

**Entomotoxicological and Thermal Factors Affecting the Development of Forensically  
Important Flies**

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# Entomotoxicological and Thermal Factors Affecting the Development of Forensically Important Flies

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## Abstract

Studies were conducted on the effects of alcohol and opioids on the development of forensically important flies. In addition different methods of degree-day calculations and development thresholds were used to determine the effects on PMI estimates. The first study determined the effects of ethanol on the development of *Phormia regina* in vitro. Ground pork loin was treated with a 1, 5, or 10% ethanol solution to give an equivalent Blood Alcohol Concentration (BAC) of 0.01, 0.04, and 0.8 % w/v. A significant difference in the time for second instars' to complete the stage was seen between the 1% treated and control. Significant differences were also found among pupal and adult weights between all treatments and the control. A significant difference was shown between growth curves of the 5% treated and control for third instar larvae using the Kolmogorov-Smirnov test.

The ethanol content of *Phormia regina* in migrating third instar larvae that fed on treated meat was examined using headspace-gas chromatography (HSGC). All larvae had a content of 0.01% w/v, including the control.

The effects of ante-mortem injection of oxycodone in pigs were examined with respect to insect succession patterns and the development rates of *Phormia regina*. Pigs were given a subcutaneous injection of oxycodone hydrochloride (3 mg/kg by weight) and antemortem blood samples were collected prior to and following drug injection. Shortly after death the carcasses were placed at an open field site and allowed to decompose in a field cage. Insect samples were collected from carcasses for seven days post-mortem and the collected data were used to develop occurrence matrices. The Simple Matching Coefficient showed that successional patterns were similar between treated and untreated animals. Loin and liver from the carcasses were used as rearing media for in vitro development studies of *Phormia regina*. Kolmogorov-Smirnov test showed that third instar *P. regina* maggots from treated loin tissue were significantly longer in length than maggots feeding on untreated loin tissue. A significant difference in time was found

among larvae on loin for the time from eclosion to completion of the second instar. Significant differences were seen in the weight of adults reared on liver and loin. A chi-square for homogeneity showed that adults were biased towards males (2:1) from untreated loin tissue.

A final study compared weather data sources, Accumulated Degree-Day (ADD) methods, and postmortem interval (PMI) estimations based on threshold and developmental data source. Four pigs were used for statistical comparisons. Pigs were taken to a test site and allowed to decompose in an experimental cage. Probes recorded ambient temperatures and body temperatures. Maggot sampling was completed every day for each pig. A three way factorial linear fit model was used to test for statistical differences. Significant differences were seen in the calculated ADD based on probe location and the development threshold used. The ADD calculated from local weather station locations: Kentland Farm, Moore Farm, and Blacksburg Airport were also compared. A significant difference in ADD was found in the main effects among locations (Airport 44.1 ADD, Kentland 37.5 ADD, Moore 48.6 ADD), as well as among the thresholds used (10, 12.2, and 14°C). Different PMI estimations also resulted when using development data from different development studies on *Phormia regina*.

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## 1. Introduction

Man has been able to associate insects (mainly flies) with carrion (animal or human) for thousands of years. For example, in Homer's *The Illiad*, the character Achilles worries over the dead body of his friend, Patrokis, fearing that flies will "breed worms" and "make the body foul" (Homer, *The Illiad*, Book 19). The *Illiad* is commonly dated back to the 9<sup>th</sup> or 8<sup>th</sup> century B.C. The first reference to blowflies was produced more than 3600 years ago in the Har-ra-Hubulla, a collection of cuneiform writings on clay. It is the oldest known book in zoology and first mentions the "green" fly and the "blue" fly (Greenberg and Kunich 2002). Some 2500 years ago the Egyptians embalmed deceased individuals in order to protect organs from insects and decay. Maggots are even referenced in the Book of the Dead, Chapter 154: "That my body will not become prey to maggots" (Hutchet 1995).

It wasn't until till the 13<sup>th</sup> century that insects associated with a deceased body were used to solve a crime. In 1247, Sung Tz'u, published *The Washing Away of Wrongs*, a training manual for death scene investigators. In his manual, Sung Tz'u tells a story of a slashing that occurred in a rural village of farmers. The wound on the victim appeared to have been made by a sickle, so the investigator had the men of the village assemble in a line with their sickles lying on the ground before them. The guilty individual was identified when the investigator noticed a cluster of flies surrounding his sickle. Although the weapon had been wiped clean, there was enough blood residue left on the blade to attract dozens of flies, thus identifying the suspect (McKnight 1981).

Sung Tz'u was the first to mention a case involving forensic entomology. Forensic entomology involves the interaction of arthropods (mainly insects) with legal activity. Three principle areas of forensic entomology are currently recognized (Hall 2001): Stored products entomology is concerned with insect infestation or damage of commercial commodities. Urban entomology involves the effect of insects on man-made structures, such as bed bugs infesting hotel rooms. Both of these areas of forensic entomology deal primarily with civil cases. Medicocriminal forensic entomology is the third area and deals with the utility of arthropods in criminal investigations, particularly those involving wrongful or unexplained death. This specific area of forensic entomology is used synonymously with the phrase 'forensic entomology'.

The scope and applications of Medicocriminal Forensic Entomology are quite broad. A forensic entomologist can use arthropod populations associated with a decedent in a number of ways including cause and manner of death (includes detection of drugs/toxins), determination of death location, placement of body after death, and identification of wound sites. However, the most valuable use for entomological data is the estimation of the postmortem interval (PMI), or the time elapsed since death (Hall 2001).

In the first 72 hours after death, the pathologist is usually able to provide a reasonably accurate determination of the time of death. Historically, this has been based upon the condition of the body and such parameters as: lividity, rigor mortis, postmortem cooling, changes in the chemical constituents of body, autolysis of tissue, and decomposition due to bacterial activity in the body (Gennard 2007). The pathologist depends on rigor mortis, livor mortis, and core temperature of the corpse to derive acceptable estimates within 24 to 36 hours of death. Insects offer a much longer time scale measured in hours, days, weeks, or even years (Greenberg and Kunich 2002).

There are two primary ways to estimate the PMI of human remains using entomological evidence. Insect succession of arthropod species found on a body provide one method of determining the post mortem interval (PMI) (Schoenly and Reid, 1987). Insects arrive at decomposing remains in predictable, successive waves based on the stage of decomposition (Reed 1958, Payne 1965). The other method utilizes the degree of development of the oldest maggots feeding on the corpse, from which one can determine a close approximation of the time since death. Insects often lay eggs within minutes or hours after death (Catts and Goff 1992), thus providing a developmental reference.

However, there are controversies regarding the way forensic entomologists calculate the PMI using accumulated degree-hours (ADH). Some of these include whether to use the maggot mass temperature in ADH calculations, what are appropriate minimum developmental thresholds, the source of developmental data used in an estimation, and the method used to calculate degree-hours or degree-days. There are also questions as to whether some of these factors interact with each other such as minimum threshold and method used to determine degree-days. Higley et al. (1986) concluded that the source of temperature data used in degree-day calculations was likely the most significant source of error.

The use of drugs prior to death can result in an inaccurate estimation of PMI based on insect development (Goff et al. 1991). For example, Bourel et al. (1999) found that morphine can cause an underestimation of the PMI in *Lucilia sericata* by 24 hours. In another study, ethanol caused significant differences in maggot length for third instars feeding on treated meat (0.15 -0.17 % w/v) compared to an untreated control in field conditions (Tabor et al. 2005). The presence of ethanol in tissue of remains can affect the development of maggots and estimates of PMI, but it is uncertain at what concentrations that significant differences will appear.

Currently, there is only one study which tested for the effect of antemortem ingestion of ethanol on succession and maggot development (Tabor et al. 2005). Currently there are no studies which test for the effect of antemortem injection of oxycodone on succession or maggot development rates. There have only been a few recent papers that address controversies of determining accumulated degree-days (ADD) (Archer 2004, Nability et al. 2006, VanLaerhoven 2008). Therefore, the objectives of this research were: 1) To examine the effects of different concentrations of alcohol on maggot development of the black blowfly, *Phormia regina* (Meigen) under laboratory conditions; 2) To test the effect of antemortem injection of oxycodone on development and successional patterns of *Phormia regina* (Meigen); 3) To test and compare the accuracy of ADD methods, inclusion of maggot mass temperatures in PMI calculations, the effect of weather station location, and the use of different developmental data sources in determining PMI.

## **2. Literature Review**

### **2.1. PMI and Fly Development**

The Postmortem Interval (PMI), or time elapsed since death, provides an important piece of information in homicide investigations and untimely deaths (Byrd and Castner 2001). PMI can be determined by various methods including the physical and chemical changes of the corpse (Henssge et al. 1995), and the succession of arthropod species found on and within the body (Schoenly and Reid 1987). Development data for primary blow flies provide the most accurate means of estimating the PMI using arthropod information (Greenberg 1991). It is presumed that the first individuals that arrive at and lay eggs on a corpse do so soon after death (Catts and Goff 1992).

In order for insect development analyses to yield accurate PMI estimations, data must be available for the species of the earliest colonizers collected from the corpse at the time of discovery. The most relevant colonizers are the oldest individuals derived from the first eggs deposited on the body. Certain species can oviposit within a few hours following death and continue to do so for at least two weeks (Smeeton et al. 1984). It is customary for investigators to collect samples for rearing, as well as for preservation (Catts and Haskell 1990). A sub-sample of the eggs/maggots/pupae collected at the time of the corpse's discovery is reared in the laboratory until adult emergence after which they can be identified using keys for adult flies (Hall and Townsend, Jr. 1977, Smith 1986, McAlpine 1987).

Insect development is temperature dependent; meaning an increase in temperature (within limits) causes an increase in the rate of development. Duration of development is treated as a linear curve so that as temperatures increase within optimum ranges the duration decreases (Anderson 2000). Accumulated Degree-Days (ADD) or Accumulated Degree-Hours (ADH) are used in many forensic cases to determine insect age. In order to calculate the ADD the minimum and maximum temperature threshold for the fly species of interest must first be determined. The temperature below a species' minimum threshold is the temperature at which development stops while the maximum temperature is the temperature at which development slows down. The temperature threshold at which growth and development do not take place is known as the base temperature. The base temperature for a particular species is determined in the laboratory from the insect's growth rate at set temperatures. Developmental thresholds and stage-specific

accumulations for forensic species can be found in work by Kamal (1958), Nishida (1982), Nishida et al. (1986), Vinogradova and Marchenko (1984), Introna et al. (1989), Greenberg (1991), and Byrd and Butler (1996, 1997, 1998). The experimental temperatures are multiplied by the time, usually in hours, taken to reach the individual life stages. The base temperature must be subtracted from the temperature at which the specimens were grown, before multiplying this figure by the time taken to pass from the egg stage to the chosen life cycle stage. Once the species has been identified and the amount of energy to reach the life cycle stage recovered from the body, the temperature data from the death scene are used. Total accumulated degree-days, reflect the time taken for the insect to develop to the stage recovered from the death scene. The formula is:  $\text{Time}_{(\text{hours})} \times (\text{temperature} - \text{base temperature}) = \text{ADD}$ . The ADD or ADH for a duration of time is calculated by reverse summation (Anderson 2000). This technique has been used in several publications (Anderson 1997, Lord et al. 1994). The relationship between temperature and development as a linear relationship is valid as long as the data used to generate the degree days are close to the temperature at which the degree day information will be applied. If there is a large difference between temperatures, errors could result in the development time estimates. Higley et al. (1986) concluded that the source of temperature data used for degree-day calculations was likely the most significant single source of error. Therefore, data from the nearest recording site need to be directly compared to maximum and minimum temperatures from the death scene to allow for possible adjustment in the estimates (Byrd and Castner 2001).

Insect stages such as eclosion, pupation, and adult emergence do not occur at definite time intervals even at constant temperature, but over a range of times. Grassberger and Reiter (2001) for example, studied the effect of temperature on *Lucilia sericata* (= *Phaenicia sericata*) under ten different temperature regimes. Data from these studies were used to construct a graph from which maggot age could be predicted based on maggot length in cases where temperatures are fairly constant.

Insect development has been studied under various temperatures to determine development rates, thresholds, and effect on mortality. Kamal (1958) collected information on the life histories of thirteen species of sarcosaprophagous diptera. Kamal discovered that the controlled temperature of  $80^{\circ}\text{F} \pm 2^{\circ}\text{F}$  tended to shorten the life span of the adult compared to fluctuating conditions. The effect of temperature on the development of *C. macellaria* (Byrd and Butler 1996) and on *C. rufifacies* (Byrd and Butler 1997) was studied under various temperature

regimes. Development curves for the eggs, larvae, and pupae were developed under both cyclic and constant temperatures. Anderson (2000) obtained minimum and maximum development rates of five forensically important Calliphoridae species at several temperatures, including *Phormia regina*, *Lucilia sericata*, *Calliphora vicina*, *Eucalliphora latifrons*, and *Lucilia illustris*.

Development rates in some insects have been shown to be the same at fluctuating temperatures as at constant temperatures, while others have been shown to differ when development rates at fluctuating temperatures are compared to those at constant temperatures. In many insect species, developmental rates were shown to be the same at natural, fluctuating temperatures, as at constant temperatures, when the temperature range is suitable, and the constant temperatures represent the mean of the fluctuating temperatures (Butler Jr. and Lopez 1980, Champlain Jr. and Butler 1967). Some insect species were reported to develop more rapidly under fluctuating temperatures than under constant temperatures (Hagstrum and Leach 1972, Richards and Suanraksa 1962). Other studies report insects taking longer to develop under fluctuating temperatures than under constant temperatures (Greenberg 1991, Byrd and Allen 2001, Clarkson et al. 2004). Greenberg (1991) reported that, *P. sericata*, *P. regina*, *Chrysomya rufifacies* (Macquart), and *Cochliomyia macellaria* (F.) development was slightly longer at fluctuating temperatures than at a mean, constant temperature.

### **2.1.1. Methods for Determining ADH and ADD**

Generally, the cooler the temperature the slower the rate of growth and development of insects and plants. Accumulated degree-days (ADD) represent heat energy units available to an organism for growth. A “base temperature” represents the temperature at which the biological process of growth essentially stops. Forensic entomologists often use a base ranging from 10°C to 14°C when they determine ADD for *Phormia regina* (Deonier 1942, Haskell 1993, Nability 2006). The simplest method used to estimate the number of Degree Days for one day is the min-max method. Other methods include the use of trapezoidal approximations, the single sine, and the double sine. All of these methods are linear methods, because the rate of development is presumed to be linear with respect to temperature (Wilson and Barnett 1983).

Allen (1988) developed a method for finding the average of a nonlinear function of a distributed variable. He noted that an environmental variable such as temperature in a field varies from location to location, therefore organisms will experience highly variable conditions at a given time point. The developmental rate function of temperature may be known, but the

organisms may be found in a variety of temperatures. If the organism is exposed to a distribution of temperatures the developmental rate function that is based on a constant average temperature will be incorrect. Allen (1988) noted that for nonlinear functions it is not generally true that the average of a function is equal to the function of the average, which means the average of the developmental rate might not equal the average we calculate in a variable environment.

Kramer et al. (1991) noted a problem when using rate for parameter estimation instead of time in a least-squares estimations of nonlinear development models. When modeling insect development, one can use either time or rate. Kramer et al. (1991) noted that entomologists traditionally use rate. The purpose of Kramer's study was to show that in some cases parameters should be estimated using a time function rather than a rate function. They tested two models; the sigmoid model by Stinner et al. (1974) and the asymptotic expansion model by Logan et al. (1976). Kramer et al. (1991) noted that prediction in time helps at low-temperature values, but for high-temperature data, the technique is not useful.

Sharpe and DeMichelle (1977) formulated a biophysical model that described the nonlinear response in development rates at both high and low temperatures, as well as the linear response at intermediate temperatures. Wagner et al. (1984) reviewed the literature on the application of various insect development modeling techniques and concluded that the biophysical model of Sharpe and DeMichele was the most suitable. The work recognizes that insect development occurs within a definite range.

### **2.1.2. Factors that Influence Carrion Insect Development**

The body temperature of a corpse can fluctuate, and carcass temperatures can differ significantly from ambient temperature due to larval fly activity. In 1940, Deonier observed blowfly activities and took temperatures of animal carcasses in the Southwest United States. He found that blowfly activity was reduced by cool weather and that a carcass in the shade cooled to a temperature nearer to ambient air temperature than one left in the sun. Carcasses left in the sun had a higher temperature than that of the surrounding air. He also noted that both the temperatures maintained by maggot masses and heat absorbed by the carcass were able to maintain larval development, even if temperatures dropped below 50°F. On the other hand temperatures as much as 70°F higher than ambient temperature were recorded (Deonier 1940).

Maggot masses, especially masses comprised mostly of later instars, can affect the temperature the larvae experience. Greenberg (1990b) packed an adult human skull with 1400 g

of ground beef and retrieved it 24 hours later, with 10,000 eggs from a cage of *P. regina*. First instars had little measurable effect on temperature, but second instars began to produce excess heat which peaked at 18 degrees above ambient. The temperatures fall rapidly when postfeeding larvae disaggregate, especially in very dispersive species (Greenberg 1990b).

Ignoring internally generated temperatures in the calculation of development rates can result in the overestimation of PMI. Slone and Gruner (2007) quantitatively described heat generation in larval feeding aggregations. Eighty pig, *Sus scrofa* L., carcasses were placed out at study sites over a period of 2.5 years in central Florida and northwestern Indiana and were studied for the growth and development of carrion-feeding calliphorid larvae. The four most common species collected were *Lucilia coeruleiviridis* (Macquart), *Cochliomya macellaria* (F.), *Chrysomya rufifacies* (Macquart), and *Phormia regina* (Meigen). Internal and external temperatures were recorded daily between 1400 and 1800 hours, from when the carcass was placed until pupation of the larvae. The investigators found that larval aggregations on the carcasses that had a volume in the range of 16-25 cm<sup>3</sup> had afternoon temperatures that were fully independent of ambient temperature. Statistical analyses showed that the volume of the larval mass had a strong influence on its temperature. The internal temperatures of masses smaller than 20 cm<sup>3</sup> on the ground were influenced by soil temperature and mass volume. Larval masses larger than 20 cm<sup>3</sup> on the carcass had strongly regulated internal temperatures determined only by the volume of the mass, with larger volumes associated with higher temperatures. Slone and Gruner (2007) also determined that the presence of rain or clouds, shape of larval aggregation, weight of the carcass, species composition of the aggregation, time since death, or season were nonsignificant factors for larval aggregation temperature.

The density study of Slone and Gruner (2007) showed that some elevation of temperature occurs even at low population densities, thus further reducing the level of accuracy of ADH postmortem interval estimates in cases of light corpse infestation by maggots. An increased rate of development may relate to temperature increases associated with the maggot feeding masses at higher densities. Cianci and Sheldon (1990) found a sharp increase in maggot mass temperature with the appearance of third instar larvae on decomposing pigs. Thompson and Holling (1976) found that the activity of *P. regina* increased with increasing density. Advantages of aggregating in large numbers may include increased efficiency of food processing, gaining a competitive advantage over potential rivals, and a reduction in the

predation level. Green et al. (2002) also found that larvae reared in groups were less susceptible to toxic effects resulting from diet-borne metabolic inhibitors. Blowfly larvae produce tryptic and alkaline secretions and excretions (Hobson, 1932; Hanski, 1987) which pre-digest the substratum, with groups producing a greater volume of exudates than isolated larvae.

The photoperiod that larvae and pupae experience can also influence development time, which in turn could affect PMI estimates through degree-day based analysis. Temperature appears to have a greater effect on development than photoperiod, however constant light has been shown to increase variation in overall adult developmental time and significantly delay development compared with cyclic light (Nabity et al., 2007). Data suggest that lower temperatures might act in concert with light to alter physiological responses (Nabity et al., 2007). For example, Nabity et al. (2007), reared *Phormia regina* on different light and temperature regimes. The results showed that developmental times from egg to pupation and pupal duration were faster under cyclic light (12:12) around 20°C, but did not differ from development around 25°C with cyclic light (12:12). However, times from egg to adult and egg to pupation were faster under cyclic light (12:12) for both temperatures (20°C and 25°C) than for constant light (24:0). The knowledge that light-triggered stimuli cause developmental rates to slow under constant light may mean that developmental rates determined in other studies using constant light on *P. regina* (Greenbeg 1991, Anderson 2000) are too slow.

Development of carrion feeding fly larvae may be influenced by when individuals hatch and what intra and interspecific competition pressures are present in the environment at the time. Hutton and Wasti (1979) studied interspecific and intraspecific competition between *Phormia regina* and *Phaenicia (Lucilia) sericata* (Meig.). They found that intraspecific competition from high density levels produced lower pupal weights, pupation, and emergence rates when compared with controls. Crowding accelerated the speed of pupal development in both species. They also found that interspecific competition eliminated *Phormia* at all density levels tested. The only change for *Phaenicia* was an extension of average time for pupal development from 11.2 days to 13.3 days.

The availability of food also plays a role in maggot development. Goodbrod and Goff (1990) discovered that maximal weights and lengths of larvae were obtained in the cultures with 2 larvae/g liver, whereas minimal measurements were found in the cultures with 40 larvae/g liver for *C. megacephala*. They also found that growth rates increased with maggot density. They

noted that temperatures in all larval feeding masses were greater than the ambient temperature. In cultures of lower density (1 and 2 larvae/g liver), temperature differences were slight and showed no detectable pattern. At densities of 4 to 40 larvae/g, strong peaks in temperature were observed approximately 18 hours before maximum larval lengths were evident. A maximum temperature of 3.8°C above ambient was observed at 36 and 42 hours in culture with 40 larvae/g.

The larval substrate can affect development time, as well as fecundity for the following generation. Green et al. (2003) reared *Phormia regina* larvae, in isolation, upon either lamb's liver or meridic diets that varied in protein- and carbohydrate-content. An artificial diet containing 53% protein (by mass of dry ingredients) resulted in the heaviest *P. regina* pupae and shortest development time when compared with larvae reared upon lamb's liver and all other diets. As protein in a diet increased with a constant level of carbohydrate (17%), there was an increase in pupal weights and a decrease in development time of isolated larvae. Insufficient dietary nutrients cause some larvae of *P. regina* to extend their larval stage in an attempt to obtain more nutrients. If *P. regina* larvae are unable to obtain sufficient nutrients they can still pupate, but develop into smaller, less fecund adults compared to those that had sufficient protein when developing. Stoffolano, Jr. et al. (1995) found that sugar-fed males inseminate a low percentage of females compared with liver-fed males. If liver-deprived males are kept with liver-fed females for 24 hours, males are able to obtain their dietary requirement for mating by feeding on female feces or vomit spots. Stoffolano, Jr. et al. (2000) also found that when small males mated with large females the percentage of successfully inseminated females was significantly reduced. Measurement of aedeagus size and head width showed that in wild flies the size of the aedeagus is positively correlated with body size.

## **2.2. *Phormia regina***

Adults are approximately 7-9 mm long and are identified readily by their dark-green, shiny body and a distinctive yellow-orange anterior spiracle (Byrd and Castner 2001). This species is most active during the spring and fall and is more abundant during cool weather (Byrd and Castner 2001). The adult female deposits her eggs during the early stages of decomposition and the larvae subsequently develop in the carrion. Crystal (1983) found that female *Phormia regina* did not mate until three days postemergence. *P. regina* also tend to pupate on or close to the food source (Norris 1959, Greenberg 1990b).

Byrd and Allen (2001) found that at 30°C, *Phormia* produced first instar larvae at 12 hours, second instar at 22 hours, third instar at 50 hours and pupae at 150 hours. At 25 degrees °C the first instar was produced at 18 hours, second instar at 26 hours, third instar at 58 hours, and pupae at 134 hours. At 20 degrees the first instar was produced at 19 hours, second instar at 36 hours, third instar at 62 hours, and pupal stage at 188 hours. Post feeding larvae and pupae occupy approximately 75% of total preadult time (Greenberg 1991). Although most blow flies are active in daylight, oviposition by *P. regina* (Greenberg 1990a) can occur at night and in dark places during the day.

Stoffolono, Jr. et al. (1995) determined if faeces feeding can support *Phormia regina* nutritionally for reproduction. They found that females fed a sugar diet were unable to develop follicles beyond stage three, whereas liver proved to be the best diet for complete egg maturation. However, females could still develop eggs if fed on various types of faeces. Since most faeces are low in protein, females may require an increased feeding exposure to produce mature eggs. Males in general do not need protein for spermatogenesis, but protein is essential for development of accessory reproductive glands, an increased level of mating activity, and the ability to inseminate females. *Phormia* probably aggregate on dung for feeding and mating, but do not use it for an oviposition substrate. Males virtually cease to feed on protein after an initial intake, whereas females continue to ingest protein throughout their lives, increasing intake during each ovarian cycle as eggs develop, and decreasing intake while carrying mature eggs. Belzer (1978) has reported that egg development depletes protein reserves and has suggested that depletion of reserves provides the signal for ingestion. The ability to learn appears to be very restricted in *Phormia regina*, and it is unlikely that they are capable of learning to return to a particular source of food (Nelson, 1971).

### **2.3. Succession**

A body will progress through a sequence of decompositional stages over time. During this decomposition, it goes through recognizable physical, biological, and chemical changes (Henssge et al., 1995, Van den Oever, 1976). Each of these stages of decomposition is attractive to a different group of insect arthropods. Bornemissza (1957) characterized the succession of organisms on guinea pig carcasses in the Mediterranean-type environment of western Australia. Five stages of decomposition were identified (initial decay, putrefaction, black putrefaction, butyric fermentation, and dry decay) and these correlated with the fauna present. Some insects

use the corpse as a food or oviposition site, whereas other species are attracted by the large aggregation of other insects that they use as a food resource (Byrd and Castner, 2001).

Studies on the successional patterns of arthropods have been conducted throughout the world and in the United States in climatically different areas using various animal models. Gruner et al. (2007) used pig carcasses (*Sus scrofa* L.) as models to determine the relative abundance and seasonality of forensically important blow flies in north-central Florida. They found that species composition in aerial collections of adult flies, preserved larval collections, and samples of larvae reared to the adult stage were all highly correlated. Calliphorid seasonality was characterized by a predominance of *L. coeruleiviridis* year-round. The relative abundance of the collected species varied significantly by day of decomposition, by season and day, season and species, and day and species. *L. coeruleiviridis*, *C. macellaria*, *C. rufifaces*, and *P. regina* were found during the entire year, two *C. vicina* specimens and 11 *C. livida*, specimens were collected from December to March, whereas *C. mecacephala* was collected from June through September.

Joy et al. (2002) used raccoon carcasses to study succession at a southwestern West Virginia site in May of 2000. One raccoon was placed in a sunlit field plot while another carcass was placed in a shaded forest plot on May 16, 2000. Carcasses at both plots were examined every 3 h after initial placement over a 153 h experimental period. A sample of 30-40 larvae was collected whenever possible at every 3-h interval. Samples indicated that *Phormia regina* was the dominant species with large numbers of third instars observed at every 3-h collection period from 81 to 153 h on both carcasses. *Sarcophaga* sp. larvae were also collected, but only in samples taken from the sunlit carcass at 81 and 93 hours. Overall, species composition was not significantly different between the two carcasses; however *P. regina* larvae at the sunlit carcass had greater mean larval lengths for their third-instar, meaning they grew faster than the larvae on the shaded carcass.

Reed (1958) conducted a comprehensive year-round study of insects associated with dog carcasses in the state of Tennessee. Forty-three carcasses were distributed among wooded and non-wooded areas and placed at study sites at different times throughout the year. Reed grouped arthropods according to the stage of decomposition during which they were found most frequently. Reed defined the stage of decomposition. Arthropods in the *Fresh stage* were primarily of Muscidae and Calliphoridae, those in the *Bloated stage*, included families Silphidae,

Histeridae, Staphylinidae, Muscidae, Calliphoridae, and Sarcophagidae; those in the *Decay stage* included families such as Silphidae, Histeridae, Piophilidae, Sepsidae, and Phoridae; and those in the *Dry stage* include the families of Cleridae, Dermestidae, and Trogidae. The insect taxonomic groups were shown to vary seasonally, as well as in different field types, i.e. wooded vs. non-wooded.

Anderson and VanLaerhoven (1996) conducted succession studies in British Columbia, Canada using pig carcasses. A database of insect succession patterns over time in an open, sunlit, rural area in summer was developed. The authors noted that some species of insects were collected earlier in the decomposition process than what is usually reported in other regions. It was also found that soil fauna changed considerably in identity and the number of species observed compared to before the studies.

Rodriguez and Bass (1983) collected successional data on human cadavers in a research facility in Knoxville, Tennessee. The cadavers were placed outside during different times of year and daily observations were made. The data showed that there was a direct correlation between the rate of decay and the succession of families and species found in association with the remains. Four separate stages of decay were also described for un-embalmed, uncovered cadavers: fresh, bloated, decay, and dry (Rodriguez and Bass 1983).

The covering of bodies may affect the insect colonization pattern of the remains. In Hawaii, a young female victim was found heavily wrapped in blankets in a rural, outdoor habitat. In order to determine the possible delay of insect colonization due to the wrapping an experiment was needed. Goff experimentally wrapped a fresh pig carcass in a similar manner and observed how long it took for insects to begin to colonize the remains (Goff, 1992). Insects were first seen on the pig carcass 2.5 days after death, indicating a probable delay in colonization of 2.5 days in the human case (Goff, 1992).

Numerous other succession studies have been performed in a variety of locales throughout the world. Sukontason et al. (2003) conducted studies in Thailand, as did Lane (1975) in England, Bharti and Sing (2003) in India, Archer and Elgar (2003) in Australia, Centeno et al. (2002) in Argentina, Bourel et al. (1999a) in France, Schroeder et al. (2003) in Germany, Martinez et al. (2006) in Colombia, and Eberhardt and Elliot (2008) in New Zealand.

Succession studies need to be conducted in as many diverse geographic areas as possible, since there can be differences in insect fauna between geographic areas. In some cases, the

insect fauna in a specific area can change over time. For example, the black soldier fly, *Hermetia illucens* L., was found for the first time in Italy as necrophagous in 1997 (Turchetto et al. 2001).

