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The Effects Hot Water Kill Time has on DNA Degradation and STR Profiling from *Lucilia sericata* (Diptera: Calliphoridae) Crop Contents

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The Effects Hot Water Kill Time has on DNA Degradation and STR Profiling from *Lucilia sericata* (Diptera: Calliphoridae) Crop Contents

Nicholas A. Haas

Thesis submitted

**To the Eberly College of Arts and Sciences
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Master of Science in
Forensic and Investigative Science**

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Morgantown, West Virginia

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Abstract

The Effects Hot Water Kill Time has on DNA Degradation and STR Profiling from *Lucilia sericata* (Diptera: Calliphoridae) Crop Contents

Nicholas A. Haas

The use of entomological specimens as a source of forensic DNA evidence has been studied since the late 20th century. Currently as it stands, to preserve both the minimum postmortem interval (mPMI) and DNA evidence within fly larvae, two standards of preservation exist. For mPMI, the use of hot water kill with near-boiling water is used to fix larvae size for accurate age estimation. For DNA evidence, the standard technique of preservation is submersion in 70% ethanol. To help reduce the number of standard preservation techniques for practitioners, this study assessed the feasibility of the hot water kill preservation technique using near-boiling water when obtaining short tandem repeat (STR) profiles from the contents of fly larvae crops (foregut). Reduction in the preservation techniques would result in the ability to collect only one set of specimens that could be used for both mPMI determination and DNA collection.

Lucilia sericata larvae were fed three different human livers, the crop tissue was removed from the larvae, and the human DNA from the crop contents was extracted. STR analysis, using standard crime laboratory procedures, was performed on the crops. Analysis of Variance (ANOVA) and Linear Regression Modeling was used to assess the variability around the means of two different preservative liquids, with five different residence times ranging from 30-300 seconds in 92-99°C water. Through the use of ANOVA, it was determined that the use of the hot water produced results that were not statistically different from the field standard using 70% ethanol as a preservation technique. The linear regression modeling found no significance in the ability to predict the proportion of alleles called in an STR profile based on the time larvae spent in the hot water kill bath. High variations in the proportions of alleles called within groups lead to weak correlation coefficients in the two linear models produced. With the ability to recover even partial STR profiles, the hot water kill technique can be utilized within forensic investigations related to cases in which a body may be missing while entomological specimen can be found or even cold cases that have already preserved fly larvae.

Studies assessing the feasibility of testing certain types of evidence like the one performed here can ultimately help practitioners by creating standard operating procedures that optimize the workflow within a laboratory.

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Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
Introduction.....	1
Chapter 1: Background.....	3
<i>1.1 Forensic Entomology.....</i>	<i>3</i>
<i>1.2 Forensic Biology.....</i>	<i>5</i>
<i>1.3 Forensic DNA Testing on Entomological Specimens.....</i>	<i>8</i>
<i>1.4 Statistical Analysis.....</i>	<i>11</i>
<i>1.5 Impact.....</i>	<i>12</i>
Chapter 2: Methodology.....	13
<i>2.1 Colony Maintenance.....</i>	<i>13</i>
<i>2.2 Thermocouple Build and Calibration.....</i>	<i>13</i>
<i>2.3 Obtaining and Profiling Reference Samples.....</i>	<i>13</i>
<i>2.4 Crop Extraction Methodology Trials.....</i>	<i>14</i>
<i>2.5 Experimental Set Runs.....</i>	<i>14</i>
<i>2.6 Statistical Analysis.....</i>	<i>16</i>
Chapter 3: Results.....	18
<i>3.1 Reference Profiles.....</i>	<i>18</i>
<i>3.2 Methodology Trials.....</i>	<i>18</i>
<i>3.3 Experimental Results.....</i>	<i>19</i>
Chapter 4: Discussion.....	27
Chapter 5: Conclusion.....	31
References.....	32
Appendix A.....	35
Appendix B.....	38

Introduction

In forensic investigations, having a small amount of strong evidence can be more beneficial than a large amount of weak evidence to gain the best understanding of the sequence of events in which the crime was committed. Historically, forensic entomology has focused on using data obtained from necrophagous insects collected at the crime scene and/or on a decedent to estimate a minimum postmortem interval (mPMI)^{1,2,3}. Over the past few decades, other disciplines within forensic investigations have been used to increase the amount of information gathered from the insects collected: these sub-fields under medicolegal entomology include entomotoxicology and DNA (deoxyribonucleic acids)-focused entomology techniques. The DNA focused techniques focus on recovering human DNA from the insects that feed on a body and have used both mitochondrial DNA (mtDNA) and nuclear short tandem repeat (STR) analysis. This happens through the isolation and purification of human nuclear DNA followed by exponential copying via polymerase chain reaction (PCR), and separation and detection through capillary electrophoresis⁴.

A major component of the STR analysis process is the use of PCR to exponentially copy the DNA in a quantity that is enough to be detected by a charged couple device (CCD) camera during the capillary electrophoresis phase. The PCR step utilizes a thermostable polymerase (*Taq* Polymerase) that runs through cycles of varying temperature to denature the DNA by breaking the hydrogen bonds holding the nitrogenous bases together, anneal the primers to the DNA, and extend the DNA out from the primers to be paired with the template strands⁴. The temperatures used can go as high as 95°C and can get as low as 4°C when the final product is being stored⁴. For STR analysis, a target of 1 ng of DNA is achievable through the use of copying the DNA via PCR⁴. While it is possible to recover a full STR profile with a starting concentration of DNA under 0.06 ng/μL, concentrations at or above that 0.06 ng/μL are the most successful at obtaining full profiles⁴. If the amount of quantified DNA is below 0.06 ng/μL partial profiles are the typical result⁴.

The DNA focused entomology techniques utilize the digestive systems of the entomological specimens being observed. Since the early 2000's various scientists have studied the recovery of both human nuclear DNA and mtDNA from the digestive systems of a variety of entomological specimens^{5,6,7,8}. For the purposes of this study fly larvae crops (foregut) are going to be the primary focus. From work performed by Zehner et al. (2000), the crops of larvae to store human DNA for casework purposes⁵. The use of the crops from larvae has made the DNA recovery possible due the crop acting as a storage vessel prior to digestion. Over the last two decades, both the preservation and extraction techniques have been researched to find the conditions that optimize the recovery of human DNA from the larvae^{6,7}. It has been reported that in most cases, as long as the larvae and crops are preserved prior to 24 hours after the larvae has stopped eating, it is still possible to obtain usable DNA⁸.

The use of necrophagous species as a source for human DNA has shown several advantages. The first advantage to the use of the necrophagous species is that they are small and are found in large quantities, this allows for the ability to collect a large amount of specimens with minimal storage space needed^{6,7}. Within the same respect, it makes it possible to preserve the large amounts at one time^{6,7}. In addition, as stated previously, most of these species have digestive systems that hold the DNA well. Like discussed with the larvae, until the insects are in

a stage where they are no longer feeding, the ability to recover human DNA from the gastrointestinal tracts of these specimens is high. It is only after the insects are finished eating that complete digestion of the meal occurs. The storage of the meal allows for the ability to recover information about what was being eaten, even if the larvae or other insects are no longer eating on a particular body. This allows for investigators to use insects found at scenes to identify any missing bodies, help connect multiple scenes together, and can even identify if the insects transferred between individuals⁸.

The extraction techniques have helped develop protocols that make crop extraction more efficient as well as safeguarding against potential contamination and DNA inhibition^{6,7}. These methods have helped ensure the integrity of the samples. Preservation techniques observed have focused on ethanol and other organic solutions like formaldehyde and Kahle's solution (a mixture of ethanol, formaldehyde, glacial acetic acid, and water)⁷. In addition to the preservative fluids, temperature was also observed and it was found that an ethanol preservation and temperatures at -70°C were best at preserving the DNA for mtDNA sequencing and STR genotyping⁷. From research like this, preservation in 70% ethanol has become the standard technique due to its ability to preserve the DNA but also because the ethanol is good at denaturing nucleases and dehydrates the specimen. However, because ethanol can cause larvae to dry out and shrivel, it is not the most effective technique to help preserve the information that can help determine the mPMI as it can cause estimations to be off more than the use of hot water kill because the shriveling can distort anatomical features that are important for identifying the stage of development⁹.

The use of hot water kill as a preservation technique has been accepted as a standard for fly larvae collection when trying to determine mPMI but not for DNA evidence⁹. The hot water kill conditions effectively denature the proteins within a larvae and help fix its size, even causing it to swell to its maximum size¹⁰. This size fixing makes it beneficial when determining larvae age based on size and weight ratios for mPMI calculations as well as helps fix anatomical features that can be used to age larvae as well¹¹. The use of the size and weight ratios as well as anatomical features, helps assess stage information which when used with accumulated degree day (ADD)/ accumulated degree hour (ADH) calculations based on environmental temperature information can help determine the mPMI range estimation^{1,2,3,11}. In addition to its abilities to fix the larvae's shape, hot water kill is inexpensive and allows for long-term storage of collected specimens⁹.

Because research on how hot water kill affects DNA preservation does not exist, it has not been adopted as a standard preservation technique, thus creating two different standard methods of preservation: one to preserve the mPMI information (hot water kill) and one to preserve DNA evidence (ethanol). As the polymerase chain reaction (PCR) methods call for similar temperatures to be used on DNA to create mass copies, it is believed that the temperature from the hot water kill will cause minimal to no greater of an effect on the human DNA within the fly larvae crops than the standard ethanol preservation currently used. In order to look at the field applicability of the hot water kill method to preserve both mPMI and DNA information from larvae, this study focused on the usage of hot water killing and its effects when applied for various timeframes on the recovery of human DNA from larval *Lucilia sericata* crops. Assessing the feasibility of using the hot water kill would allow for two preservation methods to be used as one. The study focused on *L. sericata* because the species has a wide global distribution, they are quick breeding, and there is an extensive understanding of their development³.

