanner software v1.0 (Life Technologies Inc.) and forward and reverse reads for each locus were aligned in BioEdit v7.1.3.0 (T. A. Hall, 1999).

2.2.5. Data Analysis

2.2.5.1. Genotype Variation

Genotypes for all samples across the six microsatellite loci were analyzed using GenAlEx v6.5 (Peakall & Smouse, 2006, 2012). This was used to determine the number of alleles (N_a), number of effective alleles (N_e), and calculate the observed heterozygosity (H_O) and expected heterozygosity (H_E) for each

population per locus. The N_e highlights the number of equally frequent alleles required to generate the observed level of genetic diversity. Higher N_e values are indicative of a higher heterozygosity and therefore greater genetic variation within the population while lower values indicates an imbalance in allele frequencies, caused by a dominance of one allele over the others.

2.2.5.2. Relatedness

Kinship coefficients were calculated using the software SPAGeDi (spatial pattern analysis of genetic diversity) v1.4 (Hardy & Vekemans, 2002) to determine the genetic differentiation among individuals within each population. This test determines the probability that two individuals share alleles identical by descent (IBD) or common ancestry (Queller, Strassmann, & Hughes, 1993). Estimates of coancestry were calculated between all pairs of individuals within a population sample according to Loiselle *et al.* (1995) whose estimator does not possess bias when alleles of low frequency are present in the data set. Pairwise comparisons were performed between individuals within each population sample, and coefficients for each population were determined from the pairwise averages within each sample. Values can range from -1 to +1; negative and values close to zero indicate no or limited relation between individuals while positive values denote higher degrees of relatedness. Values of 0.25 and 0.50 signify individuals are of half or full-sibling status, respectively.

2.2.5.3. Population Differentiation and Structure

Analysis of molecular variance (AMOVA) (Excoffier, Smouse, & Quattro, 1992) was used to determine genetic differentiation between the wild-caught populations collected in 2008 and 2013 from across the United States using Arlequin v3.5.1.3 (Excoffier & Lischer, 2010). This hierarchical statistical test examines the level of molecular variation within individuals of a population, between individuals of pre-determined sub-populations and between regions of populations. Here, AMOVA was used to determine the degree of variation among all individuals collectively, and also between groups of populations separated by geographical distance. Two geographical groups were tested for hierarchical population structure of *P. regina*; the first tested the variance of populations collected east and west of the Mississippi River, and the second, populations were separated into four geographic regions (West: CA1, ID1-4, WA1-2 and WY1-3; Midwest: IN1-2, OH1 and SD1; Northeast: CT1, MA1, NY1 and PA1; and South: AL1-2, FL1, NC1, TX1 and WV1).

Analysis of population differentiation was further investigated using the software STRUCTURE v2.3.4 (Hubisz, Falush, Stephens, & Pritchard, 2009; Pritchard, Stephens, & Donnelly, 2000). The software aims to determine the true number of populations, *K*, within the sample. STRUCTURE uses a Markov Chain Monte Carlo (MCMC) method to calculate *L*(*K*), the probability that the data agrees with the hypothesis of *K* genetically distinct groups or clusters, this is done based on

the genetic similarity among the different subpopulations. *L*(*K*) estimates plateau after the 'true' value of *K* has been reached (Pritchard & Wen, 2003; D. G. Smith et al., 2014). The Δ*K* is correlated with the strength of the genetic subdivisions of the populations studied (D. G. Smith et al., 2014). The admixture model was assumed with a burn-in (simulation) period of 5,000 interactions followed by 50,000 MCMC simulations. Runs were performed assuming the true value of *K* was between 1 and 15 distinct groups. Each run was replicated ten times to confirm that clusters with the greatest probabilities were formed. The probabilities of *K* were visualized using Structure Harvester (Earl & vonHoldt, 2012).

2.3. Results and Discussion

2.3.1. Microsatellite Genotyping

Following capillary electrophoresis, fragment lengths for each locus were determined (Figure 2.1) and genotypes scored (Appendix III). Of the 195 wild-caught specimens amplified, 1279 genotypes from across the six loci analyzed were characterized (94.74%). Failures may be due to polymorphisms within the primer binding region.

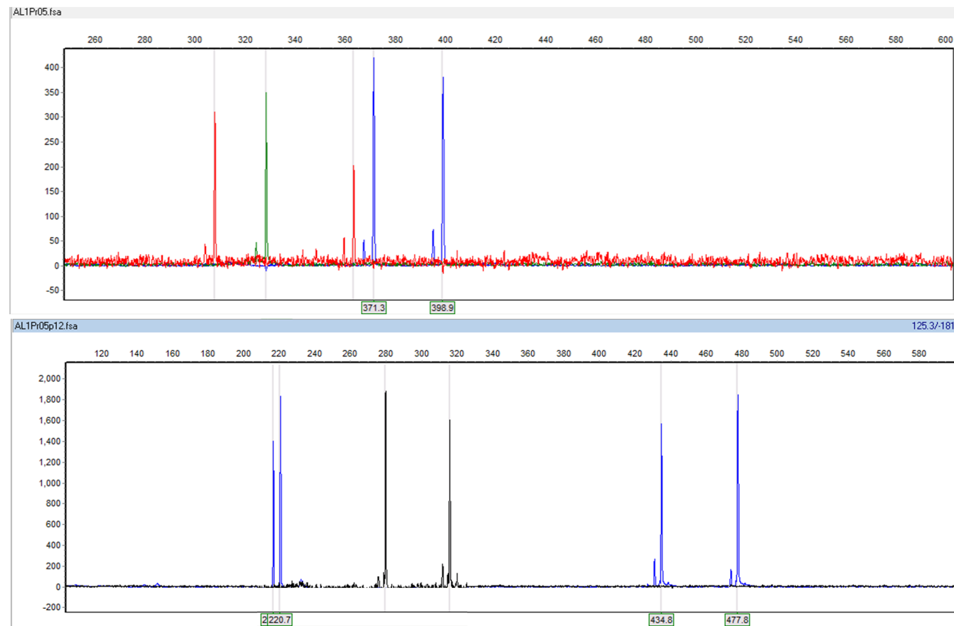

Figure 2.1: Electropherogram showing the genotypes of the 6 microsatellite loci of a specimen from the AL1 population. On top, the L3 (green), L8 (blue) and L13 (red) loci, and bottom, L9 (blue peaks on the left), L12 (blue peaks on the right) and L14 (black) loci.

A minimum of 11 alleles were observed at each locus (Table 2.3) among the 195 wild-caught *P. regina* flies. Lower observed heterozygosity versus expected values are indicative of increased levels of inbreeding within a population or due to the presence of null alleles. Null alleles may occur as a result due to the accumulation of mutations within the primer flanking regions, preferential amplification of shorter alleles causing an imbalance in allele detection or by slippage of the *Taq* polymerase during amplification (Chapuis & Estoup, 2007). The number of effective alleles is also correlated with heterozygosity; the greater the N_e, the greater the genetic variation within the population.

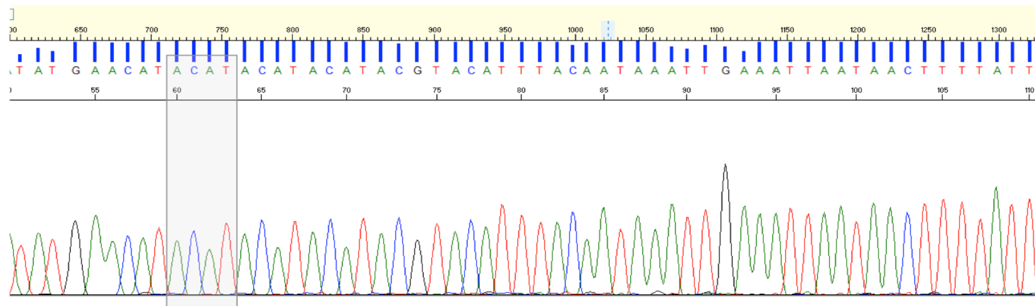

Figure 2.2: Sequencing electropherogram of a *P. regina* specimen at the L9 microsatellite locus. Locus has an ACAT motif (highlighted in grey); sequence was analyzed using Sequence Scanner v1.0.

A small number of samples, which possessed homozygous genotypes, were sequenced in order to determine the number of repeating motifs present at each locus. Forward and reverse sequences were checked using Sequence Scanner v1.0 (Figure 2.2) and forward and reverse complement sequences were aligned using ClustalW within BioEdit v7.1.3.0.

Table 2.3: Allelic variation and heterozygosity per locus as calculated using GenAlEx. N_a is the number of alleles, N_e is the number of effective alleles, H_O the observed heterozygosity, H_E the expected heterozygosity and uH_E the unbiased expected heterozygosity.

	Locus					
Parameter	**L3**	**L8**	**L9**	**L12**	**L13**	**L14**
N_a	71	43	11	77	52	86
N_e	35.651	28.710	2.049	44.946	16.095	49.679
H_O	0.557	0.880	0.536	0.529	0.638	0.614
H_E	0.972	0.965	0.512	0.978	0.938	0.980
uH_E	0.975	0.968	0.513	0.981	0.940	0.983

Kinship coefficients were calculated for the individuals within each population sample using the estimator as described by Loiselle *et al.* (1995) using SPAGeDi (Hardy & Vekemans, 2002). Values for each of the 24 wild-caught populations were calculated by determining the pairwise mean within each sample (Table

2.4). All values are close to zero, ranging from 0.033 for the WV1 population, to 0.171 for the TX1 population. As these values are close to zero, they indicate that the individuals within each sample are not strongly related. The populations which have values greater than 0.1 (AL2, CT1, TX1 and WY3) are also populations which consist of few specimens, and therefore should be considered with some caution.

Table 2.4: Kinship coefficient estimates using the estimator described by Loiselle *et al.* (1995) for the 24 wild-caught populations of *P. regina*.

Population Code	Kinship Coefficient	Population Code	Kinship Coefficient
AL1	0.052	NC1	0.068
AL2	0.134	NY1	0.086
CA1	0.043	OH1	0.064
CT1	0.163	PA1	0.058
FL1	0.059	SD1	0.069
ID1	0.051	TX1	0.171
ID2	0.065	WA1	0.064
ID3	0.043	WA2	0.087
ID4	0.081	WV1	0.033
IN1	0.036	WY1	0.091
IN2	0.062	WY2	0.057
MA1	0.062	WY3	0.134

Determination of any population structures present were conducted using AMOVA and STRUCTURE tests. The majority of the observed variation was detected within individuals of each population following AMOVA despite how the populations were grouped geographically (Table 2.5). Both configurations; an east/west Mississippi split, or four regions exhibited a very high proportion of differentiation attributable to variation among individuals within regional populations (~30%), while also finding negligible influence on variation among populations with regards to geographical distance (between regions). This

proportion of variation among populations within the regions indicates that the populations grouped within them do not primarily share the same or similar alleles across all the loci tested, inferring panmixia (random mating between individuals) among them. Therefore these data support findings observed by Picard & Wells (2009) in that individuals collected from one region are no more likely to share alleles with others collected from the same or adjacent region than one more geographically distant.

Table 2.5: Hierarchal Variation of regional wild-caught *P. regina* populations using analysis of molecular variation (AMOVA). SS = sum of squares, F_{ST} = Fixation Index of subpopulation-total population, F_{IS} = Fixation Index of individuals-subpopulation, F_{IT} = Fixation of individuals-total population.

Two Regions (East and West of the Mississippi)						
Variation Source	**SS**	**Variance Components**	**Percentage**	**Statistic**	**Value**	***P***
Among Regions	3.409	-0.0004	0%	F_{ST}	0	>0.05
Among Populations within Regions	630.084	0.8	30%	F_{IS}	0.3	**<0.001**
Within Populations	346.000	1.9	70%	F_{IT}	0.3	**<0.001**
Total	979.492	2.7	100%			
Four Regions (Northeast, Midwest, South and West)						
Among Regions	11.162	0.003	0.1%	F_{ST}	0.001	>0.05
Among Populations within Regions	622.331	0.8	29.9%	F_{IS}	0.3	**<0.001**
Within Populations	346.000	1.9	70%	F_{IT}	0.3	**<0.001**
Total	979.492	2.7	100%			

The second method employed to determine population sub-structure was by using the program STRUCTURE. An admixture model test was assumed in order to determine the true number of populations, *K*. A Delta K analysis using the mean log-likelihood with standard deviation was conducted to determine the *K*

value (Figure 2.3). The Delta K is determined based on the degree of genetic variation observed within the dataset and uses this to estimate the theorized number of populations required to generate this level of diversity. Simulations were conducted when K=1 to K=15. The Delta K distribution showed a peak when K=3, (DeltaK=5.852) suggesting the presence of three genetically distinct clusters. Three clusters have previously been observed among 11 populations of *Lu. mexicana* collected from across Texas (Archambeault, 2012). However the structure map here (Figure 2.4) found no support of a structure for *P. regina* with regards to geography but did exhibit a similar distribution of each cluster (color groupings) among all specimens and populations.

