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Effect of 17a - Ethynylestradiol on the Pupation Rate, Emergence Rate, and Sex Ratio of Phormia Regina (Calliphoridae)

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University of New Haven
Honors Program

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A thesis presented in partial fulfillment of the requirements of the Undergraduate Honors Program at the University of New Haven.

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18/Dec/19
Date

UNIVERSITY OF NEW HAVEN

HONORS PROGRAM

EFFECT OF 17α-ETHYNYLESTRADIOL ON THE PUPATION RATE, EMERGENCE RATE, AND SEX RATIO OF *PHORMIA REGINA* (*CALLIPHORIDAE*)

A THESIS

Submitted in partial fulfillment

Of the requirements of the degree of

BACHELOR OF SCIENCE IN FORENSIC SCIENCE

Bethany Hoschar

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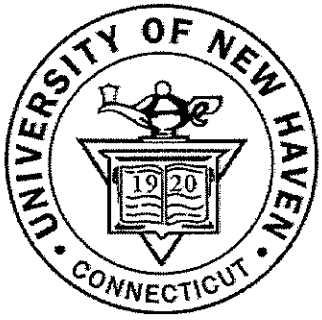

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Glossary

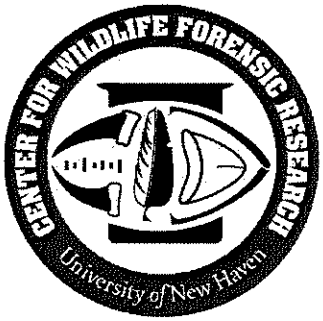
Term	Definition
PMI	Post-mortem interval. The time that has elapsed since death of an individual
Oviparous	The fly lays eggs and the eggs hatch outside of the mother
Larviparous	The fly eggs hatch inside the mother and the mother lays live maggots
Oviviparity	The process of the mother retaining eggs within her reproductive tract until they hatch
Coprophagous	The organism will feed on feces
Instar	Stage of a maggot. There are 3 instars.
Open circulatory system	Blood is pumped from the heart into body cavities
Holoptic	Two things meet or almost meet (in reference to eyes)
Dichoptic	Two things are separated (in reference to eyes)
LD100	Lethal dose of 100% of the population
Necrophagous	The organism feeds on decomposing remains
Malpghian tubules	A part of the intestinal tract of the maggot
Entomotoxicology	The study of the toxicological effects on entomological species
Eclosion	When a fly egg hatches

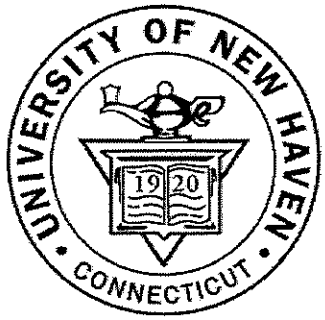
Abstract

After death, blowflies are attracted to decomposing remains for feeding and reproduction purposes. Since blowflies are usually the first species to colonize a set of remains, they are important to forensic scientists when estimating the time since death. Using the knowledge of the life cycle of a blowfly and any interferences of the environment that can affect the development, forensic entomologists can estimate the time since the arrival of blowflies, otherwise considered time since deposition. This research investigated how a specific estrogen, 17α-ethynylestradiol, present in pollutants affects the development time of blowflies, and therefore the time since death estimation. Four different concentrations of the estrogen were used, Control (no estrogen), LD25, LD50, and LD100. The Lethal dose (LD) values were calculated from the known LD100 (lethal dose to 100% of the population) value of the estrogen for rats. This research consisted of two phases, the first observing the effect of estrogen inserted into a food source such as sugar, and the second phase involved observing the development time after inserting the estrogen into a protein feed to induce oviposition. The estrogen produced a deterrent effect when placed in the sugar source for the fly colony, however there was not an observed deterrent effect when placed in the protein feed. The study conducted found an impact on pupation time with an increase of estrogen in the feed, and a decrease in percent emergence with the increase of estrogen was observed. However, replicates were limited, and further research would need to be conducted to strengthen statistics.

Chapter One:

Introduction to Entomology





1.0 Introduction to Entomology

The study of insects and their effect on organisms and the environment, otherwise known as entomology, has many applications in society. Insects, being the largest living group on earth, are important in biodiversity. The class Insecta falls under the superclass Hexapoda, which includes insects and their near relatives. To fall in this superclass, organisms must share the characteristics of three major body regions and six legs. An insect must be an arthropod, meaning it has an exoskeleton (Merritt, Courtney, & Keiper, 2012).

Insects, especially flies, can be considered beneficial in many ways. Environmentally, flies and other insects are vital decomposers, removing diseases and recycling nutrients. Medically, fly larvae were used to treat a disease before the advent of antibiotics. Agriculturally, some insects can be used as pollinators. Finally, forensically, insects such as flies can be used in time since death estimations (Anderson, 2000).