Chapter 1: Background

1.1 Forensic Entomology

The earliest recording of forensic entomology can be traced back to around the year 1235 in the book *Washing Away of Wrongs* by Sung Tz'u³. In this case, the presence of flies around a cleaned murder weapon were used to obtain a confession from the guilty party. However, it wasn't until after 1850 that forensic entomology became more widely used and researched. During a case involving infanticide, Bergeret was the first recorded scientist to study the development of entomological specimens on a corpse and actually apply it to the medicolegal field. His knowledge of the life cycle of the flies helped exonerate those charged with the crime and place the proper blame on those responsible^{2,3}. Since the mid-1800's, the use of forensic entomology has helped further determine the minimum postmortem interval (mPMI) of victims. As the years have progressed, the techniques have become more refined to give better estimates of the mPMIs¹². In addition to looking at entomology centered focus on mPMI estimation, other areas within the field of biology have tried to better understand and classify the mPMI, such as with the use of ribonucleic acid (RNA) and other molecular markers¹³. Factors that may affect the mPMI are better known and have been studied extensively for the use in casework.

1.1.1 Historical Methodology

Typical examination of entomological evidence includes the determination of the mPMI based off the assessment of life cycles of the insects found at the crime scene near a body¹.

Figure 1 displays the typical lifecycle of flies as depicted in the first edition of *Entomology and Death: A Procedural Guide*¹⁴. Through studies on many fly species, development tables have been produced to help understand the relationship between the environment, stage of development, and chronological age. Less widely used techniques rely on the relationship between some measures of body size and chronological age¹⁵.

Figure 1. The blowfly lifecycle from oviposition to mature adult. After hatching from the eggs, fly larvae have three stages prior to pupating: first, second, and third instar. The third instar stage is split between a feeding and wandering (postfeeding) phase. During the pupillary phase the larva is undergoing a metamorphosis into the adult fly.¹⁴

As poikilotherms, flies, like all insects, use thermal energy from their environment in addition to the nutrition they obtain from what they feed upon to progress through their life cycle¹. For the development to occur, the ambient temperature must be above a certain threshold temperature. This threshold temperature varies from species to species and in the same regard different species may take a shorter or longer time to progress through their lifecycle even when the threshold temperatures are reached^{1,16}. After oviposition, and when the eggs hatch, Calliphorid flies have three different larval stages: first instar, second instar, and third instar¹. Between each stage, the larva will molt and grow as it takes in more food and it is in the feeding phase of the third instar stage that the larvae accumulate 80% of its mass¹. By the end of the third instar, the larvae should be fully grown and will begin preparing for the pupal stage by digesting its food and generating the necessary energy during the wandering (postfeeding) phase¹. During the pupal stage the larvae metamorphose into the adult fly. The stages of development of the larvae are unambiguous and easy to differentiate before or after preservation based on anatomical features and whether or not the puparium has formed¹.

When necrophagous insects are eating, they secrete digestive enzymes that help break down the tissue of the body they are feeding on and ingest the tissue by scraping the tissue away from the body^{1,3}. Development of most Calliphorid species, like *L. sericata*, requires decaying meat. As the larvae feed upon the decaying meat, the tissue is stored in the crop^{1,3}. This means that any DNA within that tissue that has not otherwise been broken down can be recovered by investigators. Within this research the stages of development are vital to understanding the ability to recover human DNA from the crops of the larvae. If the larvae are in the first or second instar stage, the larvae themselves as well as the crops may be too small to recover any human DNA. Likewise, if the larvae are in or past the third instar postfeeding or wandering phase, the human DNA stored in the meal of the crop would be digested.

With different species developing at different rates and needing various threshold temperatures for development, it is important to understand what species of fly is being observed. Fly DNA can be extracted from adult flies and genotyped such that the species is known and an increased understanding of the species-specific developmental process can be applied to any mPMI calculations being made^{1,3}. The use of these DNA techniques are solely to understand the species of fly being dealt with and is different than the techniques used to recover human DNA from the insects.

1.1.2 Factors Influencing Post Mortem Interval Determination

The most significant factor affecting the rate of growth of the larvae is the temperature of the environment in which a body is found. Through the use of accumulated degree hours (ADH) and accumulated degree days (ADD), temperature data of the environment coupled with the stage of development a fly is in can help estimate the timeframe in which the fly egg could have been laid. ADH/ADD are the calculated amount of thermal energy needed over time for different species of flies to progress from one stage of development to the next. As poikilotherms, the larvae use the heat of their environment to help provide energy to function and it has been studied that there is a straightforward relationship to the thermally-driven physiological age and the chronological age for mPMI estimation. This usage of external environment temperature to help function, drives the analysis of ADH/ADD for mPMI estimation^{2,3}. ADH/ADD yield an estimation range for mPMI based on the stage of development and the temperature information.

In similar respects, the use of body size and temperature can also yield an mPMI estimation range^{2,3}.

In addition to the ADH/ADD calculations, studies have shown that the type of tissue the larvae are eating can have an effect on the development. One study in particular by Day and Wallman in 2006¹⁶, looked at how feeding on sheep brain, liver, and meat (lamb chops), would change the growth rates of the larvae of two different species (*Lucilia cuprina* and *Calliphora augur*). The authors found that the larvae growing on the liver took a longer time to reach their maximum lengths, which were also shorter, and developed smaller pupae than those growing on the sheep brain and meat¹⁶.

Fly genotypes have also been observed to have an effect on the growth cycle and maximum length of larvae. Tarone et al.¹⁷ observed *Lucilia sericata* from three different regions around the USA, each with different genotypes. It was reported that the length of the larvae and its developmental time depended on the genotype as well as the temperature¹⁵. Each factor that affects the length and development of the larvae can ultimately affect the amount of usable DNA for a profile that can be found within the crop of a maggot.

Other research looked at how the preservation techniques may affect the estimation of maggot age. Tantawi and Greenberg¹⁰ looked at 16 different preservatives typically used by entomologists to see their effects on the length of the larvae observed. Using the standard hot water kill as a control, they calculated percent shrinkage and the deviation of estimated larval age based on a growth curve versus the control. It was observed that larvae that were first killed using hot water and then transferred to a preservative solution (i.e. ethanol), did not shrink like the larvae placed directly into the preservative solutions¹⁰. The denaturing of digestive enzymes and gut flora during the hot water kill caused autolysis to be lessened resulting in the larvae maintaining their size⁸. From studies like this one, hot water killing is seen as a standard practice in the field to maintain the body size of the larvae for mPMI determination. The shrinkage, affecting the body size-based calculations, caused by the ethanol can affect the analysis with the specimens being assessed as younger than they would otherwise be assessed. In the case of ADH/ADD calculations, the shrinkage would only affect the calculation of stage if it caused the larvae to collapse and lose the anatomical stage indicators.

1.2 Forensic Biology

Forensic DNA analysis has been around since the early 1900's and has been a way to identify the person that deposited a biological fluid found within the investigation of a crime. The standard practice involved ABO blood typing at its inception⁴. In the early 1980's with the development of restriction fragment length polymorphisms (RFLP) by Ray White, the field looked at these sequence polymorphisms as a way to identify contributors to the biological samples submitted to the laboratory. The RFLP required large amounts of undegraded DNA, more than 50 ng, but was highly discriminating⁴. The drawback to this method was the subjectivity when it came to making band size calls⁴. Different analysts could make different calls based on a match window (roughly $\pm 2.5\%$). The match window was established due to: the variability between individual gels run, the resolution not being as precise as it is today (resolution down to 1 base pair with STR analysis), and there was the possibility of smearing (a sign of too much DNA present) to occur making the gel difficult to read if not done properly⁴.

In the mid- 1980's Alec Jeffreys developed a method to look at variable number tandem repeats (VNTRs) as an addition to RFLP analysis. These are locations in the DNA that would have sequences that would repeat over and over and each individual would have a different amount of repeats of a particular sequence targeted, with the exception of identical twins. With the finding of VNTRs, analysts were able to use these regions to focus the RFLP analysis on. The VNTRs used during RFLP analysis had multi-locus capabilities which allowed for the creation of a "DNA fingerprint" that could be viewed on a gel. This technique garnered international attention with the Colin Pitchfork case, where it was used to exonerate an innocent individual and put the guilty party in jail⁴.

Towards the beginning of the 1990's, short tandem repeats (STRs) were identified in the DNA sequence and are still being used by forensic DNA analysts today. Like VNTRs, the STR regions are regions of the DNA that repeat themselves and vary between individuals. All possible variations at a given locus are the alleles⁴. In the mid 1990's, the FBI created the Combined DNA Index System (CODIS), which houses DNA profiles obtained within casework and of convicted offenders. Initially, 13 core loci were needed by the FBI to participate in the usage of CODIS. In 2017, this was increased to 20 core loci to add more discrimination power when assessing likelihood of a particular profile in the population⁴.