Currently there is only one published article on forensic succession data for Southwest Virginia. Tabor et al. (2004) studied carrion-insect succession on domestic pig, *Sus scrofa* L., in the spring and summer of 2001 and 2002 in Blacksburg, VA. Investigators collected forty-seven insect taxa in the spring. Eleven families were represented (Diptera: Calliphoridae, Sarcophagidae, Muscidae, Sepsidae, Piophilidae; Coleoptera: Staphylinidae, Silphidae, Cleridae, Trogidae, Dermestidae, Histeridae). Thirty-three taxa were collected in the summer during the two years of study. The most common flies collected were: *Phormia regina* (Meigen) and *Lucilia (Phaenicia) coeruleiviridis* (Macquart). The most common beetles were *Creophilus maxillosus* L. (Staphylinidae), *Oiceoptoma noveboracense* Forster, *Necrophila americana* L., *Necrodes surinamensis* (F.) (Silphidae), *Euspilotus assimilis* (Paykull), and *Hister abbreviatus* F. (Histeridae). Statistical analyses revealed the patterns of succession to be similar between spring 2001 and 2002 and between summer 2001 and 2002. The data now provide the baseline fauna for estimating postmortem interval in cases of human death for the area.

In the future, the genetic differences among insect populations may be used in new forensic applications. Byrne et al. (1995) studied the cuticular hydrocarbons among three populations of *Phormia regina*. Discriminant analysis of the cuticular hydrocarbon profiles separated the flies according both to location and gender. This method has the possibility of being used for the determination of corpse relocation and the study of population ecology.

## **2.4. Entomotoxicology**

Forensic toxicologists qualitatively and quantitatively identify drugs and poisons which may be relevant to cause and manner of death. In most cases, toxicological specimens are collected at autopsy. Alternatively, if a body is badly decomposed, bone, hair, and insect larvae and pupae are collected and analyzed.

The use of insects and insect remnants as toxicological specimens is well documented. Insect tissue or remnants (pupal cases, frass, etc.) can be used to identify drugs and toxins present in decomposing tissues. Literature to date has cited the use of arthropods as an alternative toxicological source since 1980.

Beyer was one of the first to use maggots to qualitatively assess drug presence in a suspected suicide case. A body of a 22-year old female was found skeletonized except for the skin. Larvae were collected and homogenized with the proteins precipitated out of solution. Gas chromatography was used to identify a phenobarbital concentration of 100 µg/g in larval tissue. The larvae were identified as *Cochliomyia macellaria* (Beyer et al. 1980).

Levine et al. (2000) described a case in which an unidentified male was found by a river and was decomposed and skeletonized. An empty bottle of secobarbital was found near the body. Calf muscle and maggots were sent for toxicological analysis. No substances or drugs were detected in the calf muscle, but secobarbital was identified in the maggots by electron ionization gas chromatography/mass spectrometry.

In a similar study, Wilson et al. (1993) reared *Calliphora vicina* on human skeletal muscle from suicidal overdose victims of co-proxamol (propoxyphene and acetaminophen) and amitriptyline. Third instars were transferred to drug-free muscle or allowed to feed on drug-laden muscle for two more days prior to harvesting. The drug concentrations in the muscle food source were 0.48 µg/g amitriptyline, 0.38 µg/g nortriptyline, 0.99 µg/g propoxyphene, and 14.3 µg/g acetaminophen. The mean ratios of drug concentrations in larvae to the food source were 0.5, amitriptyline; 0.5, nortriptyline; and 0.06 for propoxyphene. In all stages no drugs or metabolites were detected in puparia, pupal cases, or adults (Wilson et al, 1993).

Malathion, an organophosphate insecticide, was identified in larvae found on a decedent thought to be a suicide victim. Malathion was detected at a concentration of 2,050 µg/g of larvae in specimens collected from the decomposing remains. Malathion exhibits low toxicity in mammals, yet a high toxicity to adult insects. In this case, the maggots were developing normally despite concentrations of malathion that were toxic to rats and adult species of blowfly (Gunatilake and Goff 1989). This case illustrates the importance of studying the effects many drugs and toxins have on insect species since drugs and toxins may affect insect development, mortality rates, and PMI.

The effects of various drugs and toxins to carrion-feeding insects have been investigated, but this area of study is still expanding. Tracqui et al. (2004) examined 29 necropsies in which various organic compounds (including benzodiazepines, barbiturates, antidepressants, phenothiazine, opiates, cannabinoids, meprobamate, digoxin, and nefopam) were detected in arthropod larvae sampled from human corpses. Larvae were collected from multiple sites on the

cadaver, weighed, washed, and dried. The larvae were mechanically homogenized and then extracted using solid or liquid phase extraction procedures. Sample extracts were then analyzed by gas or liquid-chromatography. The results indicated that the concentrations of the drugs in insect tissues tended to be lower than those of cadaveric samples, and that concentrations varied between anatomic sites (i.e. within anatomic sites when larvae were grouped according to their site of sampling). Tracqui et al. (2004) also found only weak correlations between the concentrations of drugs in biofluids at the time of death and those in the larvae sampled from the cadaver at a later time.

Goff has conducted a number of entomotoxicology experiments with various drugs (Goff et al. 1989, Goff et al. 1991, Goff et al. 1992, Goff et al. 1993, Goff et al. 1994). When Goff did his studies he administered the drug to a living animal. He did this so that known and reproducible concentrations of drugs and metabolites in animal tissue could be used to approximate amounts normally encountered in human fatal overdoses.

Hédouin et al. (1999) established concentrations of morphine in an animal model before rearing larvae on tissues. Morphine, a metabolite of heroin, was injected intravenously into rabbits. The kinetics of morphine elimination from blood after a single intravenous injection of morphine and the concentrations of morphine in tissues following a continuous perfusion were established. Morphine concentrations were determined using radioimmunoassay techniques. The rabbits that received a single injection received 2 mg/kg of morphine hydrochloride. Three rabbits received 2 mg/kg of body weight of morphine hydrochloride per hour for a period of 3 h using a continuous perfusion through a plastic catheter in the ear. Results from the continuous perfusion showed that the concentrations of morphine differed according to the organ analyzed, but were reproducible for organs between animals. This study permitted known and reproducible concentrations of morphine in the rabbit to be used as a substrate for rearing of larvae in entomological studies.

Goff used rabbits in his entomotoxicological studies of cocaine and heroin on *Boettcherisca peregrina* (Goff et al. 1989, Goff et al. 1991). The rabbits in the heroin study were given 6, 12, 18, and 24 mg of heroin by cardiac puncture. *Boettcherisca peregrina* were allowed to feed and develop on liver tissue containing heroin. From hours 18 to 96, larvae feeding on liver tissue containing heroin developed more rapidly than those feeding on the liver from the control. Time required for pupation was also greater for larvae that fed on tissue from heroin-

dosed rabbits than for the control larvae. The rates of development were sufficient to alter PMI estimates based on larval development by up to 29 hours (Goff et al. 1991).

In a similar study, three domestic rabbits received dosages of 35, 69, and 137 mg cocaine in 5 mL saline via cardiac puncture in the cocaine study. The dosages represent one-half the LD<sub>50</sub>, the normal LD<sub>50</sub>, and twice the LD<sub>50</sub>. *Boettcherisca peregrina* were allowed to feed and develop on tissues containing cocaine. From hours 30 to 70, larvae developed more rapidly on tissue containing cocaine from rabbits injected with 69 mg and 137 mg of cocaine than on tissue from rabbits injected with 35 mg of cocaine or no cocaine. Total development times required for pupation and adult eclosion were also shortened. Differences between larvae developing on cocaine-dosed rabbit tissue compared to a control were sufficient to alter PMI estimates based on larval development in decomposing human tissues by up to 24 h (Goff et al. 1989). Goff's results indicate that an opiate (e.g., heroin) and a stimulant drug (e.g., cocaine) can both increase the rate of development in the *Boettcherisca peregrina* (Goff et al. 1989, Goff et al. 1991).

Bourel et al. (1999b) administered morphine chlorhydrate to three rabbits each at a different concentration. The three concentrations were 12.5, 25.0 and 50.0 mg/h of morphine chlorhydrate via ear perfusion. A fourth rabbit was used as a control. Following administration of the drug, rabbits were sacrificed and 400 eggs of *Lucilia sericata* were placed in the eyes, nostrils, and mouth of each rabbit. Larvae were sampled daily to determine growth rate and weight. Puparia and emerging adults were also sampled. In this study, the larvae reared on the control and the rabbits that received 12.5 and 25 mg/h of morphine developed at similar rates from hours 41 to 69, while larvae reared on the carcass given 50.0 mg/h of morphine developed at a slower rate. From hour 91 to 165, the larvae from carcasses that received 12.5 and 50.0 mg/h developed at the same rate, which was slower than the control colony. Overall, the effects of morphine appear to be dose dependent as the larvae feeding on the rabbit that received the greatest dosage were the slowest to develop. Based on results from this study, between hours 91 and 165 estimations of larval age based on total length can be significantly in error if the presence of morphine in tissues is not considered. The error can be as great as 24 h for *Lucilia sericata* larvae measuring from 8 to 14 mm total length.

In another case Bourel et al. (2001) used approximately 100 larvae of *L. sericata* reared on seven 250 g portions of minced beef combined with morphine hydrochloride solutions. After egg hatch, 10 specimens of second instar, third instar, post-feeding third instar and pupae were

sampled and immediately frozen. After adults emerged, they were kept in a jar until they died and desiccated. Samples were homogenized, centrifuged, and the supernatant analyzed for morphine content using a specific radioimmunoassay. Concentrations of morphine were high in second and third instar larvae, almost proportional to concentrations in minced meat, but almost no morphine was detected in pupae. The results indicate that larvae excrete the drug during the post-feeding stage. A quantity of morphine is sequestered in the cuticle of pupae, but at minute concentrations. Morphine is sequestered in the cuticle during larval growth and in the formation of puparia (Bourel et al. 2001).

Elimination of drugs or toxins prior to metamorphosis has been shown in other studies. Sadler et al. (1995) was able to detect trimipramine, trazodone, and temazepam, in the larvae of *Calliphora vicina*, but was unable to detect the drugs in the pupae. The fact that drugs do not bioaccumulate throughout the life of the larvae suggests that elimination mechanisms are present. Drug concentrations decreased when larvae were taken from drug laden meat and placed on drug free meat. The results of these studies indicate the importance of collecting larvae for toxicological analysis from those feeding actively on a corpse.

Introna et al. (1990) reared *Calliphora vicina* larvae on liver specimens from 40 cases in which cause of death had been determined to be opiate intoxication. Analysis of larvae and liver for opiates (morphine) was accomplished by radioimmunoassay. The concentration of opiates for all cases was found to range from 8 to 1,208 µg/kg for larvae and 26 to 1,769 µg/kg for the liver specimens. A significant difference was found between the opiate liver and larval concentrations.

Goff and Lord (1994) reviewed various studies in entomotoxicology and concluded that entomotoxicological testing was essential to accurate forensic entomology conclusions. Data indicating the presence of drugs allow for corrections to the data in cases when drugs affect insect development.

#### **2.4.1. Oxycodone (OxyContin®)**

OxyContin® is a synthetic, time-release pain medication containing oxycodone HCl, which is an opioid. It is manufactured by Purdue Pharma and was approved by the Food and Drug Administration on December 12, 1995 to aid cancer patients and people with moderate to severe pain (Congress 2002). Developed in a German lab in 1916, oxycodone is a synthetic opiate and chemically resembles other opium derivatives such as heroin, morphine, codeine,

fentanyl, and methadone (Tough 2001). Opioid agonists are substances that act by binding to specific opioid receptors, mu ( $\mu$ ) receptors, which are found in the brain, spinal cord, and gastrointestinal tract (Kalb 2001). When the drug interacts with opioid receptors in the brain and spinal cord the mechanism of action is to effectively block the transmission of pain messages to the brain (e.g., the perception of pain to the subject). The drug-receptor interactions modulate analgesia or relief from pain (Congress 2002).

Oxycodone is a semisynthetic narcotic analgesic, which is derived from thebaine, that is available in oral formulations. The usual adult dose is 2.5-5 mg as the hydrochloride salt every six hours (Baselt, 1982). Recently controlled-release (CR) oxycodone has been developed with the aim of providing analgesia in a similar manner to normal-release (NR) oxycodone, but with improved compliance as it needs to be taken less frequently (Davis et al. 2003). Absorption of NR oxycodone is mono-exponential. The time to maximum serum concentrations (T-max) is 1 h, and the mean half-life by single dose pharmacokinetics is 3.5-5.65 hours (Leow et al. 1992). Oxycodone is subject to hepatic first-pass effects and is known to be metabolized by N- and O-demethylation in the liver by the cytochrome P450 enzyme, CYP2D6. One of the metabolites, oxymorphone, is a potent narcotic analgesic, while the other, noroxycodone, is relatively inactive. Oxymorphone accounts for only 10% of oxycodone metabolites (Heiskanen et al. 1998). Oxycodone is capable of producing stupor, coma, muscle flaccidity, severe respiratory depression, hypotension, and cardiac arrest in overdosage. Naloxone is a specific antidote.

OxyContin<sup>®</sup> is a Schedule II narcotic. Drugs covered under the Federal Controlled Substances Act are divided into five schedules. Schedules are indicated by roman numerals, with Schedule I having the strictest control and Schedule V having the least control. Schedule II drugs have a high potential for abuse and have a currently accepted medical use in treatment in the United States or a currently accepted medical use with severe restrictions. The Federal Controlled Substances Act requires a written prescription for the dispensing of substances listed in Schedule II. Such prescriptions may not be refilled.

The only active ingredient of OxyContin<sup>®</sup> is oxycodone. OxyContin<sup>®</sup> was marketed in 1996 as a drug that provided long lasting relief, but not as addictive as other opioids. Oxycontin<sup>®</sup> has been proven effective in chronic, severe cancer pain, and is superior to morphine because of lessened side effects (Mucci-LoRusso 1998). Purdue Pharma marketed the drug aggressively to physicians at pain management seminars. These seminars advocated use of

powerful, long-acting narcotics such as oxycodone hydrochloride in pain treatment (Charatan 2001). In fact, when OxyContin<sup>®</sup> was first approved, the FDA believed that the controlled-release characteristics of the drug formulation would result in less abuse potential since the drug would be absorbed slowly and there would not be an immediate high that would promote abuse. (Meyer 2004). However, if OxyContin<sup>®</sup> tablets are broken, the time release mechanism of the drug is disabled, leading an abuser to a euphoric, heroin-like high. OxyContin<sup>®</sup>, in a crushed form, and if taken with alcohol or other drugs, is extremely dangerous due to the synergistic effects between the two drugs (Cone et al. 2004). Prolonged use of these drugs eventually changes the brain in fundamental and long-lasting ways, making it difficult for users to quit (Congress 2002).

OxyContin<sup>®</sup> is a major prescription drug of abuse. Generally, the more the drug is prescribed the greater numbers of cases of abuse. The medical use of opioids within the United States increased 400% from 1996 through 2000 (Varga et al. 2001). Control-released oxycodone's market share grew from 10% in 1996 to 53% in 2000 (Davis et al. 2003). Abuse of Oxycontin<sup>®</sup> is for the most part, concentrated in small- to medium sized urban, suburban, and rural areas (Cicero et al. 2007). Abuse of OxyContin<sup>®</sup> in rural Maine, Kentucky, Virginia, and West Virginia brought national attention to this problem. Areas most currently affected by abuse are eastern Kentucky; New Orleans, Louisiana; southern Maine; Philadelphia and southwestern Pennsylvania; southwestern Virginia; Cincinnati, Ohio; and Phoenix, Arizona (Microgram 2002). A variety of illegal techniques are used to obtain the drug because it requires a prescription. Pharmacy diversion, dishonest physicians, "doctor shopping", fraudulent prescriptions, and robbery contribute to the drug being diverted to the illicit market (Microgram 2002). The popularity of the drug makes it tempting for people to sell their prescription pills. A 30-day prescription of 40 mg CR oxycodone costs Medicaid patients from \$1 dollar to \$35 in co-payments; however, one tablet will sell for \$40 illegally (Rosenberg 2001).

OxyContin<sup>®</sup> abuse has led to overdoses and death. In October 2001, a Drug Enforcement Agency report on autopsy data revealed evidence to suggest OxyContin's<sup>®</sup> role in the overdose deaths of 282 people over a 19-month period. In Virginia in 2001 it was estimated that there had been at least 55 deaths linked to OxyContin<sup>®</sup> (Congress 2002). The Office of the Chief Medical Examiner (OCME) for the Western Region of Virginia certified 519 drug deaths from 1997 to 2001. Thirty-four percent (n=175) of the certifications identified oxycodone (n=82) or

methadone (n=93) as being significant in the cause of death (Behonick et al. 2003). Oxycodone, the sole active ingredient of OxyContin<sup>®</sup>, accounted for one-third of all narcotic analgesics reported by forensic laboratories during January 2003 to June 2003 (Department of Justice 2003).

Legislation has been enacted to combat prescription drug abuse. In July 2001, Oxycontin<sup>®</sup> was required to carry the “black box” warning, the strongest warning for an FDA approved product. Purdue Pharma was also cited in May 2000 and January 2003 for advertisements that promoted OxyContin<sup>®</sup> in a manner that was false or misleading (Meyer 2004). In February 2001, the Virginia General Assembly established a joint subcommittee to investigate the improper prescription and illegal use and diversion of Ritalin<sup>®</sup> and Oxycontin<sup>®</sup>. The abuse of Oxycontin<sup>®</sup> also caused Virginia to eventually enact a Prescription Drug Monitoring Program in 2003 that became statewide in 2006. In 2007, Purdue Pharma executives plead guilty to misleading the public about OxyContin’s<sup>®</sup> risk of addiction. The company paid \$634.5 million dollars in fines for claiming the drug was less addictive and less subject to abuse than other pain medications (Lindsey 2007).

Methods for determining the presence of opiates have been developed and applied to medico-legal cases. One criminal case involved a 10-month-old male who went into cardiac arrest and was identified as having been murdered. The mother admitted to taking 30 mg of oxycodone tablets and 350 mg of carisoprodol tablets the day prior to her son’s death. Oxycodone extraction was performed on tissue and blood of the infant and analyzed by gas chromatography utilizing a nitrogen phosphorous detector. Oxycodone was detected in the alkaline drug screen of the liver and was confirmed by full scan electron ionization gas chromatography-mass spectrometry. The liver oxycodone concentration was 1.6 mg/kg. Investigators initially believed the infant was exposed to oxycodone via the mother’s breast milk. No other drugs were detected. In adults, blood oxycodone concentrations ranging from 0.4-2.7 mg/L have been reported in cases of oxycodone intoxication (Baselt 2002). The evidence in this case suggested that the infant was most likely not exposed to the high concentrations of oxycodone from the mother’s breast milk, but that it was purposefully administered (Levine et al., 2004).

Spiller (2003) analyzed death records from ten counties in Tennessee to determine postmortem oxycodone or hydrocodone concentrations in postmortem toxicology reports. All

postmortem blood concentrations were confirmed by gas chromatography, but no metabolites were reported. The mean and median postmortem oxycodone concentrations in blood were 1.23 mg/L and 0.43 mg/L respectively. The range was 0.12 to 1.6 mg/L, with 11 cases less than or equal to 0.5 mg/L. Spiller believed that both unintentional and intentional deaths involving oxycodone and hydrocodone may be more common than previously documented. Many of these cases were reported before OxyContin<sup>®</sup> was released and marketed.

Spiehler et al. (2004) studied the accuracy of screening postmortem whole blood for oxycodone using the ratio of the oxycodone immunoassay response to the response for the specimen obtained with a general opiate-class immunoassay. The Neogen<sup>™</sup> Oxymorphone/Oxycodone ELISA and the Neogen<sup>™</sup> Opiate Group ELISA were used. The oxycodone equivalents in ng/mL from the Oxymorphone/Oxycodone ELISA were divided by the morphine equivalents in ng/mL from the Opiates ELISA to obtain an Oxycodone/Opiates Response Ratio. This ratio was compared with GC/MS data for all specimens for opiate positive samples. It was found that specimens containing oxycodone produced a large response in the oxycodone-directed immunoassay and a positive, but weaker response in the general Opiate Group Immunoassay. The sensitivity of the ELISA response ratio for the presence of oxycodone at a response ratio cutoff of 2.0 was  $89.4\% \pm 3.8\%$  and the specificity was  $88.1\% \pm 3.2\%$ . Spiehler et al. (2004) concluded that by using relative response ratios, the Neogen<sup>™</sup> Oxymorphone/Oxycodone ELISA can be used as a second immunoassay to identify which opiate-positive specimens should be confirmed for oxycodone. Oxycodone specific ELISA kits are available from Venture Labs, Inc., Redwood City, CA. The sensitivity of the Venture Labs<sup>™</sup> Oxycodone assay is 2 ng/mL. This represents the lowest concentration of drug that can be distinguished from 0 ng/mL with a confidence level of 95%. The assay can quantitatively assess the amount of oxycodone in a sample, if the amount of oxycodone is between 0 ng/mL to 80 ng/mL.

Ishida et al. (1982) determined oxycodone metabolites excreted in urine and feces of mammalian species. Seven metabolites including oxycodone and oxymorphone were found in the urine of rabbits in both free and conjugated forms. Japanese male white rabbits weighing 2-3.5 kg were used in the study. Animals received subcutaneous injections of H-oxycodone hydrochloride (10 mg/kg as free base) dissolved in saline.

The effect of opiates on arthropods has been documented in years past (Kavaliers et al. 1987, Dyakonova et al. 2002, Bourel et al. 1999b). A recent search of the literature found no articles related to the effects of the narcotic, oxycodone, on arthropods, particularly those of forensic significance.

#### **2.4.2. Ethanol**

Ethanol, or ethyl alcohol, is the alcohol contained in beverages such as wine, beer, and spirits. It is also termed drinking alcohol or grain alcohol. Fermentation of sugars by yeast is the oldest synthetic organic chemical produced by man (Morrison and Boyd 1983). Alcohol is euphorogenic and a central nervous system (CNS) and respiratory depressant; it also induces tolerance, and physical dependence (addiction) (Garriot 1996).

Ethanol has high water miscibility. It passes through the intestinal mucosa and enters the blood quickly. Some alcohol is absorbed in the stomach, but the major site of absorption is the small intestine. After it is absorbed, it is distributed uniformly throughout the body into various organs in proportion to the water content of each. Ethanol can be excreted into the urine unchanged, exhaled from the lungs, and excreted without metabolism through the skin. The liver metabolizes ninety percent of the ethanol absorbed by the body (Fenton 2002).

Ethanol may be detected in the bodies of persons who have died regardless of the cause, but has its highest incidences in deaths from violent circumstances (Norton et al. 1982, Baselt and Cravey 1980, Garriott 1993). Ethanol is also one of the leading causes of death by poisoning (Garriott et al. 1982, Taylor and Hudson 1977, Caplan et al. 1985). Detecting alcohol in the tissues can provide important information involving the circumstances of an individual's death. Most often, ethanol in biological fluids is tested using a gas chromatographic method; either by solvent extraction, protein precipitation, distillation, direct injection, or head space techniques (Jain and Cravey 1972, Gudzinowicz and Gudzinowicz 1977, Wright 1991, Tagliaro et al. 1992).

It has been documented that microorganisms can cause an increase in the postmortem production of alcohol. Studies with rodents have shown that ethanol is produced by microorganisms during the putrefaction process (Davis et al. 1972, Iribe et al. 1974). Davis et al. (1972) studied ethanol formation in the bodies of intact conventional and germ free mice and in stored tissues. All groups were stored at 22°C in sterile, aerated, and humidified containers. Three mice were analyzed on subsequent days to determine the alcohol concentrations of the

various tissues. Davis et al. (1972) discovered that significant amounts of ethanol are detectable in as little as 48 hours in the conventional mice. There was no alcohol formation in the germ free mice. Iribe et al. (1974) also studied the neof ormation of alcohol in decomposing mice. The authors studied the formation of ethanol in mice that had been drowned and stored in water and a second group that had been strangled and allowed to decompose in air. Significant amounts of ethanol were detected in both groups with a maximal concentration of approximately 160 mg/dL reached in 8-10 days when the temperature was 25°C (Iribe et al. 1974).

Gilliland and Bost (1993) did a retrospective study in which medical examiner cases were evaluated to determine the source of ethanol production and to analyze the distribution of ethanol in tissues. Of the 286 cases examined, 55 had endogenous ethanol production, 130 had ingested ethanol, and 62 had ethanol from undetermined sources. The highest blood alcohol concentration derived from endogenous ethanol production was 0.07% in the cases with other fluids being negative. The mean blood alcohol concentration was 0.06% and ranged as high as 0.16% in cases having atypical ratios. Atypical ratios occurred when alcohol was found in blood and bile while urine and vitreous fluid were negative, or had lower concentrations in cases with endogenous production. The criteria for determining endogenous production included the presence of alcohol in blood, but not in other fluids, atypical distribution of ethanol among the fluids, and the presence of a higher alcohol (2-propanol, isopropyl alcohol, etc.)

In order to distinguish between antemortem and postmortem production, vitreous humor is analyzed for the presence of alcohol. A delay occurs in the uptake and removal of alcohol in the vitreous in comparison to blood. Sturner and Coumbis (1966), for example, showed there was an average vitreous-blood ratio of 1.1 to 1.0.

In cases where vitreous fluids, other bodily fluids, or tissue are not present or too badly decomposed, the determination of antemortem ingestion of alcohol can be difficult. If antemortem ingestion of alcohol has occurred it could affect the development of insects that colonize and feed on the body. Ethanol caused significant differences in maggot length for third instars feeding on treated meat (0.15 -0.17 % w/v) compared to an untreated control in field conditions (Tabor et al. 2005). Tabor et al. (2005) also looked at changes in succession with an ethanol dosed pig and a control pig and found no differences in successional patterns. The presence of ethanol in tissue remains can affect the development of maggots and therefore estimates of PMI, but it is uncertain at what concentration significant differences will occur.

There is also very little information concerning concentrations of ethanol in larvae that feed on ethanol treated meat.

### **3. Effect of Ethanol Treated Tissue on the Development of *Phormia regina* In vitro**

#### **3.1. Introduction**

Use of insects and insect remnants in order to identify drugs and toxins present in cadaver tissues led to the field of entomotoxicology (Introna et al. 2001). Carrion feeding insects and insect remnants (e.g. frass, puparial cases, and exuviae) can sometimes be used as sample specimens for toxicological examination when traditional specimens such as blood, urine, or muscle tissue are not available (Beyer et al. 1980, Kintz et al. 1990, Levine et al. 2000).

Another important area of entomotoxicology is the investigation of the effects of drugs and toxins on arthropod development (Goff and Lord 1994). Studies show that antemortem use of various drugs and toxins can affect maggot development rates, resulting in inaccurate estimations of postmortem intervals (PMI) based on insect development (Goff et al. 1992, Bourel et al. 1999b). Drugs such as phenobarbital, cocaine, and heroin have been detected in carrion feeding insects and insect remnants. Data are sparse on the detectability of numerous other drugs, including ethanol. There are few data on the effects of many drugs and toxins on carrion insect development. For example, we do not know at what concentration ethanol alters behavior and development of *Phormia regina*, a forensically important fly. A thorough search of the literature found only one study on the effect of antemortem ethanol ingestion on the development and successional pattern of this carrion insect (Tabor et al. 2005).

The purpose of this study was to determine how different levels of ethanol affect the development of flies. Pork loin tissue was the substrate of choice for the study because loin tissue of the domestic pig, *Sus scrofa* L., was used in a similar study involving ethanol (Tabor et al. 2005). The effects of the presence of various concentrations of ethanol in pork loin tissue on maggot development were examined under laboratory conditions for the black blow fly, *Phormia regina* (Meigen).

#### **3.2. Materials and Methods**

Studies were conducted in Blacksburg, VA, from 2005-2006 to examine the effects of varying concentrations of ethanol treated meat on the development of *Phormia regina*. Three developmental studies were conducted. The first was conducted from 7 May to 31 August 2005

(Study 1), the second was conducted from 8 May to 29 July 2006 (Study 2), and the third, 23 May to 5 August 2007 (Study 3).

*Phormia regina* (Meigen) adults were collected from pig carrion at a farm in southwest Virginia (Montgomery County) before the start of each study. Adults were given sugar and water that were held in separate containers (sugar was in a Petri dish while water was in another small container, with a dental wick inserted through a hole in the lid) for maintaining the population. The adult flies in Study 1 were given pork loin for three days prior to the start of a trial. Adult flies in Study 2 and Study 3 were given access to dry powdered milk, sugar, and water *ad libitum*.

Distilled water and three solutions of ethanol (1%, 5%, and 10%) were used to treat the ground pork loin for the three studies. The ethanol concentrations were chosen to achieve concentrations (% w/v) that Tabor et al. (2005) did not evaluate in their study (0.15- 0.17% w/v of ethanol in loin tissue). One hundred and eighty grams of ground pork loin was placed in a 32 oz Ziploc<sup>®</sup> container (S.C. Johnson & Son, Inc., Racine, WI). Distilled water or an ethanol solution was poured on the meat until it was covered. Each container was kneaded and hand mixed. After the meat was mixed, additional liquid was poured over the meat again to cover as needed. The Ziploc<sup>®</sup> containers were covered with a lid and placed in a refrigerator. After a twenty-four hour period, excess solution was poured off the meat and 180 g of tissue was divided among three plastic cups containing 60 g each in Study 1. One hundred and eighty g of tissue was divided among two plastic cups containing 90 g of tissue each in Study 2 and Study 3. The plastic cups were labeled and covered with aluminum foil. Three replicates were used for each ethanol concentration or control in Study 1 and two replicates per ethanol concentration or control for Study 2 and Study 3.

To determine ethanol concentrations for the meat in each cup, two g of meat were taken from each cup and placed into a clean vial with six mL of distilled water to form an aqueous solution. Each sample was analyzed using a head-space gas chromatograph (HSGC). The HSGC consisted of an HP 7694 HS Sampler configured to an Agilent GC-6890 Plus with a flame ionization detector (Agilent Technologies, Palo Alto, CA). The column was a Restek Rtx-BAC1, and n-propanol was used as the internal standard. The GC cycle was set to four minutes for each sample. The oven was set at 70°C, the sample valve at 85°C, and the transfer line at 95°C. The volatility of ethanol relative to the aqueous biological specimen is used to separate

the volatile from the matrix. At a given temperature, the amount of volatile in the air space above the liquid, i.e. the headspace, is proportional to the concentration of the volatile liquid in the solution.

Untreated pork loin tissue was used as a substrate for oviposition from *Phormia* in the lab colony. Clusters of eggs weighing approximately 0.02 g (approximately 150 eggs) were placed on the tissue for each cup in a replication. Aluminum foil was used to cover each cup.

Eggs were observed hourly to determine the initial hatch time ( $t = 0$ ) for each cluster (cup). Egg hatch ranged from 1.5 hours to 14 hours after eggs were placed on the meat, however the cups remained covered with aluminum foil until eclosion. At the onset of egg hatch, the cups were transferred to a round glass battery jar (153 mm diameter x 204 mm depth) with an inch of sand lining the bottom. The aluminum foil was removed from each rearing cup and a plastic Saran™ Quick Covers™ lid (S.C. Johnson & Son, Inc., Racine, WI) was placed over the jar. The plastic cover had holes to allow oxygen transfer. The containers (jars) with rearing cups were placed in an incubator at 23°C under constant light.