1.0.1 History of Forensic Entomology

Entomology used in a forensics relevance was first discussed in thirteenth century China. The first recorded case using forensic entomology involves a murder being investigated by death investigator Sung Tz'u (Rivers & Dahlem, 2014). The victim had a stab wound consistent with a common sickle, so the death investigator had all the workers report with their tools. When examining the sickles, flies were on and around only one of the sickles, and the owner eventually confessed to the murder. The reason the fly evidence was beneficial refers to flies being attracted to blood and decomposing

tissue, therefore suggesting the sickle had small traces of blood and tissue left after cleaning (Rivers & Dahlem, 2014).

From the time of Sung Tz'u's recorded use of forensic entomology to the nineteenth century, almost nothing is recorded that relates to forensic entomology, however, work in biology had an impact on the applied use of insects in legal investigations. Most significantly during this time period was Francesco Redi's realization that flies are attracted to spoiling meat and lay eggs on or near the food, rather than the thought that maggots spontaneously formed on meat. By the middle of the eighteenth century, Carolus Linnaeus developed a system of nomenclature to identify plants and animals, among which he identified and described about 2000 insects (Rivers & Dahlem, 2014).

The first known recorded use of insects in estimating the post-mortem interval (PMI), or time since death, was in 1850, when French physician Bergeret was called in to investigate a set of remains of an infant. Bergeret used the knowledge of the life cycle of a fly to establish a timeline (Rivers & Dahlem, 2014).

The beginning of the twentieth century is where forensic entomology became more prevalent. Pathologists such as Stefan von Horoszkiewicz or medical examiners like Maschka encountered cases where arthropods were responsible for post-mortem lesions and abrasions that looked similar to what would occur with sulfuric acid poisoning (Rivers & Dahlem, 2014). Later in the twentieth century, observations of scientists such as Hermann Merkel discovered the method of death or any would present could influence rate of decomposition due to flies being attracted to open wounds. This time period also

focused on insecticide development, which led to more information on insect life (Rivers & Dahlem, 2014).

Since the 1980s, forensic entomology has been gaining acceptance within judicial systems and research continues to be conducted to understand how insects can assist in criminal investigations (Rivers & Dahlem, 2014).

1.0.2 *Diptera*

The order *Diptera*, meaning "two wings," is one of the largest groups of insects (Merritt et al., 2012). There are many families of flies present in North American. Calliphorids, flies within the Calliphoridae family, are the family of flies of primary concern to forensic entomologists, tend to be easily distinguished due to their color. Some flies within the families Muscidae or Tachinidae sometimes are grouped with calliphorids due to their similar colors. Other families of flies include Sarcophagidae, or flesh flies, Rhinophoridae, or Drosophilidae, also known as fruit flies (Whitworth, 2006).

Flies can be considered oviparous or larviparous. Flies that are referred to as larviparous rely on ovoviviparity, the process of the mother retaining eggs within her reproductive tract until they hatch, where she will then lay them live. When a fly is referred to as oviparous, the process varies in that the eggs do not hatch inside the mother. The mother will lay the eggs on a deposition site, and the eggs will hatch at a later point in time (Rivers & Dahlem, 2014).

1.0.2.1 Blowflies (*Calliphoridae*)

Blowflies, named as such because the larvae develop inside of decomposing remains and cause the carrion to bloat, are attracted to dead animals and garbage. Most

blowflies are shiny blue or green, however, some are shiny black or bronze. These are easily separated from other families of flies because of their metallic luster in addition to the relatively large size of adults. Some non-metallic calliphorids are accidentally grouped into muscids, sarcophagids, or tachinids, however, keys to species within that genera would assist with identification (Whitworth, 2006). Calliphorids are considered oviparous, so the mother will lay eggs on the deposition site and the eggs will later hatch and begin to develop as larvae (Rivers & Dahlem, 2014).

The most common species of blowflies in North American consist of species within the genus *Lucilia* and the species *Phormia regina*. *P. regina*, a species commonly studied in literature, is most common in the northern United States and Canada in the summer months and is the dominant species in the southern United States in the winter. The genus *Lucilia* contains 11 species in North America, some of which have limited distributions, while others have wider distributions. *L. sericata* is one of the more common species in the genus, and detailed work has been published on the biology and larval growth and development of the species. This is the species that has been used in human and veterinary medicine since the approval of maggot therapy in 2004 by the Food and Drug Administration (Rivers & Dahlem, 2014).

1.0.2.2 Flesh flies (*Sarcophagidae*)

The sarcophagid flies that feed on decomposing remains are gray flies with red eyes, dark stripes on their thorax, and have a checkerboard pattern on their abdomen, such as *Sarcophaga bullata*. Some species that are not scavengers are coprophagous, meaning they will feed on feces. Sarcophagids are referred to as larviparous, meaning the

eggs will hatch inside the mother and the mother will lay active first instar larvae on a deposition site (Rivers & Dahlem, 2014).

In North America, one of the most important species for forensic analysis is *Sarcophaga bullata*, a species used for hundreds of years for investigations into insect physiology. However, little research is focused on *S. bullata* larval rearing at different temperatures. Another flesh fly commonly collected is *Blaesoxipha plinthopyga*, generally found in the southern United States (Rivers & Dahlem, 2014).