1.2.1 Methodology

The standard workflow in a DNA laboratory starts with the extraction of DNA and moves to quantitation, polymerase chain reaction (PCR) amplification, and finally capillary electrophoresis. Extraction focuses on removing and purifying the DNA from the cells such that it can be amplified and analyzed via capillary electrophoresis. DNA can be extracted using organic, differential, and magnetic bead protocols⁴. One kit that uses a series of buffers to isolate and purify DNA commonly used within DNA laboratories is the QIAamp® DNA Extraction Kit by Qiagen. After the DNA is removed from the cell using proteinase K and a series of buffers to break down non-nucleic acid cellular components, a spin column membrane linked with silica is used to purify the DNA¹⁸. This works due to the silica within the membrane binding with the phosphate groups within the DNA backbone. More buffers are used to wash through the membrane to remove any extra components that are not DNA bound to the silica¹⁸. A final buffer wash is used to disassociate the DNA from the membrane so that all that is left in the solution is the buffer and the purified DNA¹⁸. This kit works similarly to the DNEasy® Blood and Tissue Kit (Qiagen) with the main difference being the time spent in hot water baths in the initial cell break down.

Quantitation is necessary to measure how much DNA has been extracted. This is because most PCR amplification protocols have been designed to be the most efficient with approximately 1 ng of input DNA. Quantitation allows the analyst to see how much human DNA is present to determine if a dilution or concentration of the DNA must occur. The Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems) targets three human specific targets: a small autosomal target (80 bp), a large autosomal target (240 bp), and a Y chromosome specific target (75 bp)¹⁹. In addition to targeting these three areas, using the large and small autosomal targets a

degradation index (D.I.) value can be obtained, using **Equation 1**, to assess the usability and performance of the large autosomal target in relation to the small DNA target¹⁹.

$$D.I. = \frac{\text{Small Autosomal Target Concentration } (\frac{ng}{\mu L})}{\text{Large Autosomal Target Concentration } (\frac{ng}{\mu L})} \quad (1)$$

This equation works by recognizing that larger fragments of DNA have a higher probability of being degraded than smaller fragments, as there is more area for a break in the DNA to occur. A larger D.I. value means that the larger DNA fragments have the potential of performing more poorly than the smaller DNA fragments¹⁹.

PCR is a cyclic reaction that amplifies the DNA exponentially. Within 28 to 30 cycles one strand of DNA can become millions. The Globalfiler™ PCR Amplification Kit (Applied Biosystems) has fluorescently labeled primers that will bind to the DNA at the 20 core loci, as dictated by the FBI for CODIS, with the addition of the TPOX, SE33, D22S1045, and Amelogenin (sex determining) loci. A hot start PCR is used to combat non-specific primer binding to the DNA, followed by cycles of denaturing, annealing, and extending²⁰. In addition to the genomic DNA being amplified, the sizing standard (made up of fragments of known sizes) for capillary electrophoresis is used to help the instrument compute how large the fragments are when coming off the instrument^{19,20}.

Capillary electrophoresis is a sized based separation using the electrochemistry of DNA. Since the DNA has a negatively charged backbone, a positive charge is added at the end of a capillary to draw the DNA towards the charged couple device (CCD) camera of the genetic analyzer. The 3500 Genetic Analyzer (Applied Biosystems) uses a 505 nm laser to excite the fluorophore on the primer that was attached during PCR amplification²¹. The fluorophore becomes excited and emits light detected by the CCD camera. The light detected comes off as a data point and is converted to a base pair size using the internal sizing standard, which is then converted to the allele call using the allelic ladder²¹. This information is then viewed as an electropherogram using software such as GeneMapper™ ID-X.

1.2.2 Factors Influencing Biological Examinations

Much like estimating the age of a maggot, there are many factors which influence the ability to get a complete DNA profile. Some of those factors include: PCR inhibition, low-level DNA, and temperature. In each case, allelic dropout in a DNA profile is possible, which is a problem as it is ideal to have a full profile for identification purposes. A high amount of dropout can make it difficult to make a confident conclusion about the source and can preclude the use of CODIS.

In sufficient quantities, different natural compounds found within the human body have been found to inhibit the PCR process. The inhibitors work by impacting the polymerase directly, the DNA, or a combination of the two. The inhibition of the *Taq* polymerase causes problems with efficiency and increases the cycle threshold (C_T) as the concentration of the inhibitor increases. DNA-binding inhibitors cause changes in the melting curves and the C_T values as well. The inhibition can cause less DNA to be amplified resulting in the allelic dropout throughout an electropherogram^{22,23}. Some common inhibitors include indigo dye, hemoglobin, calcium, and

urea^{4,19,20}. These inhibitors are commonly found however, through the extraction and purification process of the DNA using commercialized kits, the impact of these inhibitors can be drastically reduced^{17,19,20}.

Temperature can also affect the amount of usable DNA because there are issues with degradation. In a study by Karni et al.²⁴, dry DNA would degrade in temperatures above 100°C and as pressure was added in addition to the temperature, DNA degradation began to occur at nearly 90°C. The authors only tested the degradation in dry and pressurized conditions, not in solution²⁴. The degradation of DNA, via the breaking of the phosphate backbone, at high temperatures is important because this can directly impact the quality of STR profiles being obtained. Studies involving how boiling may affect the degradation of human DNA were not found, however several studies in the food industry have looked at the effect of boiling water on several meats. One such study by Sakalar et al.²⁵ looked at how cooking in a dry oven and boiling affected the DNA quantification step when looking at beef, pork, and chicken DNA. The authors boiled the meats at 99°C up to 240 minutes (3½ hours). It was observed that after only 10 minutes of cooking, nearly 80% of the DNA was considered to be intact from the control groups that were not boiled for all three meat types. After 240 minutes of boiling, DNA was still present from each meat type at roughly 21%, 41%, and 67% the control concentration for beef, pork, and chicken respectively²⁵. Compared to the PCR reaction, the temperature of boiling water is only 4°C higher, however the time spent within the high temperature at 240 minutes from this study is significantly longer than the time spent at 95°C and 94°C for at most one minute at a time in PCR.

A similar study by Musto²⁶ found that between roasting, boiling, and microwaving, with a raw meat control, beef meat that was boiled for five minutes decreased the yield of DNA by nearly half the starting concentration. In this study the authors looked at the *cytochrome b* and *cathepsin b* mtDNA genes to amplify with PCR for their results²⁶. These results differ greatly from what was observed by Sakalar et al. This could be due to the differing genes and their sizes between the studies. However, by looking at both studies, even after five minutes and up to 240 minutes, a DNA profile was obtained after boiling. It can thus be inferred, in an absence of data regarding the degradation of human DNA in boiling conditions, it would be possible to get some human DNA from a cell even after it has been boiled briefly due to DNA having the same general structure between organisms.

1.3 Forensic DNA Testing on Entomological Specimens

Entomological specimens have been studied widely over the past several decades as a source of human DNA because many feed upon the blood or flesh of humans^{27,28,29,30}. Both nuclear (STR) and mitochondrial (mtDNA) have been examined. While mtDNA does not have as high of a discrimination power as STR analysis due to its matrilineal inheritance, it can still be used as an investigative tool when doing familial searches⁴.

From the early 2000's studies have looked at obtaining human genetic profiles from the gut of mosquitoes as well²⁷. Ibrahim et al.²⁸ looked at two different culicid mosquitoes grown in varying temperatures to see how this would affect the timeframe of obtaining usable STR

profiles. This research and the results obtained are concordant with other research that looked at the blood meals in mosquitoes to obtain these profiles. The use of mosquitoes and their blood meal have even been used within casework in Italy²⁹. Throughout, the research shows that profiles have been obtainable, even up to three days after the ingestion of the blood meal as detailed by Ibrahim et al²⁸.

Studies have also been testing how smaller cropped insects, such as lice, have been used to obtain usable quantities of DNA for STR profiling. In 2015, the use of *Cimex lectularius* (bed bugs) was studied in obtaining STR DNA profiles and Alu insertions after the DNA had been ingested for 48 hours²⁷. It was found that the quantities of DNA ranged from 10 ng/uL to 110 ng/uL. In all cases, there was a sufficient amount of DNA that would be used to get a profile with a target amount of 1 ng of DNA²⁷. Prior to this study the authors noted that other types of lice had been observed and similar results were found²⁷. In addition to this research, studies have been performed on beetles, crickets, cockroaches, and several other insects that could potentially be found at crime scenes, both indoor and outdoor³⁰. Although research as has been performed with other insects may not have always been forensically motivated, as with the use of larvae crops, the information acquired from these studies may be beneficial to practitioners for use in casework.

1.3.1 Fly Larvae Crop DNA Focus

Previous studies have looked at influences of techniques used to extract human DNA, as well as how the preservation of larvae, and the amount of time a maggot had been eating to study the possibility of obtaining STR and mtDNA profiles. In addition to these studies on larvae, there have been casework studies showing the usability of obtaining STR profiles from larvae crops. In 2013³¹, there was a case involving a burn victim that was beyond recognition, that involved the use of tissue found in the maggot crops and lead to an identification. The investigators had an idea of who the victim was and used a buccal swab from the victim's father as a reference sample. After DNA testing was unsuccessful from a piece of liver tissue collected from the body, the contents of the larvae found on the body were used to obtain the STR profile³¹. The analysts used the paternity testing model when doing the comparison between the profile obtained for the victim and the father. Of the 15 loci tested plus the Amelogenin locus, only three loci were not compared due to complete allelic dropout at those loci. Overall, a 99% probability was determined that the questioned DNA was offspring of the known father's sample³¹.