Maggot samples were collected every eight hours until the post feeding stage, beginning at the onset of the earliest egg hatch among cups (egg clusters) in Study 1. Maggot samples were also collected every eight hours in Study 2 and Study 3, but sampling continued until pupation. A sample consisted of four randomly selected maggots per cup for a total of 12 maggots per cup per day. The maggots were placed directly into KAA fixative (100 mL 95% ethanol, 20 mL glacial acetic acid, 10 mL kerosene) (Haskell 1990) until one hour before the next sampling time. The fixative caused the maggots to fully extend so that they could be measured to the nearest 0.25 mm. Length and instar (as determined by the number of spiracular slits) were recorded for each maggot. Maggot sampling continued until the meat substrate was exhausted or until 90% of the individuals had migrated off the meat to pupate.

Ten migrating third instars were harvested, cleaned, and frozen from each container in order to determine ethanol content for Study 1. The ten maggots were later dried superficially using paper towels and weighed. The maggots were then punctured with a scalpel, placed in a clean vial, and then ground with a pestle in 0.5 mL of deionized water. Alcohol content of the larvae was determined by Head-space Gas Chromatography (HSGC), as described previously.

In Study 2 and Study 3, pupae were removed as they appeared and placed in separately labeled 4 oz GladWare® containers (The Glad Products Company, Oakland, CA). The incubator

was checked every eight hours to determine adult emergence from pupae. The adults that emerged were placed into a mason jar with tissue paper moistened in ethyl acetate. The adults were then sexed and weighed. The number of flies, their sex, and weight was determined for each container at every sampling time. A time to complete the pupal stage was determined for all adult flies that emerged. The Spearman's Rank Correlation Coefficient was used to test the hypothesis that there was a correlation between sex and emergence time. GraphPad Prism<sup>®</sup> (GraphPad Software, San Diego, CA) was used to statistically analyze the pupal and adult weight data, as well as the sex ratio data.

Sample controls were also used to determine amounts of ethanol lost to evaporation during the test period (Figure 3.1). Two containers of each ethanol concentration and control were prepared according to the methods described earlier. One meat sample was taken from each container for a five day period. Ethanol concentrations were determined from samples utilizing the procedure described previously.

The range and mean for time from egg eclosion to completion of larval and pupal stages for *Phormia regina* was calculated by using a matrix. The sampling interval composed the horizontal axis of the matrix while the vertical axis was labeled in eight hour increments that were broken down into 0.25 hour units. A time period was highlighted with a color that designated the instar that was collected during a sampling period from time zero. For the treatments and control, every stage except for the adult stage had a minimum and maximum time to complete the stage. The time periods between each minimum and maximum were used to calculate the mean time for each development stage for the control and treated larvae.

For analysis of the development data for *P. regina*, we plotted the maggot length versus time for maggots feeding on tissue that was ethanol-treated or untreated. A second order polynomial was fitted to each of the data sets by using a trendline. Statistical differences ( $\alpha = 0.05$ ) between the distribution functions for maggot length for individuals that fed on loin tissue that was ethanol-treated or untreated were determined separately for each of the three instars and for all instars combined using a Kolmogorov-Smirnov test (Conover 1999). The Kolmogorov-Smirnov compares the fitted curves of the control to the fitted curves of the treatments to determine if there is a difference in larval lengths due to the presence of ethanol. The Kolmogorov-Smirnov test was performed by entering data in a KS-test program (<http://www.physics.csbsju.edu/stats/KS-test.html>). The null hypothesis that treated and

untreated curves were the same was rejected if  $P < 0.05$ . A One-way ANOVA ( $\alpha = 0.05$ ) was performed on the fitted data for each instar to test for similarity in development of maggots feeding on meat from treated and untreated loin tissue.

### 3.3. Results

Ethanol treated pork loin tissue had detectable concentrations of ethanol in the samples. The concentrations ranged from 0.01 to 0.11% (% wt by vol). Ethanol was also detected in post feeding third instars (Table 3.1).

The development of *P. regina* maggots feeding on tissue from the three ethanol treated and the untreated pork loin under laboratory conditions is shown in Table 3.2. Mean ( $\pm$  SD) development time to reach the pupal stage required by *P. regina* maggots feeding on untreated, 1%, 5%, and 10% treated tissues were  $167.1 \pm 16.1$ ,  $155.6 \pm 10.8$ ,  $158.3 \pm 18.0$ , and  $157.5 \pm 12.3$  h respectively. Mean ( $\pm$  SD) times to complete the pupal stage by *P. regina* maggots feeding on untreated, 1%, 5%, and 10% treated tissues were  $158.9 \pm 8.9$ ,  $158.4 \pm 9.0$ ,  $155.8 \pm 11.2$ , and  $157.4 \pm 15.9$  h respectively.

A One-way ANOVA was computed on the controls and treatments of each development stage followed by Dunnett's Multiple Comparison test. A significant difference was found among second instars' time to complete that stage ( $F(3,98) = 6.13$ ,  $P < 0.05$ ). Dunnett's Multiple Comparison test revealed there was only a significant time difference between the 1% treated and control for second instars,  $P < 0.05$ . There were no other significant differences between the means of the controls and treatments for length of time to complete the first, third, and pupal stages.

Pupal and adult weights of *P. regina* maggots feeding on ethanol-treated and untreated tissue in laboratory conditions are illustrated in Figures 3.2 and 3.3. A One-way ANOVA was computed on the controls and treatments for pupal and adult weights followed by Dunnett's Multiple Comparison test. A significant difference was found among the pupal weights ( $F(3,786) = 3.57$ ,  $P < 0.05$ ). Dunnett's Multiple Comparison test revealed a significant difference between the control and all treatment concentrations for pupal weights,  $P < 0.05$ . A significant difference was also found among adult weights ( $F(3, 917) = 15.37$ ,  $P < 0.05$ ). Dunnett's Multiple Comparison test also revealed a significant difference between the control and all treatment concentrations for adult weights,  $P < 0.05$ .

Growth under in vitro conditions for *P. regina* larvae feeding on treated and untreated loin tissue in both studies is illustrated in Figure 3.4. The second order polynomial trendlines for larvae are presented in Figure 3.5. A significant difference between growth curves was found at  $\alpha = 0.05$  using the Kolmogorov-Smirnov test in development of third instars on loin tissue between the 5% treated and control group, however, no differences were detected in first and second instars between the control and 5% treated ( $D = 0.1607$ ,  $P = 0.021$ ). Significant differences were also seen at  $\alpha = 0.05$  between the third instars of 5% and 10% treated ( $D = 0.1728$ ,  $P = 0.011$ ). However, there was no significant difference in the development of third instars between the 10% treated and the control, nor between the 1% treated and the 5% treated. There were also no significant differences between first and second instars between the controls and any of the treatments. The ANOVA did not find a significant difference between the growth of any instars feeding on treated or untreated tissue.

The calculation of chi-square (by Yates' correction) for homogeneity in the sex ratio for the control and all of the treatments shows that the flies that emerged were not biased towards one sex in any of the treatments ( $X^2 = 0.24$  for control, 0.51 for 1%, 0.67 for 5%, and 0.58 for 10%,  $df = 1$ ,  $P < 0.05$ ). Figure 3.6 shows the percentage of males and females that emerged from the control and treatment groups. There was no correlation between sex and emergence time for the control or any of the treatments according to the hypothesis testing with Spearman's Rank Correlation Coefficient ( $r_s = 0.3245$  for control males, 0.3664 for control females)(5% level).

### 3.4. Discussion

In development-based PMI estimation, it is assumed that insects feeding on carrion will develop at predictable rates dependent on environmental conditions. Tabor et al. (2005) demonstrated that maggots feeding on tissue from pigs that had been dosed with ethanol antemortem (between 0.15 or 0.17 % w/v) required  $\approx 11.9$  h more, on average, to reach the pupal stage than maggots feeding on tissue from nontreated animals. The current study showed that maggots feeding on untreated pork loin tissue did not significantly differ in the average time to reach the pupal stage than the maggots that fed on the ethanol treated tissue. The data suggest that ethanol does not increase the amount of time in the larval stage unless there are concentrations of ethanol in the muscle tissue higher than 0.10% w/v (i.e. 0.15 and 0.17% the concentrations in Tabor et al. 2005). The presence of ethanol in the meat may have an effect on

maggot feeding behavior at particular levels. The ethanol may act as a feeding deterrent until sufficient levels of ethanol have evaporated, after which the larvae resume normal feeding.

The time required by *P. regina* maggots feeding on loin tissue from untreated pigs to reach the pupal stage was shorter than has been recorded in other studies. For example, Anderson (2000) found that maggots took 217-268 h to reach the pupal stage at a constant temperature of 23°C, which was the temperature of the incubator during this study. Byrd and Allen (2001) found that maggots required an average of 359 and 320 h to reach the pupal stage at constant temperatures of 20 and 25°C, respectively. In this study it took a maximum of 197 hours to reach the pupal stage in the control pigs.

Maggots feeding on untreated pork loin and reared under fluctuating temperatures in the field took less time to reach the pupal stage than the maggots in this study, 133.6 hours compared to 167.1 (Tabor et al. 2005). Tabor et al. (2005) also found that the mean ( $\pm$  SD) duration of the pupal stage for *P. regina* that fed on tissue from treated and untreated pigs was  $147.0 \pm 11.5$  and  $143.7 \pm 7.9$  hours, respectively. In this study, the duration to complete the pupal stage was  $157.4 \pm 15.9$  for 10% treated and  $158.9 \pm 8.9$  hours for the control.

A possible explanation for the difference in time between Tabor et al.'s (2005) study and this study is due to the photoperiod used in each study. The average temperature of exposure for the time to reach pupation was 23°C in Tabor's (Tabor 2004 dissertation) study. Tabor's experiment was a field study, therefore the larvae were reared under cyclic light. The studies in this experiment were done under 24:0 (L:D) h. Nabity et al. (2007) found that developmental times from egg to pupation and pupal duration were faster under cyclic light around 20°C, but did not differ at 25°C. Developmental times from egg to adult and egg to pupation were faster under cyclic lighting for both temperatures than constant lighting. Constant light increased variation in overall adult developmental time and significantly delayed development compared with cyclic light. Therefore, developmental rates of *Phormia regina* determined by those who used constant light (Greenberg 1991, Anderson 2000) may be too slow.

Nabity et al.'s (2007) paper was published after this experiment was conducted. The ethanol study was conducted with constant light and temperature based on information from previous experiments. Other studies have been conducted in constant light and temperatures (Byrd and Allen 2001). The temperature was set at 23°C because it was the average temperature larvae experienced by *Phormia regina* larvae in the Tabor et al. ethanol study (2005). Other

studies have reported insects taking longer to develop under fluctuating temperatures than under constant temperatures (Greenberg 1991, Byrd and Allen 2001, Clarkson et al. 2004). Greenberg (1991) reported that, *P. sericata*, *P. regina*, *Chrysomya rufifacies* (Macquart), and *Cochliomyia macellaria* (F.) development was slightly longer at fluctuating temperatures than at a mean, constant temperature. Since it had been reported that *Phormia regina* took longer at fluctuating temperature the incubator was set at a constant temperature for this study.

Detectable amounts of ethanol were found in the migrating third instar maggots that were collected and used as a toxicological sample. The concentrations of ethanol in the maggots were lower than the amount in the tissues on which the maggots were feeding. Lower concentrations were expected as most of the alcohol evaporated from the tissue sample. Tracqui et al. (2004) found that there was no strong relationship between the concentrations of drugs in the biofluids at the time of death and those of the larvae sampled sometime later on the cadaver. In cases of alcohol related deaths, the blood-ethanol concentration at autopsy is a conservative estimate of the amount of alcohol the person had actually consumed due to metabolism of alcohol prior to death (Marcinkowsky and Przyblyski 1974). No reported amounts of ethanol in maggots have been found in literature to date.

The evaporation controls showed that there were detectable amounts of alcohol for up to four days in treated tissue (Figure 3.1). After this time alcohol could not be detected in samples of the media that lacked maggots. The evaporation (0.0%) control showed no detectable amounts of alcohol until the fifth day (sample). The control (0.0%) sample on the fifth day showed an amount of 0.01% ethanol. This indicates that endogenous production of ethanol by microbial synthesis occurred in this evaporation (0.0%) control (Corry 1978). The control larvae that were tested for ethanol content also averaged levels of 0.01% ethanol (Table 3.1). The data suggest that the control larvae are exposed to some ethanol that is produced by microbial synthesis.

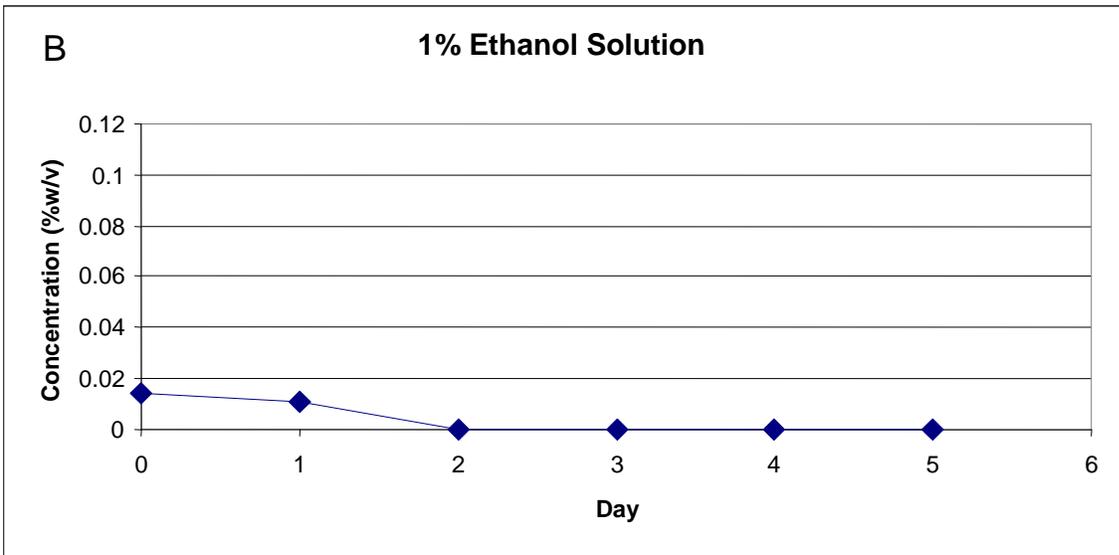
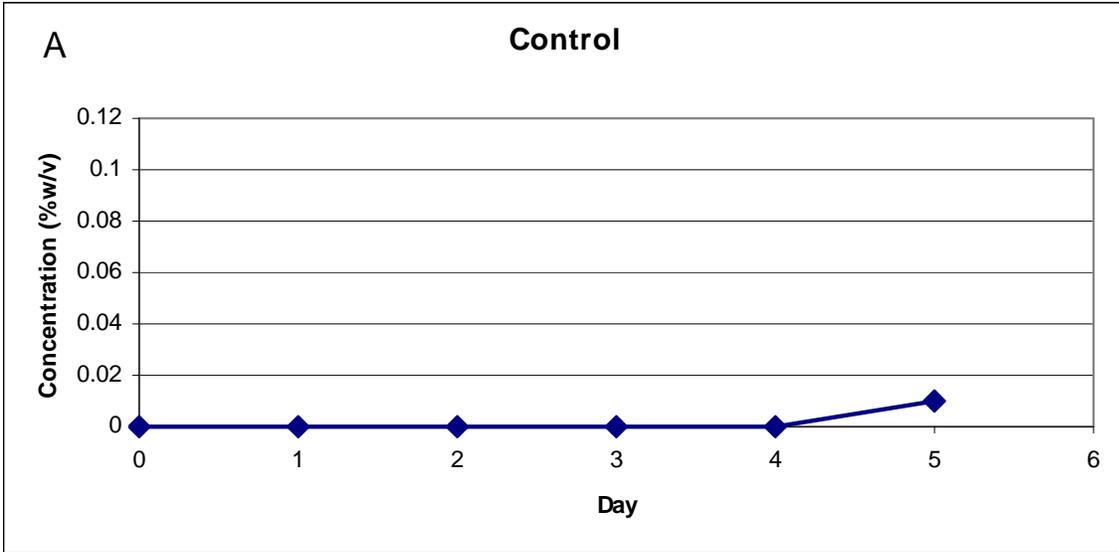
The concentrations of alcohol may have been sufficiently high initially to alter behavior of the maggots. The 1% and 5% treatments had a shorter mean time from eclosion to the end of the second instar life stage when compared to the control, however only a significant difference between the 1% and control was found for second instars with Dunnett's test. Based on the observations of the evaporation controls, the ethanol may lead to a slower development rate in the earlier instars, but once alcohol levels have diminished, the larvae start to develop at a normal

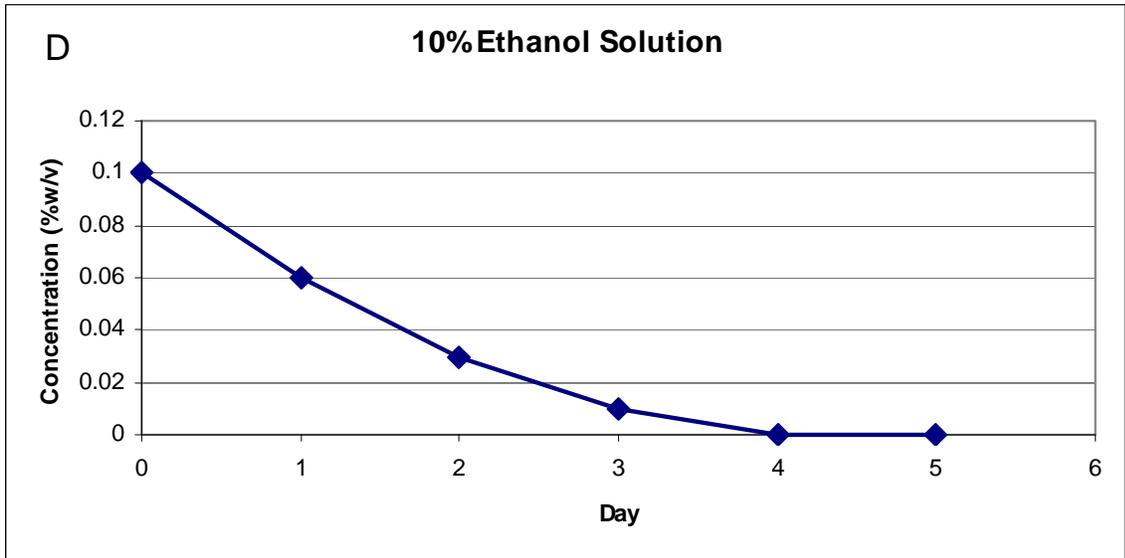
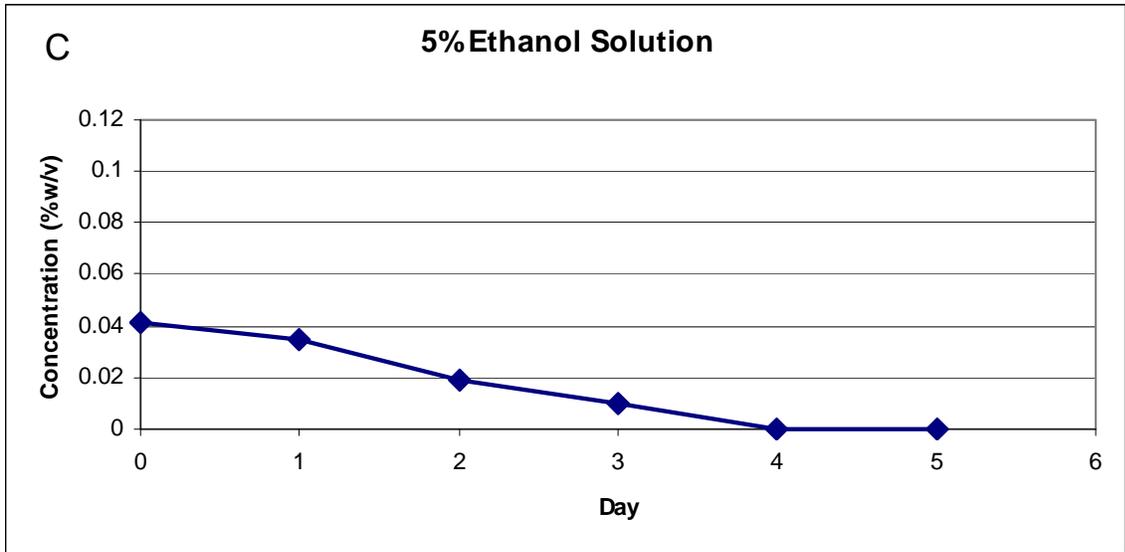
rate again. Significant differences between third instars of 5% and control were found when growth curves were compared using the Kolmogorov-Smirnov test. There was no significant difference between the mean time from eclosion to completion of the third instar stage. There were also no significant differences between the times required to complete the pupal stage for controls and all treatments.

Egg clusters weighing 0.02 g (~150 eggs) were used for all containers in both studies to ensure that density was not a factor. Overcrowding of blowfly can result in slowed larval development and smaller pupae (Ulyett, 1950; Hanski, 1987; Saunders & Bee, 1995). Green et al. (2003) showed that groups of 10 *P. regina* larvae enhanced the pupal weights and reduced development time compared to individual maggots feeding on lamb liver. The control pupal weights were significantly higher than all the treatment pupal weights. The control and treatments had the same density of maggots therefore differences between control and treatments are likely caused by the presence of alcohol. Alcohol may reduce feeding by altering maggot behavior or altering other physiological responses. Lower pupal weights would also correspond with lower adult weights seen in this study.

*Phormia regina* appears to have an unbiased sex ratio (1:1) and ethanol does not appear to alter that ratio. Some insects have shown changes in sex ratio when environmental conditions change. For example, *Lepidostoma vernale* (Trichoptera) can shift from a female biased (3:2) to unbiased (1:1) ratio with an increase in water temperature (Hogg and Williams 1996). Hermes (1928) showed that food available to the larvae had an effect on the sex ratio of *Lucilia sericata*. Hermes findings suggest that the sex ratio reversed from a large preponderance of males in groups of flies that were underfed to a preponderance of females in groups with a longer feeding period. Herpetological and ichthyological literature also includes citation temperature-dependent sex determination (Conover and Kynard 1981, Gutzke and Crews 1988). However, references on the sex ratio of *Phormia regina* or possible causes of bias are not available.

This study provides information on the effects of ethanol on the development of *P. regina*. The results suggest that ethanol levels less than 0.10% w/v have little to no apparent effect on the development of maggots, however higher levels than 0.15% w/v can affect the development of maggots and therefore estimates of PMI (Tabor et al. 2005).





**Figure 3.1: Evaporation curves for concentrations of ethanol in ground loin tissue over a five day period for a control (A), 1% ethanol solution (B), 5% ethanol solution (C), and 10% ethanol solution (D).**

**Table 3.1: Concentrations of ethanol (% weight by volume) (Mean  $\pm$  SE) in meat samples and migrating third instars. N = 23 for the control meat samples, 24 for 1%, 26 for 5%, and 30 for the 10%. N = 8 for the control larvae samples, 11 for 1%, 10 for 5%, and 11 for 10%.**

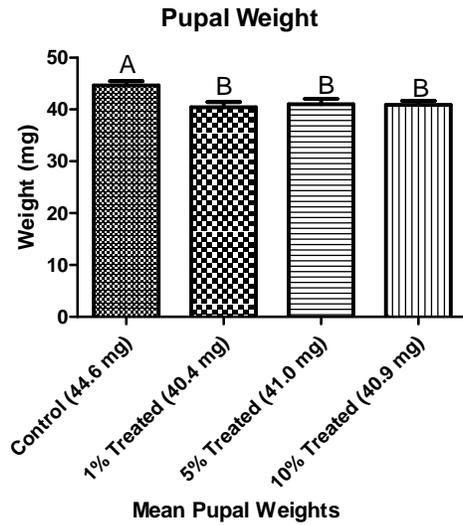
| <b>Treatments</b>  | <b>Meat Samples</b> | <b>Larvae</b>    |
|--------------------|---------------------|------------------|
| <b>Untreated</b>   | ND                  | 0.01 $\pm$ 0.003 |
| <b>1% Treated</b>  | 0.01 $\pm$ 0.001    | 0.01 $\pm$ 0.003 |
| <b>5% Treated</b>  | 0.04 $\pm$ 0.003    | 0.01 $\pm$ 0.004 |
| <b>10% Treated</b> | 0.08 $\pm$ 0.005    | 0.01 $\pm$ 0.003 |

ND, Not detected with a limit of detection (LOD) and limit of quantitation (LOQ) of 0.01%.

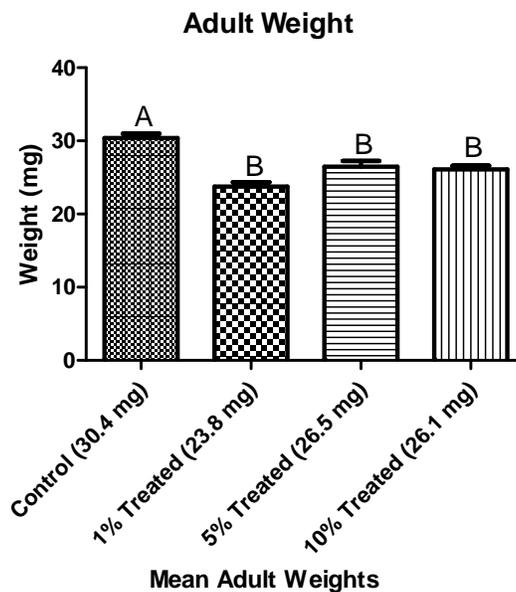
**Table 3.2: Time from egg eclosion to completion of larval stages and time to complete pupal stage for *Phormia regina* reared on ethanol-treated and untreated pork loin on a 24:0 (L:D) cycle at 23°C. (N= 67 time periods for the control, 62 for 1%, 86 for 5%, and 92 for 10%)**

| <b>Stage</b>                    | <b>Untreated tissue<br/>(range [mean ± SD] h)</b> | <b>1% ETOH-treated tissue<br/>(range [mean ± SD] h)</b> | <b>5% ETOH-treated tissue<br/>(range [mean ± SD] h)</b> | <b>10% ETOH-treated<br/>tissue<br/>(range [mean ± SD] h)</b> |
|---------------------------------|---|---|---|--|
| <b>First Instar</b>             | <b>19.0-39.0 (27.6 ± 6.8)</b>                     | <b>24.0-37.0 (29.5 ± 4.3)</b>                           | <b>24.0-58.0 (33.5 ± 10.5)</b>                          | <b>16.0-48.0 (28.3 ± 8.6)</b>                                |
| <b>Second Instar</b>            | <b>51.0-86.0 (65.1 ± 10.9)</b>                    | <b>37.0-85.0 (54.4 ± 12.6)*</b>                         | <b>40.0-98.0 (58.7 ± 14.4)</b>                          | <b>51.0-96.0 (69.5 ± 14.5)</b>                               |
| <b>Third instar<sup>a</sup></b> | <b>148.0-197.0 (167.1 ± 16.1)</b>                 | <b>141.0-173.0 (155.6 ± 10.8)</b>                       | <b>133.0-197.0 (158.3 ± 18.0)</b>                       | <b>141.0-180.0 (157.5 ± 12.3)</b>                            |
| <b>Pupal</b>                    | <b>141.8-176 (158.9 ± 8.9)</b>                    | <b>139.5-175 (158.4 ± 9.0)</b>                          | <b>136.0-183 (155.8 ± 11.2)</b>                         | <b>133-224 (157.4 ± 15.9)</b>                                |

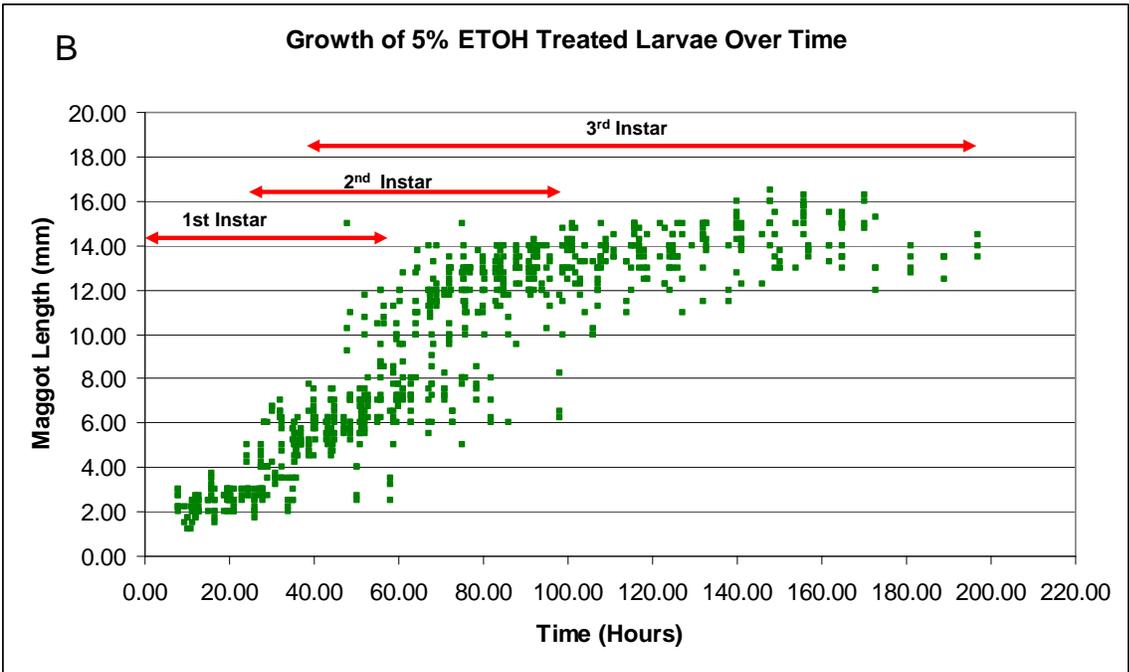
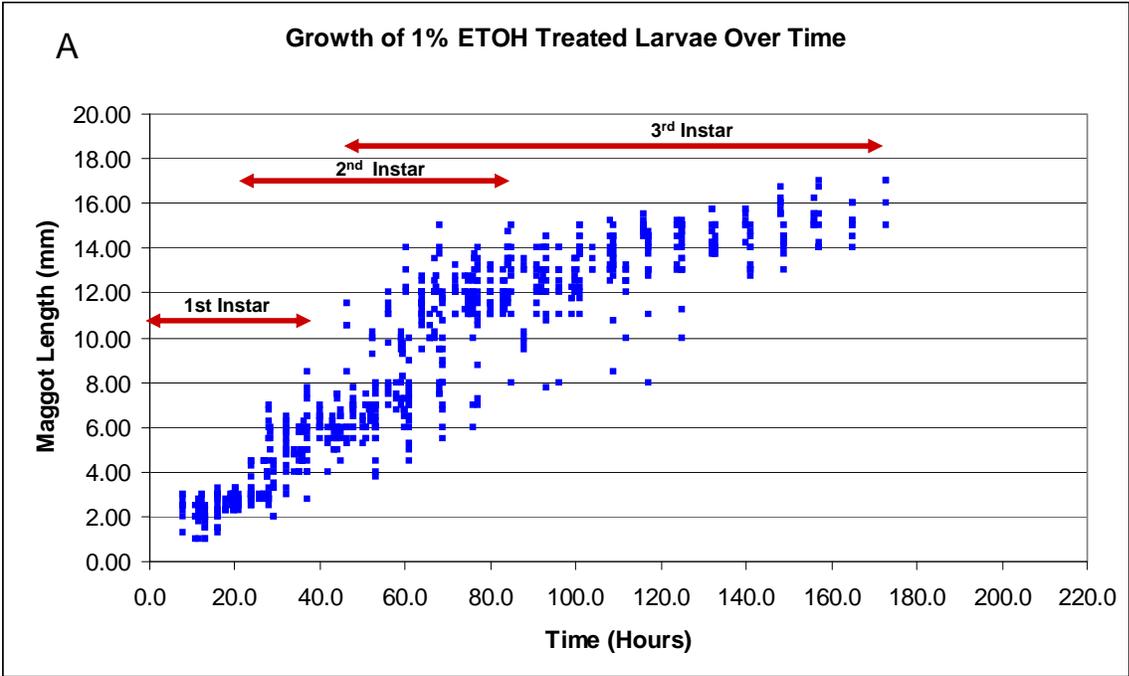
<sup>a</sup>Feeding and prepupal stages combined, \*Indicates significant difference ( $\alpha= 0.05$ ) in mean time compared to the control