1.0.2.3 Fruit flies (*Drosophilidae*)

Fruit flies, also commonly known as vinegar flies, are not normally necrophagous (Rivers & Dahlem, 2014). Fruit flies are generally attracted to ripened or fermenting fruits and vegetables. These flies are generally small, about the size of a gnat. Their bodies are tan and black and they will lay their eggs on or near the surface of a fermenting mass (Potter & Entomologist, n.d.).

The odorant detection system in a particular species of fruit fly, *Drosophila melanogaster*, is characterized in more detail than any other insect. Therefore, this species has been investigated in instances such as developing biosensors. In addition, this species has been used to study the impact of larval crowding, finding a result similar to what would be found with necrophagous flies; larval development is extended and there tends to be a depletion of nutrients (Rivers & Dahlem, 2014).

1.0.3 Morphological Features of Flies

Characteristics that define arthropods include a segmented body, jointed appendages, chitinous exoskeleton, and an open circulatory system (Rivers & Dahlem, 2014).

In the order *Diptera*, flies share several features. Adult flies usually posses large compound eyes. Depending on the species, and sometimes the sex, the eyes could be holoptic, meaning they meet or almost meet, or dichoptic, meaning they are separated. (Merritt et al., 2012).

Morphologically, the sex of a calliphorid or sarcophagid fly can only be determined using an adult fly, as opposed to the larvae. The sex is determined by looking at the head of the fly under a microscope. Males will have a small space between the eyes, while females will have a larger space (Figure 1.0-1).

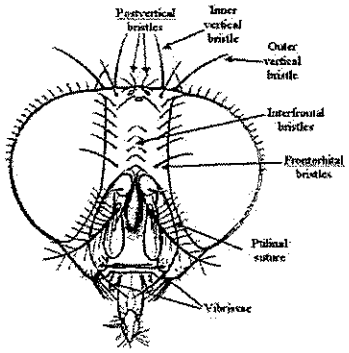


Figure 1.0-1 Diagram of a fly head ("Fly parts - morphology diagrams | Dipterists forum," n.d.)

Nearly all flies have well-developed antennae, however, the morphology of the antennae itself varies dependent on species. All adult flies will have a single pair of wings. The legs of adult flies include the basic arrangement of insect legs, with a coxa,

trochanter, femur, and tibia, however, the leg structure beyond this is variable (Merritt et al., 2012).

1.0.4 Morphological Features of Maggots

Diptera larvae can be distinguished from larvae of other insects by the lack of jointed thoracic legs (Merritt et al., 2012). The basic parts of a maggot contain a body, with midlateral welts down the length, a posterior spiracle, and an anterior spiracle (Figure 1.0-2) (Alfred, 2016).

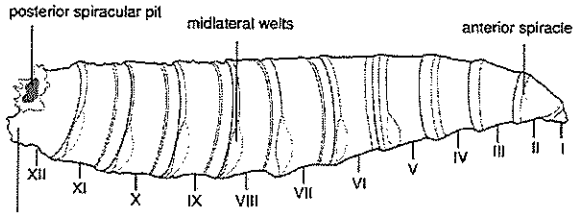


Figure 1.0-2 Labeled diagram of a maggot (Alfred, 2016)

The anterior spiracle is the front end of the maggot, where the maggot feeds on dead tissue. The posterior spiracle is the back end of the maggot and contains the posterior spiracular pit. Within this pit are the spiracles for identification of instar. There are two spiracular plates, and within each will be either one, two, or three spiracular slits, which correspond to first instar, second instar, and third instar, respectively (Alfred, 2016) (Figure 1.0-3).

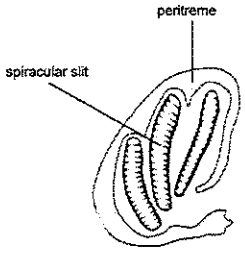


Figure 1.0-3 Typical Posterior Spiracular Plate of a maggot (Alfred, 2016)

1.0.5 The Life Cycle of a Fly

Depending on the species of fly, the basic life cycle of a fly begins as an egg or as a larva. The egg would hatch into a larva, then all larvae continue through three instar stages into a pupae stage, where the fly then emerges into an adult.