Starting around the early 2000's entomologists began looking into the ability of pulling human DNA information from the insects they were studying. While still looking at the mPMI, research looked at the possibility of obtaining both mtDNA and STR genetic profiles of the decedents the insects were feeding on. Reasons for the use of insects as a carrier for human DNA include cases of a missing body, corpse movement, connecting crime scenes together, and even sexual assault when genital lice are transferred from perpetrator to victim^{8,32}. In each case, the use of the insects can increase the information and understanding of what happened because even if the host's body has been moved, the DNA from the host can still be found within the insects found at the scene. Dr. Richard Zehner and associates found in 2004 that obtaining STR profiles from the crops (foregut) of the larvae was possible⁵. It was found that out of the larvae collected

off of 13 corpses, seven STR profiles were obtained, two STR profiles were incomplete having some allelic dropout, and four STR profiles were not obtained, having complete dropout. This study was influential; being one of the first time scientists had studied the possibility of doing STR analysis on the contents of a maggot's gut⁵.

In 2004, Linville et al.⁷ looked at how both mtDNA and STR analyses would be affected by specimen preservation techniques. The authors looked at both the preservative fluid and the temperature of storage and how these factors affected the ability of obtaining genetic profiles. The overall trend that was seen showed as time progressed from two weeks of preservation to six months, the amount of DNA that could be quantified decreased across all temperatures and preservative fluids. It was also found that when comparing the success rate of the mtDNA and STR analyses out of six larvae, the mtDNA generally had a higher success rate over all temperatures and preservative fluids. The preservative fluid that worked the best overall in both the mtDNA and STR analyses was ethanol and more specifically 70% ethanol. The formaldehyde performed the worst, only getting mtDNA results out of one maggot at two weeks and eight weeks. When temperature was used as the preservation technique, the results were comparable to those in which ethanol was used. A storage temperature, without any preservative solution, of -70°C gave the best results. Only one STR analysis failed out of the six replications at two weeks. For all other analyses, both mtDNA and STR, for this temperature (-70°C), profiles were obtained for the six larvae at each time interval. With the results, the recommendation was to use ethanol as a preservation fluid due to its ability to denature nucleases and dehydrate the specimen making it better for crop extraction.

In 2005, Campobasso et al.³³ looked at the age of the maggot, as well as the time the larvae were left alive after being removed from the food source when performing both mtDNA and STR analysis. The first observation the authors made was that prior to 2.5 days old, the larvae were too small to dissect and obtain the crop from the maggot to run mtDNA and STR analysis³⁴. For fully third instar larvae between 2.5 and 4.5 days old, it was found that this was the best range to harvest³³. The larvae that were killed and preserved immediately after being removed from the food source (2.5 to 4.5-day age) developed full mtDNA and STR results. With 24-hour starvation only mtDNA was recovered. Neither mtDNA nor STR data was recovered after 48 hours of starvation³³. In addition to these findings, it was noted that no severe enzymatic breakdown of host DNA occurred in the crop as time progressed³³. The authors stated that larger crops, in larvae still feeding, were a main factor in the ability of obtaining a profile³³.

A similar study to the 2005 study was performed looking at the ability to obtain STR profiles after starvation and larvae that were fed beef liver after feeding on the corpses from which they were obtained³⁴. It was found that STR profiles were obtained up to four days after starvation and up to two days after the larvae were fed beef after being pulled off the corpses³⁴. This contradicts the study previously mentioned. This could be due to the fact that extraction kits, as well as the instruments used for the analysis were different. The newer analysis methods were more likely to have a better optimization than the ones that came ten years prior.

1.4 Statistical Analysis

1.4.1 Analysis of Variance (ANOVA)

Analysis of Variance (ANOVA) statistical tests compare three or more independent groups at one time by assessing the mean and the variance around the mean of the data within and between each group³⁵. For ANOVA the null hypothesis (H_0) can be represented with the equation $H_0: \mu_1 = \mu_2 = \mu_3 = \dots = \mu_n$ for n groups³⁵. This null hypothesis is interpreted as the means of each group are equal to one another. To reject the null hypothesis, meaning that at least two group's means are significantly different, a p -value less than the alpha value chosen, typically .05, must be obtained³⁵. The variance of the data was assessed with an F test, by looking at both the within group and between group variances via sum of squares³⁵. When p -values show that at least two means differ, post-hoc tests like the Tukey's HSD post-hoc test can be used as a pairwise comparison to determine which exact group means differ³⁵.

To run a successful ANOVA test four assumptions must be met. The first assumption ensures that each group is normally distributed³⁵. The normal distribution can be assessed through quantile-quantile plots (Q-Q plots) which plots theoretical quantiles against sample quantiles. The second assumption is that the sample variances are equal between the groups³⁵. A residual plot is used to assess the sample variance as it plots the residuals against the fitted x values³⁵. The final assumption is that the samples within each group are randomly assigned to each group from the larger population³⁵. The final assumption ensures that the treatment groups that are being compared are independent of one another³⁵.

Within this study ANOVA is being used to determine if different times spent in a hot water kill bath has any impact on the proportion of alleles within an STR profile compared to other times and the standard 70% ethanol treatment.

1.4.2 Simple Linear Regression Modeling

Regression modeling in general, assesses how different factors affect one another and help determine if a relationship exists between two or more variables³⁵. More specifically, simple linear regression looks at how a single variable " x " may affect variable " y " in a linear (straight-line) way³⁵. Multiple linear regression and other regression models may look at how more than one variable can affect the " y " variable and/or if the relationship is non-linear. The independent variable " x " in the model is also known as the predictor variable, which means that if significant, this variable can help predict the outcome of the dependent variable " y "³⁵. **Equation 2** represents the standard linear regression equation³⁵.

$$y = \alpha + \beta x + \epsilon \quad (2)$$

The alpha value is the y -intercept, the beta value is the slope, and the epsilon value is the error in the data off the line. Written out as a function " $f(x)$ ", the epsilon value is dropped³⁵. The function is determined by looking at the residuals between the fitted line and the true data points. The residuals are squared and those squares are summed. The line that produces the smallest values from the squared residuals is used as the model best "fit" for the data³⁵. A correlation

coefficient (R^2) helps determine the degree of the relationship between the two variables. An R^2 close to a value of one means that there is a high degree of association to the relationship and as the R^2 value gets closer to zero, the lower the degree of association³⁵.

Like ANOVA, the linear model can be tested by viewing the residual plot to ensure the residual have equal variance³⁵. In addition, an F test can be used to help determine whether the model significantly reflects the data presented³⁵. Again like ANOVA, a p -value determined from the F test lower than the alpha value is determined to be statistically significant and the line is a good representation of the data.

Linear Regression is being used within this study to assess whether the amount of time spent in a hot water kill bath has a positive or negative effect on the proportion of alleles called within a STR genotype profile.

1.5 Impact

Throughout the survey of literature, little data exists as to whether human DNA will have marked degradation when boiled. Nor has it been studied from an entomological recovery standpoint. It is known that boiling water is the preservation technique that has the least damaging effect to the information that can be gleaned from larvae in regard to mPMI. As it has been shown that there is a potential for the use of human DNA analysis on the contents within the crop of a larvae, the effects of boiling the larvae is important to understand. In both settings, it can lead to understanding whether or not this practice could be used to preserve the best mPMI estimation as well as STR analysis information.

Beyond the fundamental understanding of how this preservation technique affects the human DNA within the crops of the larvae, it can help understand the used in fieldwork. As described by Wells et al. (2001) and Wells and Stevens (2008), there are several instances where it may be important to use the contents of the crop to obtain human DNA^{8,32}. Some of these include the movement of a body, cases of sexual assault where lice may be transferred from suspect to victim, and even if a body is present but larvae are found on another source of food nearby^{8,32}. In addition to these instances, there is the potential to be able to look back at cold cases for human DNA evidence in larvae that may have been preserved using hot water kill.

Chapter 2: Methodology

2.1 Colony Maintenance

A wild-type *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) colony was maintained within an Intellus Ultra C8 growth chamber (Percival Scientific). Using a 12 hr:12 hr photoperiod (LED), the *L. sericata* were kept at 25°C with 70% relative humidity. Beef liver was used to feed the larvae and the adult flies and was utilized as the oviposition substrate between generations. The average generation lifespan of the *L. sericata* was roughly three weeks.

2.2 Thermocouple Build and Calibration

A thermocouple was built at the request of a committee member no longer associated with the project using a Raspberry Pi 3 and a DS18B20 thermo sensor as a way to increase the learning outcome in regards to coding. Once operational, a NIST Traceable Thermometer (S/N: 181354674) was used for the calibration of the thermocouple. Triplicate measurements were taken using each thermometer ranging from room temperature water to around 80°C. A proportional error was indicated and the code was adjusted. Six months after the initial calibration, the thermocouple was recalibrated following the same procedure as before with the NIST Traceable Thermometer. The thermocouple was used when recording water temperatures during the hot water kill preservations.

2.3 Obtaining and Profiling Reference Samples

Three human livers were obtained from the WVU Human Gift Registry and a 25 mg sample of each liver, as well as a 10 µL blood sample from myself and an assistant during the extraction process was analyzed for reference profiles. This was to ensure that allele calls obtained during the testing matched that of the experimental samples and to analyze and exclude the assistant and myself as a contributor in the case of contamination. The 25 mg samples of each liver were obtained directly prior to liver placement in the growth chamber for larval feeding and growth. The number of allele peaks from each profile was determined to assess proportion of alleles called in the experimental trials. Homozygote peaks were considered as 1 allele peak when counting the number of allele peaks present. Liver tissue was chosen as the source of human DNA for this project due to its large size making it easier to obtain a larger quantity for optimal larval growth, and it being readily available from an existing WVU research project. Furthermore, while the liver undergoes autolysis relatively quickly, this was the human tissue able to be obtained and helped aid in understanding how quickly samples may need to be collected before too much degradation occurred for DNA recovery. In addition, prior to initial use, all utensils, microcentrifuge tubes, PCR tubes, and well plates were decontaminated under UV sterilization for 2 hours throughout the project, following a contamination event in the experimental sets half-way through the project. Any samples run prior to the contamination event were re-ran following the new sterilization protocol.