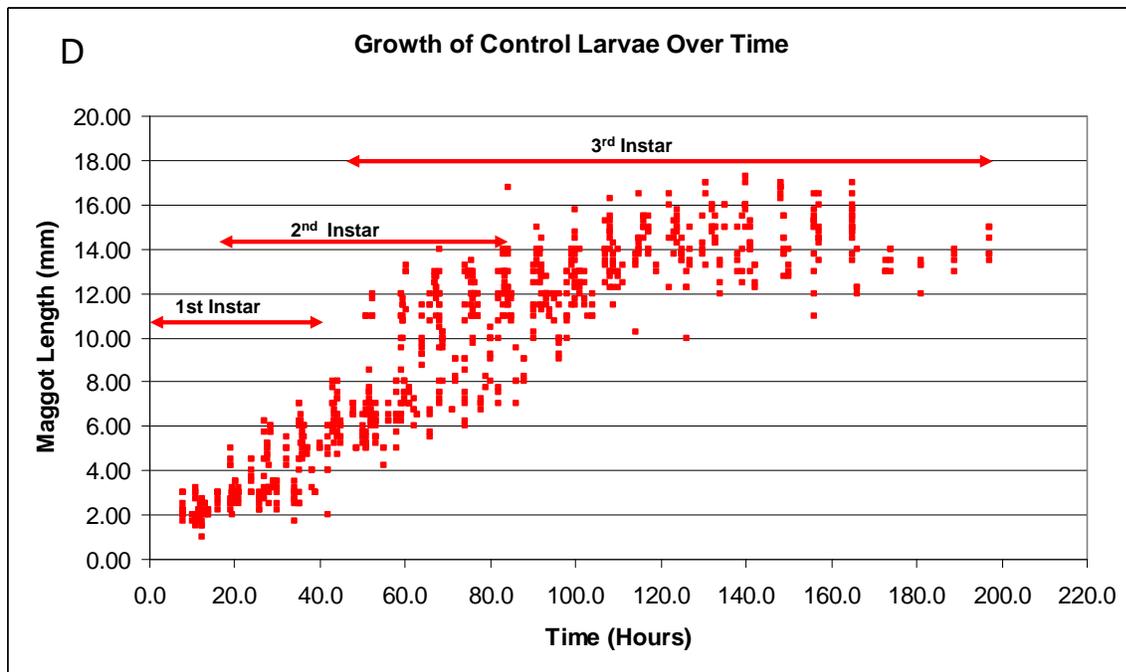
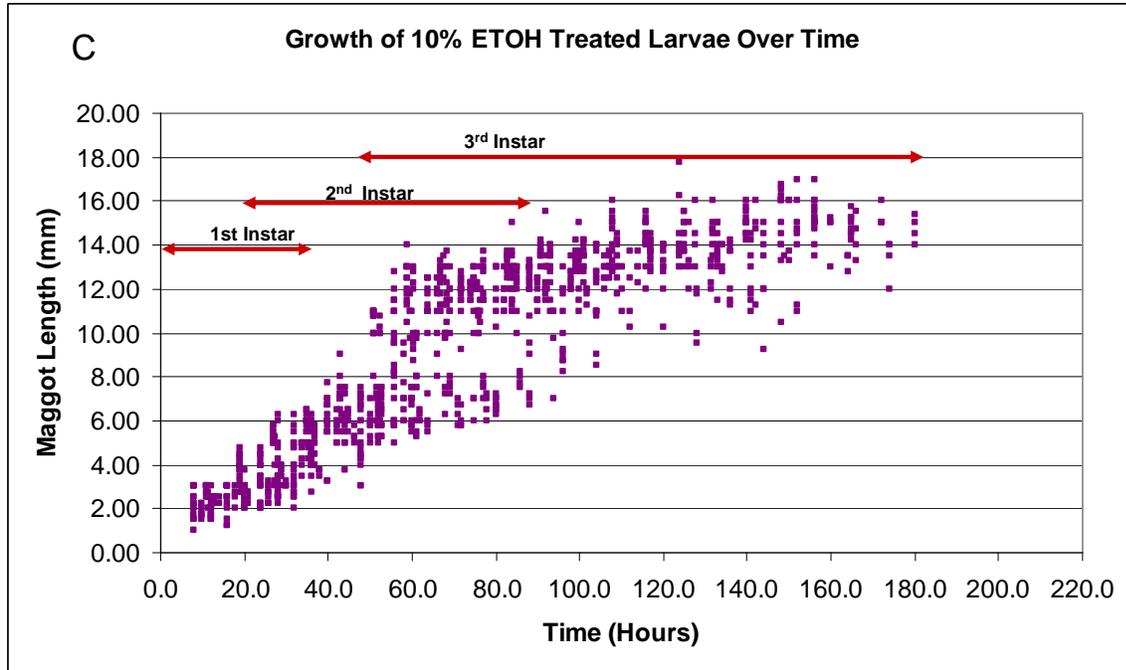


**Figure 3.2: Pupal weights (mg) (Mean  $\pm$  SE) from larvae that fed on ethanol-treated and untreated pork loin tissue. Larvae and pupae were reared on a 24:0 (L:D) cycle at 23°C. A significant difference in pupal weight was found between the control (A) and the treated groups (B). N = 155 pupae for control, 154 for 1%, 226 for 5%, and 255 for 10%.**

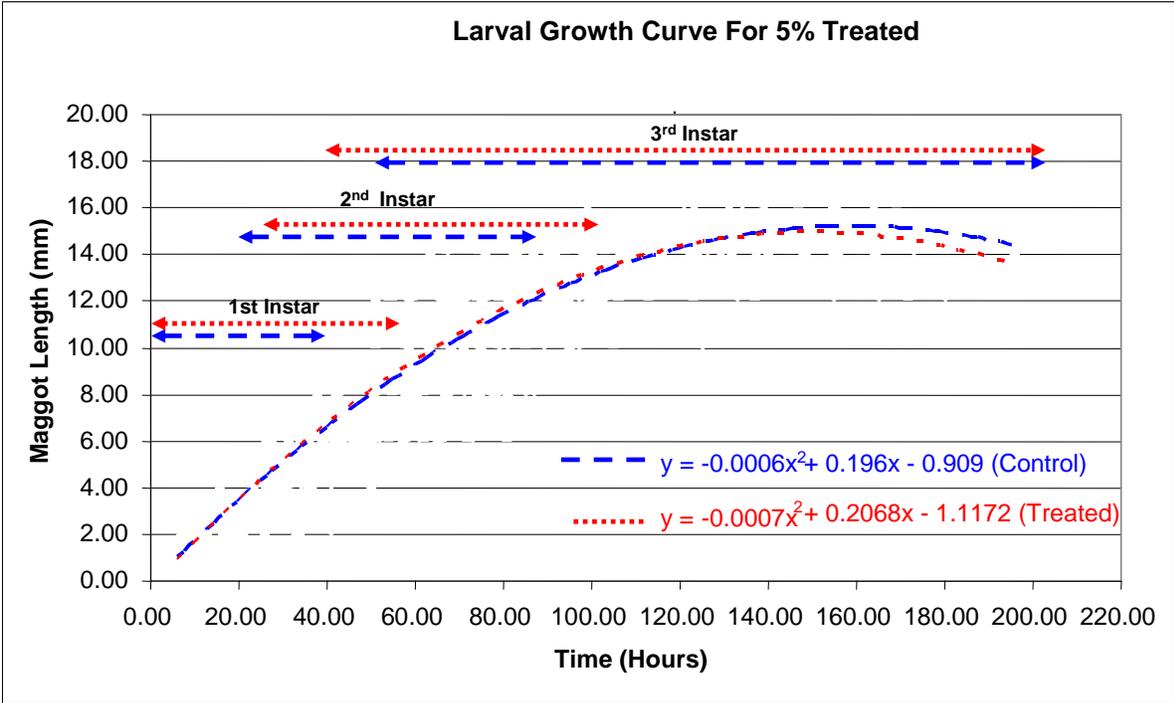
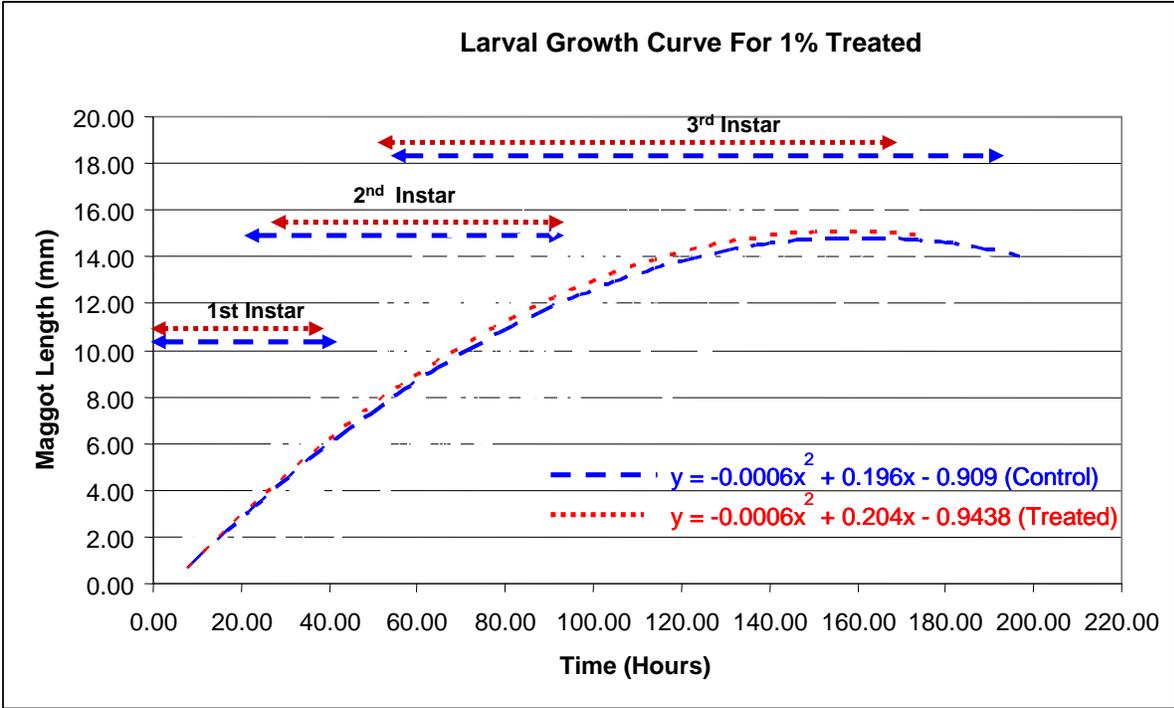


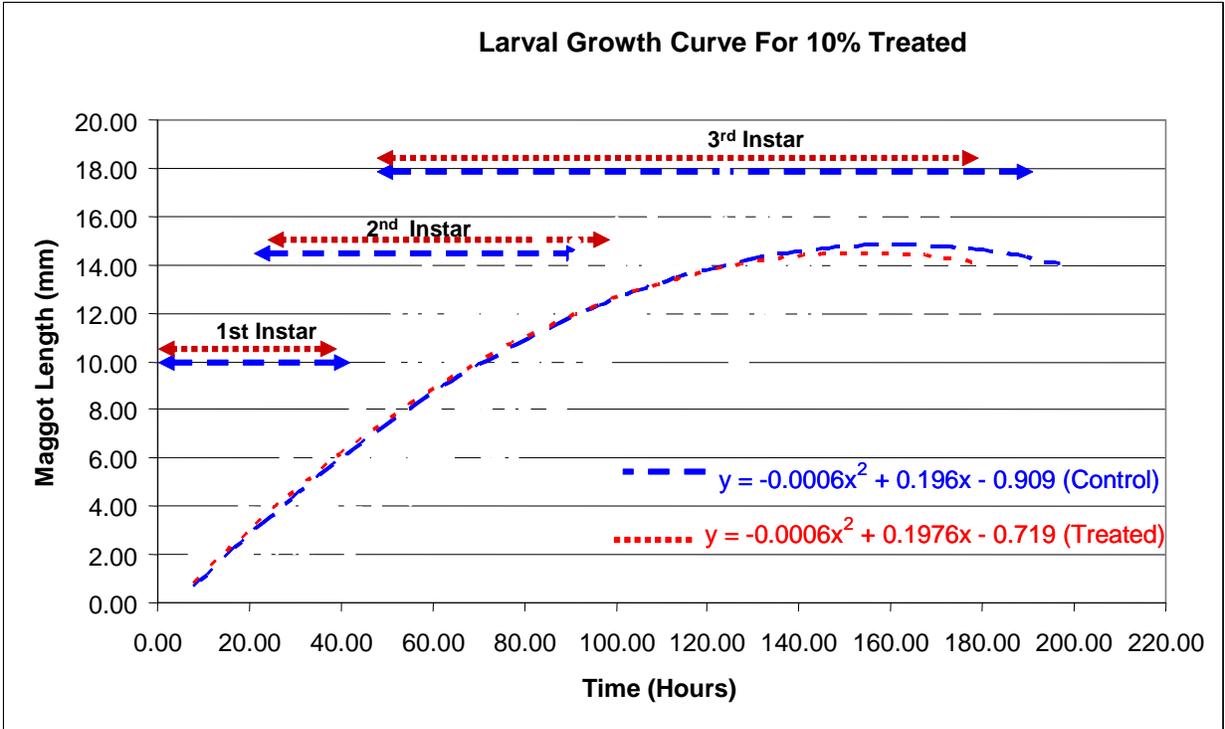
**Figure 3.3: Adult weights (mg) (Mean  $\pm$  SE) from larvae that fed on ethanol-treated and untreated pork loin tissue. Larvae and pupae were reared on a 24:0 (L:D) cycle at 23°C. A significant difference in adult weight was found between the control (A) and the treated groups (B). N = 154 adults for the control, 215 for 1%, 215 for 5%, and 337 for 10%.**



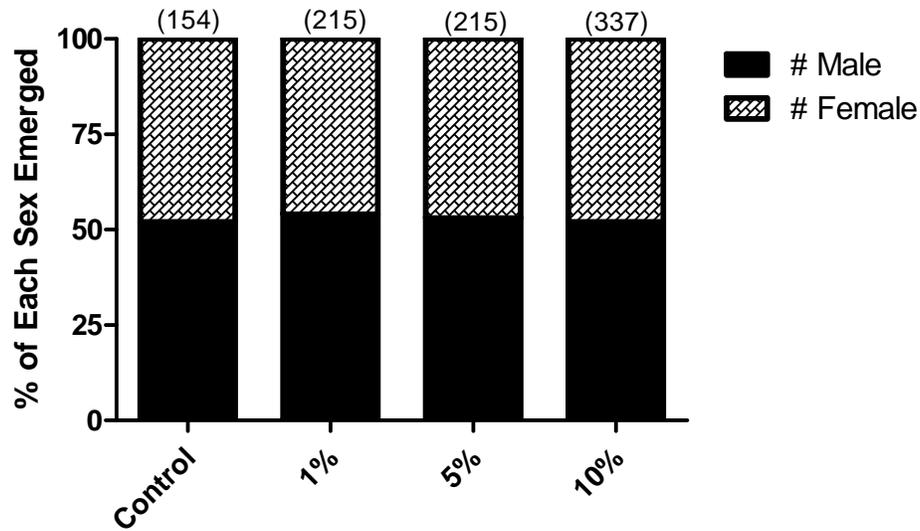


**Figure 3.4:** Lengths of maggots feeding on either a 1% (A), 5% (B), or 10% (C) ethanol-treated loin tissue or untreated (D) tissue. Larvae were reared on a 24:0 (L:D) cycle at 23°C. Four maggots were sampled every 8 hours from all treatments and the control. Maggot lengths were measured. N= 823 larvae for 1%, 860 for 5%, 1146 for 10% and 902 for the control





**Figure 3.5: Fitted curves of lengths of maggots feeding on tissue from either 1%, 5%, 10% ethanol-treated and untreated loin tissue. A second order polynomial was used as the fitted curve equation. All treatment curves were compared to the control curve.**



**Figure 3.6:** The percentage of males and females that emerged from the pupae of larvae that fed on ethanol-treated or untreated pork loin. The number in parenthesis above each bar is the total number of flies that emerged from that group. A difference among totals is due to more replications completed for some treatments. N = 154 adult flies for Control, 215 for 1%, 215 for 5%, and 337 for 10%

## **4. The Effects of Antemortem Injection of Oxycodone on Insect Successional Patterns and Development of *Phormia regina* (Diptera: Calliphoridae)**

### **4.1. Introduction**

Forensic toxicologists qualitatively and quantitatively identify drugs and toxins which may be relevant to the cause and manner of death. In most cases, toxicological specimens are collected at autopsy. Blood, vitreous humor, urine, bile, and liver are the most commonly collected specimens from a body. Other tissues such as lung, spleen, and stomach contents can also be collected and examined for evidence of drugs (Levine 2003). Alternatively, if a body is badly decomposed, bone, hair, and insect larvae and pupae are collected and analyzed.

The use of insects and insect remnants as toxicological specimens is well documented. Insect tissue or remnants (pupal cases, frass, etc.) can be used to identify drugs and toxins present in decomposing tissues. Literature citations on the use of arthropods as an alternative toxicological source date to 1980. In 1980, Beyer et al. (1980) reported a case in which a 22-year-old white female was found in a wooded area in Virginia, USA. The body was badly decomposed and skeletonized as it was found 14 days postmortem. Adjacent to the body, an empty bottle that had contained 100 phenobarbital tablets was found in a purse. No soft tissue or organs were present, but a large number of maggots were present in the hairs, in the skull, and within body cavities. The larvae were used to provide a source for qualitative identification of the drug. The larvae were collected and analyzed. A solvent extraction was done on the larvae and the presence of 100 µg phenobarbital/g of larvae was confirmed by thin-layer chromatography and gas chromatography/mass spectrometric analysis. The fly larvae collected were that of *Cochliomyia macellaria*.

This use of insects for toxicological purposes has evolved into a new area of forensic investigation called entomotoxicology. Entomological evidence is analyzed to determine whether subjects were exposed to, ingested, or were administered drugs or toxins prior to death. The first cases in this area mainly dealt with toxins. Nourteva and Nourteva (1982) found that mercury contained in food sources eaten by carrion-feeding insects could be detected at different levels in both immatures and adults. Calliphoridae larvae were reared on fish tissue containing known concentrations of mercury. The amount of mercury recovered from larval tissue was directly proportionate to the duration of the feeding period of the larvae. Larvae feeding on

mercury-containing tissue retained the heavy metal through pupation, and mercury was detected in the emerging adult flies (Nourteva and Nourteva 1982). In recent years, insects have been used to qualitatively and quantitatively assess pharmaceutical and illicit drugs (Miller et al. 1994). Entomotoxicology combines the entomological and toxicological data for the purpose of establishing the manner or cause of death and estimating the postmortem interval (PMI) (Goff and Lord 2001). The PMI is the time from when a body is found to the time when insects colonize the body (i.e. death).

Studies have shown that antemortem use of various drugs and toxins affect maggot development rate, manifesting into an inaccurate PMI estimation based on insect development (Goff et al. 1989, Goff et al. 1991, Goff et al. 1992, Goff et al. 1993, Goff et al. 1994). Errors of up to 29 h can occur in PMI estimates (overestimation) with heroin containing tissues (Goff et al. 1991) based on development of the fly *Boettcherisca peregrina*. Similar results were reported for methamphetamine (Goff et al. 1992) and amitriptyline (Goff et al. 1993). Errors of up to 24 h can occur in estimates (underestimation) with heroin on *Lucilia sericata* (Bourel et al. 1999b).

Oxycodone, an opioid, can potentially alter development and behavior in insects as well, however there are currently no published studies that analyze the effect of antemortem oxycodone exposure on insect development rates or successional patterns. OxyContin<sup>®</sup> is a major prescription drug of abuse and the sole active ingredient it contains is oxycodone. Generally, the more that OxyContin<sup>®</sup> is prescribed the greater numbers of cases of abuse. The medical use of opioids within the United States increased 400% from 1996 through 2000 (Varga et al. 2001). Abuse of OxyContin<sup>®</sup> in rural Maine, Kentucky, Virginia, and West Virginia brought national attention to this problem. A variety of illegal techniques are used to obtain the drug because it requires a prescription. Pharmacy diversion, dishonest physicians, “doctor shopping”, fraudulent prescriptions, and robbery contribute to the drug being diverted to the illicit market (Microgram 2002).

OxyContin<sup>®</sup> abuse has led to overdoses and death. In October 2001, a Drug Enforcement Agency report on autopsy data revealed evidence to suggest OxyContin's<sup>®</sup> role in the overdose deaths of 282 people over a 19-month period. In Virginia in 2001 it was estimated that there had been at least 55 deaths linked to OxyContin<sup>®</sup> (Congress 2002). The Office of the Chief Medical Examiner (OCME) for the Western Region of Virginia certified 519 drug deaths from 1997 to 2001. Thirty-four percent (n=175) of the certifications identified oxycodone (n=82) or

methadone (n=93) as being significant in the cause of death (Behonick et al. 2003). Oxycodone accounted for one-third of all narcotic analgesics reported by forensic laboratories during January 2003 to June 2003 (Department of Justice 2003). The presence of oxycodone in decedents combined with limited knowledge of the effects of this drug on insect development and succession make it an important opioid to study in forensic entomology.

This study has two major objectives: 1) to determine whether antemortem injection of oxycodone affects postmortem insect successional patterns on *Sus scrofa* L. carcasses, and 2) to examine the effect of oxycodone on maggot development of the black blow fly, *Phormia regina* (Meigen) under in vitro conditions.

## **4.2. Materials and Methods**

### **4.2.1. Animals and Oxycodone Dosing**

Studies were conducted in Blacksburg, VA, in 2006 and 2007 to examine the effects of subcutaneous injections of oxycodone hydrochloride on insect successional patterns and development of *Phormia regina*. Two successional studies were conducted. The first was conducted from August 7-14, 2006 (Study 1). The second study was conducted from August 1-7, 2007 (Study 2). A developmental study began a week after each successional study.

Eight commercially cross-bred pigs were obtained from the Swine Center at Virginia Tech for use in each successional study. The four animals in the first study were castrated males and ranged in weight from 8 kg to 12.3 kg. The four animals in the second study were also castrated males and their weight ranged from 13.5 kg to 15.5 kg. Pigs were held from feed for 12 hours prior to injection but had access to water prior to and throughout the trial. In both studies, three of the animals were administered oxycodone hydrochloride before euthanasia while the remaining pig was administered saline. The treated animals received subcutaneous injections of oxycodone hydrochloride (3mg/kg body weight as freebase) dissolved in saline. Animals received injections in the neck.

A five mL sample of blood was taken from each pig via venipuncture of the anterior vena cava prior to injection of oxycodone or saline. Following injection, antemortem blood samples were taken every hour for the next three hours. The injection and bleedings were repeated after the pigs had 24 hours to rest. Euthanasia by electrical shock occurred immediately after the final bleeding (3 hours post injection). Approximately 200 grams of loin tissue and 75 grams of liver tissue were excised from each of the pigs in Study 1 (2006) to be used as the rearing medium in

the development study of *Phormia regina* maggots in the summer of 2006. Approximately 400 grams of loin tissue and 200 grams of liver tissue were excised from each of the pigs in Study 2 (2007) and used in a similar developmental rearing study with *P. regina*.

Blood samples taken from the pigs collected during the succession studies were analyzed for oxycodone and oxymorphone by gas chromatography/mass spectrometry (GC/MS). One metabolite of oxycodone is oxymorphone, a potent narcotic analgesic, while the other, noroxycodone, is relatively inactive. Oxymorphone could potentially affect the development and succession of insects so it is important to determine levels of this drug in pig tissue. Enzyme-Linked ImmunoSorbent Assay (ELISA) was also used to provide preliminary results on presence of oxycodone in blood samples. The GC/MS procedure utilized is as follows. One mL of blood from each sample was pipetted into separately labeled test tubes. The tubes were spiked with 100  $\mu$ L of internal standard spiking stock. The internal standards were deuterated oxycodone and oxymorphone. Phosphate buffer (2 mL of 100 mM Phosphate buffer (pH 6.0)) was added before being derivatized for the first time using 500  $\mu$ L hydroxylamine hydrochloride. Solid phase (SPE) columns were prepared by adding 3 mL of methanol followed by aspiration, then 3 mL of deionized water followed by aspiration, and then 2 mL of phosphate buffer to the columns. The column was not allowed to dry out. The extracted blood samples were loaded onto columns and pulled through the columns. The columns were then washed with deionized water, followed by sodium acetate buffer, and then methanol. The columns were aspirated between washings. After the methanol washing, the column was allowed to dry for five minutes. Elution was then performed using 3 mL of methylene chloride/isopropanol/ammonium hydroxide (78:20:2) mixture. The eluted solvent was evaporated to dryness at  $<40^{\circ}\text{C}$  under a gentle stream of  $\text{N}_2$ . After the extracts were dried, the samples were derivatized a second time using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS)(Pierce, Rockford, IL). The samples were injected into the GC/MSD, a Hewlett-Packard GC 6890 and MSD 5973. The column was a HP-1MS (0.25 I.D., 30 m length, 0.25  $\mu\text{m}$  film thickness) and deuterated oxycodone and deuterated oxymorphone (Cerilliant, Round Rock, TX) were used as the internal standards. Identification and quantitation of the drugs was achieved using Selected Ion Monitoring (SIM). Total run time for each sample was 14.5 minutes at  $250^{\circ}\text{C}$  and the post temperature was  $300^{\circ}\text{C}$ . The reportable range for oxycodone and oxymorphone is 40 ng/mL to 1000 ng/mL.

Oxycodone specific ELISA kits were obtained from Venture Labs, Inc., Redwood City, CA. The sensitivity of the Venture Labs™ Oxycodone assay is 2 ng/mL. This amount represents the lowest concentration of drug that can be distinguished from 0 ng/mL with a confidence level of 95%. The assay can quantitatively assess the amount of oxycodone in a sample, if the amount of oxycodone is between 0 ng/mL to 80 ng/mL. For the ELISA method, whole blood samples are added to microtiter plate wells coated with antibodies to oxycodone. Free drug in the sample binds to the antibodies in the microtiter plate. Then enzyme conjugate, which contains the enzyme horseradish peroxidase, is added. Enzyme conjugate binds to the remaining antibody binding sites on the microtiter plate well. Unbound enzyme conjugate is removed, and a chromogenic substrate is added, causing a blue color to develop in the well. The reaction is quenched with the addition of an acidic stop solution of 3N hydrochloric acid. A yellow color is displayed in the well indicating the reaction has been stopped. The absorbance of the color in the well is measured spectrophotometrically and is inversely proportional to the drug concentration in the sample. A Dynex Technologies Triad™ (DYNEX Technologies Limited, West Sussex, United Kingdom) plate reader was used and was set at OD 450nm for reading the microtiter plates.

#### **4.2.2. Insect Succession**

After blood and tissue were extracted, the remainder of each pig's carcass was transported immediately to a field site at Moore Farm, a research facility of Virginia Tech, which is ≈4km from the Swine Center. The carcasses were placed individually under open-bottomed cages, which were located 100 m apart at the edge of an open field bordered by a thickly wooded area and allowed to decompose for seven days. Each cage measured 92 by 92 by 153 cm and was constructed with 2.5-cm steel-welded tubing and 1.27-cm hardware cloth (Tabor et al. 2004). Each cage was staked to the ground to prevent disturbance of the carcasses by scavengers.

Samples of adult insects were collected from 10 aerial net sweeps above and around the carcass, from pitfall traps, and directly off each carcass to qualitatively assess insect fauna. Adult samples were placed with a paper towel saturated with ethyl acetate into individual 3.8 liter Hefty® Onezip® plastic bags (Pactiv Corp., Lake Forest, IL). When present, several hundred eggs and maggots were collected from the carcass using soft-touch forceps. Half of each egg or maggot sample was placed directly into vials containing KAA fixative (100 ml 95% ethanol, 20

ml glacial acetic acid, 10 ml kerosene) (Haskell 1990). The remaining half of each egg or maggot sample was placed in an aluminum pouch containing ground pork (Haskell 1990). The pouches were placed into 500 ml MicroGourmet SOLO cups (Solo Cup Co., Urbana, IL) filled with 7 cm of Mica-Grow Vermiculite (PVP Industries, Inc., North Bloomfield, OH). The cups were placed into an incubator at 27°C, 75% RH where the maggots were reared to the adult stage. All collected and reared adults were pinned, labeled, and identified to genus or species.