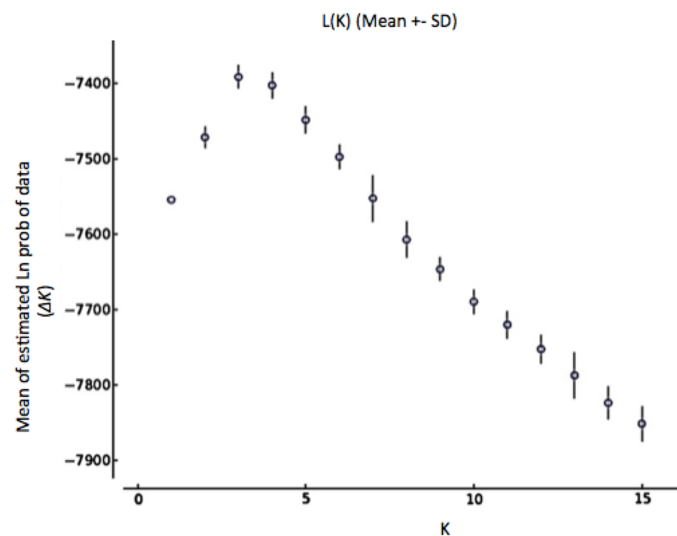

Figure 2.3: Mean likelihoods per *K* value with standard deviation to determine the estimated number of sub-populations required to generate the variation observed among the populations combined.

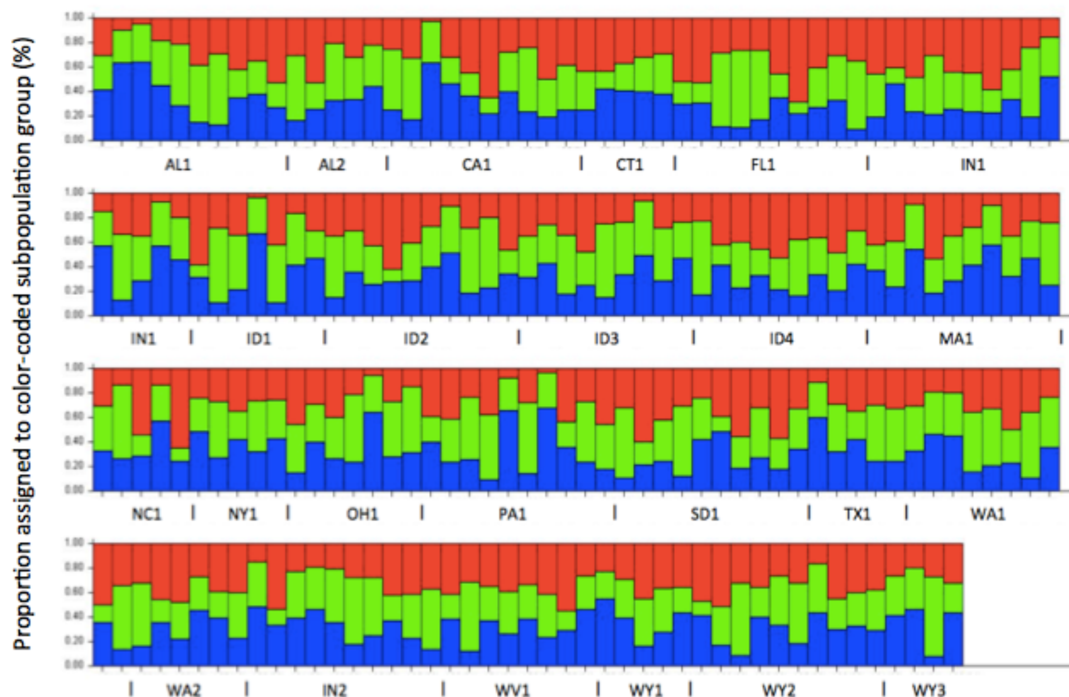

Figure 2.4: Structure map for an admixture analysis for the 24 wild-caught populations of *P. regina* for *K*=3. Each vertical bar correlates to an individual's assignment to a color-coded subpopulation when K=3. Horizontal lines are representative of the percentage assigned for each individual to a genetic subpopulation.

Failure to determine a population structure relative to geographic distance among *P. regina* populations from the contiguous United States, either by AMOVA or by STRUCTURE, may be due to the absence of a reproductive barrier. This is emphasized by the decreasing likelihood values (Δ*K*) as *K* increases (Figure 2.3), inferring that little population structure exists. Previous studies have highlighted population sub-structure between different populations; however populations inhabiting an island are more likely to become diverged from mainland populations than populations inhabiting the same landmass. Migrations between islands and greater landmasses become problematic if these distances

are larger, therefore driving divergence between them. As the populations from this study have been collected from the United States, the effect of these barriers become limited. The migratory capabilities of blow flies are well known. Mark and recapture studies by radioactively tagging flies have detected *P. regina* at a distance of four miles after 24 hours (Lindquist, Yates, Hoffman, & Butts, 1951), approximately 10 miles after 4 days (Schoof & Mail, 1953) and 28 miles after a period of 1-2 weeks (Yates, Lindquist, & Butts, 1952). Also counted were the number of alleles unique to a single population from those collected in 2008, which were subsequently also detected among the populations collected in 2013 (Table 2.6). Here, two alleles at the L3 locus were unique to populations located in the northeast (populations from 2008), subsequently detected in Indiana in 2013. No unique alleles from the Midwestern populations OH1 and SD1 (collected in 2008) were detected among either IN1 or IN2 populations collected in 2013. From these data, populations located in the South possessed the most unique alleles in 2008 to later be detected in 2013, perhaps supporting the migratory strength of blow flies. Alternatively, flies may have taken advantage of the good transport links from the South into the Midwest by hitchhiking on farm vehicles or produce and livestock transports, thus reducing their energy expenditure during migration.

Table 2.6: The number of different alleles initially detected in a single geographic region in 2008 and found in the Midwest region in 2013.

	Regions			
Locus	**Midwest**	**Northeast**	**South**	**West**
L3	0	2	2	3
L8	0	0	1	0
L9	0	0	0	0
L12	0	1	2	3
L13	0	1	1	0
L14	0	0	6	0

Additionally, the number of alleles unique to a single population collected both in 2008 and 2013 were determined (Table 2.7). At least four alleles were unique to a single population among the specimens collected in 2008, while the L3, L12 and L14 loci possessed unique alleles for specimens collected in Indianapolis in 2013. It was also determined that many of these population-unique alleles were of low frequency (<0.100) and therefore likely to be unique as a result of this. Alleles of low frequency are much less likely to become widespread throughout a population, and if gene flow occurs, into additional populations.

Table 2.7: The number of unique alleles per locus observed in a single population in the samples collected from 2008 and 2013.

	2008		2013	
Locus	**Number of Unique Alleles**	**Unique alleles with a Frequency <0.100**	**Number of Unique Alleles**	**Unique alleles with a Frequency <0.100**
L3	25	13	1	0
L8	6	2	0	0
L9	4	4	0	0
L12	27	12	3	3
L13	15	8	0	0
L14	16	9	15	12

The populations were also tested for their conformity to Hardy-Weinberg expectations using a Chi-squared test (Table 2.8). Calculations were based on

observed and expected heterozygosities from the variation and number of alleles and genotypes detected among these populations. All populations possess a χ^2 value less than α=0.05 indicating these wild populations meet the HWE criterion. Combined, the populations still maintain HWE conformity after considering the Wahlund effect (the reduction of heterozygosity resulting from the presence of subpopulations with differing allele frequencies).

Table 2.8: Chi-squared test of the 24 wild-caught populations.

Population	N loci	df	$\Sigma\frac{(O-E)^2}{E}$	Population	N loci	df	$\Sigma\frac{(O-E)^2}{E}$
AL1	6	5	0.413	NY1	6	5	0.323
AL2	6	5	0.986	OH1	6	5	0.632
CA1	6	5	0.543	PA1	6	5	0.482
CT1	6	5	0.911	SD1	5	4	0.428
FL1	6	5	0.454	TX1	6	5	1.026
IN1 (F0)	6	5	0.609	WA1	6	5	0.526
ID1	6	5	0.209	WA2	6	5	0.536
ID2	6	5	0.665	IN2	6	5	0.617
ID3	6	5	0.514	WV1	6	5	0.325
ID4	6	5	1.102	WY1	6	5	0.460
MA1	6	5	0.636	WY2	6	5	0.525
NC1	6	5	0.128	WY3	6	5	0.531

Conformation to HWE as well as an unclear population structure among populations of the USA may infer that these microsatellites may be applied to determine population differentiation of *P. regina* between North American and European populations. Additionally, in order to determine the true variation among wild populations, it is essential to make collections from multiple sites as well as revisiting sites later as blow flies have been shown to be variable in relation to both time and space.

2.3.2. Primer Specificity to other Calliphorid Species

The primers designed for these microsatellite loci were made using the draft *P. regina* genome, as a result of this, and due to the limited genetic data available among the Calliphoridae, a test of species specificity of the primers was conducted to determine whether these primers amplified *P. regina* alone or not. PCR reactions for the six loci were performed on six different species belonging to the Calliphorid subfamilies Chrysomyinae and Luciliinae (Figures 2.5-2.7); two specimens of *Protophormia terraenovae*, three of *Lucilia sericata*, two individuals of *Lucilia illustris*, and one each of *Lucilia coeruleiviridis*, *Cochliomyia macellaria* and *Ch. rufifacies* were tested. *Pr. terraenovae*, taxonomically the most closely related species to *P. regina,* was the only species to be amplified at the L3, L8 and L14 loci. The L13 locus amplified the *Ch. rufifacies* sample, while all species

were amplified at the L8 locus (Table 2.9). The loci were scored based on the ability of the primers to generate products at an annealing temperature of 56 °C.

Table 2.9: Cross-species test of the six microsatellite loci on six different Calliphorid species. + indicates amplification at the locus, - denotes no amplification.

Locus	L3	L8	L9	L12	L13	L14
Ch. rufifacies	-	+	-	-	+	-
Co. macellaria	-	+	-	-	-	-
Lu. coeruleiviridis	-	+	-	-	-	-
Lu. illustris	-	+	-	-	-	-
Lu. sericata	-	+	-	-	-	-
Pr. terraenovae	+	+	-	+	-	+

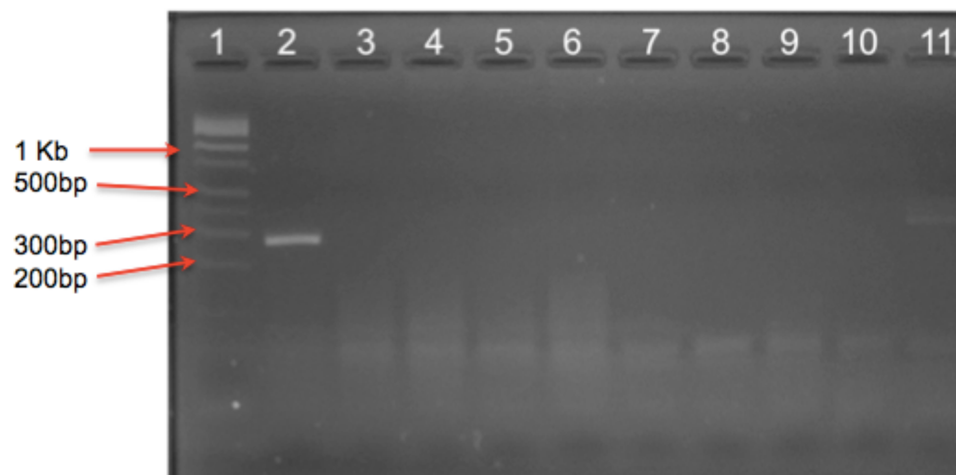

Figure 2.5: 2% agarose TBE gel of different blow fly species after amplification for the L3 locus. Lane 1: DNA marker, lane 2: *P. terraenovae* sample, lane 3 & 4: *Lu. sericata* samples, lane 5 & 6: *Lu. illustris* samples, lane 7: *Co. macellaria* sample, lane 8: *Ch. rufifacies* sample, lane 9: *Lu. coeruleiviridis* sample, lane 10: *Lu. sericata* sample, lane 11: positive control *P. regina* sample.