2.4 Crop Extraction Methodology Trials

Three trials were run to optimize the crop extraction process which would lead to having quantifiable human DNA that was sufficient to profile. In each trial, a five-gram piece of human liver was used as the oviposition substrate. When eggs were observed, the liver was removed and placed into a Ziploc container containing an additional 55 g piece of liver and vermiculite. During the first trial, as soon as first instar larvae were observed, the larvae were given an additional 80 hours to develop before being removed and preserved. It was determined that 80 hours post-hatching was too much time and the feeding period had concluded and the crop contents had all been digested. For the second trial, the 80 hours began as soon as the liver was placed as an oviposition substrate. With this trial, the crops were still visible in the third instar larvae, indicating that the larvae had not made it to the wandering stage as in the first trial. When extracting the crops, a scalpel was used and the crops were often nicked and the contents spilled. The third trial followed the second trial with the substitution of iris scissors over the scalpel to obtain the crops. Crops were extracted by cleaving off the posterior end of the larvae followed by a posterior-anterior incision to expose the crop. Once the crop was exposed, forceps were used to cleanly separate the crop from the larvae. The human DNA from the crops in all trials was extracted using the DNEasy® Blood and Tissue extraction kit (Qiagen). In addition, samples from the last trial were concentrated using the Microcon® 30k filters and the samples were quantified using the Quantifiler™ Trio Kit (Applied Biosystems). The quantitation values were compared using ANOVA and Tukey's HSD post-hoc to determine if the mean concentration differed between the various crop extraction trials.

2.5 Experimental Set Runs

Three human livers donated by the West Virginia University Human Gift Registry were used for experimental groups. One liver was male (Liver 1) and the other two were female (Livers 2 and 3). Approximately 30 grams of each liver was placed in the growth chamber and presented to the colony as an oviposition substrate. When eggs were observed on the oviposition substrate, the substrate was added to roughly 230 grams of each liver placed into a Ziploc container with vermiculite. After 80 hours in the growth chamber, when third instar feeding larvae were observed with full crops, larvae were removed, weighed, and sorted into the different vials for the preservation techniques. A final group of five larvae were immediately preserved via refrigeration at 2°C for 96 hours after weighing without using 70% ethanol or the hot water kill bath. **Figure 2** shows how the larvae were separated into experimental groups.

The hot water kill was carried out in a thermos and with water that had a starting temperature of 99°C. The thermocouple was used to track the water temperature throughout the time intervals. All temperatures stayed within the range of 92-99°C for the time intervals up to 90 seconds. For the 300 seconds intervals the lowest ending temperature was 84°C. For each time interval, all ten larvae were preserved simultaneously. Five larvae were placed in a vial with air and the other five were placed into the 70% ethanol. When all larvae were preserved, the vials were stored in a refrigerator at 2°C for 96 hours. A time of 96 hours was chosen to give some passage of time between preservation and crop extraction as to show some effects of storage time like in casework samples.

Figure 2. Larvae Preservation Workflow. Five larvae were used for each preservation group including a fourth group which was only refrigerated at 2°C for 96 hours. A total of 60 larvae were used for each liver for a total of 180 larvae sampled.

At the end of the 96-hour storage time, samples were removed from the refrigerator and placed in one milliliter of 20% bleach for two hours to decontaminate and sterilize any exogenous DNA from the outside of the larvae following protocol discussed in Linville and Wells (2006)⁶. At the end of the two hours the larvae were submerged in a beaker of distilled water to wash away the bleach. The larvae were measured for total length and crop length in millimeters. The average and standard deviation of the lengths and weights measured for the three livers was taken and two-tailed T-tests were used to compare the groups. Crops were dissected by removing the posterior end of the larvae, followed by an incision from posterior to anterior on the ventral side of the larvae using iris scissors⁶. The larvae were opened via the incision and a pair of forceps was used to recover and move the crops from each larva to a clean microcentrifuge tube. All utensils used were decontaminated between crop samples using a beaker of 100% ethanol followed by a wipe down using 20% bleach.

To isolate and purify the DNA within the crop, manufacturers guidelines were followed using a silica-based column from the DNEasy® Blood and Tissue extraction kit (Qiagen). With 200 µL as the final elution volume, all samples were then concentrated using Microcon® - 30k filters (Millipore). Prior to the concentration, the centrifugal filters and filtrate flow through tubes were sterilized under UV radiation for 2 hours. This was to ensure a sterile filter due to the filters coming marked as “non-sterile”. Quantitation of each sample was carried out in duplicate using the Quantifiler™ Trio Kit (Applied Biosystems) on the 7500 Real-Time PCR System (Applied Biosystems) according to manufacturer’s protocol. The duplicate measures were used to get a better representation of the concentration of DNA present per sample. Concentrations (ng/µL) of DNA were assessed at three targets which helped determine: degradation index values, if any male contamination was present in female samples, and the small autosomal target was used to estimate DNA concentration for amplification purposes.

The two samples from each preservation method with the highest quantitation values across the three targets were further concentrated using the Microcon® - 30k filters and sent on to amplification. A second round of quantitation was not run on the samples that were re-concentrated due to limited resources and the initial and final volumes of the samples were relatively known allowing for an estimation of quantity based on volumes and initial concentration using the $C_1V_1=C_2V_2$ equation. While the quantities were not truly known having the estimation, helped understand that the full volume of DNA would be needed for a 1 ng of total DNA target during the PCR reaction. The Globalfiler® PCR Amplification Kit was used to target as close to 1 ng of DNA in each sample according to manufacturer's protocol. Due to low quantitation values, the largest amount of DNA (15 µL) was added to the PCR reactions. Amplification was carried out using the GeneAmp® PCR System 9700 (Applied Biosystems) with temperature cycle settings shown in **Table 1**.

Table 1. Globalfiler® PCR Amplification temperature cycles as directed via the Globalfiler® PCR Amplification User Guide.

<i>Initial Incubation</i>	<i>Denature</i>	<i>Anneal/Extend</i>	<i>Final Extension</i>	<i>Final Hold</i>
Hold 95°C 1 min	Cycle 29 times 94°C 10 sec 59°C 90 sec		Hold 60°C 10 min	Hold 4°C Up to 24 hours

Capillary Electrophoresis was carried out utilizing a 3500 Genetic Analyzer (Applied Biosystems). Solutions comprised of 9.6 µL HiDi Formamide, 0.4 µL GeneScan LIZ 600 Size Standard and 1 µL of each sample, positive control DNA, PCR Negative, or Allelic ladder were loaded into 96 well trays followed by a brief vortexing and centrifugation. The DNA was denatured into single strands using an incubation at 95°C for three minutes followed by a three-minute snap cool with an ice block. **Table 2** at the beginning of the next page displays the run parameters utilized. STR profiles were analyzed and observed using the GeneMapper® ID-X software with an analytical threshold of 250 relative fluorescent units (RFU). Any peaks having a peak height lower than 250 RFU were not called in the electropherograms.

Table 2. Run Parameters for capillary electrophoresis on the 3500 Genetic Analyzer (Applied Biosystems).

Oven Temp	PreRun Voltage	PreRun Time	Injection Voltage	Injection Time	Run Voltage	Run Time
60.0°C	15.0 kV	180 sec	1.2 kV	15 sec	13.0 kV	1550 sec

2.6 Statistical Analysis

Average and standard deviations for larvae total and crop length, larvae weights, quantitation concentration of all samples within a liver, and the proportions of alleles called after capillary electrophoresis were recorded. T-tests were run on the average lengths and weights to

compare any differences between the lengths and weights of the larvae collected between the livers. ANOVA was run twice on the proportions of alleles called for each preservation technique in Liver 1 and Liver 3; Liver 2 was omitted from the analysis due to high degradation leading to sparse genotypic profiles. In addition, it was noted that the decedent supplying Liver 2 had begun the bloat stage of decomposition: a green coloration on the skin and the peritoneal cavity being expanded and filled with gas. The first run compared each treatment group individually with separation of the livers. Based on those results, the second run compared each treatment group with the combined proportions from both livers. Finally, two simple linear regression models were obtained. Both showed how residence time within a hot water kill bath (independent variable) predicts the proportion of alleles called during STR analysis (dependent variable). One model looked at the groups where only hot water kill was used; and the other looked at the addition preservation with 70% ethanol after the hot water kill.

Chapter 3: Results

3.1 Reference Profiles

The average quantitation concentrations of each reference sample from the livers was recorded in **Table 3**. Liver 1 and Liver 3 each had over 100 ng/ μ L concentrations, whereas Liver 2 had a significantly lower concentration around 20 ng/ μ L. The degradation index value for each was assessed and Liver 2 had the highest degradation index value of the three at 1.61, followed by Liver 3 at 1.31 and finally Liver 1, at 1.13. As indicated in the Quantifiler® Trio Quantitation kit user manual, these degradation index values show minimal to moderate degradation.

Table 3. Target concentrations and degradation index value observed in the liver reference profile samples using the Quantifiler® Trio Quantitation Kit. Liver 2 and Liver 3 were both female samples resulting in a concentration of 0 ng/ μ L for the Y (male) target.