#### **4.2.3. Developmental Study**

*Phormia regina* (Meigen) adults were collected from carrion at a farm in Southwest Virginia before the start of this study for the purposes of starting a laboratory colony. Egg clusters were collected from pork loin that was placed in the lab colony cage. Two hundred grams of loin tissue and 75 grams of liver tissue removed from each of the four pigs in the first succession study were placed in 473-ml cups, for a total of 12 cups (i.e., nine cups with tissue from oxycodone-treated pigs, six loin and three liver; and three cups with tissue from the untreated pig, two loin and one liver). An individual loin cup contained 90 grams of loin tissue and a liver cup contained 60 grams of liver tissue in Study 1. An egg cluster weighing 0.02 g (approximately 150 eggs) was placed on the loin tissue in each cup and an egg cluster weighing 0.015 g (approximately 100 eggs) was placed on the liver tissue in each cup in Study 1. Four hundred grams of loin tissue and 200 grams of liver tissue were removed from each of the four pigs in the second succession study and placed in 473-ml cups, for a total of 16 cups (i.e., 12 cups with tissue from oxycodone-treated pigs, six loin and six liver; and four cups from the untreated pig, two loin and two liver). An individual loin cup contained 90 grams of loin tissue and a liver cup contained 90 grams of liver tissue in Study 2. An egg cluster weighing 0.02 grams (approximately 150 eggs) was placed on the loin/liver tissue in each cup in Study 2.

Eggs were observed hourly to determine the initial hatch time ( $t = 0$ ) for each cluster (cup). The cups were covered with aluminum foil until start of eclosion. Egg hatch ranged from 3 hours to 16.5 hours after the eggs were placed on the meat. At the onset of egg hatch, the cups containing loin tissue and eggs were transferred to a round glass battery jar (153 mm diameter x 204 mm depth) with an inch of sand lining the bottom. After initial hatch the aluminum foil was removed from the cup and a plastic Saran<sup>™</sup> Quickcover<sup>®</sup> (S.C. Johnson & Son, Inc., Racine, WI) was placed over the jar. Holes were placed in the plastic cover to allow oxygen transfer. The containers encasing the rearing cups were placed in an incubator at 23°C with constant light.

Samples were collected every eight hours until pupation, beginning at the onset of the earliest egg hatch among cups (egg clusters). A sample consisted of four randomly selected maggots per cup for a total of 12 maggots per cup per day. The maggots were placed directly into KAA fixative (100 mL 95% ethanol, 20 mL glacial acetic acid, 10 mL kerosene) (Haskell 1990) until one hour before the next sampling time. The fixative caused the maggots to fully extend so that they could be measured to the nearest 0.25 mm. Length and instar (as determined by the number of spiracular slits) were recorded for each maggot.

Maggot sampling continued until  $\approx 90\%$  of the individuals in each cup had either been removed or pupated. Pupae were removed as they appeared and placed in separately labeled 4 oz GladWare<sup>®</sup> containers (The Glad Products Company, Oakland, CA). The time to complete the pupal stage also was recorded for each maggot.

Pupae were weighed as they appeared, and adults were weighed and identified to sex after they had emerged. GraphPad Prism<sup>®</sup> (GraphPad Software, San Diego, CA) was the statistical program used to analyze the pupal and adult weight data, as well as the sex ratio data.

#### **4.2.4. Data and Statistical Analysis**

The Simple Matching Coefficient (SMC) similarity metric was used to test the differences in successional patterns of insect taxa on oxycodone-treated and untreated pigs using the similarity matrices derived from species occurrence matrices. Within each study, data were combined for the three treated and one untreated pig to develop two succession diagrams and corresponding occurrence matrices for each study (one for treated, one for untreated), for a total of four occurrence matrices for the two studies (two for treated and two for controls). As is typical in this type of analysis (e.g., Cheverud et al. 1989, Manly 1997), the null hypothesis of no similarity between patterns of succession in the occurrence matrices for similar study periods between treated and untreated pigs was tested.

In an occurrence matrix, 1 and 0 represent the occurrence and absence, respectively, of a taxon within a sampling interval. For each of the occurrence matrices developed in this study, Simple Matching Coefficients were used to describe temporal changes in the between-sample similarities in the composition of insects within the study period. The similarity coefficient is

written 
$$C_{jk} = \frac{a + d}{a + b + c + d} \quad 0.0 \leq C_{jk} \leq 1.0. \quad (\text{Romesburg 2004})$$

The symbol  $C_{jk}$  stands for any quantitative resemblance coefficient that measures the resemblance between objects  $j$  and  $k$ . In this equation  $a$  = cases of 1-1 matches (attributes for which both objects are coded “1”);  $b$  = cases of 1-0 matches (attributes for which object  $j$  is coded for “1” and  $k$  is coded “0”);  $c$  = cases of 0-1 matches (attributes for which object  $j$  is coded for “0” and  $k$  is coded for “1”); and  $d$  = cases of 0-0 matches (for which both objects are coded “0”). The equation includes  $d$  in the numerator and denominator, and it is insensitive to the direction of coding. This coefficient takes into account the proportion of 1-1 and 0-0 agreements in the “ $n$ ” comparisons of attributes. Its maximum value of  $C_{jk} = 1.0$  indicates perfect similarity and occurs when  $b = c = 0$  (Romesburg 2004).

The range and mean for time from egg eclosion to completion of larval and pupal stages for *Phormia regina* was calculated by using a matrix. The sampling interval composed the horizontal axis of the matrix while the vertical axis was labeled in eight hour increments that were broken down into 0.25 hour units. A time period was highlighted with a color that designated the instar that was collected during a sampling period from time zero. For the treatments and control, every stage except for the adult stage had a minimum and maximum time to complete the stage. The time periods between each minimum and maximum were used to calculate the mean time for each stage for the control and treated larvae.

For analysis of the development data for *P. regina*, the maggot length versus time for maggots feeding on tissue from drug laden and untreated pigs was plotted. A second order polynomial was fitted to each of the data sets by using a trendline. Statistical differences ( $\alpha = 0.05$ ) between the distribution functions for maggot length for individuals that fed on loin and liver tissue from oxycodone treated and untreated pigs were determined separately for each of the three instars and for all instars combined using a Kolmogorov-Smirnov test (Conover 1999). The Kolmogorov-Smirnov compares the fitted curves of the control to the fitted curves of the treatment to determine if there is a difference in larval lengths due to the presence of oxycodone. The Kolmogorov-Smirnov test was performed by entering data in a KS-test program (<http://www.physics.csbsju.edu/stats/KS-test.html>). The null hypothesis that treated and untreated curves were the same was rejected if  $P < 0.05$ . A Student’s t-test ( $\alpha = 0.05$ ) was performed on the fitted data for each instar to test for similarity in development of maggots feeding on meat from treated and untreated pigs.

### 4.3. Results

Concentrations of oxycodone and the metabolite oxymorphone were found in samples of antemortem blood taken from treated pigs. Results from blood tested using GC/MSD are shown in Tables 4.1 and 4.2. Results from blood tested using the Venture Labs Oxycodone Assay is shown in Table 4.3. The blood was tested for oxycodone to determine the levels of drug larvae were exposed to when feeding on tissue from the succession and development studies. In total, eight pigs were used for the two studies and they had an average weight of  $10.6 \pm 1.9$  kg (mean  $\pm$  SD) in Study 1 and  $14.5 \pm 0.8$  kg (mean  $\pm$  SD) in Study 2. Loin and liver tissue was excised from all pigs after death in both studies after the series of injections and bleedings for use in the development studies. The remainder of the pig was used in the succession studies.

Twenty-six and twenty-one insect taxa were observed on treated and untreated carcasses respectively in the summers of 2006 and 2007. The earliest visitors to both carcass types were dipterans in the families Calliphoridae, Sarcophagidae, and Muscidae. Succession diagrams for oxycodone-treated and untreated pig carcasses during the seven sampling intervals in the summers of 2006 and 2007 in Blacksburg, VA are shown in Figures 4.1 and 4.2.

Dipterans were represented by 20 taxa in five families. Six of the eight blow fly species were collected from both treated and untreated carcasses, with *Phormia regina* (Meigen) (Calliphoridae), *Lucilia coeruleiviridis* (Macquart) (Calliphoridae), and *Cochliomyia macellaria* being the most abundant. *Calliphora livida* was only collected on treated carcasses in 2006 and *Cyanopsis cadaverina* was only collected on treated carcasses in 2007. Similarly, *Muscina assimillis* was collected on both treated and untreated carcasses in 2006 while one taxa of *Ravinia* species was only collected on both treated and untreated carcasses in 2007. *Sepsis* species were also only found on treated carcasses for both summers.

Twelve taxa of Coleopterans in eight families were represented in the succession studies. Only four beetle species were found on both treated and untreated carcasses for both summers. Those species were *Creophilus maxillosus* L. (Staphylinidae), *Necrophila americana* (Silphidae), *Hister* species (Histeridae), and *Omosita* species (Nitidulidae).

In the succession studies, the Simple Matching Coefficient (SMC) means ( $\pm$  95% CL) were  $0.625 \pm 0.0993$  and  $0.658 \pm 0.1801$  for the treated animals and  $0.720 \pm 0.1907$  and  $0.714 \pm 0.1490$  for untreated animals in Study 1 and Study 2, respectively. The null hypothesis of no similarity between the patterns of succession in the occurrence matrices between treated and

untreated pigs was tested using the SMC. Results indicate that there was no significant difference in insect succession patterns between treated and untreated pig carcasses in 2006 and 2007 ( $P < 0.05$ ).

The development of *P. regina* maggots feeding on tissue from the oxycodone treated and untreated pigs under laboratory conditions is shown in Tables 4.4 and 4.5. Mean  $\pm$  SD time required by *P. regina* maggots feeding on loin tissue from oxycodone treated and untreated pigs to complete the pupal stage was  $161.3 \pm 12.7$  and  $162.9 \pm 11.47$  hours, respectively. Mean  $\pm$  SD time required by *P. regina* maggots feeding on liver tissue from oxycodone treated and untreated pigs to complete the pupal stage was  $158.4 \pm 15.4$  and  $158.4 \pm 10.2$  hours, respectively.

An unpaired t-test with Welch's correction was conducted to account for the stages that had uneven variance between the control and treated for time to complete each development stage, followed by an F test to compare variances. A significant difference was found among larvae that fed on loin tissue for the time from eclosion to the completion of the second instar  $t(15) = 5.03$ ,  $P < 0.05$ , however there was no significant difference between the variance of treated and control larvae  $F(15)=1.498$ ,  $P = 0.8241$ . There were no other significant differences between the means of the control and treatment for time to complete life stages in loin and liver tissue, however there was a significant difference between the variances of the third instars in loin tissue  $F(12)=3.58$ ,  $P = 0.0287$ .

Growth under in vitro conditions for *P. regina* larvae feeding on loin and liver tissue from treated and untreated pigs in both studies is illustrated in Figures 4.3A-4.3D. The fitted lines are presented in Figure 4.4A and 4.4B. A significant difference at ( $\alpha = 0.05$ ) was found only in the development of third instars on loin tissue from treated and untreated pigs ( $D = 0.2583$  with a  $P = 0.013$ ). There were no differences in the first instar stage, second instar stage, or entire curve between larvae from treated and untreated loin tissue according to the Kolmogorov-Smirnov test. No differences were seen in development of any instars feeding on treated or untreated liver as well. The Student's t-test ( $\alpha = 0.05$ ) did not find a significant difference between the growth of any instars feeding on treated or untreated tissue.

Pupal weight of *P. regina* maggots feeding on oxycodone treated and untreated tissue in laboratory conditions is shown in Figure 4.5. The mean values of the treatments were compared to the mean value of the control using an unpaired t-test at  $P < 0.05$ . There was no significant difference in the mean pupal weight of treated larvae compared to a control for both the larvae

that fed on loin or liver tissue ( $t(368) = 0.66$ ,  $P < 0.05$  for loin tissue and  $t(111) = 1.41$ ,  $P < 0.05$  for liver tissue). A One-way ANOVA comparing all the columns indicated a significant difference between the pupal weights,  $F(3,479) = 86.4$ ,  $P < 0.05$  and Bonferroni's Multiple Comparison Test showed there was a significant difference between untreated loin tissue and untreated liver tissue, untreated loin tissue and treated liver tissue, treated loin tissue and untreated liver tissue, and treated loin tissue and treated liver tissue.

Adult weights of *P. regina* maggots feeding on oxycodone treated and untreated tissue in laboratory conditions is shown in Figure 4.6. The mean values of the treatments were compared to the mean value of the control using an unpaired t-test at  $P < 0.05$ . There was a significant difference in the mean adult weight of treated larvae compared to a control for larvae that fed on loin tissue ( $t(547) = 3.90$ ,  $P < 0.05$ ). The adult weight for the larvae that fed on untreated loin was significantly higher than the adult weight for larvae that fed on treated tissue. There was also a significant difference in the mean adult weight of treated larvae compared to a control for larvae that fed on liver tissue ( $t(162) = 3.57$ ,  $P < 0.05$ ), however the adult weight for the treated was greater than the adult weight of the untreated for larvae that fed on liver tissue. A One-way ANOVA comparing all the columns indicated a significant difference between the adult weights,  $F(3,709) = 111.5$ ,  $P < 0.05$  and Bonferroni's Multiple Comparison Test showed there was a significant difference between all pairs (untreated loin and untreated liver, untreated loin and treated liver, treated loin and untreated liver, treated loin and treated liver, untreated loin and treated loin, and untreated liver and treated liver).

The calculations of chi-square (by Yates' correction) for homogeneity in the sex ratio for the adults that emerged from the control and oxycodone treated loin tissue show that the flies emerging are biased towards one sex (males) by 2:1 in the untreated loin tissue ( $X^2 = 5.85$ ,  $df = 1$ ,  $P < 0.05$ ) (Figure 4.7). The calculations of chi-square (by Yates' correction) for homogeneity in the sex ratio for the adults that emerged from the oxycodone treated loin tissue, control liver tissue, and oxycodone treated liver tissue show that the flies emerging are not biased towards either sex.

#### **4.4. Discussion**

Taxa described in this study were typical for carrion-succession studies performed in Southwest Virginia (Tabor et al. 2004). The average monthly temperatures in August for the summers of 2006 and 2007 were 26°C and 27°C (<http://www.wunderground.com/>). In hot

weather, decomposition and hence taxonomic composition, progresses more rapidly than in cooler weather.

Oxycodone injection to pigs does not appear to alter insect succession patterns on decomposing carcasses. The Simple Matching Coefficient (SMC) ( $\pm$  95% CL) for both treated and untreated animals were  $0.625 \pm 0.0993$  and  $0.658 \pm 0.1801$  for the treated animals and  $0.720 \pm 0.1907$  and  $0.714 \pm 0.1490$  for untreated animals in Study 1 and Study 2, respectively, indicating high similarity among intervals within each study. The species collected in this study were similar to those collected in other studies conducted in Southwest Virginia (Tabor et al. 2004). Insect visitation and colonization occurred at the same rate for treated and untreated pigs. Similarly, there were no apparent differences in decomposition rates between treated and untreated animals.

Tabor et al. (2004) collected more species of insects during their succession studies in the spring and summer of 2001 and 2002 in Blacksburg, VA than in this study. Tabor et al. used pigs that weighed 41-45 kg in the spring studies and 23-27 kg in the summer studies. The pigs used by Tabor et al. (2004) weighed more than any of the pigs used in this study (12.3 to 15.5 kg). Carcass size and type can influence decomposition rates and successional patterns (Watson and Carlton 2003). Since the pigs in this study were not as large as the ones used in the baseline successional study used by Tabor, not as many species were able to colonize the carcasses. Catts and Goff (1992) suggested that the most appropriate animal model for use in forensic successional studies is the domestic pig, *Sus scrofa* L., weighing  $\approx$ 23 kg. However, it has been shown that the patterns of succession do not differ significantly between pig carcasses with moderate differences in size (Hewadikaram and Goff 1991). There were only moderate differences in the size of pigs used in both succession studies (8 kg to 12.3 kg for Study 1)(13.5 to 15.5 kg for Study 2) at Moore Farm.

Opioid receptors, known as mu ( $\mu$ ) receptors, are found in both vertebrates and invertebrates. In humans, opiates such as morphine and opioids such as hydrocodone and oxycodone bind to the same sites as the endogenous opioid peptides (Lord et al. 1977). In general, opioids cause analgesia and sedation, respiratory depression, and slowed gastrointestinal transit in humans (Karch 2007). However, research involving insects and opiates indicate that they act as a feeding stimulant in some species (Kavaliers et al. 1987, Goff et al. 1991). Goff et al. (1991) had colonies of *Boettcherisca peregrina* feed on tissues containing heroin (as

morphine) and showed differences in larval development rates sufficient to alter postmortem interval estimate by up to 29 hours, and if based on puparial development, by 18 to 38 hours. The morphine treated rabbit livers that sarcophagid flies fed on in Goff's study showed an increased rate of development compared to those that fed on untreated rabbit livers. However, the duration of the pupal stage was significantly longer for larvae reared on tissues containing the drug than for the controls. Bourel et al. (1999b), in contrast to Goff's study, presented data that suggested *Lucilia sericata* larvae feeding on rabbits that were administered morphine chlorhydrate had a slower growth rate than the controls. The data also showed that the presence of morphine in tissues was dose dependent, as the highest dosed tissue produced the slowest developing larvae. An underestimation of the postmortem interval of up to 24 hours is possible if the presence of morphine is not considered according to Bourel et al. (1999b). Studies involving opioids and insects seem to show that different invertebrates including necrophagous flies can have different responses to opiate drugs and rates of development can be increased, retarded, or perhaps remain unchanged.

The time larvae grew from eclosion to completion of the second instar stage on loin tissue excised from oxycodone treated pigs was found to be significantly different from the time maggots took to grow feeding on tissue from a control. No significant differences were detected in first and third instars that fed on treated or untreated loin. No significant differences were detected between any of the instars feeding on treated liver tissue. These results are similar to a study by Goff et al. (1991) who demonstrated that the effect of heroin (as morphine) on maggot growth was significant during hours 18-96 in larval development, but not during the hours 0-12. In Goff et al.'s (1991) study four domestic rabbits (weighing no more than 3.18 kg) were given dosages of 6, 12, 18, and 24 mg of heroin in 5 mL of saline by cardiac puncture. The animals were sacrificed 20 minutes after administration of drug and a 2-mL blood sample was taken from each rabbit. The blood sample from the rabbit dosed with 6 mg of heroin had morphine present at 436 ng/mL, the rabbit dosed with 12 mg had 1148 ng/mL of morphine, the rabbit dosed with 18 mg of heroin had 1451 ng/mL morphine, and the rabbit dosed with 24 mg of heroin showed 2216 ng/mL of morphine in the blood. The concentrations of heroin (morphine) in the blood of the rabbits were much higher than the concentrations of oxycodone found in this study involving pigs, but Goff was using higher dosages than the human therapeutic dosage used in this oxycodone study. Sarcophagid larvae were reared on the rabbit livers in Goff et al.'s study

(1991). As mentioned before, Goff et al. (1991) observed significant differences in the rates of development of larvae from the heroin-containing colonies (heroin treated was developing more rapidly) when compared with the control colony beginning at hour 18. The difference continued through hour 96, when the maximum total lengths were recorded from all the colonies. Larvae from the heroin colonies were also significantly larger than larvae from the control colony in Goff et al.'s (1991) study. Goff et al. (1991) also observed all the heroin treated colonies reach the prepupal stage faster than the control, however the duration of the pupal stage was significantly greater for colonies that fed on tissues containing heroin than for the control colony. In this study where larger animals and lower dosages of an opioid were administered, the treated larvae feeding on loin tissue had faster mean development times for completing the first through third instar development stages, however only the second instar time was significantly different (45-67 hours for treated compared to 68-80 hours for untreated). The larvae feeding on treated liver generally had longer mean development times for completing the first through third instar development stages, however none of those times were significantly different. Another difference between this study and Goff et al.'s (1991) study is that pupation and adult emergence times were similar in maggot clusters that fed on tissue from treated versus untreated pigs in this study.

The presence of oxycodone in loin tissue may eventually affect the adult weight of those larvae that fed on treated tissues. The weight of the adults that fed on treated loin tissue when they were larvae (mean and SE for larvae on treated loin =  $38.8 \pm 0.3$  mg) was significantly less than the adult weight of larvae that fed on the control (mean and SE for larvae on untreated loin =  $41.1 \pm 0.4$  mg) ( $t(547) = 3.90$ ,  $P < 0.05$ ). In contrast to the adult weights from loin tissue, the adult weight of larvae that fed on treated liver tissue (mean and SE for larvae on treated liver =  $29.9 \pm 1.0$  mg) was significantly larger than the adult weight of larvae that fed on the control (mean and SE for larvae on untreated liver =  $22.5 \pm 1.3$  mg) ( $t(162) = 3.57$ ,  $P < 0.05$ ). There is a significant difference between adult weight of larvae when comparing the two tissues,  $F(3, 709) = 111.5$ ,  $P < 0.05$ . The Bonferroni Multiple Comparison Test showed that all the adult weights were significantly different for all pairs (untreated loin and untreated liver, untreated loin and treated liver, treated loin and untreated liver, treated loin and treated liver, untreated loin and treated loin, and untreated liver and treated liver).

All cups in this experiment contained 0.02 g of eggs (~ 150 eggs) so competition from high density levels should not have affected adult weights. In loin tissue, the mean adult weight for the control flies was only slightly higher than the treated flies, however it was significant. The larvae that fed on treated liver tissue, however, weighed dramatically more (133%) when they emerged as adults than the control group (29.9 mg compared to 22.5 mg). Liver contains sufficient protein for the larvae to develop. The higher adult weight could signify that the oxycodone present in the liver stimulated increased feeding causing the larvae on treated liver tissue to develop into larger flies than those in the control group. This evidence is not conclusive as there were no significant differences in pupal weight or time to complete the pupal stage between treated and untreated tissues. Another possibility for the difference between treated and untreated weights could be an effect of feeding on the different tissues.

*Phormia regina* appears to have an unbiased sex ratio (1:1) and oxycodone does not appear to alter that ratio. The calculation of chi-square (by Yates' correction) for homogeneity in the sex ratio for the adults that emerged from the oxycodone treated loin tissue, control liver tissue, and oxycodone treated liver tissue show that the flies emerging are not biased towards one sex, however flies that emerged from untreated loin tissue were biased towards males (2:1). Some insects have shown changes in sex ratio when environmental conditions change. For example, *Lepidostoma vernale* (Trichoptera) can shift from a female biased (3:2) to unbiased (1:1) ratio with an increase in water temperature (Hogg and Williams 1996). Hermes (1928) showed that food available to the larvae had an effect on the sex ratio of *Lucilia sericata*. Hermes findings suggest that the sex ratio reversed from a large preponderance of males in groups of flies that were underfed to a preponderance of females in groups with a longer feeding period. It is unclear why there is a bias for more males in the untreated loin tissue group.

A toxicological investigation typically begins with the preliminary identification of drugs or chemicals present in post-mortem specimens. Confirmatory testing is then performed to conclusively identify the substance(s) present in the post-mortem specimens (Karch 2007). The next step is generally to determine the quantity of substance in the appropriate specimens. Identifying drugs in bile and urine is useful, but has limited interpretive value. Drug quantification in peripheral blood, along with quantification in samples from other specimens may be more meaningful. Occasionally, mere detection of a drug is sufficient (Karch 2007). The oxycodone specific ELISA kit was used in this study to first identify oxycodone's presence

in blood samples. The ELISA kit was used to qualitatively assess oxycodone in the blood tissue of treated pigs. The GC/MSD was used to quantitatively assess the amount of oxycodone and oxymorphone in those blood samples. GC/MSD data can be used for both identification and confirmation testing (Karch 2007).

Results may have been more significant if a higher dosage of oxycodone hydrochloride had been used. Many studies have tested the pharmacokinetic/pharmacodynamic properties of oxycodone. The pigs used in our study were given a human therapeutic dosage of 3 mg/kg. Renzi and Tam (1979) reported that six human subjects given oxycodone hydrocodone orally averaged 18 ng/mL in plasma at one hour, 9 ng/mL at four hours, and 5 ng/mL at eight hours.

The oxycodone levels of the pigs observed in this study are similar to cases involving deaths of persons with oxycodone in their system. The antemortem oxycodone concentration of the pigs from which the loin and liver tissue used was not determined, but at the last blood draw the highest concentration of oxycodone was 81 ng/mL and 390 ng/mL for Study 1 and Study 2 respectively. The levels of oxycodone detected in blood samples from the pigs in this study are within the range reported by Wolf et al. (2005) when they looked at oxycodone in postmortem toxicological studies in eight cases where oxycodone was the only drug detected. The oxycodone concentrations in Wolf et al. (2005) ranged from 0.21 mg/L (210 ng/mL) to 1.22 mg/L (12,200 ng/mL) (mean 0.55 mg/L or 550 ng/mL). Baselt (2002) reviewed six deaths involving oxycodone, three of which involved at least one other depressant drug. Postmortem oxycodone levels in these cases ranged from 0.4 mg/L to 14.0 mg/L. Spiller (2003) reported 24 deaths attributable to oxycodone alone, with no other drugs detected in postmortem analysis. The postmortem blood oxycodone concentrations in those cases ranged from 0.12 mg/L (120 ng/mL) to 8 mg/L (8000 ng/mL) (mean 1.23 mg/L), with 13 cases having oxycodone concentrations less than or equal to 0.5 mg/L.

Oxymorphone was also found at detectable levels in this study. The highest concentration of oxymorphone in the pig blood was 139 ng/mL. These levels are high compared to other studies. Pöyhiä et al. (1992) studied the pharmacokinetics and metabolism of oxycodone in nine healthy human volunteers. Each subject received oxycodone chloride once intramuscularly ( $0.14 \text{ mg kg}^{-1}$ ) and twice orally ( $0.28 \text{ mg kg}^{-1}$ ) at intervals of two weeks. Pöyhiä et al. (1992) found that oxymorphone found in human urine was mostly in a conjugated form and plasma concentrations of unconjugated oxymorphone were negligible. It is possible that

differences between human and pig physiology account for higher concentrations of oxycodone in blood tissue for this study compared to Pöyhiä et al. (1992).

This study provides information on the effect of oxycodone hydrochloride on insect successional patterns and development rates of *Phormia regina*. Results indicate that while oxycodone had no apparent effect on successional patterns, a statistically significant difference in development was seen in second instar maggots feeding on loin tissue from oxycodone treated pigs. The estimated age of an immature insect that has fed on a body provides a minimum post mortem interval (PMI) for investigators at a death scene (Byrd and Castner 2001). Normally, temperature is the most important factor regarding the rate of development in insects for estimating PMI, however chemicals in or on the victim, such as might be seen in a drug overdose or suicide, may have a variety of effects on carrion insects (Goff and Lord, 1994). The amount of oxycodone given to the pigs in this study was not near the lethal dose, however the drug showed indications of causing the larvae to develop more rapidly as indicated by larvae completing the second instar stage more rapidly than control larvae when feeding on treated loin tissue. Experiments conducted involving opiates and insects indicates that an opiate can affect invertebrates inversely to vertebrates, meaning that opiates generally stimulate the nervous system of invertebrates, but depress the nervous system of vertebrates (Kavaliers et al. 1987, Goff et al. 1991). It is possible that PMI can be overestimated if oxycodone is present in a body that carrion fly larvae developed on by 13 to 23 hours if the body was found when only early instars are present. There needs to be more research done with oxycodone before it can be conclusively proven that oxycodone will sufficiently alter PMI. For example, a range of oxycodone dosages need to be tested, especially those that are lethal dosages, before oxycodone's affects on PMI are fully known.