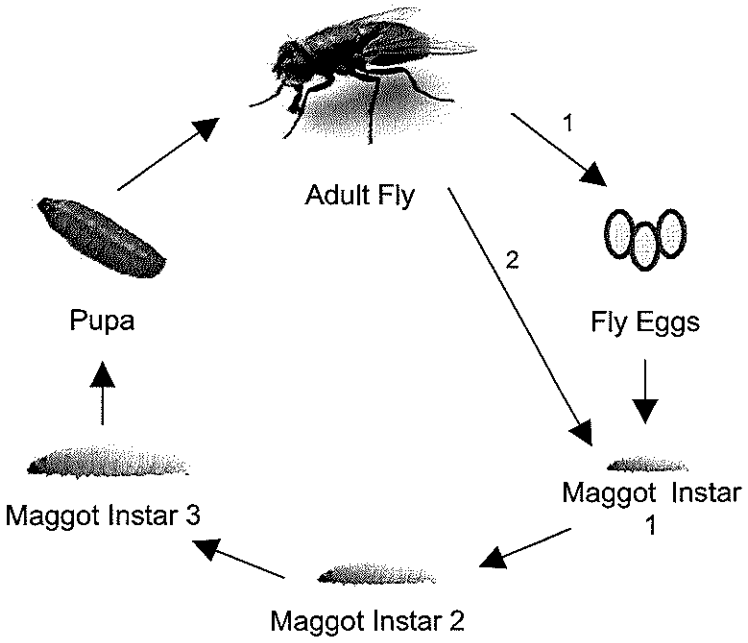


Figure 1.0-4 Life Cycle of *Diptera*

The life cycle of a fly follows two paths (Figure 1.0-4). For oviparous species that lay eggs, such as calliphorids, they would follow the (1) arrow from adult fly, following to eggs and then into the instar stages. For larviparous species that hatch inside the mother, they would follow the (2) arrow after the adult fly and begin maggot development as a larvae (Rivers & Dahlem, 2014).

To begin this life cycle, oviposition must occur. Oviposition is the term used to describe the laying of eggs or larvae. This process includes the selection and attraction to the oviposition site, the laying of the eggs or larvae, and the initial protection of the eggs and larvae when laid. Selecting a suitable oviposition site could involve visual or chemical cues, depending on the type of fly. Some entomological species may have strategies to ensure that the development of the larvae can proceed, such as coatings on the egg to protect from environmental stresses. (Lancaster, Downes, Lancaster, & Downes, 2013). Numerous factors can interfere with the process of oviposition, such as rain and temperature in the environment, or accessibility to an oviposition site. In addition, some entomological species may be affected by daylight (Amendt, Zehner, & Reckel, 2008). Once oviposition occurs, the eggs will hatch, and then proceed to the instar larval stages.

In the first instar stage, maggots are still young and smaller in size, but as they begin to grow into their second instar stage, maggot activity will increase. This second instar stage is usually where maggots will form maggot masses, which in turn, produces heat due to the friction of the maggots against each other during feeding. During the third instar, feeding continues, and they will begin to migrate away from the body to pupate in

the soil (Ahmad & Omar, 2018). This is where it reaches the pupa stage, where it will form a casing around itself. When the fly emerges, the pupae casing will be left behind.

At about room temperature, eggs will hatch within several hours. Once the egg hatches, it will spend approximately a day in the first instar stage, then proceed into the second instar stage, where it will stay for approximately two days. Once in the third instar stage, it may start to wander away to get ready to pupate, and this stage could take about four to five days. Once in the pupae stage, the insect remains there for at least a week, possibly more, where the insect will then emerge as an adult (Byrd & Allen, 2001).

1.1 Introduction to Forensic Entomology

From a forensic science standpoint, entomology has been used to study the life cycle of insects and relate back to the post-mortem interval (PMI). Forensic entomology focuses on blowflies, due to their relatively predictable behavior and distribution across the world (Heaton, Sc, et al., 2014). To establish the postmortem interval (PMI) in a death investigation, an understanding of the process of corpse decomposition is crucial. An estimate of how long an individual has been dead can be determined by the sequence of postmortem changes in the body, in addition to modifications of the decomposition process, such as scavenging (Campobasso, Vella, & Introna, 2001).

1.1.1 Flies in the Decomposition Process

The stages of decomposition have been described by various researchers, two of which are O'Brien (2008) and Galloway (1996). O'Brien (2008) describes five stages of decomposition: fresh, bloat, putrefaction, advanced, and skeletonization. The 'fresh'

stage begins when the animal is sacrificed and ends at the first observation of bloat. In the 'bloat' stage, bloating is visible in any part of the body, and continues until the body is at least partially collapsed. 'Putrefaction' stage begins once the body has collapsed and consists of fluids leaching from the body into the soil. The 'advanced' stage begins once skeletal elements are visible and mummification of the skin starts. The 'skeletonization' stage is classified as beginning when large amounts of skeletal elements are exposed, as well as large amounts of the soft tissue removed or mummified (O'Brien, 2008). These stages were developed specifically for experimental research purposes, and they were largely visually based. These stages were based around a similar outline of the stages of decomposition set by Galloway (1996). These stages proceeded as follows: fresh, early decomposition, advanced decomposition, skeletonization, and extreme decomposition. Early decomposition starts at the sight of bloat, and advanced decomposition involves the caving of the abdominal cavity. Skeletonization starts when bones are visible with desiccated or mummified tissue, and extreme decomposition involves processes similar to bone-weathering (Galloway, 1996).

Research has been conducted to analyze and discuss what could interfere in the process of decomposition (Campobasso et al., 2001). The rate of decomposition can be affected by both intrinsic factors, concerning the carcass, or extrinsic factors, concerning the environment. Within the corpse, age, weight, or injuries can all influence the time it takes the body to decompose, or how the body progresses through the decomposition process. In the environment, temperature, as well as humidity, play a large role in leading the decomposition process. Also present from environmental factors, animal predators can have a predominant role in accelerating the rate of decomposition. These animal

predators can include scavengers such as foxes, cats, or birds, or animals that may colonize on the remains, such as flies, or necrophagous arthropods (Campobasso et al., 2001).