Liver	Target	Concentration (ng/ μ L)	Degradation Index Value
L1	Large Autosomal	115	1.13
	Small Autosomal	130	
	Y	130	
L2	Large Autosomal	18	1.61
	Small Autosomal	29	
	Y	0	
L3	Large Autosomal	111	1.31
	Small Autosomal	146	
	Y	0	

The number of allele peaks present for each liver sample was determined to be 41 for both Liver 1 and Liver 3 and 39 for Liver 2. With each individual on the planet having a different combination of alleles being heterozygous or homozygous, the profiles obtained did not look out of the norm. Genotype profiles for the reference samples can be found in **Appendix A**.

3.2 Methodology Trials

The average small autosomal concentration (ng/ μ L) for four trials was assessed using ANOVA. The averages of the four trials each with $n=5$ can be found in **Table 4**.

Table 4. The average small autosomal target quantity per trial ($n=5$) with standard deviation. For Trial 1 and 2 the full larvae were used after the crops were not able to be cleanly isolated when using a scalpel. Trial 3 crops were isolated using iris scissors. Trial 4 crops were isolated using iris scissors and the DNA extract was concentrated.

Trial	Average Small Autosomal Quantity (ng/ μ L)
Trial 1 (Full Larvae)	0.002 ± 0.004
Trial 2 (Full Larvae)	0.002 ± 0.004
Trial 3 (Crop Extracted)	0.018 ± 0.011
Trial 4 (Crop Extract + DNA concentrated)	0.035 ± 0.016

With a $p=0.000132$ and an $\alpha=0.05$, it can be stated that at least two trial mean quantitation values were different. Using Tukey's HSD Post-Hoc test, four significant differences between the means appeared between: Trial 1 and Trial 3 ($p=0.0481$), Trial 1 and Trial 4 ($p=0.0001$), Trial 2 and Trial 4 ($p=0.0002$), and Trial 3 and Trial 4 ($p=0.0404$). **Table 5** displays the results of ANOVA and The Tukey's HSD Post-hoc test.

Table 5. ANOVA summary table for the four trials ($N=20$) with the Tukey's HSD Post-hoc results. With $\alpha=0.05$ and $p=0.00132$, Tukey's HSD Post-Post hoc determined Trial 1v3, Trial 1v4, Trial 2v4, and Trial 3v4 each have significant differences.

ANOVA					
Source of Variation	d.f.	SS	MS	F	<i>p</i> -value
Between	3	0.0038	0.0013	13.257	0.000132
Within	16	0.0015	0.0001		
Total	19	0.0053			
Tukey's HSD		<i>p</i> -value			
Trial 1v2		0.9981			
Trial 1v3		0.0481			
Trial 1v4		0.0001			
Trial 2v3		0.0623			
Trial 2v4		0.0002			
Trial 3v4		0.0404			

3.3 Experimental Results

3.3.1 Weight, Length, and Quantitation of Experimental Samples

The weight, total length, and crop length of each larva were recorded and **Table 6** summarizes the averages and standard deviations for each liver as a whole. The lengths and weights were utilized as a rough estimate for developmental stage. Statistical difference was seen between Livers 1 and 2 ($p=1.18e-11$) and Livers 2 and 3 ($p=4.99e-12$) for weight, between Livers 1 and 2 ($p=2.24e-4$) and Livers 2 and 3 ($p=3.04e-7$) for total length and between Livers 1 and 2 ($p=1.38e-8$) and Livers 1 and 3 ($p=1.02e-4$) for crop length as summarized in **Table 7**.

Figure 3 shows the distributions of weights and lengths measured for each liver.

Table 6. Average weight, total length, and crop length with standard deviation of the larvae within each liver, $n=60$ for each liver with $N=180$.

Liver	Average Weight (g)	Average Total Length (mm)	Average Crop Length (mm)
L1	0.053 ± 0.007	13.1 ± 0.7	2.6 ± 0.3
L2	0.043 ± 0.007	12.2 ± 0.8	3.5 ± 0.4
L3	0.053 ± 0.007	13.5 ± 0.8	3.2 ± 0.5

Table 7. Two-tailed *t*-tests comparing the weights and lengths to see if there were any statistical differences between the collected samples between livers. Statistical differences were found between the weights for Liver 1 and 2 and Liver 2 and 3, between the total lengths for Liver 1 and 2 and Liver 2 and 3, and between the crop lengths for Liver 1 and 2 and Liver 1 and 3 when $\alpha=0.05$.

T-test Comparison	<i>p</i> -value (Weight)	<i>p</i> -value (Total Length)	<i>p</i> -value (Crop Length)
L1vL2	1.18e-11	2.24e-4	1.38e-8
L1vL3	0.87	0.06	1.02e-4
L2vL3	4.99e-21	3.04e-7	0.06

Figure 3. Histograms comparing the frequency distributions of the sizes and weights determined for the larvae. “W” indicates weights (top row), “T” indicates total lengths (middle row), and “C” indicates the crop lengths (bottom row). The numbers 1 (left column), 2 (middle column), and 3 (right column) indicate the associated measurements from that liver.

Based on the quantitation results (**Table 8**), the average quantity (as a concentration) of DNA in each larva was near zero. In a majority of the samples, the large autosomal target concentration was 0.00 ng/μL, which made calculating the degradation index value impossible, as dividing by zero produces an indeterminate solution (**Equation 1**, pg. 6). As seen, by the averages in **Table 8**, the concentration of DNA in the samples stayed around 0.02 ng/μL. In some instances, a large amount of DNA was shown as in the case of one of the samples in Liver 3 where the Y target concentration was 1.43 ng/μL. This could not have been from the liver itself as it was female and both the large and small autosomal target concentrations were around 0.01

ng/ μ L. The concentration ranges were 0.00-0.04 ng/ μ L, 0.00-0.21 ng/ μ L, and 0.00-0.31 ng/ μ L for the large autosomal, small autosomal, and Y targets of Liver 1 respectively. For Liver 2, the concentrations ranged between 0.00-0.05 ng/ μ L, 0.00-0.09 ng/ μ L, and 0.00-0.09 ng/ μ L for the large, small, and Y targets respectively. Finally, for Liver 3 the concentrations ranged between 0.00-0.02 ng/ μ L, 0.00-0.07 ng/ μ L, and 0.00-1.43 ng/ μ L for the large, small, and Y targets respectively. The maximum concentration values explain the high standard deviations seen in **Table 8**, as the higher values were primarily isolated to one sample.

Table 8. Average quantity (ng/ μ L) for each DNA Target using the Quantifiler® Trio Quantitation Kit (Applied Biosystems), $n=60$ for each liver with $N=180$. Small autosomal yields were used for capillary electrophoresis.

Liver	DNA Target	Average Concentration (ng/ μ L)
L1	Large Autosomal	0.00 ± 0.01
	Small Autosomal	0.02 ± 0.03
	Y	0.02 ± 0.05
L2	Large Autosomal	0.01 ± 0.01
	Small Autosomal	0.02 ± 0.02
	Y	0.01 ± 0.02
L3	Large Autosomal	0.00 ± 0.00
	Small Autosomal	0.01 ± 0.01
	Y	0.03 ± 0.09

3.3.2 Genotyping and Allele Call Proportion ANOVA Analysis

When the genotype profiles were observed in each, degradation was apparent due to a decrease in the peak heights for the larger STR loci. The average proportion of alleles called per preservation technique can be found in **Table 9**. Based on the table, most of the average proportions called were around 60%. The variance in the data was higher with many standard deviations sitting around 20%. A 60 second hot water kill with the addition of 70% ethanol had the highest proportion of alleles called at 80.5% of the alleles.

Table 9. Average proportion (as a percent) of alleles called per preservation method. Each preservation technique had $n=4$ samples, with $N=48$. Samples within a treatment group between livers were combined.

Preservation Method	Average Proportion Called	Average Proportion Called
2°C	47.5 ± 12.0	
70% Ethanol	65.3 ± 25.3	
Time (seconds)	Hot Water Kill	Hot Water Kill + Ethanol
30	37.8 ± 22.9	53.0 ± 20.5
60	63.5 ± 20.4	80.5 ± 18.6
75	37.0 ± 13.7	59.8 ± 15.1
90	63.5 ± 5.2	62.3 ± 28.5
300	62.5 ± 12.7	70.3 ± 19.8

Figure 4 is the genotype of the sample from Liver 3 that resulted in a full profile. This sample and one other from the same liver, 70% ethanol was used to preserve both the source larvae, were the only two samples to have all alleles called. There were four samples from Liver 2 with zero alleles called. Unlike Liver 1 and Liver 3, Liver 2 had a higher degree of degradation and allelic dropout within the profiles. The highest proportion of alleles called (44%) in a sample from Liver 2 was from a sample preserved by hot water kill for 300 seconds followed by the 70% ethanol treatment for 96 hours at 2°C. The average alleles called per liver were around 55%, 15%, and 60% for Liver 1, Liver 2, and Liver 3 respectively. **Figure 5** represents a partial profile from Liver 2 that had a proportion of alleles called just above the average for the liver at 17% of alleles. **Appendix B** shows the sample profiles with the highest, closest to average, and lowest proportion of alleles called for each liver.

Based on the first run through ANOVA, there was not enough evidence to reject the null hypothesis ($p=0.185$) meaning there was no statistical difference between the mean proportions of alleles called for the different preservation types between both Liver 1 and Liver 2. **Table 10** presents the summary statistics of the first ANOVA test. While higher averages were seen in the samples placed in ethanol following hot water kill the higher amounts were not statistically significant.

Table 10. ANOVA summary table with the preservation groups from each liver being observed separately. $n=2$ samples were seen in each group, with $N=48$ samples overall. With $p=0.185$ there is not enough evidence to reject the null hypothesis and it can be stated that there is no significant difference between the mean proportions of alleles called between the groups.