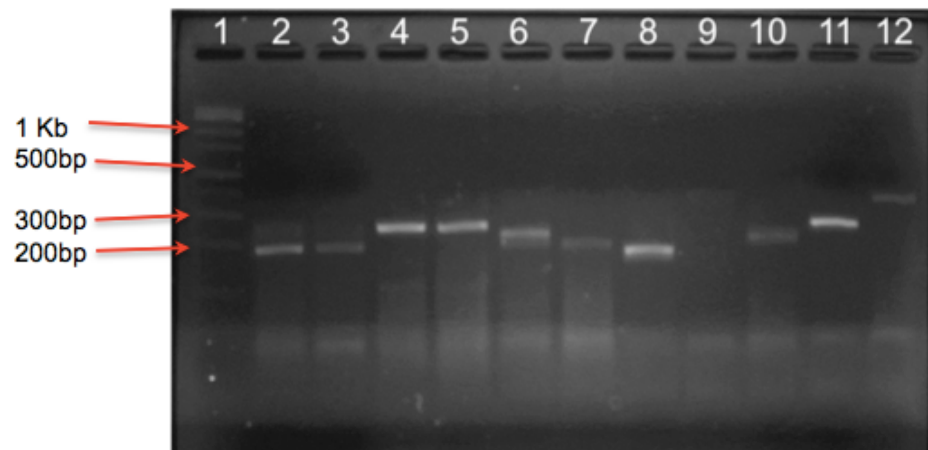

Figure 2.6: 2% agarose TBE gel of different blow fly species after amplification for the L8 locus. Lane 1: DNA marker, lane 2 & 3: *P. terraenovae* samples, lane 4 & 5: *Lu. sericata* samples, lane 6 & 7: *Lu. illustris* samples, lane 8: *Co. macellaria* sample, lane 9: *Ch. rufifacies* sample, lane 10: *Lu. coeruleiviridis* sample, lane 11: *Lu. sericata* sample, lane 12: positive control *P. regina* sample.

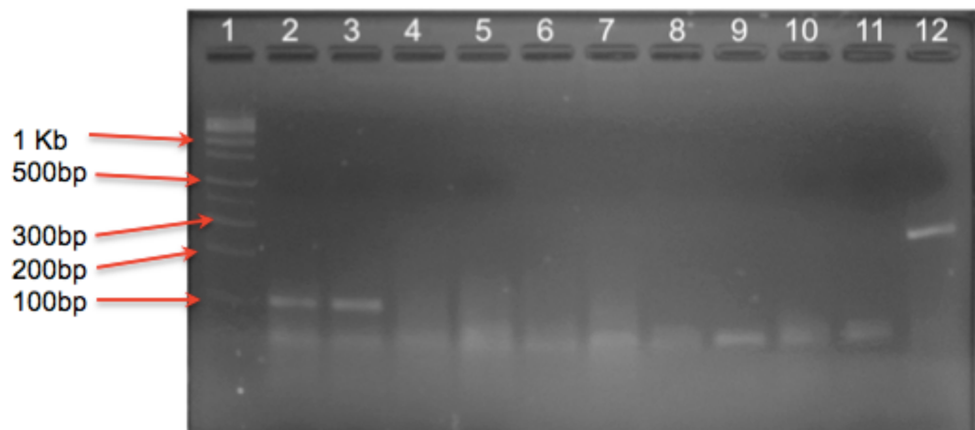

Figure 2.7: 2% agarose TBE gel of different blow fly species after amplification for the L14 locus. Lane 1: DNA marker, lane 2 & 3: *P. terraenovae* samples, lane 4 & 5: *Lu. sericata* samples, lane 6 & 7: *Lu. illustris* samples, lane 8: *Co. macellaria* sample, lane 9: *Ch. rufifacies* sample, lane 10: *Lu. coeruleiviridis* sample, lane 11: *Lu. sericata* sample, lane 12: positive control *P. regina* sample.

From this analysis, five of these primers can also amplify other Calliphorid species; four of which amplified a single species (either *Pr. terraenovae* or *Ch. rufifacies*) while one amplified each of the six species tested. If we assume these

fragments are the same loci within the respective genomes as with *Phormia regina*, analysis on a greater number of specimens from each species would be required to determine whether the microsatellite motifs may also be polymorphic in length, and also whether they enter the same expected fragment size ranges.

3. THE CORRELATION BETWEEN GENETIC AND DEVELOPMENT RATE VARIATION

3.1. *P. regina* Development

Insect development is dependent on temperature, and as such it becomes essential to know and understand their relationship with one another. In order to calculate a PMI, one is required to accurately identify the species of insect(s) present and their life stage at the time of collection (Pai, Jien, Li, Cheng, & Yang, 2007). Using known development data at certain temperatures for the insect in question, one can estimate its age – the minimum time required for the collected specimens to reach the stage or size at the time of collection.

P. regina is one of the most common blow fly species in North America (Byrd & Castner, 2009) and is therefore of great forensic importance being a primary colonizer of carrion as it is often encountered on corpses at crime scenes. Studies of *P. regina* development from the USA and Canada (Anderson, 2000; Byrd & Allen, 2001; Greenberg, 1991; Kamal, 1958; Nabity et al., 2006; Nunez-Vazquez et al., 2013) have exhibited variation in development rate between them. These studies did examine development at different temperatures, but this

can be corrected by converting to physiological time units known as accumulated degree hours (ADH) and accumulated degree days (ADD). When this correction is accounted for, variation between data still persists. Additionally, previous studies of *Lu. sericata* (Gallagher, Sandhu, & Kimsey, 2010; Tarone et al., 2011) and *Co. macellaria* (Owings et al., 2014) have observed differences in development rate under the same conditions between populations of different geographic origin. Since these studies have identified these variations, it stands to reason that an underlying genetic component to the development of these insects, perhaps controlling size in order to meet specific biological requirements.

In addition to this, the high relatedness among samples as identified by Picard and Wells (2009) highlights a potential problem with the current protocols conducted to generate such developmental data. Laboratory colonies have often been used to engender such data as seen in Anderson (2000), Byrd & Allen (2001) and Grassberger & Reiter (2001, 2002), therefore the cohort of flies used may be inbred and of low genetic variation from the study's inception. Therefore if this founding population is compromised i.e. inbred, low population size etc. then the validity of the data may be questioned as the individuals used to collect such data do not represent nor possess a broad range of the (genetic) variation within the overall population.

The aim of this chapter was to determine the effect of reduced genetic variation due to colonization (monitored using the microsatellite loci described in Chapter

2) on larval development of *P. regina.* Particular attention was applied to the third instar stage of larval development since during this phase, a larva will feed almost continuously until it is ready to pupate, this stage in the life cycle is also often encountered in death investigations. One would might therefore expect that a reduction in the genetic variation would result in a narrower spread of physical characters such as length.

3.2. Methods

3.2.1. *P. regina* Collection, Species Identification and Colony Maintenance

Adult blow flies were collected from six different locations in Indianapolis, Indiana, USA (Marion County, IN). Collections were made on multiple days throughout the weeks beginning May 20th 2013 (population IN2) and June 3rd 2013 (population IN1) (Table 3.1). Some locations were revisited more than once to account for temporal variation. Decayed chicken liver was used as an attractant and adult flies were collected by sweep net. Adult flies were identified using a taxonomic key to North American blow fly species (Whitworth, 2006).

Table 3.1: Location and date of collection of *P. regina* specimens in 2013.

	Population	
Location	**IN1**	**IN2**
Canal Walk	5th June	23rd May
IUPUI Campus	4th June 7th June	21st May 27th May
Lockefield Common	3rd June 8th June	22nd May
Military Park	3rd June 7th June 8th June	21st May 24th May 27th May
University Park/Veterans Memorial Park	-	23rd May
White River State Park	4th June	24th May

P. regina adults were transferred to a BugDorm rearing and observation cage (BioQuip Products, Rancho Dominguez, CA). Flies were provided with distilled water in a plastic bottle, granulated sugar in a petri dish *ab libitum* and fresh chicken blood (= protein meal) for ovary maturation (Figure 3.1). Colonies were housed in a temperature controlled room at approximately 25 °C, 40-60% relative humidity and 12:12 photoperiod.

Figure 3.1: Insect rearing cage set-up.

3.2.2. Development Conditions and Sampling

Following protein meal, fresh chicken liver was made available for oviposition and was removed after three hours ensuring eggs collected were of a similar age. This was also conducted for each subsequent generation. The offspring of the wild-caught (F0), F5 and F10 adults were monitored for their development. Egg batches laid by these generations were homogenized and groups of approximately 100 eggs were transferred to 36 separate 95 mL plastic cups each containing 75 g fresh chicken liver. This ratio of eggs-liver was used to prevent overcrowding of the substrate which can result in increased development rates and undersized larvae and adults (Ireland & Turner, 2006). Each cup was placed into an individual mason jar that was half-filled with fine pine shavings (Lanjay Inc., Montreal, QC). Each jar was covered with a WypAll X60 sheet (Kimberly-Clark, Roswell, GA) and secured in place with the jar lid. The 36 jars were randomly placed into a Percival® environmental chamber (Perry, IA), in the arrangement of 12 jars per shelf (Figure 3.2). The incubator was programmed to 25 °C, 65% relative humidity and 12:12 light/dark photoperiod. Each jar was moved down one shelf daily to avoid shelf bias on immature development. Larvae were otherwise allowed to develop undisturbed until third instar.

Figure 3.2: Arrangement of the 36 mason jars inside the incubator.

During third instar, at least twice daily, three randomly selected jars were removed and sampled (one prior to mid-morning and one in the late afternoon or early evening), and the final three jars served as development controls and allow for the monitoring of adult eclosion. All larvae from the sampled jars were hot water killed (HWK) for one minute using boiled water from a standard kitchen kettle, and transferred to containers filled with 70% ethanol. Body lengths of each larva were recorded to the nearest 0.25 mm using a metric ruler visualized under a stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL). Mean lengths and standard deviations were calculated from each jar and time point in the F1, F6 and F11 generations.

3.2.3. Extraction of DNA, Amplification and Fragment Length Analysis

All DNA samples were extracted, amplified and electrophoresed using the 3500 Genetic Analyzer as described in Chapter 2.

3.2.4. Data Analysis

3.2.4.1. Analysis of Larval Length and Eclosion

Analysis of variance (ANOVA) was used to determine the level of differentiation in larval length and mean eclosion time between the three generations (F1, F6 and F11) using R. Following ANOVA, a Tukey's Honest Significant Difference (HSD) test was performed in order to determine between which sample means the significant difference is located. Tukey's HSD is a post-hoc test and should be performed following ANOVA. The purpose of an ANOVA is to identify whether there is a difference between the sample means, while a Tukey's HSD test is more informative in that it identifies between which populations the difference lies. A Kruskal-Wallis test was also performed on the length data using R. This test is analogous to ANOVA, but differs in that it does not assume the sample populations are of equal size nor possess a normal distribution. To determine whether the timing of, and duration of eclosion was significant between each sample, an ANOVA test was performed using R.

3.2.4.2. Genetic and Genotype Analysis

Determination of allele frequencies, heterozygosity and kinship were conducted using GenAlEx and SPAGeDi respectively, as described in Chapter 2. The adult specimens derive from the wild-caught IN1 population, F5 and F10 cohorts.

3.3. Results and Discussion

3.3.1. Analysis of Larval Development

Larval development throughout the third instar stage, both actively feeding and post-feeding, was measured for the offspring of the F0, F5 and F10 generations. The aim was to determine the effect of reduced genetic variation on length, the quantitative measurement used by forensic entomologists when estimating specimen age and subsequently PMI. All larvae were sampled and measured. Lengths of all larvae measured can be found in Appendix IV.

Table 3.2: Sampling times throughout *P. regina* development in the three generations studied.

	Generation		
	F1	**F6**	**F11**
Time in hours since oviposition	67h	69h	75h
	73h	75h	93h
	90h	90h	100h
	95h	95h	116h
	98h	99h	125h
	116h	114h	141h
	122h	124h	148h
	141h	140h	165h
	146h	148h	170h
	163h	163h	189h
	170h	171h	195h

The lengths of *P. regina* larvae recorded were consistently shorter throughout the colonized generations (F6 and F11) in comparison to the larvae measured from the F1 generation. Each colonized cohort progressively consisted of fewer individuals of the larger lengths. To begin to examine this trend, the maximum larval length was recorded for each generation and compared to the maximum length observed at a similar time in development in the other two generations tested (Table 3.3). The time point at which the maximum length was recorded in F11 (148 h), larvae of similar length were also recorded among both F1 and F6 generations. Likewise, at 114 h, the time point when the longest larva was recorded in F6, larvae of similar length were also observed in F1.

Table 3.3: Maximum length of larvae for each generation and time into development length was recorded. *Indicates the length of the longest specimen measured in that generation.