The order *Diptera* are the most prominent organisms present during decomposition of a corpse. Calliphorids are among the first to colonize remains (Figure 1.1-1), and are therefore important for PMI estimations (Rodriguez & Bass, 1983).

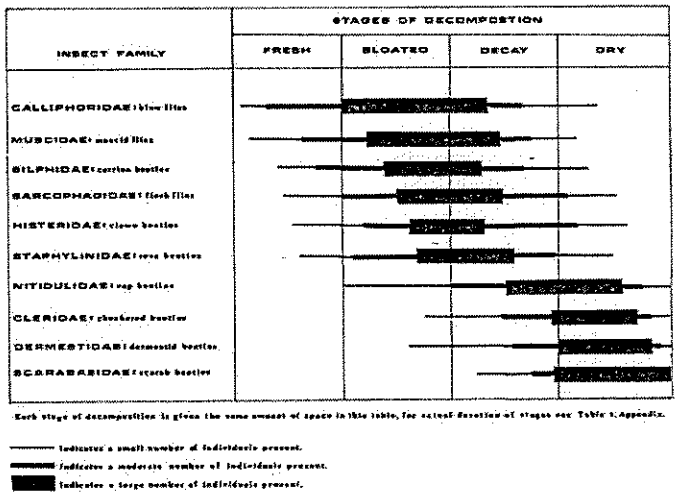


Figure 1.1-1 The Ten Major Insect Families Observed and Their Approximate Spring/Summer Distribution During Stages of Decay (Rodriguez & Bass, 1983).

Necrophagous arthropods like certain types of flies are attracted to the odors released during the process of decomposition (Campobasso et al., 2001). These odors are the chemical cues that lead a forensically important fly to an oviposition site, such as a body (Lancaster et al., 2013). Therefore, within minutes, insects will begin to interact with exposed remains and flies will begin to lay eggs on the remains. As the maggots begin to grow, they will form maggot masses, groups of maggots feeding in the same

place on remains. The interference of the insect community, through these maggot masses, in the decomposition process accelerates the rate of decomposition (Campobasso et al., 2001).

1.2 Influences on Fly Development

Since insects are sensitive to changes in the environment, interferences could change the life cycle as they attempt to adapt to survive. Research has been conducted to analyze the development behavior of blowflies in conditions thought to interfere, including both environmental and chemical interferences.

1.2.1 Environmental Conditions that affect Fly Development

Insect development is primarily governed by temperature, so the age of a specimen can be determined by the level of development and the thermal history during its development. Since temperature of the surroundings plays a large role in the development, the relationship between temperature and development can be quantified for a species (Gasz, 2016). Byrd and Allen (2001) looked at the egg eclosion rate and size of the larval body over time of *Phormia regina*, the dominant species of blowflies in the northern climates of the US during the summer months, at different temperatures. The researchers found that at lower temperatures, the peak eclosion hour was larger, meaning the eggs generally took longer to hatch. The average total time spent in the larval stages of *P. regina* at approximately room temperature was a total time of 110-210 hours, with more time spent in the later stages (Byrd & Allen, 2001). Blowfly activity around decomposing carcasses has been shown to reduce with decreasing temperature, however,

temperature is not the only factor that can affect development or activity of maggots (Monthei & Paulson, 2009).

Currently, estimation of the age of a maggot is based on time for a specific species to progress through developmental landmarks such as length, weight, and life cycle stage in relation to temperature. Life cycle stage for age estimation is a preferred method compared to age based on length and weight due to potential interferences in development that can affect the size of the maggot. Issues like diet and competition could limit the size that a maggot could grow (Gasz, 2016). Reference data that can assist in age estimations would include the duration of development of specific life cycle stages such as egg eclosion, larval instars, pupation, and emergence at different constant or fluctuating temperatures. A common occurrence at crime scenes, fluctuating temperature conditions has an effect on developmental rates. Some studies have indicated that development rate under fluctuating temperatures does not correlate to the resulting mean constant temperature, however, most reference data is produced using constant temperatures (Gasz, 2016).

Sunlight has been shown to interfere with maggot development. A carcass will have a higher temperature if left in the sun rather than if left in the shade, and the temperature of a carcass can either assist or restrain maggot development. In addition, the photoperiod maggots are present in can affect how their feeding pattern and in turn, their development on decomposing remains. Constant light has been shown to increase the variation in the time of development (Monthei & Paulson, 2009).

Factors within the maggot mass itself can interfere in the life cycle of maggots. Crowding of maggots within masses can accelerate the speed of pupal development in certain species, however, combining species showed an extension of pupation time. This plays into the role of food availability for the maggots. As the maggot density increases, growth rates have been shown to increase as well (Monthei & Paulson, 2009).