ANOVA					
Source of Variation	d.f.	SS	MS	F	p -value
Between	23	11,676	507.7	1.453	0.185
Within	24	8,388	349.5		
Total	47	20,064			

When the proportions from both livers were combined in their respective preservation groups, ANOVA indicated that through the combination, there was still no significant difference ($p=0.0866$) between the mean proportions of alleles called for the preservation types. **Table 11** displays the summary statistics from the second run of ANOVA.

Table 11. ANOVA summary table with the preservation groups from each liver being observed separately. $n=4$ samples were seen in each group, with $N=48$ samples overall. With $p=0.0866$ there is not enough evidence to reject the null hypothesis and it can be stated that there is no significant difference between the mean proportions of alleles called between the groups.

ANOVA					
Source of Variation	d.f.	SS	MS	F	p -value
Between	11	7,173	652.1	1.821	0.0866
Within	36	12,891	358.1		
Total	47	20,064

Figure 4. Genotype profile from Liver 3 sample that was hot water killed for 90 seconds then placed in 70% ethanol at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had a 100% proportion of alleles called compared to the reference profile obtained. The red line in the first panel indicates degradation through the negative slope.

Figure 5. Genotype profile from Liver 2 sample that was hot water killed for 60 seconds then placed in 70% ethanol for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and red. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had a 17% proportion of alleles called compared to the reference profile obtained.

Figure 6 displays the Q-Q plot, residual plot, and a box and whisker plot showing the mean and variance within each group when the proportions from both livers were combined. Based on the quantile-quantile (Q-Q plot) and the residual plots produced, both normality and homogeneity of variance was maintained.

Figure 6. Three plots used to help assess the assumptions of ANOVA with the proportions of alleles called from Liver 1 and Liver 3 combined for each group tested. **A.)** The Quantile-Quantile plot helps assess normality in the data and with most of the points close to the red line depicted normality can be assumed. There is a little deviation from the line at the top but this is not affecting the assumption of normality. **B.)** The residual plot helps assess the homogeneity of the variance. With the points showing no uniform shape and a random distribution across the plot, homogeneity is maintained. **C.)** A box and whisker plot showing the means and variances within the treatment groups. 2°C refers to the samples preserved at 2°C for 96 hours, E refers to samples preserved in 70% ethanol for 96 hours, and for the rest, the number in front indicates how long the samples spent in hot water kill with the H indicating just the use of hot water kill followed by storage at 2°C for 96 hours and HE indicating the storage in 70% ethanol for 96 hours following hot water kill.

3.3.3 Linear Regression of Proportion of Alleles Called Over Time

Using simple linear regression, the proportion of alleles called over the time spent in hot water kill was assessed for both samples just hot water killed and those samples hot water killed and placed in 70% ethanol for 96 hours. For the samples that were placed within hot water and preserved for 96 hours at 2°C in air, a linear model equation (**Equation 3**) was produced with an adjusted $R^2=0.012$. An F-statistic with 18 degrees of freedom was produced $F=1.238$ along with $p=0.2840$ to assess the significance of the linear model. With $p>\alpha=0.05$, the predictive power of time for the proportion of alleles called is insignificant.

$$y = 0.049x + 50.491 \quad (3)$$

The second linear regression run was on the samples that were placed in 70% ethanol at 2°C for 96 hours produced a linear model (**Equation 4**) with an adjusted $R^2 = -0.0337$. An F-statistic with 18 degrees of freedom was produced $F = 0.3798$ along with $p = 0.5454$ to assess the significance of the line produced. With $p > \alpha = 0.05$, the predictive power of time for the proportion of alleles called is insignificant

$$y = 0.030x + 61.768 \quad (4)$$

Figure 7 displays the data with the proposed linear regression lines with the red points and line indicating those of the hot water kill samples placed in air at 2°C for 96 hours and the blue points and line indicating those of the hot water kill samples placed in 70% ethanol at 2°C for 96 hours. As indicated by **Equation 3** and **Equation 4**, the samples treated with ethanol had a higher y-intercept while the samples not treated with ethanol had a higher slope. Again, looking at the p-values neither were significant, thus the two lines and the data are similar to one another and there is no significant difference between the use of ethanol after hot water kill.

Figure 7. Linear regression analysis was run on the data points of proportions of alleles called versus time. Samples that were only exposed to hot water kill (HWK) can be seen in the red points. The linear model for the data had an equation of $y = 0.049x + 50.491$ and an adjusted $R^2 = 0.012$, indicated by the red line. Samples that were treated with 70% ethanol following hot water kill (HWK + EtOH) can be seen in the blue points. The linear model for the data had an equation of $y = 0.030x + 61.768$ and an adjusted $R^2 = -0.0337$, indicated by the blue line.

Chapter 4: Discussion

With no statistical difference between the treatment groups, it can be stated that the length of time in the hot water kill between 30-300 seconds has no better or worse impact on the DNA recovery from contents within the crops of the fly larvae. The information provided helps support the hypothesis that the heat from the hot water kill does not have an increased degradation effect on the DNA contents within the crops versus the standard 70% ethanol preservation treatment. As the hot water bath temperature was set to 99°C, which is just below boiling at standard pressure, hot water kill temperatures up to this point can be safely used to preserve the DNA. The use of 70% ethanol following hot water kill as an additional step of preservation showed to not have an effect different than when ethanol was not used. While the average proportion of alleles called were higher, they were not significant and thus it would be recommended that a laboratory use the 70% ethanol after hot water kill, since it has not shown to affect mPMI information and since it helps keep the larvae in a preservation solution until they can be examined and as it is already considered a standard practice⁹. In addition, this would allow for the denaturing of nucleases that would keep the DNA within the crops from being degraded.

In all cases but two within this study, partial profiles were all that could be obtained. It is important to note that partial profiles are still useful by helping to support exclusion. In the cases where there may not be much more information based on the evidence, this can still supply useful information. While it was never explicitly stated whether the STR profiles obtained within the study were full or partial, the results from the study by Linville et al. (2004) were similar to those found in this study in regards to the use of 70% ethanol and temperature alone as the preservation techniques⁷. With both the 70% ethanol and near freezing temperatures used for preservation, there were instances where STR profiles were unable to be obtained⁷. In this study, of the 72 samples profiled, two samples had full profiles, 66 had a partial profile and four resulted in no profile at all. In addition, while two different techniques were used to assess the quantity of DNA between the studies (Quantiblot® in Linville et al. (2004)⁷ and Quantifiler® Trio in this study), the 2004 study showed higher concentrations of DNA. Furthermore, if the determination of a success in the Linville et al (2004)⁷ study was a full profile, this may have also been due to the fact that they used spleen tissue rather than liver tissue being that the two tissues undergo autolysis at different rates thus the liver having further DNA degradation than the spleen. Beyond the effect autolysis may have had, the Promega *GenePrint*® PowerPlex™ 1.2 system used in 2004 looked at 16 loci versus the 24 loci looked at with the Globalfiler® kit, which are the original 16 loci with an additional 8 new loci being observed⁷. The likelihood of obtaining the full profile from only 16 loci versus 24 is greater and thus could be the reason for a higher “success” of obtaining a full profile.

In regard to looking at the length of time spent in hot water kill and its effect on the profile, based on the linear model there appears to be no effect between 30-300 seconds in the hot water kill bath. The two slopes present were both positive, however only slightly. Again, the results of the linear models showed that in both cases time was not a statistically significant predictor of the proportion of alleles called in an STR profile. This is most likely due to the high variance in the proportions of alleles called at each time interval. Regardless of the fit of the regression model, it should be stated that whether at 30 seconds or 300 seconds, at least a partial

profile could still be obtained. The ability to obtain even a partial profile after 300 seconds means that if the larvae are placed in the hot water bath and forgotten about or larger specimens need longer time in the hot water, this will have no more adverse effects on the DNA inside larvae.

One source of the allelic dropout could be from the natural degradation of the samples used over time. The samples used in this study came from donated cadavers and were all in various stages of decomposition prior to the recovery of the livers being used for testing. This can be seen in the initial quantitation results from the reference sources. Liver 2 had much lower quantitation values compared to Liver 1 and Liver 3, which were both similar in DNA concentration. That means prior to the livers being re-exposed to a non-freezing temperature, there was already higher degradation in Liver 2. Thus when pulled out of freezing temperatures and into a warm environment the degradation of the DNA in that liver was already at a deficit compared to the other two livers.

It is probable that the quick autolysis or rapid-onset of postmortem cellular rupture, of liver tissue was a factor resulting in the low DNA yield from these subjects. Some degradation had likely already occurred to the liver samples with further degradation when the samples were removed from freezing temperatures. Rates of autolysis differed between organs in a study from 1911, when comparing the autolytic rates between the kidneys, liver, and pancreas, due to the high enzymatic levels in the pancreas and kidney, the rate of autolysis was quicker³⁶. While the pancreas had the highest rate of autolysis, the liver cells degraded faster than the kidney cells³⁶. It was noted that at 37°C, the liver cells that were not a part of the connective tissue had mostly broken down and the overall organ structure was in a state of necrosis by 72 hours³⁶. While the study from 1911 had a higher incubation temperature, the rate of autolysis can be explained as well as the reason that the DNA was still able to be obtained after further incubation at 25°C for 80 hours. While the liver goes through autolysis quickly, the use in this study helps present information in how quickly the DNA could be degraded in larvae found at a scene. In a chapter by Gill-King (1997)³⁷, Gill-King outlined the typical order of tissue decomposition. In this chapter the autolytic rate of the kidneys is typically slower than what was found in 1911. However, the liver was described in the group that autolyzed the quickest along with the stomach and pancreas because of the high hydrolytic enzyme activity within these tissues³⁷. According to Gill-King, skeletal muscle and connective tissues typically autolyze at the slowest rate³⁷.