	F1	**F6**	**F11**
Length of longest larva per generation and time in development	18.25mm (116 h)	18.00mm (114 h)*	16.00mm (116 h)
	18.75mm (122 h)*	17.25mm (124 h)	16.25mm (125 h)
	17.50mm (146 h)	17.00mm (148 h)	17.25mm (148 h)*

As well as a reduction in the maximum length recorded, the colonized generations also possessed a lesser proportion of individuals of longer length (Table 3.4). The F1 generation possessed 35 individuals (1.23%) of all larvae measured of 18.00 mm or greater. This number was reduced to one and then zero for F6 and F11 respectively. Meanwhile, the modal length for each generation was 16.00 mm in F1 (n=212, 7.46%), 15.00 mm in F6 (n=283, 8.84%) and 15.00 mm in F11 (n=304, 8.11%).

Table 3.4: Proportion of individuals from each generation to measure 15.00, 15.50, 16.00, 17.00 or 18.00mm or greater per generation. n=number of individuals, %=percentage of total cohort.

	≥15.00mm		**≥15.50mm**		**≥16.00mm**		**≥17.00mm**		**≥18.00mm**	
Generation	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
F1 (n=2843)	1452	51.07	1135	39.92	872	30.67	302	10.62	35	1.23
F6 (n=3202)	753	23.52	363	11.34	173	5.40	19	0.59	1	0.03
F11 (n=3749)	781	20.83	373	9.95	190	5.07	8	0.21	0	0.00

Across the three generations studied, the mean maximum (μ_{max}) length of the larvae measured during each 24-hour period of sampling progressively reduced from a μ_{max} of 16.67 mm in F1, 14.44 mm in F6, to 14.57 mm in F11 (Figure 3.3). The mean length for larvae measured from 139 hours until pupation seemed to plateau at around 14.00 mm, while data for both F1 and F6 follow the normal insect immature development curve (Higley & Peterson, 1994) where length increases before decreasing as the larvae begin to migrate away from the food source. Analysis of variance (ANOVA) between each generation was determined using the R statistical package (R-Core-Team, 2014). The variation in length

mean during each 24-hour period between the three generations were highly significant (Table 3.5), to the p<0.001 level for the 67-90, 91-114, 115-138 and 139-162 hour periods in development and to the p<0.01 level for the 163-186 hour period (Figure 3.3). In order to determine where the significant difference lies, a Tukey's HSD test (Table 3.6) was performed using R. During the first 4 development periods, each generation were highly significant from one another (F1-F6, F1-F11 and F6-F11), however, the area of greatest different for the 163-186 hour period was between the F1 and F11 generations.

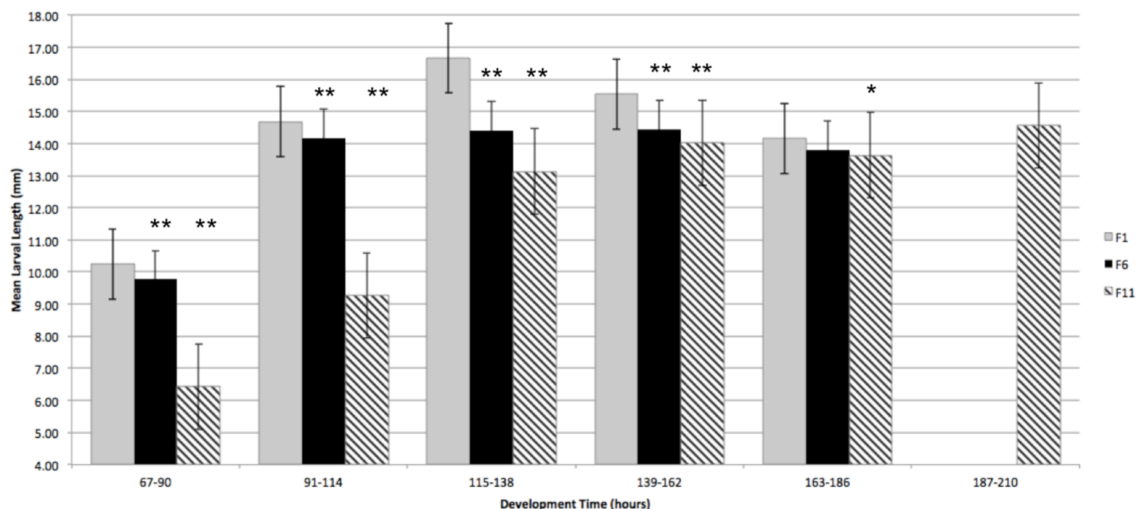

Figure 3.3: Mean length of larvae for F1, F6 and F11 during each 24-hour sampling period with standard error. *p<0.01, **p<0.001.

Table 3.5: ANOVA of mean larval length across each generation during each 24-hour period.

Development Period		Df	Sum of Squares	Mean Sq.	F Value	p Value	Result
67-90 hours	Generation	2	4147	2073.6	307.9	$<2 \times 10^{-16}$	p < 0.001
	Residuals	2359	15887	6.7			
91-114 hours	Generation	2	12496	6248	2652	$<2 \times 10^{-16}$	p < 0.001
	Residuals	2297	5411	2			
115-138 hours	Generation	2	4270	2135.2	8928	$<2 \times 10^{-16}$	p < 0.001
	Residuals	1629	3896	2.4			
139-162 hours	Generation	2	780	390.1	214.6	$<2 \times 10^{-16}$	p < 0.001
	Residuals	2050	3726	1.8			
163-186 hours	Generation	2	18.2	9.079	5.171	0.0058	p < 0.01
	Residuals	970	1703.1	1.756			

Table 3.6: Tukey's HSD test to determine where the greatest difference lies between each generation. **p*=<0.01, ***p*=<0.001.

Development Period	Pairwise Comparison	Difference	p Value	Length Difference
67-90 hours	F6-F1	-0.4703	0.0001627**	F1 longer
	F11-F1	-3.8148	<0.001**	F1 longer
	F6-F11	3.3445	<0.001**	F6 longer
91-114 hours	F6-F1	-0.5027	<0.001**	F1 longer
	F11-F1	-5.4086	<0.001**	F1 longer
	F6-F11	4.9059	<0.001**	F6 longer
115-138 hours	F6-F1	-2.254	<0.001**	F1 longer
	F11-F1	-3.543	<0.001**	F1 longer
	F6-F11	1.288	<0.001**	F6 longer
139-162 hours	F6-F1	-1.092	<0.001**	F1 longer
	F11-F1	-1.515	<0.001**	F1 longer
	F6-F11	0.424	<0.001**	F6 longer
163-186 hours	F6-F1	-0.351	0.133	F1 longer
	F11-F1	-0.514	0.008*	F1 longer
	F6-F11	0.162	0.209	F6 longer

In addition, a Kruskal-Wallis one-way analysis of variance test was performed on the dataset using R (Table 3.7). The Kruskal-Wallis analysis is a non-parametric equivalent to an ANOVA test and is also suitable when testing two or more independent samples which may have varying sample sizes. The data suggests that the differences between the means across the three generations during the first four 24-hour sampling periods are highly significant prompting the rejection of the null hypothesis, while the fifth has a p-value greater than 0.05.

Table 3.7: Kruskal-Wallis one-way analysis of variance test across each generation during each 24-hour period throughout third instar development.

Development Period	Kruskal-Wallis χ^2 Value	df	p-value
67-90 hours	801.899	2	$<2.2 \times 10^{-16}$
91-114 hours	1375.585	2	$<2.2 \times 10^{-16}$
115-138 hours	1073.12	2	$<2.2 \times 10^{-16}$
139-162 hours	375.239	2	$<2.2 \times 10^{-16}$
163-186 hours	5.709	2	0.0576

The fewer number of longer larvae among the colonized generations results in a larger proportion of individuals of a similar, shorter length (Figure 3.4). This observation may be an adaptive response to the reduction in genetic variation, in that the optimum fitness for survival is geared toward larvae of median length, while mortality for the longer extremes increases. Alternatively, the differences in total development time and reduced larval length may be attributed to a reduction in genetic variation within the colonized population as a result of multiple inbreeding events with ever-increasingly related individuals. Consequently, alleles controlling or affecting increased length may be lost from the population as the genetic variation of the available breeding individuals decreases. This supports findings from previous studies which intimate that there may be an underlying genetic influence on blow fly development (Gallagher et al., 2010; Owings et al., 2014; Tarone et al., 2011).

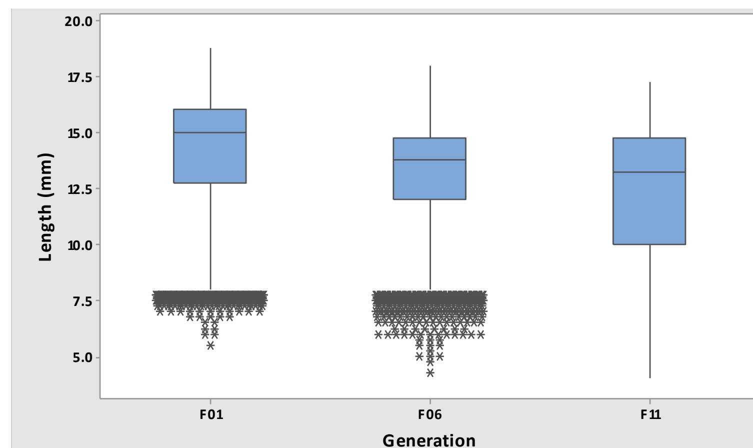

Figure 3.4: Boxplot of length from all larvae measured in each cohort. Bars represent the distribution in lengths recorded, stars represent outlying data points.

The apparent loss of variation appears to have limited the spread of the larval lengths recorded. The longest larva measured decreased from 18.75 mm to 17.25 mm in F1 to F11, while the proportion of larvae greater than or equal to 15.00 mm decreases from 51% in F1 to 21% in F11. This greater spread of length and proportion of longer individuals among the F1 larvae may be due to their wild and panmictic nature, while the F11 larvae are of more similar lengths, potentially due to their limited genetic make-up. The apparent reduction in genetic variation via colonization is supported by the increase in the R values between individuals of each population. An R value greater than +0.25 is indicative of at least half-sibling status among individuals. The importance of this observation is that if a larva is collected from a cadaver, and its age is estimated from development data of colonized flies, its age may be over-estimated. This would undoubtedly decrease the accuracy in the estimation of a minimum postmortem interval. Therefore wild blow flies, and their larvae, are more likely to possess more variation prior to colonization than populations that have experienced one or more inbreeding events. Therefore, for these data, it is of great importance to make collections of flies from more than one event to ensure that data generated may contain as much of the variation that would be experienced within a naturally wild population.

Table 3.8: Duration mean and standard deviation of eclosion for the three control jars for F1, F6 and F11 generations.

	Generation		
Jar Number	**F1**	**F6**	**F11**
1	298.00 (11.56)	305.17 (11.25)	314.83 (10.86)
2	296.17 (10.61)	307.99 (13.64)	348.24 (9.85)
3	298.04 (11.18)	313.48 (13.50)	344.38 (10.43)
Overall Mean	297.46 (11.14)	308.86 (13.30)	336.42 (17.90)

Additionally, duration of development was monitored from F1 through to F11 (Table 3.8), the time until and duration of adult eclosion from the three control jars were observed to steadily increase. For F1, eclosion was first observed 284 h after oviposition and ceased after 313 hours. This is considerably shorter from the F11 generation where eclosion of one jar began at 290 h and ended at 340 h, while the eclosion duration for the other two jars were from 328-358 h and 332.5-358 h respectively.

Table 3.9: ANOVA of eclosion of the control jars from each generation.

	Df	**Sum of Squares**	**Mean Sq.**	**F Value**	**p Value**	**Result**
Generation	2	277,201	138,600	651.6	$<2 \times 10^{-16}$	$p < 0.001$
Residuals	1030	219,103	213			

3.3.2. Genetic Diversity Following Colonization

Florin and Gyllenstrand (2002) detected a loss of genetic diversity among colonized blow fly populations within the laboratory in a few number of generations. The genetic variation among the colonized flies of *P. regina* was identified (Table 3.10). Allele analyses of the inbred cohorts exhibit a large reduction in allelic variation from the wild-caught flies (F0) through to the tenth generation progeny (F10) emphasizing that few inbreeding events are required in order to observe loss of diversity.

Table 3.10: Allelic variation and observed and expected heterozygosity between wild-caught adult *P. regina* flies, fifth, and tenth generation progeny. N_a is the number of alleles, N_e is the number of effective alleles, H_O the observed heterozygosity, H_E the expected heterozygosity and uH_E the unbiased expected heterozygosity.