**Table 4.1: Concentration of oxycodone (ng/mL) in blood tissue from pigs on first and second day of bleedings for both Study 1 and Study 2. There was not enough blood collected from pigs in Study 1 to quantify using GC/MSD for the second day of bleedings. The treated animals received subcutaneous injections of oxycodone hydrochloride (3mg/kg of 20 mg/mL oxycodone HCl) dissolved in saline. The control pigs received only saline. Blood was drawn from the animals prior to injection and each hour for three hours following injection. Extract samples were analyzed by GC/MSD. Identification and quantitation of the drugs are achieved using Selected Ion Monitoring (SIM). The reportable range for these drugs are 40 ng/mL to 1000 ng/mL. No detectable amounts of oxycodone were found in any blood samples from pigs prior to them receiving the drug. ND = Not detectable. NS = No sample.**

| <b>Day 1</b> |                        |                  |                            | <b>Concentration of Oxycodone (ng/mL) in Blood Tissue of Pigs</b> |                            |                            |
|--------------|------------------------|------------------|----------------------------|---|----------------------------|----------------------------|
| <b>Pig</b>   | <b>Pig Weight (kg)</b> | <b>Treatment</b> | <b>Dosage Administered</b> | <b>Blood Draw</b>   | <b>Blood Draw</b>          | <b>Blood Draw</b>          |
|              |                        |                  | <b>Dosage (3 mg/kg)</b>    | <b>1 hr Post Injection</b>  | <b>2 hr Post Injection</b> | <b>3 hr Post Injection</b> |
| 1            | 10.6                   | Oxycodone        | 31.9 mg                    | 273 ng/ml   | 136 ng/ml                  | 14 ng/ml                   |
| 2            | 12.3                   | Oxycodone        | 36.8 mg                    | 291 ng/ml   | 160 ng/ml                  | 81 ng/ml                   |
| 3            | 11.4                   | Oxycodone        | 34.1 mg                    | 325 ng/ml   | 159 ng/ml                  | 34 ng/ml                   |
| 4            | 8                      | Saline           | 0                          | ND  | ND                         | ND                         |
| 5            | 14.5                   | Oxycodone        | 43.5 mg                    | NS  | 286 ng/ml                  | 135 ng/ml                  |
| 6            | 14.5                   | Oxycodone        | 43.5 mg                    | 693 ng/ml   | 275 ng/ml                  | 132 ng/ml                  |
| 7            | 13.5                   | Oxycodone        | 40.5 mg                    | 319 ng/ml   | 200 ng/ml                  | 104 ng/ml                  |
| 8            | 15.5                   | Saline           | 0                          | ND  | NS                         | NS                         |

| <b>Day 2</b> |                        |                  |                            | <b>Concentration of Oxycodone (ng/mL) in Blood Tissue of Pigs</b> |                            |                            |
|--------------|------------------------|------------------|----------------------------|---|----------------------------|----------------------------|
| <b>Pig</b>   | <b>Pig Weight (kg)</b> | <b>Treatment</b> | <b>Dosage Administered</b> | <b>Blood Draw</b>   | <b>Blood Draw</b>          | <b>Blood Draw</b>          |
|              |                        |                  | <b>Dosage (3 mg/kg)</b>    | <b>1 hr Post Injection</b>  | <b>2 hr Post Injection</b> | <b>3 hr Post Injection</b> |
| 5            | 14.5                   | Oxycodone        | 43.5 mg                    | 233 ng/ml   | 112 ng/ml                  | 50 ng/ml                   |
| 6            | 14.5                   | Oxycodone        | 43.5 mg                    | 553 ng/ml   | 285 ng/ml                  | 58 ng/ml                   |
| 7            | 13.5                   | Oxycodone        | 40.5 mg                    | 277 ng/ml   | 136 ng/ml                  | 68 ng/ml                   |
| 8            | 15.5                   | Saline           | 0                          | NS  | NS                         | NS                         |

**Table 4.2: Concentration of oxymorphone (ng/ml) in blood tissue from pigs on the first and second day of bleedings for both Study 1 and Study 2. There was not enough blood collected from pigs in Study 1 to quantify using GC/MSD for the second day of bleedings. The treated animals received subcutaneous injections of oxycodone hydrochloride (3mg/kg of 20 mg/mL oxycodone HCl) dissolved in saline. The control pigs received only saline. Blood was drawn from the animals prior to injection and each hour for three hours following injection. Extract samples were analyzed by GC/MSD. Oxymorphone is a metabolite of oxycodone. Identification and quantitation of the drugs are achieved using Selected Ion Monitoring (SIM). No detectable amounts of oxymorphone were found in any blood samples from pigs prior to them receiving oxycodone. The reportable range for these drugs are 40 ng/mL to 1000 ng/mL. ND = Not detectable. NS = No sample.**

| <b>Day 1</b> |                        |                  |                            | <b>Concentration of Oxymorphone (ng/mL) in Blood Tissue of Pigs</b> |                            |                            |
|--------------|------------------------|------------------|----------------------------|---|----------------------------|----------------------------|
| <b>Pig</b>   | <b>Pig Weight (kg)</b> | <b>Treatment</b> | <b>Dosage Administered</b> | <b>Blood Draw</b>   | <b>Blood Draw</b>          | <b>Blood Draw</b>          |
|              |                        |                  | <b>Dosage (3 mg/kg)</b>    | <b>1 hr Post Injection</b>  | <b>2 hr Post Injection</b> | <b>3 hr Post Injection</b> |
| 1            | 10.6                   | Oxycodone        | 31.9 mg                    | 139 ng/ml   | 93 ng/ml                   | 22 ng/ml                   |
| 2            | 12.3                   | Oxycodone        | 36.8 mg                    | 36 ng/ml  | 49 ng/ml                   | 51 ng/ml                   |
| 3            | 11.4                   | Oxycodone        | 34.1 mg                    | 73 ng/ml  | 65 ng/ml                   | 30 ng/ml                   |
| 4            | 8                      | Saline           | 0                          | ND  | ND                         | ND                         |
| 5            | 14.5                   | Oxycodone        | 43.5 mg                    | NS  | 62 ng/ml                   | 49 ng/ml                   |
| 6            | 14.5                   | Oxycodone        | 43.5 mg                    | 108 ng/ml   | 117 ng/ml                  | 106 ng/ml                  |
| 7            | 13.5                   | Oxycodone        | 40.5 mg                    | 58 ng/ml  | 61 ng/ml                   | 48 ng/ml                   |
| 8            | 15.5                   | Saline           | 0                          | ND  | NS                         | NS                         |

| <b>Day 2</b> |                        |                  |                            | <b>Concentration of Oxymorphone (ng/mL) in Blood Tissue of Pigs</b> |                            |                            |
|--------------|------------------------|------------------|----------------------------|---|----------------------------|----------------------------|
| <b>Pig</b>   | <b>Pig Weight (kg)</b> | <b>Treatment</b> | <b>Dosage Administered</b> | <b>Blood Draw</b>   | <b>Blood Draw</b>          | <b>Blood Draw</b>          |
|              |                        |                  | <b>Dosage (3 mg/kg)</b>    | <b>1 hr Post Injection</b>  | <b>2 hr Post Injection</b> | <b>3 hr Post Injection</b> |
| 5            | 14.5                   | Oxycodone        | 43.5 mg                    | 52 ng/ml  | 51 ng/ml                   | 40 ng/ml                   |
| 6            | 14.5                   | Oxycodone        | 43.5 mg                    | 119 ng/ml   | 156 ng/ml                  | 49 ng/ml                   |
| 7            | 13.5                   | Oxycodone        | 40.5 mg                    | 49 ng/ml  | 53 ng/ml                   | 41 ng/ml                   |
| 8            | 15.5                   | Saline           | 0                          | NS  | NS                         | NS                         |

**Table 4.3: Blood tissue of pigs were tested for oxycodone using an ELISA oxycodone assay. These samples are from the first and second day of bleedings for the pigs in Study 2. There was not enough blood collected from pigs in Study 1 to analyze using ELISA. The treated animals received subcutaneous injections of oxycodone hydrochloride (3mg/kg of 20 mg/mL oxycodone HCl) dissolved in saline. The control pigs received only saline. Blood was drawn from the animals prior to injection and each hour for three hours following injection. Oxycodone is quantified with the units of ng/mL. The ELISA assay can quantify amounts between 0 to 80 ng/ml. ND = Not detectable, NS = No sample, NEG = no oxycodone found, and POS = sample with an absorbance reading lower than assay cutoff calibrator. Samples were diluted in a (1:10 ratio) (1 part sample: 9 parts buffer wash) therefore the samples that are positive have a higher concentrations of oxycodone than indicated. All blood samples taken from all the pigs prior to injection of drug tested negative for the presence of oxycodone.**

**Day 1**

| Pig | Pig Weight (kg) | Treatment | Dosage Administered | Blood Draw          | Blood Draw          | Blood Draw          |
|-----|-----------------|-----------|---------------------|---------------------|---------------------|---------------------|
|     |                 |           | Dosage (3 mg/kg)    | 1 hr Post Injection | 2 hr Post Injection | 3 hr Post Injection |
| 5   | 14.5            | Oxycodone | 43.5 mg             | POS                 | 66.7 ng/ml          | 31 ng/ml            |
| 6   | 14.5            | Oxycodone | 43.5 mg             | POS                 | POS                 | 61 ng/ml            |
| 7   | 13.5            | Oxycodone | 40.5 mg             | 64.6 ng/ml          | 47.5 ng/ml          | 25 ng/ml            |
| 8   | 15.5            | Saline    | 0 mg                | NEG                 | NS                  | NS                  |

**Day 2**

| Pig | Pig Weight (kg) | Treatment | Dosage Administered | Blood Draw          | Blood Draw          | Blood Draw          |
|-----|-----------------|-----------|---------------------|---------------------|---------------------|---------------------|
|     |                 |           | Dosage (3 mg/kg)    | 1 hr Post Injection | 2 hr Post Injection | 3 hr Post Injection |
| 5   | 14.5            | Oxycodone | 43.5 mg             | 47 ng/ml            | 36.9 ng/ml          | 10.9 ng/ml          |
| 6   | 14.5            | Oxycodone | 43.5 mg             | POS                 | POS                 | 18.3 ng/ml          |
| 7   | 13.5            | Oxycodone | 40.5 mg             | 53.8 ng/ml          | 27.5 ng/ml          | 9.8 ng/ml           |
| 8   | 15.5            | Saline    | 0 mg                | NEG                 | NS                  | NS                  |

**Table 4.4: Time from egg eclosion to completion of larval stages and time to complete pupal stage for *Phormia regina* reared on oxycodone-treated and untreated pork loin on a 24:0 (L:D) cycle at 23°C. These data are combined data from both studies (2006 and 2007). (N = 62 time periods for oxycodone-treated and 38 for untreated)**

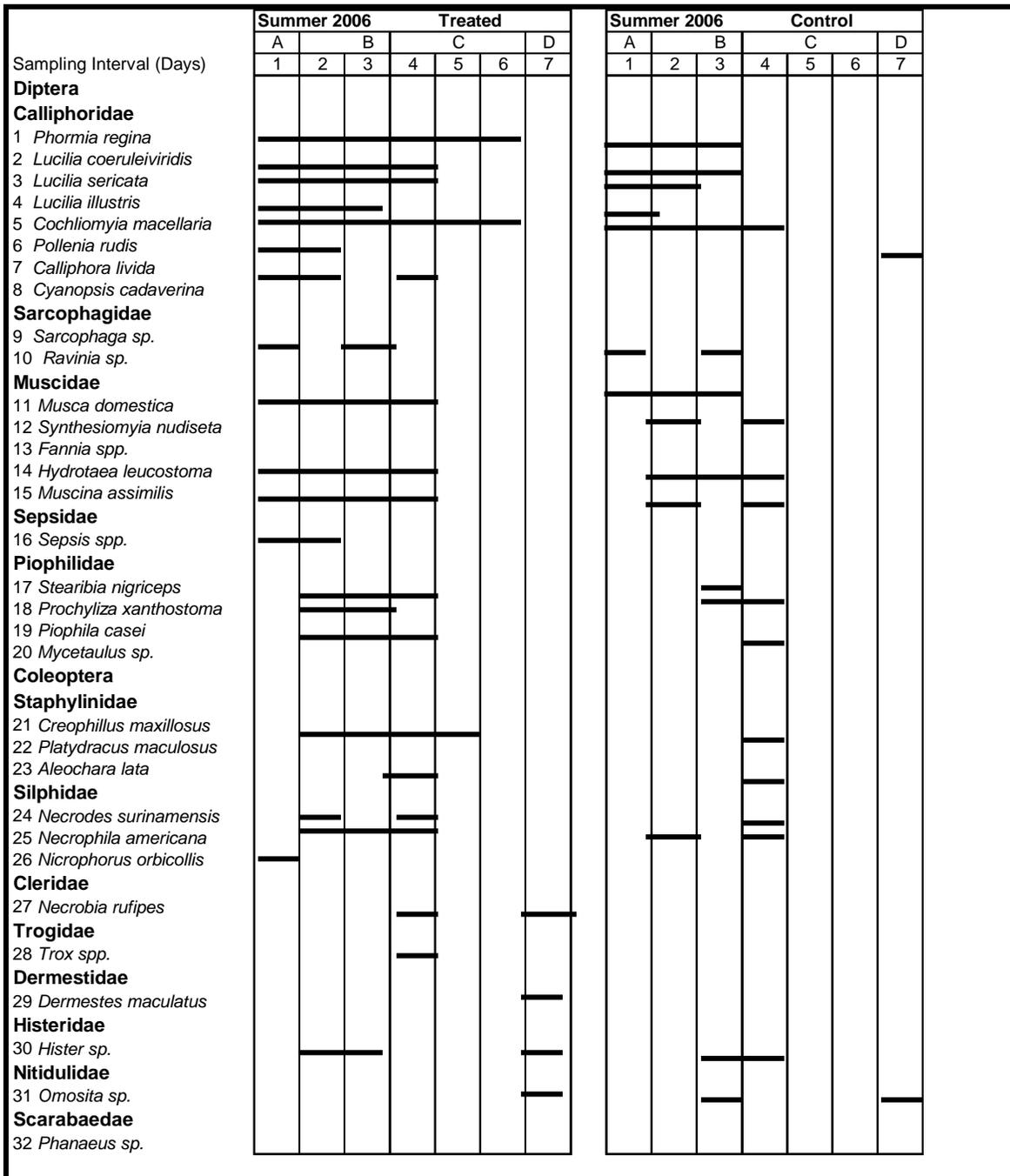
| <b>Stage</b>                    | <b>Untreated tissue<br/>(range [mean ± SD] h)</b> | <b>Oxycodone-treated tissue<br/>(range [mean ± SD] h)</b> |
|---------------------------------|---|---|
| <b>First Instar</b>             | <b>32-34 ( 33 ± 1.4 )</b>                         | <b>21-34 ( 28.3 ± 5 )</b>                                 |
| <b>Second Instar</b>            | <b>68-80 (75.3 ± 5.5 )</b>                        | <b>45-67 (56.6 ± 6.7)*</b>                                |
| <b>Third instar<sup>a</sup></b> | <b>140.5-218 (175.3 ± 27.3 )</b>                  | <b>144-194 (161.8 ± 14.4)</b>                             |
| <b>Pupal</b>                    | <b>144-186 (162.9 ± 11.5 )</b>                    | <b>130-181 (161.3 ± 12.7 )</b>                            |

<sup>a</sup>Feeding and prepupal stages combined, \*Indicates significant difference ( $\alpha = 0.05$ ) in mean time compared to the control

**Table 4.5: Time from egg eclosion to completion of larval stages and time to complete pupal stage for *Phormia regina* reared on oxycodone-treated and untreated pork liver on a 24:0 (L:D) cycle at 23°C. These data are combined data from both studies (2006 and 2007). (N = 47 time periods for oxycodone-treated and 14 for untreated)**

| <b>Stage</b>                    | <b>Untreated tissue<br/>(range [mean ± SD] h)</b> | <b>Oxycodone-treated tissue<br/>(range [mean ± SD] h)</b> |
|---------------------------------|---|---|
| <b>First Instar</b>             | <b>34-37 (35.5 ± 2.1 )</b>                        | <b>36-45 (39 ± 4.1)</b>                                   |
| <b>Second Instar</b>            | <b>82-101 (91.3 ± 7.5)</b>                        | <b>76-103 (86.8 ± 9.7)</b>                                |
| <b>Third instar<sup>a</sup></b> | <b>162-173 (167.5 ± 7.8)</b>                      | <b>158-202.5 (176.1 ± 16.1 )</b>                          |
| <b>Pupal</b>                    | <b>144-168 (158.4 ± 10.2)</b>                     | <b>137.5-192 (158.4 ± 15.4)</b>                           |

<sup>a</sup>Feeding and prepupal stages combined



**Figure 4.1: Succession Diagram for Oxycodone-treated and Untreated pig carcasses during 7 day sampling intervals in summer 2006 in Blacksburg, VA. Stage of decomposition are shown and are (A) Fresh, (B) Bloat, (C) Active Decay, and (D) Advanced Decay**

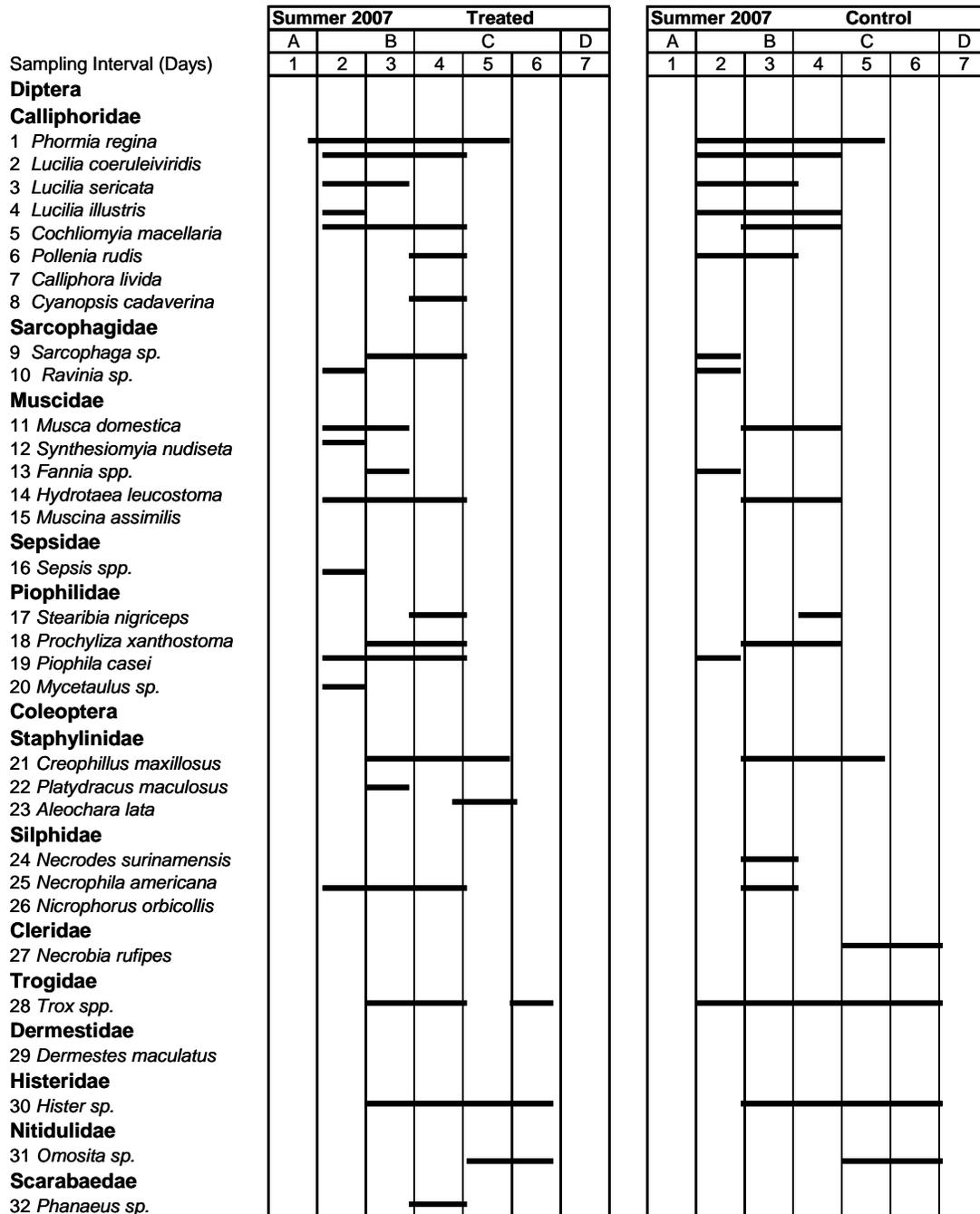
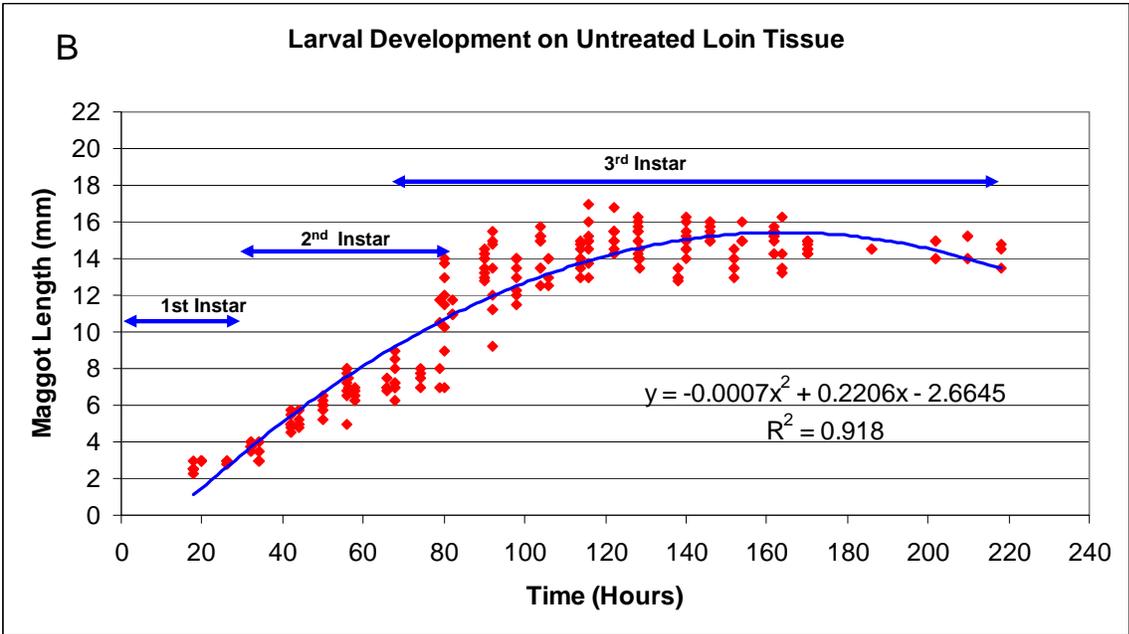
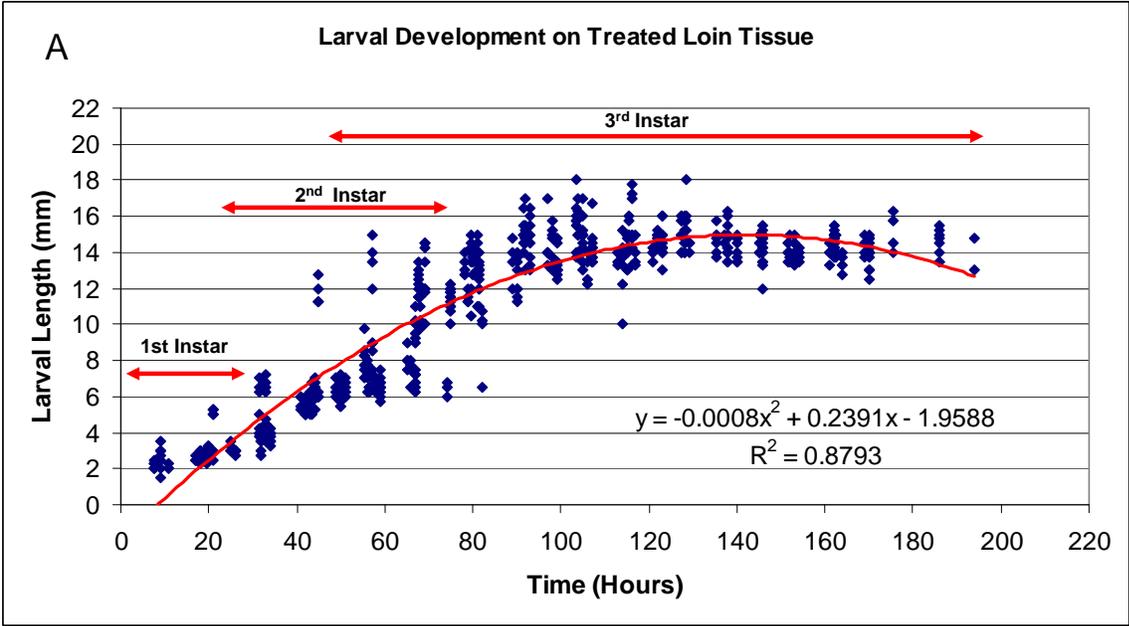
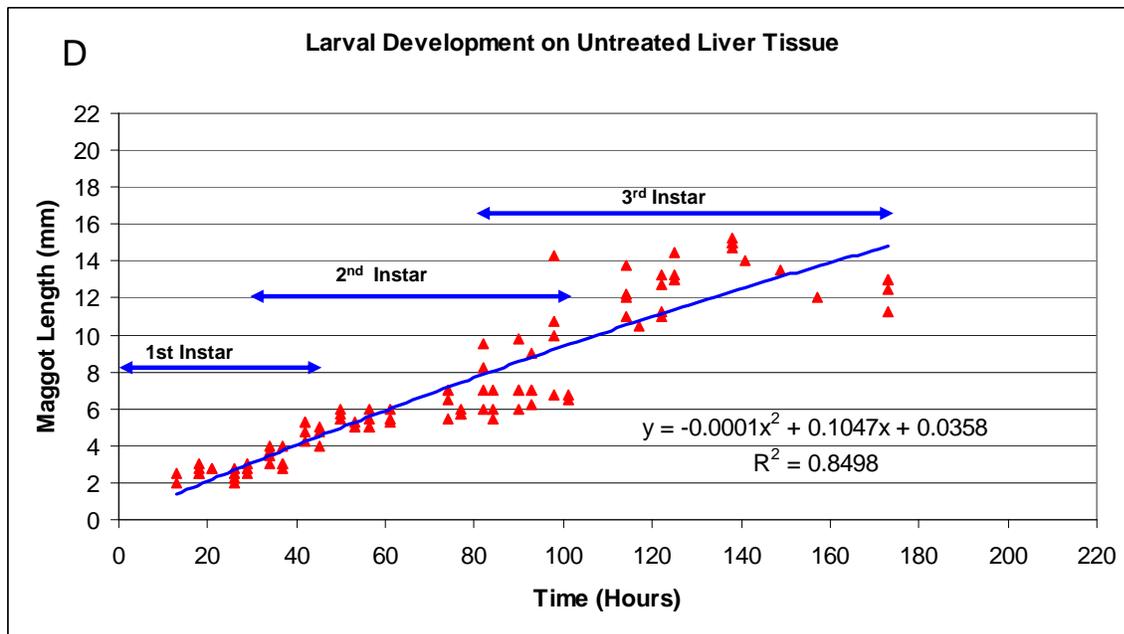
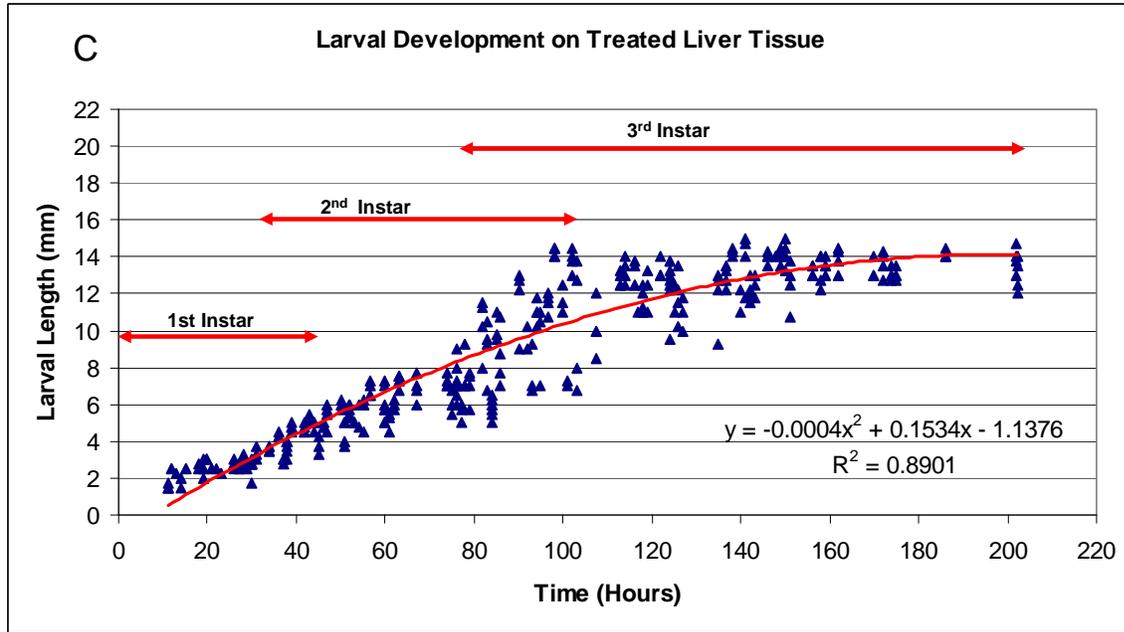
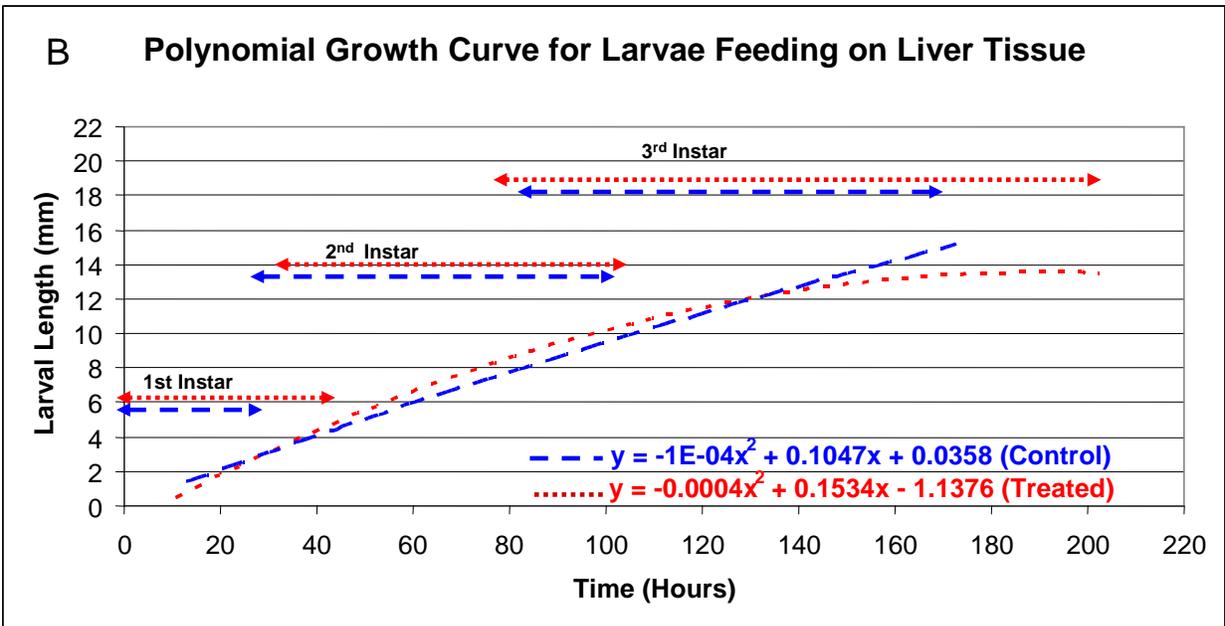
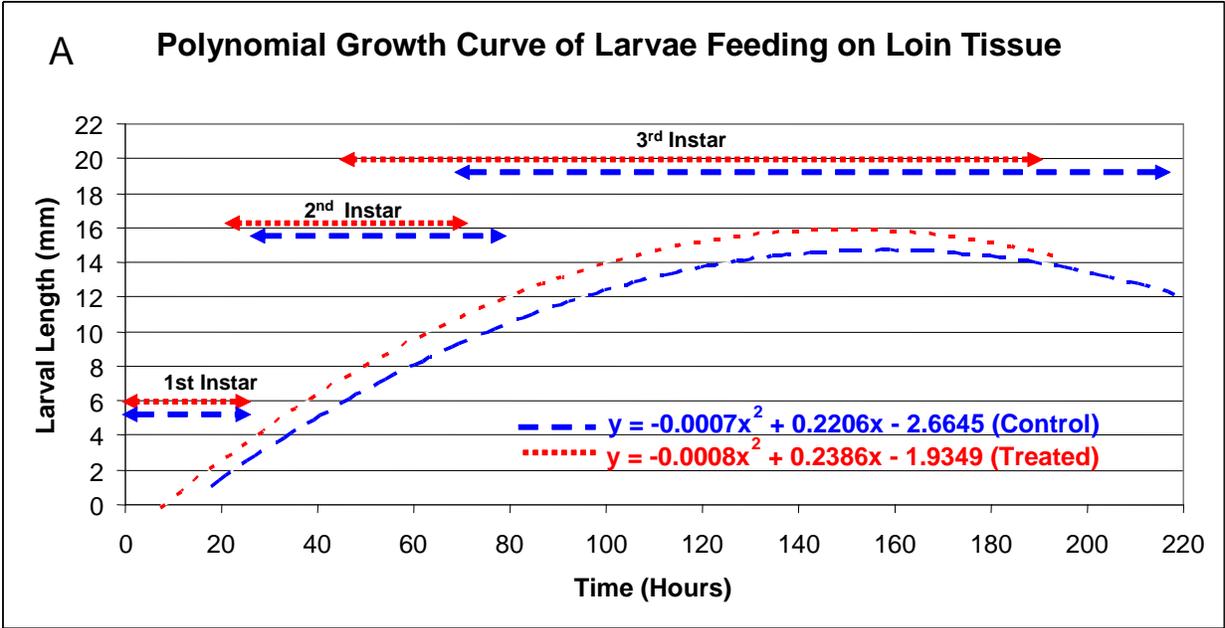


Figure 4.2: Succession Diagram for Oxycodone-treated and Untreated pig carcasses during 7 day sampling intervals in summer 2007 in Blacksburg, VA. Stage of decomposition are shown and are (A) Fresh, (B) Bloat, (C) Active Decay, and (D) Advanced Decay

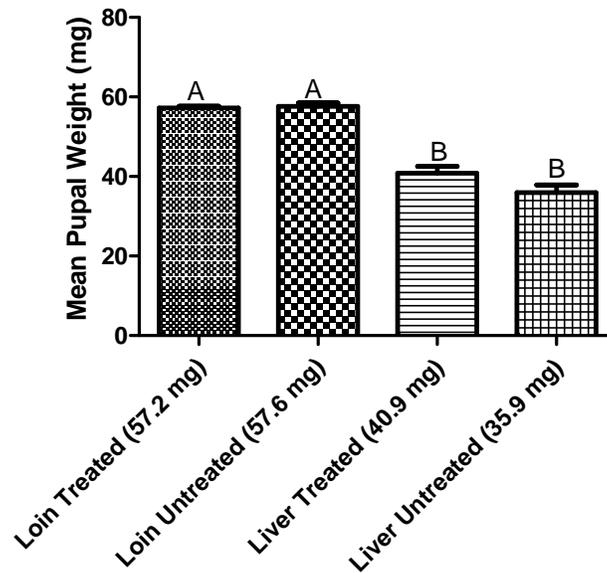




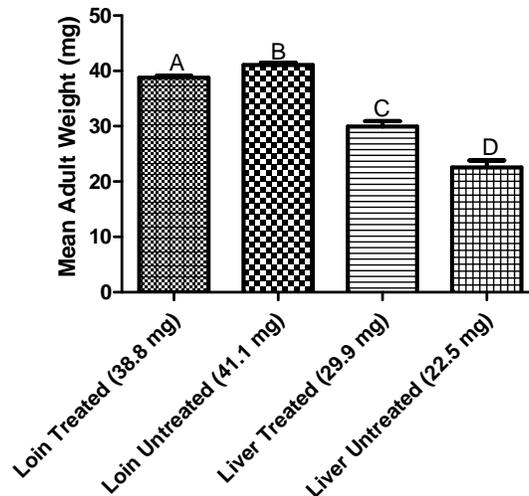
**Figure 4.3:** Lengths of maggots feeding on either (A) oxycodone-treated loin tissue, (B) untreated loin tissue, (C) oxycodone-treated liver tissue, or (D) untreated liver tissue. Larvae were reared on a 24:0 (L:D) cycle at 23°C. Four maggots were sampled every eight hours from all treatments and the control. Maggot lengths were measured. A second order polynomial was used as the fitted curve equation. N= 670 larvae for treated loin, 242 for untreated loin, 358 for treated liver, and 96 for the untreated liver.