A study from 2016³⁸ investigated the autolytic process in various organs that were refrigerated at 2°C³⁸. At temperatures just above freezing, autolysis still occurred within these organs, the liver included. While autolysis continued even at these cold temperatures, the rate was significantly lower than what was noted in 1911³⁸. Similar to the 1911 study it was found that the connective tissue of organs broke down slower than the rest of the organ's cells³⁸. In the liver it took up to 22 days for the full structure of the liver to be broken down, however by day 8 most of the blood vessels as well as hepatocytes began to lose their structure³⁸. With the results from this study, the degradation of any DNA and liver cells prior to the placement of the livers in the growth chamber can be explained. In addition, with the source of Liver 2 having the visual cues of being in the bloat stage at time of the liver retrieval further corroborates the amount of DNA seen within the reference samples. Each liver was in an unknown state of degradation prior to retrieval as it was not discussed how long the decedent had been deceased or how long the individual had been in and out of the refrigeration unit.

Looking at the average larval crop weights, total lengths, and crop lengths, the weights and lengths did not look different from one another. However, the t-tests showed statistical differences between the weights and lengths between livers. Looking specifically at the crop length, while Liver 2 had on the largest crops on average, the proportion of alleles within those samples were the closest to zero with an average proportion of alleles called around 14%. This contradicts the remarks made by Campobasso et al. (2005) that the larger crops lead to higher rates of profiling success³². While this in general may be true, it should be added that the degree of decomposition the substrate being fed upon is just as important of a factor as the size of the crop³². If the substrate is degraded, then it does not matter how large the crop may be.

One objective from this study was also to determine degradation through calculating a degradation index value (**Equation 1**) using the information gained through the use of the Quantifiler® Trio Quantitation kit¹⁹. Through the study it became apparent that this index should only be used as a guide. For a majority of the samples, the degradation index could not be calculated due to the large autosomal DNA having a concentration of 0.00 ng/uL. In the field when ground truth is not known this can be a useful tool; however, it has its limitations like those seen within this study with the absence of a concentration for the large autosomal target. Within this study, the extent of degradation was only apparent when the profile was viewed after capillary electrophoresis. The low quantitation values, in relation to the reference samples, helped indicate the extent of degradation. But if the reference samples were not available, the extent would have been hard to think through. In addition, with the reference samples, Liver 2 technically displayed “minimal to moderate” degradation based on the degradation index value. However, when compared to the other two livers, it was apparent that there was a greater deal of degradation in this sample by the quantitation values alone.

Beyond the results of the experimental sets, it should be stated that midway through the project a contamination event occurred, where male DNA was found within the profiles where a female was the donation source of the liver. Due to the low quality and low number of alleles present within the profiles it was not certain where the contamination came from. This prompted a restart of the experimental sets with new livers and a reworking of the methods. Because the Microcon® - 30k filters are labeled as “non-sterile”, the extra step of using 2 hours of UV radiation to decontaminate the filters was taken. The potential for contamination in the filters is not uncommon and have been reported in crime labs across the United States³⁹.

In addition, all distilled and nuclease free water was replaced with clean, uncontaminated water. Furthermore, disposable sleeve covers as well as a surgical mask was worn to ensure contamination did not occur. It should be noted that even with these extra precautions, there was still notable contamination in a few samples from the quantitation step but it was not as widespread as the first instance. The contamination appeared in the female liver samples with Y-DNA being quantified. In most of the cases the Y-DNA was minimal, however there was an instance where the instrument registered a quantity of 1.43 ng/μL. This was determined not to be true contamination since the small and large autosomal targets were around 0.01 and 0.02 ng/μL. Furthermore, contamination was not seen in the STR profiles after capillary electrophoresis. This meant that the sources were not contaminated, rather there was contamination just in the few samples within the quantitation process and could have been due to individual qPCR tubes not be decontaminated via UV radiation well enough, or even the investigator not being careful enough when pipetting sample and contaminating the reactions.

In future studies it may be beneficial to see if larvae feeding on different bodily structures plays a role in obtaining higher proportions of allele calls in the DNA profiles due to differential decomposition. By running these studies, practitioners can begin to understand how to optimize the recovery of viable evidence that can help in an investigation. As for the findings from this study, it can be deduced that there is no different effect on the DNA profiles from the use of hot water killing versus the standard 70% ethanol preservation and thus can be utilized in field-work to preserve both mPMI and DNA evidence. This can save on resources by having practitioners using one method of preservation versus two to obtain these two types of evidence.

Chapter 5: Conclusion

With the high expense that goes into STR profiling, it is important to understand the viability of testing certain pieces of evidence so that resources are not squandered when funding can be limited. The viability of STR typing the DNA within the crops of fly larvae after being preserved via hot water kill was assessed and determined to have statistically similar results as the standard 70% ethanol used in field work. It has also been discovered that even after 300 seconds in near boiling conditions, partial STR profiles can be obtained. The use of 70% ethanol as an added preservation method after the hot water kill, while having higher averages did not have statistically significant higher averages and would be up to a laboratories discretion on whether or not it would be used to further preserve the evidence. It should be noted that in most cases the profiles obtained were partial, however in a few instances full profiles were obtained and thus it is possible to obtain full profiles.

This information can be applied to field work and standard operating procedures can be modified such that only one method be used to preserve the mPMI and the DNA evidence versus the current two method standard of both hot water kill for mPMI and 70% ethanol for DNA. It is recommended that when preserving the larvae, the best route would be to use the hot water kill preservation followed by submersion in 70% ethanol. This would lead to the combination of the size and anatomical fixing effects of the hot water kill with the nuclease degrading effects of the ethanol. In addition to use in fieldwork and cases that may come in in the future, cases with entomological evidence already preserved using the hot water kill technique have the potential to be looked at with a new perspective. More specifically this information can help in cold cases and cases where a body may be missing.

Although the statistics in this may not have high power due to the low sample number, it should not be overlooked that both partial profiles and full profiles can be obtained. In the future it would be recommended to look at other soft tissue as a substrate that does not undergo autolysis as quickly as livers to see if this can increase the amount of quantifiable human DNA as well as decrease the allelic dropout occurring in the profiles.

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Appendix A

Figure 8. Genotype profile from Liver 1 reference sample used to base the proportions of alleles called. Five panels can be seen each with the loci along the top of the panels in green and red. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. A total of 41 alleles are present.

Figure 9. Genotype profile from Liver 2 reference sample used to base the proportions of alleles called. Five panels can be seen each with the loci along the top of the panels in green and red. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. A total of 39 alleles are present.

Figure 10. Genotype profile from Liver 3 reference sample used to base the proportions of alleles called. Five panels can be seen each with the loci along the top of the panels in green and red. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. A total of 41 alleles are present.

Appendix B

Figure 11. Genotype profile from Liver 1 sample that had the lowest proportion of alleles present. The sample was hot water killed for 75 seconds then stored in 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had 22% of the alleles called compared to the reference profile obtained.

Figure 12. Genotype profile from Liver 1 sample that had the closest to the average (55%) proportion of alleles present. The sample was stored at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had 56% of the alleles called compared to the reference profile obtained.

Figure 13. Genotype profile from Liver 1 sample that had the highest proportion of alleles present. The sample was hot water killed for 300 seconds then stored in 70% ethanol at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had 71% of the alleles called compared to the reference profile obtained. The sample preserved by 30 seconds in the hot water kill bath then stored at 70% ethanol at 2°C for 96 hours, had the exact same alleles called, just with varying peak heights for the alleles.

Figure 14. Genotype profile from Liver 2 sample that had the lowest proportion of alleles present. The sample was hot water killed for 90 seconds then stored at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Due to all peaks being below the analytical threshold of 250 RFU no alleles were called. This particular genotype profile had 0% of the alleles called compared to the reference profile obtained. Three other samples also had 0% of the alleles called from this liver: one stored at 2°C for 96 hours, one preserved in 70% ethanol at 2°C for 96 hours, and the last hot water killed for 300 seconds then stored at 2°C for 96 hours.

Figure 15. Genotype profile from Liver 2 sample that had the closest to the average (15%) proportion of alleles present. The sample was hot water killed for 30 seconds then stored at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had 14% of the alleles called compared to the reference profile obtained.

Figure 16. Genotype profile from Liver 2 sample that had the highest proportion of alleles present. The sample was hot water killed for 300 seconds then stored in 70% ethanol at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had 44% of the alleles called compared to the reference profile obtained.

Figure 17. Genotype profile from Liver 3 sample that had the lowest proportion of alleles present. The sample was hot water killed for 30 seconds then stored at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had 12% of the alleles called compared to the reference profile obtained.

Figure 18. Genotype profile from Liver 3 sample that had the closest to the average (62%) proportion of alleles present. The sample was hot water killed for 30 seconds then stored in 70% ethanol at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had 63% of the alleles called compared to the reference profile obtained.

Figure 19. Genotype profile from Liver 3 sample that had the highest proportion of alleles present. The sample was stored in 70% ethanol at 2°C for 96 hours. Five panels can be seen each with the loci along the top of the panels in green and yellow. Allele call boxes are found beneath the peaks with the allele call, peak height (RFU), and base pair size being expressed from top to bottom. An analytical threshold of 250 RFU was used. This particular genotype profile had 100% of the alleles called compared to the reference profile obtained. The other sample that gave 100% alleles called can be seen in **Figure 4**.