		Locus					
	Parameter	**L3**	**L8**	**L9**	**L12**	**L13**	**L14**
IN1 (F0)	N_a	17	20	6	12	14	22
	N_e	12.250	16.667	2.273	8.229	9.389	16.071
	H_O	0.571	1.000	0.533	0.500	0.385	0.800
	H_E	0.918	0.940	0.560	0.878	0.893	0.938
	uH_E	0.952	0.972	0.579	0.917	0.929	0.970
F5	N_a	17	14	5	7	9	16
	N_e	13.235	9.574	1.654	5.760	4.780	11.250
	H_O	0.933	0.933	0.400	0.167	0.571	0.600
	H_E	0.924	0.896	0.396	0.826	0.791	0.911
	uH_E	0.956	0.926	0.409	0.862	0.820	0.943
F10	N_a	3	5	1	4	3	5
	N_e	2.761	3.169	1.000	3.039	2.542	3.982
	H_O	0.733	0.733	0.000	0.500	0.600	0.333
	H_E	0.638	0.684	0.000	0.671	0.607	0.749
	uH_E	0.660	0.708	0.000	0.696	0.628	0.775

The greatest number of alleles observed among the F10 generation was five at both the L8 and L14 loci, while the L9 locus became mono-allelic. At least 67% of all alleles have been lost at each locus among the F10 progeny when compared to the number of alleles present in the IN1 population. All variation has been lost at the L9 locus, while an increase in allele frequencies are also observed among the F10 population (Table 3.11).

Table 3.11: Most common alleles and their frequencies among the colonized flies. Where there are two most common alleles, both alleles share the same frequency.

	Most Common Allele(s) (bp)			Frequency of Common Allele		
Locus	**IN1**	**F5**	**F10**	**IN1**	**F5**	**F10**
L3	328	328	362	0.179	0.167	0.367
L8	371 & 395	384 & 403	395	0.100	0.167	0.433
L9	217	217	217	0.633	0.767	1.000
L12	505	514	439	0.250	0.250	0.393
L13	261	261	300 & 332	0.231	0.357	0.433
L14	328	268	352	0.133	0.167	0.400

Colonized populations exhibited high relatedness values following the kinship test using SPAGeDi; from 0.036 for the wild-caught IN1 population, to 0.055 for F5 and 0.261 for F10, thus highlighting the loss in genetic variation and the increase in shared alleles between individuals. This pairwise data infers that individuals within a sample have progressed from unrelatedness in the IN1 population to half siblings among F10 individuals. As evidenced here and within previous studies, inbreeding reduces genetic variation, but what is seldom studied is whether this results in a reduction in the variation of another trait. Diversity is essential for a population. Among larger populations, allele

frequencies are influenced by a combination of pressures such as selection and drift etc., and the frequencies of the alleles at a particular locus do not fluctuate much from one generation to the next. However, smaller populations, where diversity is limited, drift plays a much greater role in the frequencies of these alleles. Depending upon the degree of variation within the founding population, alleles can be fixed or lost rapidly. Starting with a population of adequate or appropriate variation for analysis is essential.

From these data, we identify that for both genetic analysis and development studies of *P. regina*, and most likely other blow fly species, that specimen collection is required from several events over space and more importantly, time in order to capture as much of the variation present. Collections made from few events and for a short duration will exhibit characteristics of a founder event whereby the individuals collected and/or sampled are representative of a small proportion of the much larger population from which they came. From this, if any further colonization were to occur, this window of diversity would only become narrower. Few studies have also examined the temporal effect on genetics of specimens from a particular location. While individuals at this location will change over time, their genetic identity may still be represented among the 'new' individuals. It is largely unknown how genetically different individuals collected at the exact same location, but collected at different times throughout the year, or subsequent years that follow. Therefore, in order to derive the most accurate conclusions, as much of the wild data must be sampled as possible.

4. CONCLUSION

The present study has focused on the population genetic surveys of *P. regina* from across the contiguous United States using multiple microsatellite loci, determining whether a population structure exists in relation to geography. Additionally, this thesis has sought to determine the effect of reduced genetic variation on *P. regina* development with particular emphasis on the third instar life stage. This study presents the first microsatellite survey of a blow fly species from several populations from across the USA.

The use of microsatellites here has identified significant differentiation, ~30%, between population samples of *P. regina* indicating the presence of population structure and supporting the data as previously reported by Picard and Wells (2009) when using AFLP markers. This temporal structure is likely due to the manner in which the population samples were collected; from one location over a limited period of time. Many of the previous genetic studies of the Calliphoridae have utilized the mtDNA COI gene, particularly for the purpose of species identification and the detection of different haplotypes. Some studies have detected population differentiation in relation to geographic distance among different species, most often the economical pest *Co. hominivorax*. The most

extensive population analyses on *P. regina* have been with the use of the dominant marker of AFLP's, however, they do not possess the suitable capacity to determine divergences from Hardy-Weinberg expectations. Since microsatellites are codominant, and can detect heterozygotes and homozygotes, these divergences can be tested. The only previous microsatellite analysis on *P. regina* performed to date has been conducted on specimens from Canada. Additionally, from this one study, dinucleotide motifs were the primary microsatellite tested and all analysis was performed with the specimens grouped together as one metapopulation regardless of geographic origin. This study not only tested tetranucleotides, a more stable motif, but also analyzed the genotypes and allele frequencies within and between population samples separated by both geographic and temporal distance. Here, individual populations exhibited many alleles at each locus, however the observed heterozygosity was not representative of this variation. Lower observed heterozygosities than expected may be indicative of inbreeding between related individuals within each population sample or also the presence of null alleles at the screened loci.

Wild-caught populations exhibited moderate R values; all greater than zero, ranging from 0.033 to 0.171, indicating that they derive from few colonization events where individuals develop, eclose and seek new resources during a comparable timeframe within the same ecological zone. A single female has the potential to lay up to 300 eggs, each one being a full sibling to one another, if this

is done in one sitting, then all eggs will develop and hatch around the same time since they all experienced the same climatic conditions. Once hatched, the larvae will also progress through development at a similar rate as they feed upon the same resource, before leaving the corpse again around the same time. Finally each larva will also enter the pupal stage at the same time before the eclosion occurs and a new adult fly emerges. As newly emerged adults, together they will be attracted to similar odor cues for essential resources and may still be within close proximity when they are ready to mate. This highlights a major problem when conducting both genetic and development studies, in that if a study population is formed from a single collection, it will contain many related individuals simply due to the biology of these flies and the manner in which they grow and develop.

Throughout colonization, *P. regina* cohorts lost genetic variation as recorded by the loss in the number of genotypes observed. This number dropped rapidly between generations F5 and F10. The loss of genetic variation also appears to have affected development, particularly that of the F11 generation where all larvae measured were consistently of shorter length than the larvae of comparable age sampled in both F1 and F6. Both cohorts of colonized larvae also consisted of fewer proportions of larvae of longer length, greater than 15.00 mm, compared to F1, indicating a potential adaptive response to reduced genetic variation where larvae of median length possess the greatest fitness. For individuals to reach the maximum lengths as detected in F1, they would have to

feed for longer, potentially exposing themselves to predators and the weather conditions for a longer period of time. Failure to meet these lengths prevents this extended exposure.

As well as length, duration of larval development was also lengthened for the F11 generation, further indicating an underlying genetic effect on development. Further analysis should be conducted in order to determine whether this was a significant observation and has any ties associated with the limited genetic variation present within this generation. The measurement of the longest larva plays a key role in forensic investigations; it is accepted that the longest larva (of a particular species) is the oldest larval specimen on a corpse. However, there is some variation to this since larvae reduce in size prior to entering the pupal stage. If the larvae of the F11 generation do not meet these maximum lengths as observed in the F1 generation, then the window for misestimating its age increases both as it increases in length while feeding, but also postfeeding prior to pupation. Further implications are that if development data derived from colonized flies, such as F11, were used in a real life case scenario, calculation of a specimen’s age (that was found on a corpse and that would follow the development trajectory of an F1 larva) may be over-estimated, therefore resulting in a less accurate PMI. Colonies of flies, to date, are often collected from a single collection event, in some cases over an hour or less for both genetic and development analyses. Consequently, based on these observations, development of *P. regina*, and perhaps other necrophageous fly species, ought

to be conducted from the most wild and diverse group of flies, with as little colonization as possible, with the founding population composed of more than temporal collection event.

Genetic analysis of the colonized flies exhibited increased levels of homogeneity. Individuals from the F10 generation were highly related, likely due to the multiple inbreeding events. Both the genetic and length data here identifies a narrowing range of variation from one generation to the next. While data is available on the reduction in genetic diversity as a result of inbreeding, and this has been observed in *P. regina* for the first time, the effect of inbreeding on other traits and the reduction in the spread of this data is not well studied. From this, we accentuate the importance of collecting flies from more than one collection event, either varying temporally, geographically, or both but with greater emphasis on temporal distance. This is of importance when studying both development and genetics to ensure as much of the natural variation may be observed.

Recently, the argument has been whether geographic or temporal distance has the greatest effect on population variation of these flies. Based on the growing amount of data (like that of AFLP in *P. regina, Lu. sericata* and *Ch. megacephala*), and of that presented in this thesis, evidence suggests distances of time play a more significant role than geography. The high proportion of variation among individuals with population samples according to AMOVA analysis supports this.

Since larval development seems to be affected by genetics, future work and analyses may be focused on the genetic role in development; identifying genes and/or markers associated with larval length or even the formation of adult body structures during pupariation. Additionally, the genetic effect on immature development of other Calliphorid species may differ from *P. regina*, and therefore may be studied to determine whether they are influenced in the same way. As well as this analysis, additional microsatellite loci may be utilized to further analyze the population structure of *P. regina* and determine whether shifts in allele (and their frequencies) can be correlated to geography. From the data presented in this thesis, it would be my advice to all forensic entomologists, who undertake any study, but particularly those on development, to move away from collecting flies over a very short period of time and making it common practice to construct a founding population derived from multiple temporal collections to maximize its diversity.

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APPENDICES

Appendix I – Materials and Equipment

Equipment:

0.1-2.5µl pipettor	1-10µl pipettor	2-20µl pipettor
20-200µl pipettor	100-1000µl pipettor	Applied Biosystems® 3500 Genetic Analyzer
Applied Biosystems® Veriti Thermocycler	Denver Instrument Scales	Electrophoresis Tank
Eppendorf Centrifuge 5424	Eppendorf Mastercycler Pro Thermocycler	Fisher Scientific PCR Workstation Cabinet
Fisher Scientific Vortex Mixer Model: 945404	Fotodyne Foto/UV 26 Transillumintor with Canon PowerShot S95 Digital Camera	Haier Microwave Oven
Isotemp Freezer	Isotemp Heatblock	Isotemp Heated block/Magnetic Stirrer
Kenmore Refrigerator	Leica Microsystems Stereomicroscope with KL 200 LED, IC80 HD and M60 attachments	Major Science Minis-300 Powerpack
MicroAmp® 96-well plate	Qubit 2.0 Fluorometer	Micro Pestles

Consumables:

20mL Disposable Scintillation Vials; Product code V6880	Fisherbrand 0.2mL PCR tubes; Product code 14230225
Fisherbrand 1.5mL microcentrifuge tubes; Product code 05-408-129	Fisherbrand SureOne™ Filter Tip Reload Pipet Tips 0.1-5µl; Product code 02-707-472
Fisherbrand SureOne™ Filter Tip Reload Pipet Tips 1-10µl; Product code 02-707-474	Fisherbrand SureOne™ Filter Tip Reload Pipet Tips 2-20µl; Product code 02-707-476
Fisherbrand SureOne™ Filter Tip Reload Pipet Tips 20-200µl; Product code 02-707-478	Fisherbrand SureOne™ Filter Tip Reload Pipet Tips 100-1000µl; Product code 02-707-480
Fisherbrand SureOne™ Non-Filtered Tip Pipet Tips 0.1-10µl; Product code 02-707-454	Fisherbrand SureOne™ Non-Filtered Tip Pipet Tips 5-300µl; Product code 02-707-447
Fisherbrand SureOne™ Non-Filtered Tip Pipet Tips 100-1000µl; Product code 02-707-405	IBI Scientific Molecular Biology Grade Agarose; Product code IB70042
Qiagen DNeasy Blood & Tissue DNA Extraction Kit; Product code 68506	Qubit Assay Tubes; Product code Q32856
Qubit Molecular Probe dsDNA BR Assay Kit; Product code Q32850	Thermo Scientific™ 0.2mL strip tubes with caps; Product code AB-1182

Solutions:

- 10X Tris-Borate-EDTA (TBE) buffer; Product code FER B52 (Thermo Scientific).
- SYBR Safe gel stain solution; Product code S33102 (Life Technologies).