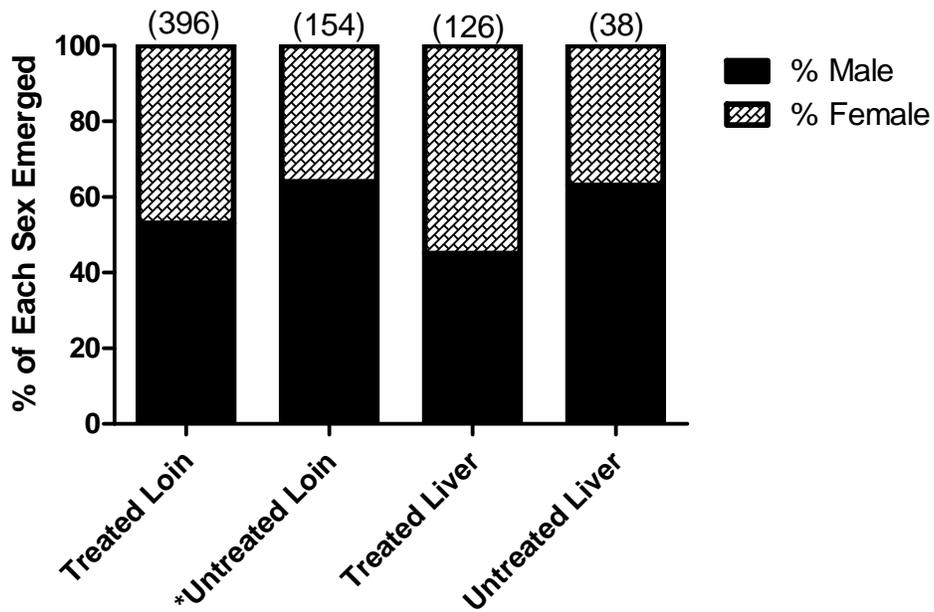
**Figure 4.4:** Fitted curves of lengths of maggots feeding on tissue from oxycodone-treated and untreated (A) loin and (B) liver tissue. A second order polynomial was used as the fitted curve equation.



**Figure 4.5:** Mean pupal weight (mg) (Mean  $\pm$  SE) of larvae that fed on liver or loin from oxycodone-treated and untreated tissue. Larvae and pupae were reared on a 24:0 (L:D) cycle at 23°C. N= 259 pupae for treated loin and 111 for untreated loin. N= 89 pupae for treated liver and 24 for untreated liver. No significant difference was seen between pupal weights of larvae that fed on treated and untreated loin (A) as well as between larvae that fed on treated and untreated liver (B).



**Figure 4.6:** Adult weight (mg) (Mean  $\pm$  SE) of larvae that fed on liver or loin from oxycodone-treated and untreated tissue. Larvae and pupae were reared on a 24:0 (L:D) cycle at 23°C. N= 396 adults for treated loin and 153 for untreated. N= 126 adults for treated liver and 38 for untreated. A significant difference was seen between adult weights of larvae that fed on treated loin (A) and untreated loin (B) and between adult weights of larvae that fed on treated liver (C) and untreated liver (D).



**Figure 4.7:** The percentage of males and females that emerged from the pupae of larvae that fed on oxycodone-treated or untreated pork loin and the percentage of males and females that emerged from the pupae of larvae that fed on treated and untreated pork liver. A difference among totals is due to more replications being run because of more tissue available (i.e more treated pigs than untreated) N = 396 adults for Treated Loin, 154 for Untreated Loin, 126 for Treated Liver, and 38 for Untreated Liver. \*Indicates a biased sex ratio.

## **5. Comparison of Thermal Factors and PMI Estimates Using Forensically Important Flies**

### **5.1. Introduction**

The Postmortem Interval (PMI), or time elapsed since death provides an important piece of information in homicide investigations and untimely deaths (Byrd and Castner 2001). PMI can be determined by various methods including the physical and chemical changes of the corpse (Henssge et al. 1995), as well as the succession of arthropod species found on and within the body (Schoenly and Reid 1987). Development data for primary blow flies provide the most accurate means of estimating the PMI using arthropod information (Greenberg 1991). It is presumed that the first individuals arriving and laying eggs on a corpse do so soon after death (Catts and Goff 1992).

In order for insect development analysis to yield an accurate estimate of PMI, data must be available for the earliest colonizer species collected from the corpse at the time of discovery. Therefore, the most relevant colonizers are the oldest individuals derived from the first eggs deposited on the body. Certain species can oviposit within a few hours following death and continue to do so for at least two weeks (Smeeton et al. 1984). It is customary for investigators to collect samples for rearing, as well as for preservation (Catts and Haskell 1990). A sub-sample of the eggs/maggots/pupae collected at the time of the discovery of the corpse is reared in the laboratory until adult emergence, after which they can be identified using keys for adult flies (Hall and Townsend, Jr. 1977, Smith 1986, McAlpine 1987).

After identifying specimens from the body, the next step is to link this information to the temperature at the death scene. Obtaining representative data from the body discovery site is essential for estimating minimum PMI. Temperature determines larval growth and insect succession rates (Goff and Lord 1994). Normally ambient temperatures obtained from a weather station nearest to the site of discovery of the body are used in the estimation of PMI. However, there may be significant differences between the temperatures experienced at the discovery site and the weather station. The effect of season, distance from the weather station, sun and shade, and indoor and outdoor settings may all impact the accuracy of a PMI estimation. Weather station data can be 'corrected', using a correction factor calculated from the weather station data and hourly temperature readings recorded at the death scene for 3-5 days after the body was

discovered. These corrected values provide an estimate of the temperature at the crime scene before the corpse was found. Validation studies show that weather data that are retrospectively corrected provide a more accurate representation of site temperatures for a period of time than uncorrected data (Archer 2004).

A number of other factors need to be taken into account when calculating PMI to reduce errors in the estimates. For example, it is important to consider using the temperature of the maggot mass as the temperature for larval development in particular instars since it may be the highest temperature experienced by the larvae (Higley and Haskell, 2001). Experimental growth data needs to be available for the species found on the body and, if no data are available, then that research must be done to support PMI estimates. Currently, there are a few published papers that provide data on *Phormia regina* development (Anderson 2000, Byrd and Allen 2001, Greenberg 1991, and Kamal 1958), but the data vary among the studies. In addition, appropriate base temperatures must be used for each individual species. Oliveira-Costa and de Mello-Patiu (2004) point out that calculations incorporating an inappropriate base temperature will overestimate or underestimate accumulated degree hours (ADH).

There is a lack of data comparing ambient site temperatures and the temperatures experienced by maggots on a corpse. The present study was designed to investigate the methods used by experts in forensic entomology and to determine the validity of those practices. This study makes comparisons among weather data sources, Accumulated Degree-Day (ADD) methods, and PMI estimations based on developmental threshold and the developmental data source. *Phormia regina*, a ubiquitous and forensically important fly, was the species of choice for the study. This species was also the most abundant fly collected during the study. Baseline developmental temperature data for *Phormia* has given a minimum threshold that has ranged from 0 to 14 degrees (Higley and Haskell 2001, Byrd and Allen 2001, Haskell 1993, Marchenko 2001).

Individually, some factors in estimating PMI such as weather station data source (Archer 2004), degree-day method (VanLaerhoven 2008), and minimum threshold (Nabity et al. 2006) have been investigated. This study has three objectives: 1) to determine whether three factors; location of probe (body, inside cage, or ambient), threshold (10,12.2, or 14°C), and degree-day method (averaging, single sine, or single triangle) interact with each other and determine if each factor was significant individually; 2) to determine whether three factors; weather station

location (Moore Farm, Kentland Farm, or Blacksburg Airport), minimum threshold used in degree-day calculation (10,12.2, or 14°C), and degree-day method (averaging, single sine, or single triangle) interact with each other and to determine if each factor was significant individually, and 3) to make comparisons among estimates of PMI made using different developmental data sources (i.e. Byrd and Allen (2001) or Anderson (2000)), thresholds(10,12.2, or 14°C), and weather station data (Moore Farm, Kentland Farm, or Blacksburg Airport).

## **5.2. Materials and Methods**

### **5.2.1. Study Site**

Studies were conducted in the summers (June to August) of 2007 and 2008 at Moore Farm (N37° 13.436', W80° 27.674'), a research facility of Virginia Polytechnic Institute and State University (Virginia Tech) located at Blacksburg in Montgomery County in southwest Virginia. Temperature data for the wider area around the study site were obtained from a weather station at Kentland Farm (N37° 11.853', W80° 34.236') another research facility of Virginia Tech located  $\approx$  6.5 miles from Moore Farm, and from the Blacksburg Airport (N37° 12.349', W80° 24.556') located  $\approx$  3.5 miles from Moore Farm. The distance between the Blacksburg Airport and Kentland Farm is  $\approx$  8.9 miles.

### **5.2.2. Animals and Cages**

Six stillborn pigs (*Sus scrofa* Linnaeus) ranging from 0.91-3.63 kg were used as the model species in the 2007 study and four pigs ranging from 1.0-1.72 kg were used in the 2008 study. The pigs had an average weight of  $2.18 \pm 1.03$  kg (mean  $\pm$  SD) in 2007 and  $1.41 \pm 0.31$  kg (mean  $\pm$  SD) in 2008.

The pigs were obtained from the Swine Center at Virginia Tech. Shortly after birth, the pigs died of natural causes and were frozen until used. Approximately half of the dead fetal pigs had a hernia which was the most likely cause of death. No other readily visible signs of trauma or defects were observed.

Pigs were allowed to thaw individually in a sealed garbage bag in a refrigerator for 24 hours prior to being placed in the test cage. The pigs were allowed to reach room temperature for approximately four additional hours in the investigational test cage before placement in the field. Placement in the field was used as the time of death for PMI calculations.

Each test cage measured 40.5 x 42 x 51 cm and consisted of four plexiglass sides and two thick nylon mesh sides. The bottom of the cage had a 40.5 x 20 cm rectangular opening lined

with a wire mesh to allow for fluid drainage. The top of the cage also had circular holes 15 cm in diameter that were covered with insect screening for ventilation. The screening was normally too small for most flies and beetles to pass through. Nine holes approximately 2 cm in diameter were drilled 16.5 mm equidistant from each other to allow for the passage of three probes attached to metal rods. When the remaining holes were not in use they were covered with tape. A hinged door measuring 29 x 21.5 cm was constructed on one of the plexiglass sides. The modified test cage protected the small carcass from large predators, but still allowed the carcass to be colonized by insects.

Thawed pigs were taken to Moore Farm and were examined carefully for signs of fly oviposition before each was placed inside a test cage where the cage was opened to allow easy access for blowfly colonization. The test pig was placed inside the cage by opening the hinged door and propping (Figure 5.1 A) it open for 24 hours to allow easy access for adult blowflies, and until a sufficient number of eggs were present to generate a maggot mass.

Two HOBO<sup>®</sup> H8 Outdoor/Industrial 4-Channel External loggers (Onset Computer Corporation, Onset, MA) were used to record the temperature data every 12 minutes from the time the cage was placed at Moore Farm till the end of each replication. The HOBO<sup>®</sup> H8 Outdoor/Industrial 4-Channel External loggers were both attached to a separate wire cage that measured 92 x 92 x 153 cm and consisted of a metal frame constructed with 2.5 cm steel-welded tubing, which was enclosed with 1.27 cm mesh hardware cloth. The data loggers were also covered with Ziploc<sup>®</sup> bags (S.C. Johnson & Sons, Inc., Racine, WI) to protect the loggers from moisture. One of the data loggers contained four probes while the other had three probes. Two probes recorded ambient temperature, two probes recorded the internal temperature of the cage, and three probes recorded the temperature of the body/maggot mass (one placed on the head, thoracic, and abdominal area). The three probes that recorded body/maggot mass were attached to the metal rods and held in place by clamps at the top of the cage (Figure 5.1 B). Studies were ended when the pig was skeletonized and little to no soft tissue was left.

Once larval aggregations started, the probes were positioned in the aggregations and adjusted daily to stay in contact with the larvae until the larvae dispersed for pupation. Temperature measurements for the three body areas (head, thoracic area, and abdominal area) were obtained by gently inserting the rod with the probe into the aggregation approximately two cm.

### 5.2.3. Sampling Protocol

The design of the test cage prevented the collection of adults from aerial net sweeps above and around the carcass. During each trial, maggots were sampled from the body at least once a day. Half of the maggots collected were preserved in glass vials filled with K.A.A. fixative (10 ml kerosene, 80-100 ml 95% ethanol, and 20 ml acetic acid) (Catts and Haskell 1990). The other half of the maggots collected were reared to the adult stage. Maggots were reared in 500 ml MicroGourmet<sup>®</sup> SOLO cups (Solo Cup Co., Urbana, IL). The maggots were placed in pouches made with aluminum foil containing ground pork (Catts and Haskell 1990). Each pouch was then placed in a rearing cup containing  $\approx 7$  cm of vermiculite in which migrating maggots could pupate. A large hole was cut in the lid of each cup to provide aeration and a piece of mesh cloth was placed under the lid to prevent maggots from escaping. Rearing cups with maggots were placed in an incubator set at 23°C at constant light. Adults that emerged were killed after a few days by placing them in Ziploc<sup>®</sup> bags containing paper towels moistened with ethyl acetate. These adults were then pinned, labeled, and identified to species. The minimum age (i.e. instar) of fly larvae were determined by the number of spiracular slits. The species of the larvae were either all *Phormia regina* or a mixture of *Phormia regina*, *Lucilia coeruleiviridis*, *Lucilia sericata*, and *Cochliomyia macellaria*. *Phormia regina* was the most abundant species collected.

The cage and probes were cleaned after a pig was skeletonized and the procedure was repeated for the next pig.

### 5.2.4. Statistical Comparisons

Temperature data for ten pigs were collected over the two summers, however only data from four pigs were used to make statistical comparisons because the pigs from these studies matched up closely in type of weather conditions and start date between summers. Some pigs were not used to make statistical comparisons because of certain weather factors (i.e. several days of rain or low temperatures), species composition on the body (i.e. *Phormia regina* was not present on the pig or was not found as a third instar), or because of a recording error (i.e. a broken probe that did not record temperatures). Pig 1 and 2 weighed 3.63 kg and 2.72 kg respectively and were both from the summer of 2007. Pig 3 and 4 weighed 1.36 kg and 1.54 kg respectively and were both from the summer of 2008.

Accumulated Degree-Days (ADD) (Arnold 1959) was calculated from daily maximum and minimum temperatures recorded by the probes for each of the four pigs and from the weather stations at two external sites: the Blacksburg Airport and Kentland Farm. The ADD was calculated by hand using the averaging (rectangular) method. Averaging and rectangular are the same method and the terms are used interchangeably throughout the chapter.

The development thresholds were set at 10, 12.2, and 14°C for the lower and 45°C for the higher values, following the estimates provided by Byrd and Allen (2001), Nabity et al. (2006), and Wigglesworth (1967). According to Anderson (2000), 68.6 – 77.5 degree-days are required for *Phormia regina* to reach a feeding third-instar for a 10°C threshold, and 57 - 64.4 and 47.5 - 53.6 degree-days for a 12.2 and 14°C threshold, respectively. According to Byrd and Allen (2001), 28.8 – 42.5 degree-days are required for *Phormia regina* to reach a feeding third-instar for a 10°C threshold, and 20.2 – 33.2 and 15.5 – 25.5 degree-days for a 12.2 and 14°C threshold. Calculations for the two other methods, triangle and sine, were performed with the Degree-Day Utility software (UC IPM 1990) using a horizontal cutoff. The endpoint for each pig was standardized to the time when a feeding third instar was first collected from the body, because it is difficult to determine the first pre-pupal (wandering stage) or pupal stage with the experimental setup under field conditions.

Statistical comparisons of probe location, degree-day method, and minimum threshold were conducted using a three-way factorial linear fit model using standard least squares and Tukey's HSD test for mean separations within JMP<sup>®</sup> 7.0.2 (SAS Institute, Cary, NC). Statistical comparisons were also made of the three climate data locations: Moore Farm, Kentland, and Blacksburg Airport, with a three-way factorial linear fit model using standard least squares and Tukey's HSD test for mean separations. The three-way factorial tested for interactions between the method and probe location, method and development threshold, probe location and development threshold, as well as significant differences within the method, probe location, and development threshold. The program also compared the degree-day methods, probe location, and threshold individually. The data that were entered into the program were the ADD calculated from the time third instars were first collected from each individual pig to the known time that the pig was placed in the field.

PMI estimates were calculated using the averaging model (rectangular method) of DD calculations with a minimum developmental threshold of 10, 12.2, and 14°C and Anderson's

(2000) developmental data. The estimate was compared with the actual PMI. Additional estimates were calculated using Byrd and Allen's (2001) developmental data. The developmental data provided both the minimum and maximum time to reach the third-instar developmental stage, therefore both were used to calculate a range for the minimum PMI estimate. The data used to calculate PMI were from the ambient temperature data from Moore Farm, the Blacksburg Airport, and Kentland Farm. Since these were ambient temperature data no maggot mass temperatures were included in any of the calculations. Another comparison was made using the temperature data of accumulated degree-days with the probe placed in the head of each of the four pigs. PMI intervals were calculated using the averaging method with Anderson's (2000) and Byrd and Allen's (2001) development data as well (Table 5.1). No statistical test was conducted for PMI.

### 5.3. Results

There were significant differences according to the ANOVA, ( $F = 8.44$ ,  $df = 44,135$ ,  $P < 0.0001$ ). There were no significant interactions. The only significant differences in ADD occurred for the main effects of probe location ( $F=28.07$ ,  $df = 4, 175$ ,  $P<0.0001$ ; Figure 5.2) and development threshold ( $F= 45.8$ ,  $df= 2.177$ ,  $P < 0.05$ ; Figure 5.3).

Statistical comparisons of ADD calculated from the temperature readings at three locations: Moore Farm, Kentland, and Blacksburg Airport were also tested with a three-way factorial linear fit model using standard least squares. This model tested for interactions between the method and location, method and development threshold, location and development threshold, as well as significant differences within the method, location, and development threshold. The data that were entered into the program was the ADD calculated from the known time of placement for that pig to the time third instars were first collected from each individual pig. There were significant differences according to the ANOVA, ( $F = 11.83$ ,  $df =26,81$ ,  $P < 0.05$ ). However, there were no significant interactions (Method\*Location, Method\*Development Threshold, Location\*Developmental Threshold, and Method\*Location\*Developmental Threshold) in the model. The only significant differences ADD occurred for the main effects of location ( $F = 17.63$ ,  $df =2,105$ ,  $P < 0.0001$ ; Figure 5.4) and the development threshold ( $F = 61.0$ ,  $df = 2,105$ ,  $P < 0.0001$ ).

Different PMI estimations can result by using development data from different sources. For all pigs, Anderson's (2000) data overestimated PMI using the rectangular method with the

weather data from all three locations, whereas Byrd and Allen's (2001) data tended to slightly underestimate PMI. However, most of the estimates made with their data provided ranges that included the actual date of death (Table 5.1). More specifically, Byrd and Allen's (2001) data underestimated Pig 2 and 3's PMI at all three weather data locations when at thresholds of 12.2 and 14°C were used. Byrd and Allen's data gave a more accurate estimate for Pigs 1 and 4 especially at a threshold 10°C for all three weather data locations. For all pigs, Anderson's data (2000) gave the most accurate PMI with the temperature data from the head probe and her data provided a range that included the actual date of placement for all the pigs. Byrd and Allen's (2001) development data underestimated the PMI by a day for all four pigs when using temperature data from the head probe (Table 5.2).

#### **5.4. Discussion**

There is some debate among forensic entomologists about how PMI should be calculated and this is due in part to a lack of studies that validate the principles and techniques. One area subject to disagreement is whether maggot mass temperature should be used in the Degree-Day calculations. A large number of larvae in the same location can generate a temperature that is higher than the ambient, causing a "maggot mass" effect (Cianci and Sheldon 1990, Goodbrod and Goff 1990, Greenberg 1991, Turner and Howard 1992). The higher temperatures are due to many carrion fly larvae having a metabolic and feeding rate greater than most immature insects (Hanski 1976, Levot et al. 1979). Because maggots feeding in a mass can increase the temperature to a level higher than ambient air temperature, it may increase the developmental rate of the flies compared with the expected development rate of individuals exposed to ambient air temperatures. However, in some situations the oldest maggots may migrate away from the body prior to the development of elevated temperatures by the maggot mass. In such cases maggot mass temperatures should not be incorporated into the ADH or ADD calculations for PMI (Dillon 1997).

In this study significant difference in the ADD calculated from the temperatures recorded by the probes inserted in the body of the pigs (i.e., the probes in head, and thoracic, and abdominal areas) and the internal cage temperatures were observed. Significant difference were also observed in the ADD from the internal cage temperatures and the outside (ambient temperature) temperatures, as well as between all body temperatures and the outside temperature. The data suggest that the temperatures maggots experienced on the body are higher than the

ambient air temperature, a difference that is most likely due to either a maggot mass and/or heating of the body by the sun. The study cage with the dead pigs was exposed to direct sunlight for several hours each day. A corpse in the sun will lose biomass more rapidly than a body in the shade and will progress through the decompositional stages faster (Dillon 1997, Reed 1958, Shean et al. 1993). The cage itself provided some insulation as the air temperatures in the cage were higher than the ambient air temperatures; however the temperatures on the body were significantly higher than the cage ambient temperatures. The temperature probes inside the cage were approximately 15 cm above the body, and averaged temperatures that were less than the temperatures from the body probes, but had slightly higher temperatures than the ambient temperatures had acquired. The higher body temperatures resulted in the highest number of ADD. Such differences between temperatures associated with the corpse/maggot mass and ambient temperatures can result in incorrect estimations of PMI.

Another common consideration in forensic studies and the estimation of PMI is choosing which weather station location to use to obtain temperature data, and whether or not to correct such data to approximate the temperature at the body discovery site. Normally, when insect collecting at a death scene is completed, weather data from one to two weeks prior to a rough estimate when death occurred, and to three to five days past the time the body was discovered, is obtained from the nearest weather station (Byrd and Castner 2001). However, there may be significant differences between the temperatures experienced at the death scene (site) and from the weather station (Catts 1992). Considerable differences can occur even within short spatial distances. The investigator can obtain corrected death scene temperatures by plotting a scatter diagram of the meteorological temperatures (x axis) against the death scene temperatures (y axis) recorded for 3-5 days after the body was discovered. A regression equation is then developed and used to correct each of the weather station readings to generate predictions of death scene temperatures (Gennard 2007). The correction of weather data using the correlation method has been reported to provide a more accurate representation of site temperatures than uncorrected data (Archer 2004). In this study, Tukey's HSD test showed a significant difference between the ADD for all the weather station locations. This study also considered the ambient temperature recorded by the HOBO<sup>®</sup> data logger at the Moore Farm study site to be a weather station location. The shortest distance between the three locations is the distance between Moore Farm and the Blacksburg Airport which is approximately 3.5 miles. Even for this relatively short

distance there is a significant difference between the ADD. values. For example, total ADD hour difference for Fig 1 between Blacksburg Airport and Moore farm using a 10°C threshold is 3.1 ADD, a 12.2°C threshold is 4.6 ADD, and a 14°C threshold is 6.4 ADD. Differences between the sites could result in an inaccurate PMI unless that station data is corrected with the death scene site using a linear regression.

The use of minimum developmental thresholds at which different species of flies cease to develop is also controversial. In this study, the mean ADD for the thresholds 10°C, 12.2°C, 14°C were 69.3, 60.2, and 52.8, respectively. Tukey's HSD test revealed that the mean ADDs for all three thresholds were significantly different from each other,  $P < 0.05$ . The developmental minima, maxima, and thermal constant are determined by regressing  $1/\text{developmental time}$  versus temperature and by using the x-intercept of this regression as a base temperature for calculating the thermal constant. The thermal constant is  $\text{Time}_{(24 \text{ hours})} \times (\text{temperature} - \text{base temperature}) = \text{ADD}$ . The ADD to reach each life stage of the insect can be determined with the thermal constant. The maximum threshold is less well-defined but is the temperature at and above which the rate of development begins to decrease (Zalom et al. 1983). The maximum threshold is probably close to the temperature that is lethal to the species (Byrd and Castner 2001). The minimum and maximum thresholds are different for each species. For example, Wigglesworth (1967) suggested the maximum temperature for *Calliphora* spp. larvae was 39°C, while for *Phormia* spp. it was 45°C. Upper threshold temperatures are rarely experienced at death scenes as an ambient temperature, although if temperatures do remain near the maximum for a long period of time, it will affect the PMI estimate (Gennard 2007). Byrd and Allen (2001) reported a maximum threshold of 40°C for the black blow fly *Phormia regina*. More variation occurs with the minimum threshold. Recently, Nabity et al. (2006) determined that the minimum developmental temperature to be higher (14°C) than that generated under the x-intercept method (5.46°C) by using data from oviposition to adult emergence. Greenberg (1991) and Anderson (2000) used 0°C, whereas Byrd and Allen (2001) used and observed 10°C to calculate thermal constants for *Phormia regina*. Other minimum threshold temperatures include 12.5°C (Haskell 1993) and 11.4°C (Marchenko 2001). The results of the statistical analysis done in this study indicate that there are significant differences in the ADD values where different thresholds are used. The three thresholds used in this study (10, 12.2, and 14°C) were chosen because 10°C was the minimum threshold observed by Byrd and Allen (2001) for

*Phormia regina*, 14°C was the observed minimum threshold by Nabity et al. (2006), and 12.2°C fell in the middle of those thresholds. *Phormia regina* was the species used to calculate ADD and determine PMI as it was found on all pigs in this study and it was the most abundant fly collected. The lower the minimum threshold, the more accumulated degree-days (Wilson and Barnett 1983). Use of an inappropriate minimum threshold invalidates the basic assumption of linearity in degree-day models. When values higher than the x-intercept are used, fewer ADD are needed for development than is actually the case. Also, when values are lower than the x-intercept, more ADD are needed than the true value. Therefore using observed minimum thresholds or investigator selected temperatures for calculations results in underestimating or overestimating actual degree-days, which transfers into the inaccurate estimates of PMI (Nabity et al. 2006). Some authors have suggested that geographic variation may lead to differences in developmental times and thus developmental minimums (Greenberg 1991, Grasberger and Rieter 2001). Developmental studies combined with molecular methods may find a link between populations of a given fly species and differences in minimum developmental thresholds.

One area that does not seem to be as controversial is the method used to calculate degree-days from temperature records. The most commonly used method is the averaging method (Higley and Haskell 2001). There are other linear approximation methods that can be used such as single triangle, double triangle, single sine, and double sine. In this study we compared the ADD of the methods and found there was no significant interaction or difference between the methods of the averaging (rectangular), single triangle, and single sine. Table 5.3 shows the ADD for all three methods for Pig 1 using all three thresholds. Other studies have made comparisons with degree-day methods and have found them to be very similar to each other in terms of ADH or ADD (Fry 1983, VanLaerhoven 2008). From all of the published research studies examined it appears that in situations where daily temperatures fall entirely between developmental thresholds, any of the methods of degree-day calculation would be acceptable. It is when temperatures fall outside the developmental thresholds that noticeable and significant differences between methods could arise.