1.2.2 Chemical Interferences in Fly Development

Once a set of remains begins to decompose and blowflies colonize the carcass, maggots will then ingest the dead tissue. Contaminants present in or on the tissues and organs of the remains, such as drugs, can have a potential effect on the maggots feeding on the remains. Since the maggots on a set of remains and their stage in their developmental cycle is sometimes used in PMI estimations, this issue has led to the emergence of the field of entomotoxicology, the analysis of toxins in arthropods (Monthei & Paulson, 2009).

1.2.2.1 Toxicology

For contaminants to be present in the tissues and organs, the drug would have to distribute throughout the body. One of the more common drugs, ethanol, and the pharmacokinetics of the absorption, distribution and metabolism of it has been studied by researchers to understand the process in the body. Ethanol is a water-soluble molecule that is present and consumed in alcoholic drinks. After ingestion, ethanol will be absorbed through the stomach or through the small intestine. Several factors can affect the rate of absorption, including information on the person ingesting it, as well as what is being ingested (Paton, n.d.). The weight and sex of the person consuming the alcohol, in

addition to what they have eaten or are eating, are important factors in the rate of absorption. The concentration of alcohol in the drink served and if it is served with carbonated drinks can also influence the rate of absorption. After the alcohol begins to absorb into the bloodstream, it is distributed through the water in the body, so the heart, brain and muscles will be exposed to the same concentration that is present in the blood. However, very little alcohol is distributed to fat due to the poor solubility. The body will then begin to metabolize the alcohol by oxidation, starting to decrease the concentration. After metabolism, the majority of ethanol is eliminated by the liver, however, some is eliminated through sweat, urine, and breath (Paton, n.d.). This entire process generally follows the 'bell curve,' where the concentration of ethanol in the blood increases for a time after ingestion of the drink, and then decreases after reaching a peak (Figure 1.2-1).

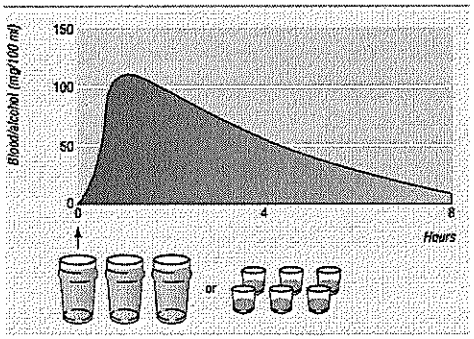


Figure 1.2-1 Concentration of alcohol in the blood after 6 units of alcohol (Paton, n.d.)

Drugs other than ethanol will go through the same stages within the body: absorption, distribution, metabolism, and then elimination. The rate of absorption and elimination is dependent on the specific drug and sometimes the mode of consumption. For example, when considering the drug ketamine, the drug is typically administered in high doses, and shows a rapid tolerance (McDougall et al., 2019).

If death occurs at any point during the absorption, distribution or metabolism stages, the drug is likely still present when blowflies colonize the remains. Understanding the process of the drug within the body is important to know the concentration of the drug that the maggot would ingest, and therefore assist in understanding how that drug could affect the development of the maggot.

While the interpretation of post-mortem drug concentrations was a static approach in the past, in which it was thought that the concentration would stay the same, the current approach shows otherwise. The process a drug goes through in the body after death is complicated due to the process of post-mortem drug redistribution. Certain areas of the body may have different concentrations of the drug than others, for example, heart blood concentration tends to have a higher concentration. In addition, as the PMI increases, the concentration of the drug tends to increase (*Postmortem redistribution*, 2019).

1.2.2.2 Entomotoxicology

The introduction of forensic entomotoxicology was based around the use of insects as toxicological samples when other means of sampling are not present. Since species belonging to the order *Diptera* are among the first to colonize decomposing remains, specimens of this order are recommended for collection for entomotoxicological analysis (Gosselin et al., 2011). The toxicological specimens collected are used for both qualitative and quantitative analysis of drugs present in the corpse at the time of death (Monthei & Paulson, 2009).

Qualitatively, studies have shown that larvae sampled from a carcass with a drug present can detect what specific drug is present with gas chromatography. However, the qualitative studies referenced by Monthei and Paulson (2009) could only obtain qualitative results from larvae, and not pupae, suggesting a possibility of elimination of drugs prior to metamorphosis (Monthei & Paulson, 2009).

Quantitatively, the concentration of drugs in larval tissues tend to be lower than the concentration of drugs in tissues of the feed (Monthei & Paulson, 2009). While some studies have shown a possible correlation between the drug concentration in a substrate, or a set of remains, and the concentration in a specific larval stage, this idea is still under controversy. Some authors believe a quantitative relationship is hard to determine due to interfering factors that have not been studied in detail. These potential interfering factors involve the process a drug goes through within a larvae (Gosselin et al., 2011).