Appendix II – Concentration of DNA of each Specimen

Table S2.1: Concentration of DNA in nanograms per microlitre for each *P. regina* specimen from each population following Qiagen extraction kit protocol.

Sample ID	Population ID	DNA Concentration (ng/µl)	Sample ID	Population ID	DNA Concentration (ng/µl)
AL1Pr01	AL1	34.5	ID4Pr01	ID4	62.2
AL1Pr02	AL1	70.0	ID4Pr02	ID4	32.7
AL1Pr03	AL1	67.0	ID4Pr03	ID4	86.3
AL1Pr04	AL1	23.6	ID4Pr04	ID4	24.4
AL1Pr05	AL1	67.1	ID4Pr05	ID4	111.0
AL1Pr06	AL1	151.4	ID4Pr06	ID4	103.2
AL1Pr07	AL1	11.0	ID4Pr07	ID4	108.4
AL1Pr08	AL1	65.9	ID4Pr08	ID4	48.0
AL1Pr09	AL1	19.5	ID4Pr09	ID4	125.0
AL1Pr10	AL1	41.4	MA1Pr01	MA1	94.9
AL2Pr01	AL2	70.2	MA1Pr02	MA1	111.5
AL2Pr02	AL2	224.8	MA1Pr03	MA1	80.2
AL2Pr03	AL2	43.1	MA1Pr04	MA1	182.8
AL2Pr04	AL2	39.0	MA1Pr05	MA1	77.0
AL2Pr05	AL2	65.2	MA1Pr06	MA1	29.0
CA1Pr01	CA1	50.0	MA1Pr07	MA1	89.3
CA1Pr02	CA1	58.8	MA1Pr08	MA1	169.6
CA1Pr03	CA1	82.0	MA1Pr09	MA1	54.5
CA1Pr04	CA1	69.3	MA1Pr10	MA1	33.0
CA1Pr05	CA1	148.2	NC1Pr01	NC1	50.1
CA1Pr06	CA1	287.8	NC1Pr02	NC1	72.2
CA1Pr07	CA1	278.0	NC1Pr03	NC1	102.2
CA1Pr08	CA1	654.8	NC1Pr04	NC1	48.3
CA1Pr09	CA1	50.9	NC1Pr05	NC1	40.9
CA1Pr10	CA1	240.3	NY1Pr01	NY1	75.8
CT1Pr01	CT1	133.8	NY1Pr02	NY1	104.4
CT1Pr02	CT1	111.9	NY1Pr03	NY1	65.0
CT1Pr03	CT1	108.0	NY1Pr04	NY1	130.5
CT1Pr04	CT1	43.3	NY1Pr05	NY1	79.7
CT1Pr05	CT1	51.7	OH1Pr01	OH1	Value could not be determined
FL1Pr01	FL1	54.0	OH1Pr02	OH1	Value could not be determined
FL1Pr02	FL1	56.5	OH1Pr03	OH1	Value could not be determined

FL1Pr03	FL1	24.7	OH1Pr04	OH1	Value could not be determined
FL1Pr04	FL1	124.7	OH1Pr05	OH1	Value could not be determined
FL1Pr05	FL1	147.9	OH1Pr09	OH1	198.9
FL1Pr06	FL1	506.4	OH1Pr10	OH1	153.9
FL1Pr07	FL1	56.0	PA1Pr01	PA1	66.0
FL1Pr08	FL1	130.6	PA1Pr02	PA1	74.7
FL1Pr09	FL1	56.9	PA1Pr03	PA1	70.2
FL1Pr10	FL1	325.2	PA1Pr04	PA1	60.6
F0Pr01	F0	6.8	PA1Pr05	PA1	67.8
F0Pr02	F0	6.8	PA1Pr06	PA1	41.0
F0Pr03	F0	4.4	PA1Pr07	PA1	181.6
F0Pr04	F0	5.1	PA1Pr08	PA1	72.8
F0Pr05	F0	5.8	PA1Pr09	PA1	41.4
F0Pr06	F0	5.2	PA1Pr10	PA1	149.8
F0Pr07	F0	15.2	SD1Pr01	SD1	90.0
F0Pr08	F0	9.7	SD1Pr02	SD1	365.0
F0Pr09	F0	14.3	SD1Pr03	SD1	162.6
F0Pr10	F0	11.7	SD1Pr04	SD1	114.1
F0Pr11	F0	13.0	SD1Pr05	SD1	175.8
F0Pr12	F0	9.8	SD1Pr06	SD1	87.2
F0Pr13	F0	14.7	SD1Pr07	SD1	80.6
F0Pr14	F0	13.8	SD1Pr08	SD1	887.0
F0Pr15	F0	15.3	SD1Pr09	SD1	826.6
F5Pr01	F5	10.4	SD1Pr10	SD1	1401.7
F5Pr02	F5	15.5	TX1Pr01	TX1	Sample volume low
F5Pr03	F5	11.6	TX1Pr02	TX1	Sample volume low
F5Pr04	F5	6.1	TX1Pr03	TX1	Sample volume low
F5Pr05	F5	15.3	TX1Pr04	TX1	Sample volume low
F5Pr06	F5	12.1	TX1Pr05	TX1	Sample volume low
F5Pr07	F5	12.6	WA1Pr01	WA1	56.9
F5Pr08	F5	8.6	WA1Pr02	WA1	79.0
F5Pr09	F5	5.4	WA1Pr03	WA1	42.7
F5Pr10	F5	6.5	WA1Pr04	WA1	46.1
F5Pr11	F5	13.5	WA1Pr05	WA1	51.9
F5Pr12	F5	14.3	WA1Pr06	WA1	18.0
F5Pr13	F5	14.5	WA1Pr07	WA1	16.8
F5Pr14	F5	8.2	WA1Pr08	WA1	162.6
F5Pr15	F5	15.9	WA1Pr09	WA1	140.5

F10Pr01	F10	14.4	WA1Pr10	WA1	130.7
F10Pr02	F10	11.0	WA2Pr02	WA2	189.5
F10Pr03	F10	14.1	WA2Pr06	WA2	179.1
F10Pr04	F10	14.0	WA2Pr07	WA2	97.1
F10Pr05	F10	11.6	WA2Pr08	WA2	53.2
F10Pr06	F10	10.8	WA2Pr09	WA2	156.1
F10Pr07	F10	11.3	WA2Pr10	WA2	74.0
F10Pr08	F10	11.4	IN2Pr01	IN2	21.6
F10Pr09	F10	12.3	IN2Pr02	IN2	53.2
F10Pr10	F10	9.1	IN2Pr03	IN2	40.7
F10Pr11	F10	12.7	IN2Pr04	IN2	41.2
F10Pr12	F10	8.3	IN2Pr05	IN2	27.4
F10Pr13	F10	6.9	IN2Pr06	IN2	28.2
F10Pr14	F10	10.9	IN2Pr07	IN2	29.3
F10Pr15	F10	11.1	IN2Pr08	IN2	28.7
ID1Pr01	ID1	73.0	IN2Pr09	IN2	34.7
ID1Pr02	ID1	41.0	IN2Pr10	IN2	29.9
ID1Pr03	ID1	81.2	WV1Pr01	WV1	61.5
ID1Pr04	ID1	27.3	WV1Pr02	WV1	40.9
ID1Pr05	ID1	87.4	WV1Pr03	WV1	63.2
ID1Pr06	ID1	57.7	WV1Pr04	WV1	22.8
ID1Pr07	ID1	213.5	WV1Pr05	WV1	102.9
ID2Pr01	ID2	118.3	WV1Pr06	WV1	1031.6
ID2Pr02	ID2	49.1	WV1Pr07	WV1	24.1
ID2Pr03	ID2	66.6	WV1Pr08	WV1	17.0
ID2Pr04	ID2	20.2	WY1Pr06	WY1	148.0
ID2Pr05	ID2	143.5	WY1Pr07	WY1	263.4
ID2Pr06	ID2	72.6	WY1Pr08	WY1	289.1
ID2Pr07	ID2	51.7	WY1Pr09	WY1	303.2
ID2Pr08	ID2	298.9	WY1Pr10	WY1	451.9
ID2Pr09	ID2	149.2	WY2Pr01	WY2	193.7
ID2Pr10	ID2	103.1	WY2Pr02	WY2	42.6
ID3Pr01	ID3	85.4	WY2Pr03	WY2	82.4
ID3Pr02	ID3	76.8	WY2Pr04	WY2	114.4
ID3Pr03	ID3	86.4	WY2Pr05	WY2	125.1
ID3Pr04	ID3	61.5	WY2Pr06	WY2	59.6
ID3Pr06	ID3	212.1	WY2Pr07	WY2	141.4
ID3Pr07	ID3	158.1	WY2Pr08	WY2	97.2
ID3Pr08	ID3	158.4	WY2Pr09	WY2	49.9

ID3Pr09	ID3	172.8	WY2Pr10	WY2	262.1
ID3Pr10	ID3	106.1	WY3Pr01	WY3	176.1
			WY3Pr02	WY3	80.1
			WY3Pr03	WY3	122.4
			WY3Pr04	WY3	74.3

Appendix III – Microsatellite Genotypes

Table A3.1: Microsatellite genotypes, of fragment length, at each locus for each specimen within each sample. -1 denotes missing data.

	Genotypes											
Sample Name	**Pair 3**		**Pair 8**		**Pair 9**		**Pair 12**		**Pair 13**		**Pair 14**	
AL1Pr01	280	364	368	423	217	217	509	509	308	387	-1	-1
AL1Pr02	364	364	356	379	217	237	493	525	371	371	352	352
AL1Pr03	364	384	328	348	217	217	518	518	288	355	308	308
AL1Pr04	341	369	356	368	221	225	-1	-1	324	324	316	316
AL1Pr05	328	328	371	399	217	221	435	478	308	363	280	316
AL1Pr06	309	309	383	435	217	229	521	568	261	261	316	356
AL1Pr07	335	335	379	391	213	217	466	490	300	351	-1	-1
AL1Pr08	392	439	375	404	217	225	494	514	308	316	292	448
AL1Pr09	474	478	368	423	217	217	411	411	316	316	400	408
AL1Pr10	290	346	336	407	217	217	497	525	316	367	316	344
AL2Pr01	-1	-1	391	391	213	221	470	522	-1	-1	-1	-1
AL2Pr02	420	420	-1	-1	217	217	520	520	261	281	308	312
AL2Pr03	334	334	375	411	217	229	435	544	292	292	340	364
AL2Pr04	372	372	391	399	217	217	415	415	383	462	272	396
AL2Pr05	349	349	371	383	217	221	545	545	-1	-1	284	368
CA1Pr01	340	364	395	449	217	217	557	557	261	265	320	320
CA1Pr02	340	340	379	438	221	221	521	521	261	336	280	356
CA1Pr03	372	400	399	403	229	333	517	517	265	324	308	400
CA1Pr04	350	353	352	399	217	225	494	576	375	379	304	304
CA1Pr05	369	396	383	395	217	221	450	458	312	359	332	336
CA1Pr06	419	419	387	438	217	217	509	536	261	320	-1	-1
CA1Pr07	323	323	421	434	217	217	509	509	355	355	332	372
CA1Pr08	467	467	367	391	217	217	439	439	300	308	368	368
CA1Pr09	338	344	375	450	217	221	494	498	312	312	296	296
CA1Pr10	-1	-1	332	383	217	217	-1	-1	399	450	260	344
CT1Pr01	329	329	352	383	217	217	521	521	426	426	280	432
CT1Pr02	369	376	387	446	217	217	427	458	269	269	288	480
CT1Pr03	361	361	450	482	217	217	529	529	364	395	360	464
CT1Pr04	361	387	446	474	217	217	587	587	344	360	282	420
CT1Pr05	372	372	447	470	217	217	435	435	277	277	312	396
FL1Pr01	337	437	387	478	217	221	490	529	359	367	300	440
FL1Pr02	352	388	375	431	217	217	599	599	308	375	244	244
FL1Pr03	325	353	380	477	213	217	505	505	261	340	376	404
FL1Pr04	341	341	391	462	229	229	521	541	261	261	368	454