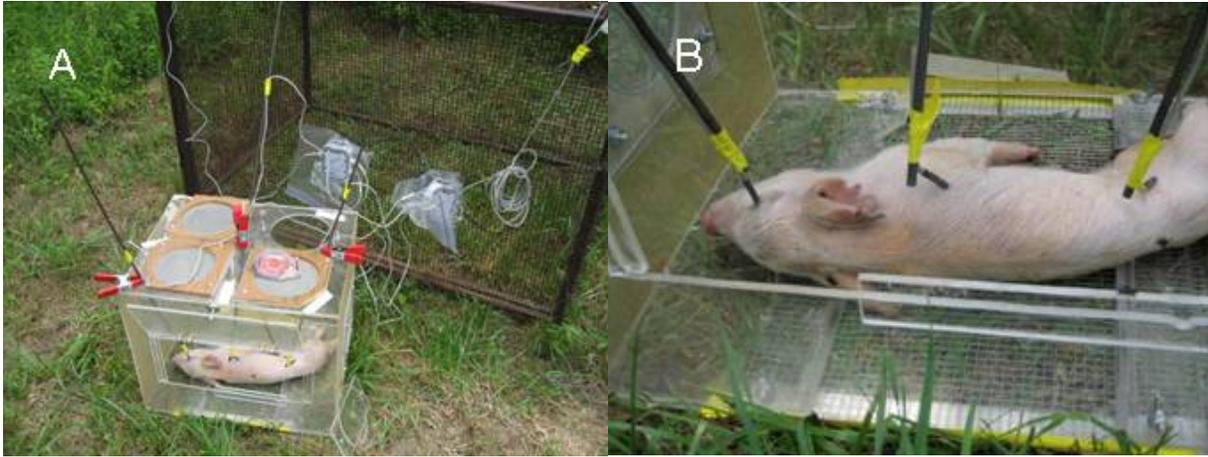
If one puts the factors in the context of PMI; minimum threshold, developmental data source, and weather station source are more important factors that affect PMI than the degree-day method used. Using Anderson's (2000) developmental data set overestimated the PMI for all the pigs for all three weather data locations. These results mean that for all the pigs, the

estimated time of death would have occurred before any of the pigs were placed out at Moore Farm. Byrd and Allen's (2001) data set provides a more accurate PMI for all of the pigs, however some PMI intervals were underestimated especially when higher thresholds were used, 12.2 and 14°C (Table 5.1). One interesting observation to note is that the Kentland data provided the most accurate PMI estimates with Byrd and Allen's (2001) data set for all thresholds used in determining PMI with weather station data, however it is the furthest weather station source from the death scene. Conversely, Anderson's data (2000) gave the most accurate PMI with the temperature data from the head probe for all four pigs and her data provided a range that included the actual date of death for all the pigs (Table 5.2). Byrd and Allen's (2001) development data underestimated the PMI by a day for all four pigs when using temperature data from the head probe. A possible explanation why Anderson's source data does not provide accurate estimations for a PMI with ambient temperature data is that in her developmental study she reared the maggots in masses within large jars, so there was the effect of the maggot mass temperature taken into account when calculating development using these data (Anderson 2000). Byrd and Allen (2001) purposefully kept a ratio of 1.5 larvae per 1.0 g of pork by adding meat to their rearing cups. They did this so that the metabolic heat generated by the feeding larvae would not be higher than that of the growth chamber settings, thus negating a maggot mass effect.

The choice of an incorrect threshold temperature in PMI determinations combined with some developmental data sources, can lead to problems with providing accurate PMI intervals. One can correct weather station data, and one can factor in a maggot mass temperature into degree-day calculations, if applicable to the age of insects found on a body at the death scene. In this study all three probes that were in contact with the body and with the maggot mass led to significantly higher temperatures than the ambient temperature, therefore including maggot mass temperatures with ambient temperature data would have been reasonable. However, confusion and controversy surrounding the selection of a proper threshold is due to a lack of information about the development of fly species like *Phormia regina*. For example, some evidence points to *Phormia regina* having a unique minimum threshold and development rate for each stage. Nability et al. (2006) conducted a development study with *Phormia regina* and observed egg hatch at 11.7°C, cessation of larval development at and below 12°C (larvae died), and at a temperature of 12.2°C larvae pupated, but did not emerge. They observed complete egg to adult

development at 14°C. Using a linear regression analysis, they determined a minimum threshold of 5.4°C. The incongruence with this study is that the authors observed development at 14°C and higher, but one would obtain a less accurate PMI if one used a threshold higher or lower than 5.4°C with these development data. The minimum threshold observed by Nabity et al. (2006) is also different from what Byrd and Allen's (2001) observed threshold of 10°C. The difference in observed thresholds points to the possibility that there may be geographic variation among fly populations which would account for differences in developmental times and minimums. More developmental studies with *Phormia* from different geographic locations could help in finding the best threshold to use when calculating ADD for flies in specific geographic areas.

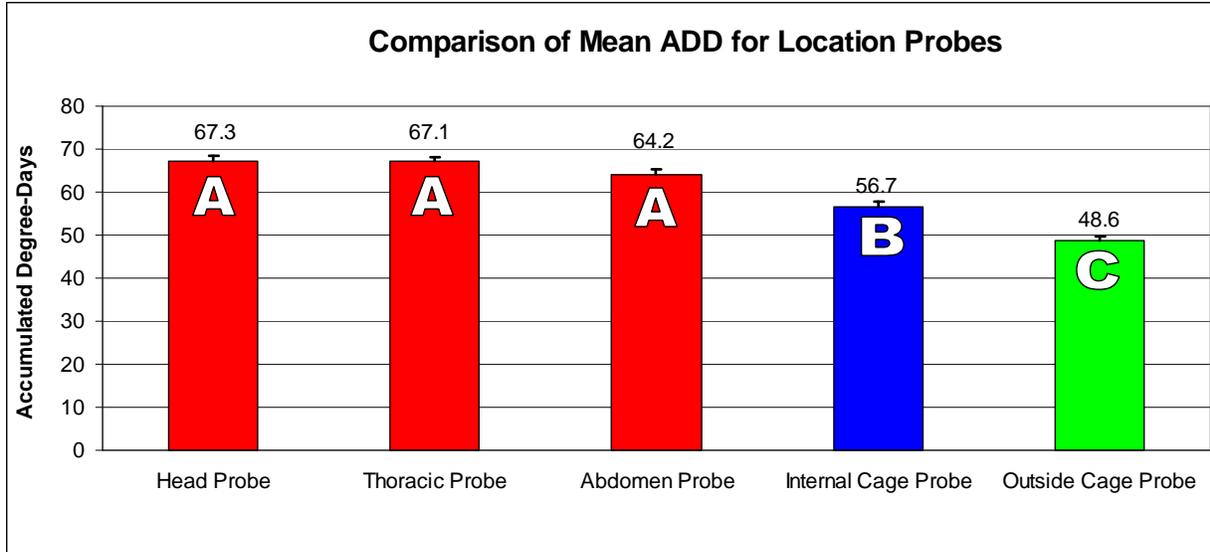
Validating studies of PMI estimates based on entomological evidence are needed because entomologists have been questioned on the accuracy of PMI estimates. The 2002 homicide case of Danielle van Dam and the subsequent Westerfield trial in San Diego, CA, USA (People v. Westerfield) illustrated the point that there needs to be consistency among forensic entomologists in determining PMI estimates. During the Westerfield trial, four entomologists (Neil Haskell, David Faulkner, Madison Lee Goff, and Robert Hall) provided PMI estimates, each based on slightly different methods and gave PMI estimates that either overlapped or came within four days of overlapping ([http://www.courtstv.com/trials/westerfield/timeline/time\\_of\\_death.html](http://www.courtstv.com/trials/westerfield/timeline/time_of_death.html)). If multiple forensic entomologists cannot come up with the same PMI estimates, a juror in the trial may feel that it is not good science, but attorneys can challenge the accuracy of those estimates as well. Validating methods and techniques using the scientific method will not allow PMI estimates to be thrown out of court on the grounds that those estimates are not accurate.



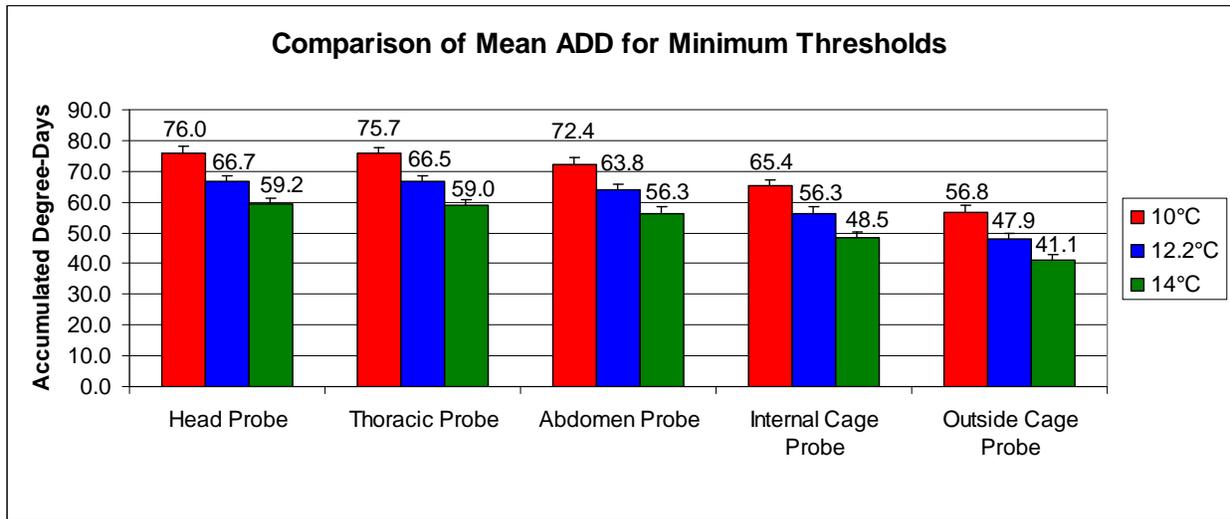
**Figure 5.1 A, B. Experimental cage set up and placement of probes.**

**Table 5.1: Weather data from all three weather station locations: Blacksburg Airport, Kentland Farm, and Moore Farm were used to calculate ADD from the time each pig was placed in the field (i.e. approximate time of death) to the time a third instar was first collected using the Averaging method and 10, 12.2, and 14°C developmental thresholds. A PMI was then calculated for each location using Anderson’s (2000) and Byrd and Allen’s (2001) developmental data sets.**

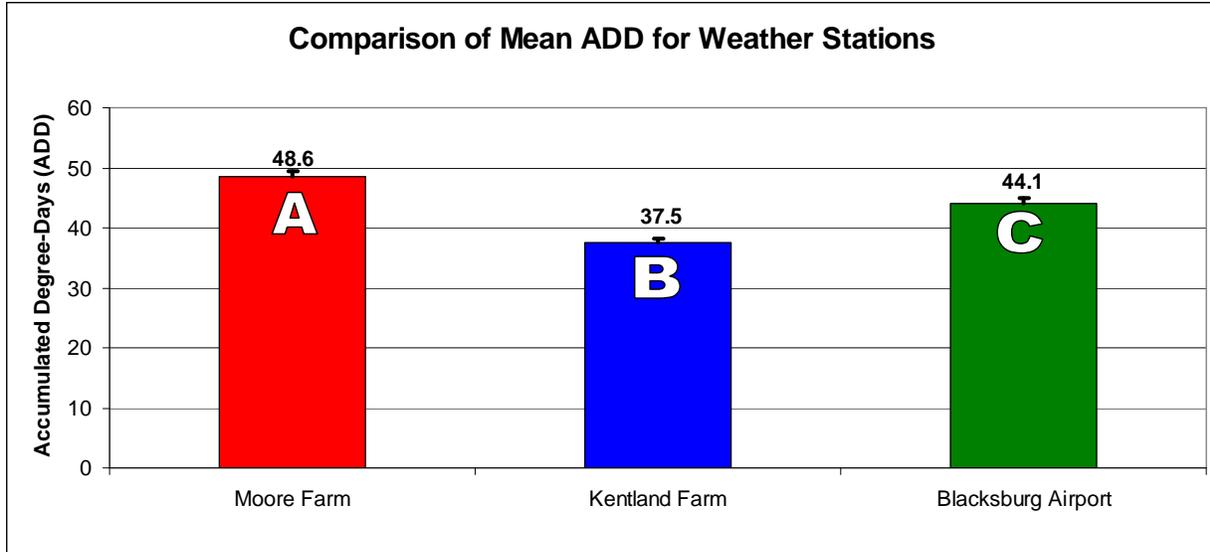
| Actual date of death and PMI |                | Moore Farm                      |          |                     |          |                     |          |                     |          |
|------------------------------|----------------|---------------------------------|----------|---------------------|----------|---------------------|----------|---------------------|----------|
|                              |                | Pig 1                           |          | Pig 2               |          | Pig 3               |          | Pig 4               |          |
|                              |                | 29-Jun-07                       | 3 days   | 14-Jun-07           | 3 days   | 25-Jun-08           | 3 days   | 13-Jul-08           | 4 days   |
| Developmental data source    | Threshold (°C) | Estimated date of death and PMI |          |                     |          |                     |          |                     |          |
|                              |                | Date                            | PMI      | Date                | PMI      | Date                | PMI      | Date                | PMI      |
| <b>Anderson</b>              | <b>12.2</b>    | Before 29 June (07)             | 4-5 days | Before 14 July (07) | 4-5 days | Before 25 June (08) | 4-5 days | Before 13 July (08) | 5-6 days |
|                              | <b>14</b>      | Before 29 June (07)             | 4-5 days | Before 14 July (07) | 4-5 days | Before 25 June (08) | 3-4 days | Before 13 July (08) | 5-6 days |
|                              |                | Before 29 June (07)             | 4-5 days | Before 14 July (07) | 4-5 days | Before 25 June (08) | 3-4 days | Before 13 July (08) | 4-5 days |
| <b>Byrd and Allen</b>        | <b>10</b>      | 29-30 June (07)                 | 2-3 days | 14-15 July (07)     | 2-3 days | 26 June (08)        | 2 days   | 14-15 July (08)     | 2-3 days |
|                              | <b>12.2</b>    | 29-30 June (07)                 | 2-3 days | 15-16 July (07)     | 1-2 days | 26-27 June (08)     | 1-2 days | 14-15 July (08)     | 2-3 days |
|                              | <b>14</b>      | 29-30 June (07)                 | 2-3 days | 15-16 July (07)     | 1-2 days | 26-27 June (08)     | 1-2 days | 14-15 July (08)     | 2-3 days |
|                              |                | <b>Kentland Farm</b>            |          |                     |          |                     |          |                     |          |
| <b>Anderson</b>              | <b>10</b>      | Before 29 June (07)             | 5-6 days | Before 14 July (07) | 5-6 days | Before 25 June (08) | 5-6 days | Before 13 July (08) | 5-6 days |
|                              | <b>12.2</b>    | Before 29 June (07)             | 5-6 days | Before 14 July (07) | 5-6 days | Before 25 June (08) | 5-6 days | Before 13 July (08) | 6-7 days |
|                              | <b>14</b>      | Before 29 June (07)             | 5-6 days | Before 14 July (07) | 5-6 days | Before 25 June (08) | > 3 days | Before 13 July (08) | 6-7 days |
| <b>Byrd and Allen</b>        | <b>10</b>      | 28-29 June (07)                 | 3-4 days | 14-15 July (07)     | 2-3 days | 25-26 June (08)     | 2-3 days | 13-14 July (08)     | 3-4 days |
|                              | <b>12.2</b>    | 28-29 June (07)                 | 3-4 days | 14-15 July (07)     | 2-3 days | 25-26 June (08)     | 2-3 days | 13-14 July (08)     | 3-4 days |
|                              | <b>14</b>      | 29-30 June (07)                 | 2-3 days | 14-15 July (07)     | 2-3 days | 25-26 June (08)     | 2-3 days | 13-14 July (08)     | 3-4 days |
|                              |                | <b>Blacksburg Airport</b>       |          |                     |          |                     |          |                     |          |
| <b>Anderson</b>              | <b>10</b>      | Before 29 June (07)             | 5-6 days | Before 14 July (07) | 5-6 days | Before 25 June (08) | 5-6 days | Before 13 July (08) | 6-7 days |
|                              | <b>12.2</b>    | Before 29 June (07)             | 5-6 days | Before 14 July (07) | 4-5 days | Before 25 June (08) | 4-5 days | Before 13 July (08) | 4-5 days |
|                              | <b>14</b>      | Before 29 June (07)             | 5-6 days | Before 14 July (07) | 5-6 days | Before 25 June (08) | 4-5 days | Before 13 July (08) | 4-5 days |
| <b>Byrd and Allen</b>        | <b>10</b>      | 29-30 June (07)                 | 2-3 days | 14-16 July (07)     | 1-3 days | 25-26 June (08)     | 2-3 days | 14-15 July (08)     | 2-3 days |
|                              | <b>12.2</b>    | 29-30 June (07)                 | 2-3 days | 15-16 July (07)     | 1-2 days | 26-27 June (08)     | 1-2 days | 14-15 July (08)     | 2-3 days |
|                              | <b>14</b>      | 29-30 June (07)                 | 2-3 days | 15-16 July (07)     | 1-2 days | 26-27 June (08)     | 1-2 days | 14-15 July (08)     | 2-3 days |



**Figure 5.2:** The mean Accumulated Degree-Days (ADD) for each probe location at Moore Farm were analyzed for significant differences using Tukey’s HSD test. Columns with different colors and letters are significantly different ( $\alpha = 0.05$ ). Mean ADD for each probe is listed above the column. Standard error for all probes was 1.14.



**Figure 5.3:** The mean Accumulated Degree-Days (ADD) for each minimum threshold used (10°C, 12.2°C, and 14°C) to calculate ADD for each probe location at Moore Farm were analyzed for significant differences using Tukey’s HSD test. The test revealed significant differences between the ADD between all three thresholds for all probe locations. Mean ADD for each probe is listed above the column. Standard error for all probes was 1.98.



**Figure 5.4:** The mean Accumulated Degree-Days (ADD) for each weather station location were analyzed for significant differences using Tukey's HSD test. Columns with different colors and letters are significantly different ( $\alpha = 0.05$ ). Mean ADD for each location is listed above the column. Standard error for all locations was 0.08.

**Table 5.2: Weather data from Moore Farm were used to calculate ADD from the time each pig was placed in the field (i.e. approximate time of death) to the time a third instar was first collected using the Averaging method and 10, 12.2, and 14°C developmental thresholds. A PMI was then calculated for each location using Anderson’s (2000) and Byrd and Allen’s (2001) developmental data sets. These data are from the body probe that monitored temperatures in the head.**

|                                 |                | Pig 1                  |          | Pig 2           |          | Pig 3           |          | Pig 4           |          |
|---------------------------------|----------------|------------------------|----------|-----------------|----------|-----------------|----------|-----------------|----------|
| Actual date of death and PMI    |                | 29-Jun-07              | 3 days   | 14-Jun-07       | 3 days   | 25-Jun-08       | 3 days   | 13-Jul-08       | 4 days   |
| Estimated date of death and PMI |                | Date                   | PMI      | Date            | PMI      | Date            | PMI      | Date            | PMI      |
| Developmental data source       | Threshold (°C) |                        |          |                 |          |                 |          |                 |          |
| Anderson                        | 10             | 28-29 June (07)        | 3-4 days | 13-14 July (07) | 3-4 days | 24-25 June (08) | 3-4 days | 13 July (08)    | 4 days   |
|                                 | 12.2           | 28-29 June (07)        | 3-4 days | 13-14 July (07) | 3-4 days | 24-25 June (08) | 3-4 days | 13-14 July (08) | 3-4 days |
|                                 | 14             | 29 June (07)           | 3 days   | 14 July (07)    | 3 days   | 25 June (08)    | 3 days   | 13-14 July (08) | 3-4 days |
| Byrd and Allen                  | 10             | 30 June (07)           | 2 days   | 15-16 July (07) | 1-2 days | 26-27 June (08) | 1-2 days | 15 July (08)    | 2 days   |
|                                 | 12.2           | 30 June - 01 July (07) | 1-2 days | 15-16 July (07) | 1-2 days | 26-27 June (08) | 1-2 days | 15-16 July (08) | 1-2 days |
|                                 | 14             | 31 June - 01 July (07) | 1-2 days | 15-16 July (07) | 1-2 days | 26-27 June (08) | 1-2 days | 15-16 July (08) | 1-2 days |

**Table 5.3: Accumulated Degree-Days (ADD) are given for Fig 1. The Degree-Days were calculated from the time the pig was placed in the field (i.e. approximate time of death) to the time a third instar was first collected for Fig 1. The temperature data was from the outside ambient probe at Moore Farm. Accumulated Degree-Days were calculated using three methods: Averaging, Single triangle, and Single sine using 10, 12.2, and 14°C as developmental thresholds. Statistical analysis indicated no significant difference in the ADD between ADD methods for all pigs.**

| Date                                     | Daily temperature (°C) |         | Degree-Day Methods |                 |             |
|--|------------------------|---------|--------------------|-----------------|-------------|
| Developmental threshold = 10 °C          | Minimum                | Maximum | Averaging          | Single triangle | Single sine |
| 7/2/2007                                 | 11.00                  | 21.90   | 6.45               | 6.45            | 6.45        |
| 7/1/2007                                 | 10.60                  | 35.10   | 19.30              | 19.30           | 19.30       |
| 6/30/2007                                | 14.5                   | 31.9    | 32.5               | 32.5            | 32.6        |
| 6/29/2007                                | 18.7                   | 33.5    | <b>48.6</b>        | <b>48.6</b>     | <b>48.7</b> |
| <b>Developmental threshold = 12.2 °C</b> |                        |         |                    |                 |             |
| 7/2/2007                                 | 11.00                  | 21.90   | 4.90               | 4.30            | 4.40        |
| 7/1/2007                                 | 10.60                  | 35.10   | 16.40              | 15.00           | 15.20       |
| 6/30/2007                                | 14.5                   | 31.9    | 27.4               | 26              | 26.2        |
| 6/29/2007                                | 18.7                   | 33.5    | <b>41.3</b>        | <b>39.9</b>     | <b>40.1</b> |
| <b>Developmental threshold = 14 °C</b>   |                        |         |                    |                 |             |
| 7/2/2007                                 | 11.00                  | 21.90   | 4                  | 2.9             | 3.1         |
| 7/1/2007                                 | 10.60                  | 35.10   | 14.6               | 12              | 12.5        |
| 6/30/2007                                | 14.5                   | 31.9    | 23.8               | 21.2            | 21.7        |
| 6/29/2007                                | 18.7                   | 33.5    | <b>35.9</b>        | <b>33.3</b>     | <b>33.8</b> |

## 6. Summary

The most important function of entomological evidence is its role in the estimation of the postmortem-interval (PMI). The two primary ways in which insects are used to estimate the PMI are by analyzing the degree of development of the insects derived from the first eggs deposited on a corpse and by analyzing successional patterns of insects visiting a corpse. Generally temperature plays the most important factor in insect development, however other biotic and abiotic factors such as individual species characteristics, weather, maggot mass, food type, presence of drugs and toxins, and geographic region can affect insect development and succession patterns (Byrd and Castner 2001).

The use of insect development for PMI estimation requires development data for the species of flies collected from a corpse. In most locations, these species are flies in the families Calliphoridae, Sarcophagidae, and Muscidae. A number of laboratory and field studies have been conducted on a variety of forensically relevant fly species (eg. Bishopp 1914, Melvin 1934, Kamal 1958, Greenberg and Szyska 1984, Liu and Greenberg 1989, Greenberg 1991, Davies and Ratcliffe 1994, Byrd and Butler 1996, Byrd and Butler 1997, Anderson 2001, Byrd and Allen 2001, Grassberger and Reiter 2001, Grassberger and Reiter 2002).

Forensic entomological studies were conducted in Southwest Virginia to obtain data on the effect of alcohol and opiates on the development of insects, as well as add more information on the accuracy of calculating degree-days. The first study determined the effects of a range of ethanol solutions on the development of the black blow fly, *Phormia regina* in vitro. This study was completed to expand on the data collected from Tabor et al.'s (2005) study. Tabor et al. (2005) conducted a study with ethanol in vivo using *Sus scrofa* (Linnaeus) as the test animal. Their data indicated that growth of third instar maggots feeding on loin tissue removed from ethanol-treated pigs was significantly different from growth of maggots feeding on tissue from untreated pigs. The BAC (Blood Alcohol Concentration) of the loin tissue used in Tabor et al.'s (2005) study was 0.15 and 0.17 (% w/v) for the treated pigs. In this current study, ground pork loin was treated with either a 1, 5, or 10% ethanol solution to give the meat an equivalent content of 0.01, 0.04, and 0.08 % w/v. *Phormia* eggs were placed on treated and untreated meat to grow and develop. Dunnett's Multiple Comparison test revealed there was a significant time difference between the 1% treated and control for second instars' time to complete that stage ( $P < 0.05$ ).

Significant differences were also found among pupal and adult weights between all treatments and controls. A significant difference between growth curves was found at  $\alpha = 0.05$  using the Kolmogorov-Smirnov test in development of third instars on loin tissue between the 5% treated and control group. No differences were detected in first and second instars between the controls and any of the treatments. Calculation of chi-square (by Yates' correction) for homogeneity in the sex ratio showed the sex ratio was homogeneous for the control and all treatments.

The ethanol contents of *Phormia regina* in migrating third instar larvae that fed on treated meat were examined using head-space gas chromatography (HSGC). Ten migrating third instars were harvested, cleaned, and frozen from each container in order to determine ethanol content. The ten maggots were later dried superficially using paper towels and weighed. The maggots were then punctured with a scalpel, placed in a clean vial, and then ground with a pestle in 0.5 mL of deionized water. All larvae had a content of 0.01 % w/v, including the control.

The effects of ante-mortem injection of oxycodone to pigs were examined with respect to insect succession patterns and the development rates of *Phormia regina*. Pigs were given a subcutaneous injection of oxycodone hydrochloride (3 mg/kg by weight). Antemortem blood samples were collected one time prior to injection of drug and three times following injection, each an hour apart. After euthanasia, the carcasses were placed at an open field site and allowed to decompose. Insect samples were collected from carcasses for 7 days post-mortem and the collected data were used to develop occurrence matrices. The Simple Matching Coefficient (SMC) was used to test the null hypothesis of no similarity between successional patterns of taxa from treated and untreated pigs. The SMC showed that the successional patterns were similar between treated and untreated pigs. Loin and liver tissue from the carcasses were used as a rearing medium for laboratory studies of the black blow fly, *Phormia regina*. Development rates of third instar *P. regina* maggots feeding on loin tissue from treated pigs were significantly different from development rates of maggots feeding on loin tissue from untreated pigs. No significant differences were detected in first and second instars or with the development of maggots feeding on treated liver tissue. A significant difference was found among larvae that fed on loin tissue for the time from eclosion to the completion of the second instar. There was no significant difference in pupal weights, however there was a significant difference in adult weights for adults in the liver and loin groups. The calculations of chi-square (by Yates' correction) for homogeneity in the sex ratio for the adults that emerged from the control and

oxycodone treated loin tissue show that the flies that emerged were biased towards one sex (males) by 2:1 in the untreated loin tissue.

A final study compared weather data sources, Accumulated Degree-Day (ADD) methods, and postmortem interval (PMI) estimations based on threshold and developmental data source. Six stillborn pigs (*Sus scrofa* Linnaeus) ranging from 0.91-3.63 kg were used as the model species in the first summer (2007) of this study and four pigs ranging from 1.0-1.72 kg were used in the second summer (2008). Pigs were taken to a test site and allowed to decompose in an experimental cage. Probes recorded ambient temperatures and body temperatures. Maggot sampling was completed daily for each pig. The statistical program JMP<sup>®</sup> 7.0.2 was used to test a three way factorial linear fit model using standard least squares. This tested for interactions between the method and probe location, method and development threshold, probe location and development threshold, as well as significant differences within the method, probe location, and development threshold. There were significant differences according to the ANOVA, however there were no significant interactions. The only significant differences in ADD occurred for the main effects of probe location and development threshold.

A comparison of Accumulated Degree Days was also made using data from different weather station locations: Kentland Farm, Moore Farm, and Blacksburg Airport. There were significant differences according to the ANOVA, however, there were no significant interactions (Method\*Location, Method\*Development Threshold, Location\*Developmental Threshold, and Method\*Location\*Developmental Threshold). The only significant differences in ADD occurred for the main effects of location and the development threshold.

Different PMI estimations can result by using development data from different sources. Anderson's (2000) data overestimated PMI for all pigs with the weather data from all three locations, whereas Byrd and Allen's (2001) data tended to slightly underestimate PMI. However most of the estimates made with their data provided ranges that included the actual date of death. More specifically, Byrd and Allen's data underestimated Pig 2 and 3's PMI at all three weather data locations at thresholds 12.2 and 14°C. Byrd and Allen's data gave a more accurate estimate for Pigs 1 and 4 especially at 10°C for all three weather data locations. Anderson's data (2000) gave the most accurate PMI with the temperature data from the head probe for all pigs and her data provided a range that included the actual date of death for all the pigs. Byrd and Allen's (2001) development data underestimated the PMI by a day for all four pigs when using

temperature data from the head probe. Overall, the study indicates that using an inappropriate minimum threshold combined with the inappropriate developmental data source could lead to incorrect estimations of a postmortem interval. Correcting weather station data and incorporating maggot mass temperatures in ADD calculations should be done on a case by case basis where it is appropriate to use the techniques.

In conclusion, this research provides entomotoxicological and thermal information to the database of forensic literature. The entomotoxicological factors in these studies were ethanol and oxycodone. Ethanol is a common drug, but has not been thoroughly investigated in forensic entomology. The effects of ethanol on fly development in vitro, expands on studies already completed on the subject (Tabor et al. 2005). The evidence from the in vitro studies suggest that low amounts of ethanol (0 - 0.1 % w/v) does not affect larval development of *Phormia regina*, however it can cause differences some aspects of development (i.e. lower pupal and adult weights). Additionally, it was determined that ante-mortem injection of oxycodone affects the growth rate of maggots, thereby having the potential of skewing PMI estimates based on development in death cases involving oxycodone. Oxycodone does not affect successional patterns of insects, therefore PMI estimates made using this method would not be skewed. Finally, thermal factors were investigated and it was determined when forming PMI estimates using degree-days, it is recommended that maggot mass temperatures be included, weather station data corrected to death scene temperatures, and that appropriate minimum thresholds and developmental data sources be used.

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