There is little published information on the pharmacokinetics of drugs in insects, however, what is known references a few factors that could potentially influence the metabolism of a drug in the insect. These factors include species, developmental stage, and feeding activity. Larvae with different feeding activities have produced differences in drug concentrations. In addition, drug concentrations in pupae tend to be lower than drug concentrations in larvae, however they are less variable (Gosselin et al., 2011). From the studies that have shown no bioaccumulation of drugs in the larvae, producing no presence of the drug in the pupae, elimination mechanisms are likely present. Elimination mechanisms increase the importance of collecting specimens for toxicological analysis when at the scene, so as to determine presence of a drug at the earliest possible point (Monthei & Paulson, 2009).

The physiological mechanism of excretion of drugs within a larvae is unknown, however, the Malpighian tubules seem to be a likely site for active secretion (Parry, Linton, Francis, Donnell, & Toop, 2011). The Malpighian tubules are a part of the intestinal tract of the maggot. These tubules allow fluid to pass through them, which then in turn gets eliminated. This mechanism was likely designed for instances where the maggot ingests too much of a particular ion, however, some studies suggest that this mechanism is likely the case for why some drugs do not have a significant effect on certain species of larvae (O'donnell, 2009).

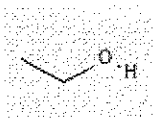
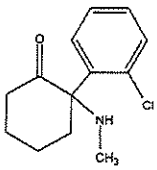
In addition to using entomological specimens for toxicological samples, the drug present may influence the blowfly larvae feeding on the remains, therefore interfering with PMI estimations. Understanding how toxins present in or on the body would affect development of larvae is important for more accurate PMI estimations in forensic cases. The study of toxicological effects on entomological species is a relatively new field, but a review of the field describes the traditional set up of an entomotoxicological study, using artificial foodstuff or post-mortem inoculated animal organs/meat to rear insects (Gosselin et al., 2011). Some studies analyze the effects by administering the dose to a living animal, so that the drugs and metabolites are both present in the tissue, enabling a connection to human fatal overdoses (Monthei & Paulson, 2009).

Monthei (2009) analyzed effects of ethanol treated protein feeds on factors in the development of *P. regina*. The researcher soaked pork (*Sus scrofa*) loin in different ethanol concentrations, and used the pork to set up rearing dishes. Data was collected on pupation time, emergence time, weight and sex. The results of the study found that the

presence of ethanol may have had a deterrent effect on feeding behavior of *P. regina* (Monthei & Paulson, 2009).

Other chemicals, such as ketamine, have been observed on a different blowfly, *Lucilia sericata*. The researchers placed rations of the lethal dose of ketamine in the fly's protein feed. There was a significant difference in body length and weight that was found between concentrations of ketamine, also showing a shortening of larval stages in the species, meaning the chemical accelerated the development of the maggot (Zou et al., 2013).

Table 1.2-1 Two chemicals and their effect on humans and insects

Chemical	Structure	Effect on Humans	Effect on insects	Reference
Ethanol		Sedative Mild anesthetic	Deterrent on feeding behavior	("Ethanol," n.d.) (Paton, n.d.) (Monthei & Paulson, 2009)
Ketamine		Anesthetic Psychedelic effects	Shortening of larval stages Acceleration of development	(Lee, Kunalan, Fahmi, & Abdullah, 2016) ("Ketamine - Alcohol and Drug Foundation," n.d.) (Zou et al., 2013)

1.2.3 Endocrine Disruptors

The drugs and chemicals that influence blowfly larvae may be present on the body through pollutants. Many common pesticide and herbicide pollutants have been identified as endocrine disruptors, such as atrazine, most commonly used in the US and Australia (Mnif et al., 2011). Endocrine disruptors can be present in man-made chemicals such as synthetic estrogens, polychlorinated biphenyls, dioxin-like compounds, insecticides like DDT or other pesticides. The specific estrogen 17α-ethynylestradiol, a common endocrine disruptor, is common in medication for humans, as well as in agricultural activity (Aris, Shamsuddin, & Praveena, 2014). This synthetic estrogen is prevalent mainly from hormonal contraceptive pills. It can be excreted in urine and feces in its active free form, or as an inactive conjugate of the drug. The drugs resistance to degradation leads the drug to be a main hormonal pollutant (Bovier, Rossi, Mita, & Digilio, 2018).

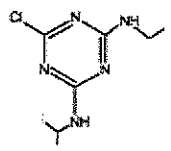
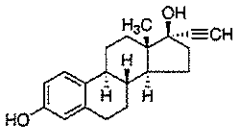
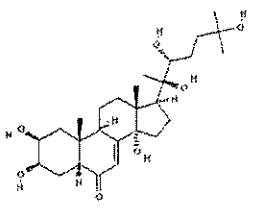
1.2.3.1 Process of Endocrine Disrupting Chemicals in the body

Endocrine disrupting chemicals (EDCs) are compounds that alter the normal functioning of the endocrine system. There are three different outcomes that can come from an endocrine disrupting chemical being present in the body (USGS, 1998). The effects of an endocrine disruptor can decrease or increase normal hormone levels, by disturbance of the Control of certain hormone levels. EDCs do this by binding to hormone receptors without activating them, inhibiting the action of the receptor. EDCs also interfere with synthesis, transport, and elimination of hormones. To humans, EDCs have also been shown to disrupt reproductive and sexual development (Mnif et al., 2011).