FL1Pr05	353	353	375	395	217	229	544	556	261	352	264	396
FL1Pr06	368	368	432	435	217	225	-1	-1	296	316	344	448
FL1Pr07	417	417	336	387	217	217	560	560	332	375	468	484
FL1Pr08	342	390	391	391	217	217	458	509	328	328	488	492
FL1Pr09	325	357	344	387	217	225	556	556	328	332	328	328
FL1Pr10	333	333	391	427	217	217	470	505	261	261	300	300
F0Pr01	310	310	395	462	217	233	415	505	320	320	368	404
F0Pr02	384	384	387	446	217	217	513	534	348	426	352	352
F0Pr03	378	403	387	439	217	221	-1	-1	261	261	456	464
F0Pr04	366	370	372	376	221	221	469	469	261	261	264	508
F0Pr05	329	329	356	395	217	217	509	513	340	340	260	304
F0Pr06	330	389	375	383	217	221	599	599	261	261	322	396
F0Pr07	321	368	336	360	217	225	-1	-1	332	332	324	404
F0Pr08	358	382	395	407	217	217	455	455	-1	-1	320	480
F0Pr09	317	344	348	415	217	229	505	505	324	324	376	504
F0Pr10	-1	-1	356	368	225	237	549	549	-1	-1	306	448
F0Pr11	392	392	348	399	217	217	505	514	281	304	256	308
F0Pr12	343	343	367	411	217	228	-1	-1	355	399	404	404
F0Pr13	329	372	371	415	217	217	579	610	284	300	320	352
F0Pr14	348	350	363	419	217	217	517	610	265	304	328	328
F0Pr15	329	396	371	423	217	221	505	505	347	347	328	328
F5Pr01	357	392	403	486	217	217	513	513	261	307	408	448
F5Pr02	364	368	360	368	212	227	513	513	265	265	284	284
F5Pr03	329	392	348	435	217	225	509	509	-1	-1	320	320
F5Pr04	353	357	364	462	217	217	517	517	261	261	336	404
F5Pr05	328	396	384	395	217	225	517	517	261	261	268	348
F5Pr06	397	423	384	395	217	217	-1	-1	261	308	268	387
F5Pr07	329	396	383	486	217	217	411	517	261	261	268	348
F5Pr08	352	357	376	435	217	221	-1	-1	332	332	319	387
F5Pr09	357	392	368	383	217	221	-1	-1	308	308	300	388
F5Pr10	362	362	332	420	217	217	439	506	265	281	320	324
F5Pr11	348	360	367	403	217	217	537	537	261	308	348	348
F5Pr12	360	366	383	403	217	217	537	537	261	265	344	356
F5Pr13	328	368	407	485	217	221	513	513	316	340	268	268
F5Pr14	368	391	367	403	217	221	439	439	308	340	448	448
F5Pr15	295	329	395	403	217	217	411	411	284	300	352	352
F10Pr01	360	368	293	380	217	217	439	439	332	332	444	448
F10Pr02	328	360	395	403	217	217	411	513	300	332	264	264
F10Pr03	328	360	395	403	217	217	411	513	300	332	264	264
F10Pr04	368	368	380	403	217	217	439	439	300	336	444	448

F10Pr05	328	328	395	403	217	217	411	513	300	332	352	352
F10Pr06	328	360	395	395	217	217	411	513	300	332	352	352
F10Pr07	328	360	304	395	217	217	411	513	300	300	260	260
F10Pr08	328	360	395	395	217	217	411	411	300	300	352	352
F10Pr09	360	368	380	403	217	217	-1	-1	332	336	444	448
F10Pr10	360	368	380	380	217	217	439	439	332	332	444	448
F10Pr11	328	360	395	403	217	217	411	411	332	332	352	352
F10Pr12	360	360	380	395	217	217	439	439	300	300	260	260
F10Pr13	328	328	395	395	217	217	509	513	300	332	352	352
F10Pr14	360	368	380	403	217	217	439	439	300	336	444	448
F10Pr15	328	360	395	403	217	217	411	439	332	336	352	352
ID1Pr01	354	354	375	431	217	217	-1	-1	320	320	304	352
ID1Pr02	333	370	379	427	217	217	-1	-1	261	261	320	436
ID1Pr03	383	425	364	387	217	221	458	458	261	328	376	388
ID1Pr04	361	435	399	399	217	217	517	549	324	336	328	340
ID1Pr05	345	345	427	427	217	217	482	621	261	344	256	300
ID1Pr06	365	400	399	434	217	217	-1	-1	261	344	328	372
ID1Pr07	349	470	379	383	217	245	605	605	332	387	268	352
ID2Pr01	329	341	395	411	217	217	486	486	261	351	356	404
ID2Pr02	386	386	434	454	217	237	505	583	320	324	344	408
ID2Pr03	323	342	399	431	217	225	479	479	261	320	384	404
ID2Pr04	314	427	336	427	217	217	580	580	269	320	336	336
ID2Pr05	313	313	367	427	217	217	478	572	308	336	288	288
ID2Pr06	399	399	344	415	217	217	548	548	328	328	424	456
ID2Pr07	341	341	340	340	217	217	-1	-1	265	308	340	340
ID2Pr08	423	423	363	363	217	217	482	482	261	348	324	392
ID2Pr09	329	329	399	399	217	225	423	423	261	352	372	376
ID2Pr10	362	368	415	434	217	217	525	540	336	375	300	324
ID3Pr01	333	333	395	431	217	233	434	529	363	371	312	312
ID3Pr02	404	429	411	419	217	221	537	537	308	324	266	266
ID3Pr03	313	392	372	395	217	217	458	478	261	261	271	287
ID3Pr04	346	346	336	360	217	217	509	509	328	328	260	380
ID3Pr06	316	373	363	375	217	217	-1	-1	344	355	320	320
ID3Pr07	348	353	363	375	217	217	435	435	261	332	232	232
ID3Pr08	339	353	356	419	217	225	517	517	340	344	368	392
ID3Pr09	376	464	328	328	217	221	447	447	261	281	340	344
ID3Pr10	301	367	407	419	217	221	454	568	442	469	328	336
ID4Pr01	370	373	379	379	217	217	423	423	261	261	344	344
ID4Pr02	337	429	339	446	217	220	502	502	387	387	256	256
ID4Pr03	396	396	371	371	217	237	-1	-1	320	344	260	260

ID4Pr04	381	381	332	371	217	225	462	462	261	316	352	352
ID4Pr05	352	352	364	375	217	217	498	498	367	379	296	404
ID4Pr06	-1	-1	391	395	217	217	498	498	308	340	264	264
ID4Pr07	370	405	360	367	221	225	462	578	340	395	292	292
ID4Pr08	426	426	387	395	217	221	521	521	261	320	456	460
ID4Pr09	342	364	403	442	217	225	475	475	273	273	336	416
MA1Pr01	377	377	375	383	217	236	462	548	296	340	312	364
MA1Pr02	341	376	391	442	217	217	443	478	328	387	312	312
MA1Pr03	342	342	339	423	217	221	466	486	371	371	-1	-1
MA1Pr04	319	357	379	415	217	217	450	454	281	320	356	404
MA1Pr05	364	408	356	391	217	221	497	544	261	261	292	292
MA1Pr06	398	398	371	383	217	217	435	435	-1	-1	276	276
MA1Pr07	394	394	363	399	217	237	431	486	300	371	348	384
MA1Pr08	436	436	344	391	217	225	501	501	328	379	292	356
MA1Pr09	349	415	383	407	-1	-1	537	537	265	265	268	268
MA1Pr10	324	324	371	434	217	221	466	482	328	343	292	292
NC1Pr01	442	490	396	411	217	229	525	529	293	317	384	388
NC1Pr02	341	341	332	332	217	233	411	466	343	343	328	368
NC1Pr03	456	460	392	423	217	217	462	537	281	332	464	468
NC1Pr04	382	434	399	423	217	221	466	466	371	418	288	288
NC1Pr05	409	438	383	435	217	217	529	561	367	367	296	342
NY1Pr01	399	399	403	466	217	217	574	612	308	363	248	248
NY1Pr02	307	353	383	411	217	236	490	490	312	340	280	280
NY1Pr03	340	352	355	430	217	217	530	534	399	399	272	308
NY1Pr04	337	369	359	367	-1	-1	431	431	261	340	384	396
NY1Pr05	353	361	391	454	221	229	514	612	375	395	276	276
OH1Pr01	345	407	368	415	221	225	500	533	261	261	420	420
OH1Pr02	337	388	442	486	217	217	458	505	316	360	328	328
OH1Pr03	370	390	352	438	217	217	486	573	308	308	324	324
OH1Pr04	326	326	364	364	217	229	521	564	356	407	308	344
OH1Pr05	364	364	356	403	217	217	-1	-1	304	371	284	284
OH1Pr09	326	402	379	379	217	221	-1	-1	304	304	336	356
OH1Pr10	391	399	399	403	217	233	478	482	344	344	-1	-1
PA1Pr01	380	380	487	491	217	221	470	470	436	452	304	304
PA1Pr02	320	320	372	372	217	217	569	573	261	261	352	432
PA1Pr03	341	398	332	395	217	233	470	569	328	360	264	264
PA1Pr04	344	344	383	436	217	217	466	466	261	261	348	428
PA1Pr05	376	380	360	403	217	221	517	517	371	418	-1	-1
PA1Pr06	344	413	356	415	229	249	478	478	261	301	328	344
PA1Pr07	364	364	361	432	221	225	517	517	257	324	-1	-1

PA1Pr08	-1	-1	384	407	217	221	-1	-1	-1	-1	364	468
PA1Pr09	333	333	360	368	217	225	470	490	352	383	280	312
PA1Pr10	357	439	387	407	217	217	533	564	261	261	268	324
SD1Pr01	288	345	367	379	-1	-1	466	466	343	343	324	356
SD1Pr02	329	387	415	426	-1	-1	462	502	320	320	292	464
SD1Pr03	329	329	371	383	-1	-1	522	522	308	340	311	328
SD1Pr04	317	317	355	395	-1	-1	470	521	308	351	324	324
SD1Pr05	324	324	407	407	-1	-1	529	544	300	300	460	384
SD1Pr06	351	408	403	434	-1	-1	-1	-1	288	288	352	352
SD1Pr07	309	309	375	387	-1	-1	442	474	284	316	296	311
SD1Pr08	330	344	343	343	-1	-1	552	556	304	304	456	456
SD1Pr09	337	409	387	427	-1	-1	462	486	261	320	311	311
SD1Pr10	-1	-1	367	399	-1	-1	470	525	284	316	291	301
TX1Pr01	361	361	411	445	217	221	-1	-1	324	324	340	340
TX1Pr02	413	413	462	462	217	217	556	556	339	372	398	398
TX1Pr03	392	410	387	403	217	217	494	545	348	399	380	380
TX1Pr04	403	403	356	356	217	217	466	505	296	340	280	280
TX1Pr05	341	364	375	375	217	217	-1	-1	310	310	372	376
WA1Pr01	331	396	391	403	217	217	454	538	300	340	400	400
WA1Pr02	348	348	372	387	217	217	466	537	312	415	284	340
WA1Pr03	333	338	388	399	217	217	435	576	324	348	288	288
WA1Pr04	345	345	328	328	225	229	474	513	261	261	312	356
WA1Pr05	325	325	379	446	221	225	537	537	261	261	264	280
WA1Pr06	439	439	387	442	217	229	455	455	261	316	312	312
WA1Pr07	315	315	387	387	217	217	443	466	261	316	412	412
WA1Pr08	333	348	356	372	217	217	-1	-1	336	383	392	456
WA1Pr09	368	368	438	450	217	217	-1	-1	296	316	300	300
WA1Pr10	342	458	391	482	217	229	462	565	261	261	316	372
WA2Pr02	416	416	391	399	217	217	564	564	308	403	320	404
WA2Pr06	352	352	371	387	217	217	415	415	-1	-1	416	440
WA2Pr07	337	394	344	431	201	217	447	537	261	336	324	428
WA2Pr08	364	368	407	434	217	225	423	510	328	418	248	248
WA2Pr09	380	384	383	403	217	245	478	478	-1	-1	304	356
WA2Pr10	-1	-1	-1	-1	217	225	596	596	-1	-1	324	324
IN2Pr01	362	366	355	363	217	220	423	423	324	355	304	304
IN2Pr02	375	407	383	438	215	215	459	459	320	339	288	288
IN2Pr03	373	373	355	359	216	224	435	435	324	351	355	355
IN2Pr04	348	348	359	407	216	228	427	474	304	347	436	472
IN2Pr05	440	440	379	411	216	220	490	517	328	328	290	290
IN2Pr06	377	440	332	355	213	217	521	541	332	343	273	277

IN2Pr07	334	350	387	411	216	224	447	447	261	261	319	319
IN2Pr08	321	376	351	351	216	220	-1	-1	363	363	395	424
IN2Pr09	345	360	375	414	216	224	529	533	304	343	295	298
IN2Pr10	423	423	367	383	220	224	466	560	261	261	250	287
WV1Pr01	352	352	403	438	217	217	533	544	352	352	292	364
WV1Pr02	463	463	376	391	217	229	-1	-1	332	344	412	452
WV1Pr03	359	359	340	387	217	221	537	605	261	324	256	356
WV1Pr04	402	402	368	383	217	221	463	535	261	304	244	244
WV1Pr05	309	313	395	407	217	221	517	574	300	308	300	300
WV1Pr06	338	352	419	435	217	217	-1	-1	261	261	324	380
WV1Pr07	344	390	383	462	217	217	502	502	332	407	304	304
WV1Pr08	362	370	435	466	217	225	537	544	281	348	356	476
WY1Pr06	303	356	328	399	225	237	-1	-1	357	357	332	352
WY1Pr07	340	350	371	383	217	225	-1	-1	399	407	396	480
WY1Pr08	-1	-1	368	415	217	217	498	498	261	261	380	380
WY1Pr09	340	340	391	442	217	217	580	580	312	312	384	500
WY1Pr10	351	370	359	359	217	221	474	513	375	422	-1	-1
WY2Pr01	329	356	438	446	217	221	455	455	371	391	304	304
WY2Pr02	345	345	395	427	217	217	435	602	320	367	316	360
WY2Pr03	332	332	391	427	217	233	620	620	261	261	376	376
WY2Pr04	434	434	431	442	217	217	459	459	316	359	384	384
WY2Pr05	349	403	356	360	217	217	435	462	261	261	308	368
WY2Pr06	346	404	340	380	217	233	587	587	284	351	260	348
WY2Pr07	340	348	348	411	217	233	498	548	304	304	268	376
WY2Pr08	369	380	435	450	217	221	462	462	261	308	408	456
WY2Pr09	283	298	383	458	217	221	505	573	328	360	300	316
WY2Pr10	-1	-1	-1	-1	217	233	534	534	-1	-1	-1	-1
WY3Pr01	374	392	336	344	217	221	404	521	324	348	-1	-1
WY3Pr02	-1	-1	348	411	217	217	529	529	300	300	328	328
WY3Pr03	317	317	391	411	217	217	478	540	261	261	344	396
WY3Pr04	359	372	411	529	217	217	527	527	336	360	268	268

Appendix IV – Measurements of Larval Length

Table A4.1: Length of each F1 larval specimen in millimetres during each 24-hour sampling period.

F1				
Sampling Period (Hours)				
67-90	**91-114**	**115-138**	**139-162**	**163-186**
5.50	8.50	13.50	9.50	11.50
6.00	9.00	13.75	11.75	11.75
6.00	10.00	13.75	12.25	12.75
6.25	10.75	13.75	12.50	12.75
6.25	11.00	14.00	12.75	13.00
6.50	11.25	14.00	12.75	13.00
6.50	11.25	14.00	12.75	13.00
6.75	11.50	14.50	13.00	13.00
6.75	11.50	14.50	13.00	13.00
6.75	11.75	14.50	13.00	13.00
6.75	12.00	15.00	13.00	13.25
6.75	12.00	15.00	13.00	13.25
7.00	12.00	15.00	13.25	13.25
7.00	12.00	15.00	13.25	13.50
7.00	12.00	15.00	13.25	13.50
7.00	12.00	15.00	13.25	13.50
7.00	12.00	15.00	13.25	13.75
7.00	12.00	15.00	13.50	13.75
7.00	12.25	15.00	13.50	13.75
7.00	12.25	15.00	13.50	13.75
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7.00	12.50	15.25	13.50	13.75
7.25	12.50	15.25	13.50	14.00
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7.25	12.50	15.25	13.50	14.00
7.25	12.50	15.25	13.50	14.00
7.25	12.50	15.25	13.75	14.00
7.25	12.50	15.25	13.75	14.00
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7.25	13.00	15.75	14.00	14.75
7.25	13.00	15.75	14.00	14.75
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7.50	13.00	15.75	14.00	15.00
7.50	13.00	15.75	14.00	15.25
7.50	13.00	15.75	14.00	15.50
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Table A4.2: Length of each F6 larval specimen in millimetres during each 24-hour sampling period.

F5				
Sampling Period (Hours)				
67-90	**91-114**	**115-138**	**139-162**	**163-186**
4.25	7.75	11.00	11.00	10.50
4.75	8.00	11.50	11.00	10.75
5.00	8.00	11.50	11.25	11.00
5.00	8.50	12.00	11.25	11.25
5.00	9.00	12.00	11.25	11.25
5.25	9.00	12.00	11.50	11.50
5.50	9.00	12.00	11.75	11.50
5.50	9.50	12.00	12.00	11.50
5.50	10.00	12.25	12.00	11.50
5.75	10.00	12.25	12.00	11.75
5.75	10.00	12.50	12.00	11.75
5.75	10.00	12.50	12.00	12.00
6.00	10.00	12.50	12.00	12.00
6.00	10.00	12.75	12.00	12.00
6.00	10.25	12.75	12.25	12.00
6.00	10.25	12.75	12.25	12.00
6.00	10.50	12.75	12.50	12.00
6.00	10.50	12.75	12.50	12.00
6.00	10.50	13.00	12.50	12.25
6.00	10.75	13.00	12.50	12.25
6.00	10.75	13.00	12.50	12.25
6.00	11.00	13.00	12.50	12.25
6.00	11.00	13.00	12.50	12.25
6.00	11.00	13.00	12.75	12.25
6.00	11.00	13.00	12.75	12.25
6.25	11.00	13.00	12.75	12.25
6.25	11.00	13.00	12.75	12.50
6.25	11.00	13.00	12.75	12.50
6.25	11.00	13.00	12.75	12.50
6.25	11.00	13.25	12.75	12.50
6.25	11.00	13.25	12.75	12.50
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6.50	11.00	13.25	13.00	12.50
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6.50	11.00	13.25	13.00	12.75
6.50	11.00	13.25	13.00	12.75
6.50	11.00	13.25	13.00	12.75
6.50	11.00	13.25	13.00	12.75
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6.75	11.75	13.75	13.00	13.00
6.75	11.75	13.75	13.25	13.00
6.75	11.75	13.75	13.25	13.00
6.75	11.75	13.75	13.25	13.00
6.75	11.75	13.75	13.25	13.00
6.75	11.75	13.75	13.25	13.00
6.75	11.75	13.75	13.25	13.00
7.00	11.75	13.75	13.25	13.00
7.00	11.75	13.75	13.25	13.00
7.00	11.75	13.75	13.25	13.00
7.00	11.75	13.75	13.25	13.25
7.00	12.00	13.75	13.25	13.25
7.00	12.00	14.00	13.25	13.25
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7.75	13.00	16.75	14.25	14.75
7.75	13.00	17.00	14.25	14.75
7.75	13.00	17.00	14.25	14.75
7.75	13.00	17.25	14.25	14.75

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7.75	13.25		14.25	15.75
7.75	13.25		14.25	16.00
7.75	13.25		14.25	16.25
7.75	13.25		14.25	16.25
7.75	13.25		14.25	16.50
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8.00	13.50		14.25	Pupa
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8.25	13.75		14.50	Pupa
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Table A4.3: Length of each F11 larval specimen in millimetres during each 24-hour sampling period.

F11					
Sampling Period (Hours)					
67-90	**91-114**	**115-138**	**139-162**	**163-186**	**187-210**
4.00	5.50	6.00	8.00	8.75	10.00
4.00	5.50	6.50	8.00	8.75	10.00
4.25	6.00	6.75	8.25	9.00	10.50
4.25	6.00	6.75	8.75	9.50	11.00
4.75	6.00	7.00	9.00	9.50	11.25
4.75	6.00	7.00	9.00	9.50	11.25
5.00	6.25	7.00	9.00	9.50	11.75
5.00	6.25	7.00	9.00	9.75	12.00
5.00	6.25	7.00	9.25	9.75	12.00
5.00	6.25	7.00	9.25	9.75	12.50
5.00	6.50	7.25	9.50	9.75	12.50
5.00	6.50	7.25	9.50	9.75	12.50
5.00	6.50	7.25	9.50	10.00	12.50
5.00	6.50	7.25	9.75	10.00	12.75
5.00	6.50	7.25	9.75	10.00	12.75
5.25	6.50	7.50	10.00	10.00	12.75
5.25	6.50	7.50	10.00	10.00	12.75
5.25	6.50	7.50	10.00	10.25	12.75
5.25	6.50	7.50	10.00	10.25	12.75
5.25	6.50	7.50	10.00	10.50	13.00
5.25	6.50	7.50	10.00	10.50	13.00
5.25	6.50	7.75	10.00	10.50	13.00
5.25	6.50	7.75	10.00	10.50	13.00
5.25	6.50	8.00	10.25	10.75	13.00
5.25	6.75	8.00	10.25	10.75	13.00
5.25	6.75	8.00	10.25	10.75	13.00
5.25	6.75	8.00	10.50	10.75	13.00
5.25	6.75	8.00	10.50	10.75	13.00
5.25	6.75	8.00	10.50	10.75	13.00
5.25	6.75	8.00	10.50	10.75	13.25
5.50	6.75	8.25	10.50	10.75	13.25
5.50	6.75	8.50	10.50	11.00	13.25
5.50	6.75	8.50	10.75	11.00	13.25
5.50	6.75	8.50	10.75	11.00	13.25
5.50	6.75	8.50	10.75	11.00	13.25

5.50	6.75	8.50	10.75	11.00	13.50
5.50	6.75	8.50	10.75	11.00	13.50
5.50	6.75	8.50	11.00	11.00	13.50
5.50	6.75	8.50	11.00	11.00	13.50
5.50	6.75	8.50	11.00	11.00	13.50
5.50	6.75	8.50	11.00	11.00	13.50
5.50	6.75	8.75	11.00	11.00	13.50
5.50	6.75	8.75	11.00	11.00	13.50
5.50	7.00	8.75	11.00	11.00	13.50
5.50	7.00	8.75	11.00	11.25	13.50
5.50	7.00	8.75	11.00	11.25	13.50
5.50	7.00	8.75	11.00	11.25	13.50
5.50	7.00	8.75	11.00	11.25	13.50
5.50	7.00	8.75	11.00	11.25	13.50
5.50	7.00	9.00	11.25	11.25	13.50
5.50	7.00	9.00	11.25	11.25	13.50
5.75	7.00	9.00	11.25	11.25	13.50
5.75	7.00	9.00	11.25	11.50	13.50
5.75	7.00	9.00	11.25	11.50	13.50
5.75	7.00	9.00	11.25	11.50	13.75
5.75	7.00	9.00	11.25	11.50	13.75
5.75	7.00	9.00	11.25	11.50	13.75
5.75	7.00	9.00	11.25	11.50	13.75
5.75	7.00	9.25	11.25	11.50	13.75
5.75	7.00	9.25	11.25	11.50	13.75
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5.75	7.00	9.50	11.25	11.75	13.75
5.75	7.00	9.50	11.50	11.75	13.75
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5.75	7.00	9.75	11.50	12.00	13.75
5.75	7.00	9.75	11.50	12.00	13.75
5.75	7.00	9.75	11.50	12.00	13.75

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5.75	7.25	10.00	11.75	12.00	14.00
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6.00	7.50	11.50	12.25	12.50	14.25
6.00	7.50	11.50	12.25	12.75	14.25
6.00	7.50	11.50	12.25	12.75	14.25
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6.25	7.50	12.00	12.75	13.00	14.50
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6.75	8.00	12.50	13.00	13.00	14.75
6.75	8.00	12.50	13.00	13.00	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.50	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75

6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.25	14.75
6.75	8.00	12.75	13.00	13.50	14.75
6.75	8.00	12.75	13.00	13.50	14.75
6.75	8.00	12.75	13.00	13.50	14.75
6.75	8.25	12.75	13.00	13.50	14.75
6.75	8.25	12.75	13.00	13.50	14.75
6.75	8.25	12.75	13.00	13.50	14.75
6.75	8.25	12.75	13.00	13.50	14.75
6.75	8.25	12.75	13.00	13.50	14.75
6.75	8.25	12.75	13.00	13.50	14.75
7.00	8.25	12.75	13.00	13.50	14.75
7.00	8.25	12.75	13.25	13.50	14.75
7.00	8.25	12.75	13.25	13.50	14.75
7.00	8.25	12.75	13.25	13.50	14.75
7.00	8.25	13.00	13.25	13.50	14.75
7.00	8.25	13.00	13.25	13.50	14.75
7.00	8.25	13.00	13.25	13.50	14.75
7.00	8.25	13.00	13.25	13.50	14.75
7.00	8.25	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	14.75
7.00	8.50	13.00	13.25	13.50	15.00
7.00	8.75	13.00	13.25	13.50	15.00

7.00	8.75	13.00	13.50	13.50	15.00
7.00	8.75	13.00	13.50	13.75	15.00
7.00	8.75	13.00	13.50	13.75	15.00
7.00	8.75	13.00	13.50	13.75	15.00
7.00	8.75	13.00	13.50	13.75	15.00
7.00	8.75	13.00	13.50	13.75	15.00
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