Mnif et al (2011) includes tables showing the structure of common EDC found in the environment and the effect they have on the endocrine system.

Other than binding to a hormone receptor to block the response, endocrine disruptors can also mimic the naturally occurring hormone, which can potentially produce overstimulation by the hormone. An endocrine disruptor can also alter the natural production of hormones, for example, altering the metabolism in the liver (Mnif et al., 2011).

Table 1.2-2 Three endocrine disrupting chemicals and their effect on humans

EDC	Structure	Effects in humans	Reference
Atrazine		Weak estrogenic effect. Induction of aromatase activity and increased estrogen production. Reduction of steroid hormone metabolism	(Mnif et al., 2011)
17α-ethynylestradiol		Synthetic estrogen used in estrogen replacement therapy – act as estrogen.	("17α-Ethynylestradiol ≥98% | Sigma-Aldrich," n.d.)
20- Hydroxyecdysone (hormone important in fruit fly development)		Not considered hazardous Not investigated thoroughly	("20-Hydroxyecdysone ≥93% (HPLC), powder | Sigma-Aldrich," n.d.)

1.2.3.2 Desmasculinization of Vertebrates with Endocrine Disruptors

Endocrine disruptors present in the water have been investigated to analyze the effect on biota present. The effluent from a wastewater treatment plant contains variable mixtures of synthetic and biogenic endocrine disruption chemicals (EDCs), some steroidal estrogens like 17α-ethynylestradiol. These EDCs present in wastewater effluent were discovered to feminize male fish in Colorado. Controlled effects of the effluent on adult male fathead minnows (*Pimephales promelas*) found sex characteristics were demasculinized, such as a decrease in sperm count, after short exposure. When the fish were exposed to dilutions of the effluent equivalent to what would be found in the waterways downstream of the wastewater plant, definitive adverse effects were found, including the demasculinization (Vajda et al., 2011).

1.2.3.3 Effect of Endocrine Disrupting Chemicals on Invertebrates

Despite a large amount of research conducted on how EDCs affect wildlife species, the effect on invertebrates has been less studied (Bovier et al., 2018). To observe effects of endocrine disruptors on flies, Marcus and Fiumera (2016) analyzed pupation and emergence rate of fruit flies exposed to atrazine. The study found that the proportion of larvae that pupated and emerged were significantly reduced by atrazine exposure, however, there was an accelerated emergence rate for the exposed flies (Marcus & Fiumera, 2016).

Research conducted on aquatic invertebrates, such as embryos of apple snails (*Ampullariidae*), showed stimulated egg production at low doses, but inhibitory effects at

higher doses (Aris et al., 2014). Researchers have also introduced 17α-ethynylestradiol (EE2) in the food source for a species of fruit flies, *Drosophila melanogaster*. The researchers created concentrations of EE2 by dissolving the estrogen in ethanol and diluting with DI water. The results indicated that mean lifespan, maximum lifespan, and 50% survival time decreased with an increase of EE2 concentration (Bovier et al., 2018).

1.3 Forensic relevance

Endocrine disrupting chemicals have been found as pollutants in waterways, which increases the concern for the biota present in the water. Research has exposed potential demasculinization of fish due to EDCs, as well as effects on egg production of invertebrates. The effects of biota exposed to endocrine disruptors like synthetic estrogens or pesticides with endocrine disrupting qualities has raised a concern in forensic investigations dealing with bodies that have been exposed to these chemicals in the water. The demonstrated effect of other chemicals on the development of maggots leads to a question on if endocrine disrupting chemicals will also influence maggot development. Maggots present at a scene are sometimes used in post-mortem interval (PMI) estimations and any influence on the development of the maggot will influence the estimation.

A study done by the United States Geological Survey in 1998 sampled various waterways to find potential sources of endocrine disrupting chemical pollutants or waterways that have already been contaminated. A presence of vitellogenin, a hormone, in male carp or bass, is an indicator of exposure to endocrine disrupting chemicals. After analyzing vitellogenin in male carp from US waterways, one location contained a large

number of male carp (53%) with the hormone: Lake Luzerne, N.Y. Other waterways that have shown vitellogenin levels higher than the threshold include Poughkeepsie, N.Y. and Mohawk River, N.Y. These three locations are all along the Hudson River in New York (USGS, 1998).

From 2005 to 2014, there was an average of 3,500 non-boating related drowning deaths annually in the United States. More than half of the drownings for individuals aged 15 or older occurred in natural water settings, such as lakes, rivers, or oceans (CDC, 2016). In addition, the state of New York is fourth in the U.S. for drowning deaths per year, and over 50% of drowning deaths occur in lakes and rivers ("Stats - End Drowning Now," n.d.).

Any deaths that would occur in contaminated waters such as the Hudson River where the body then washes onto shore, could affect any entomological activity.

1.4 Research Question

The research conducted for this thesis looks to examine the questions; does the literature value of the LD50 of 17α-ethynylestradiol for rats correlate to flies, and does different concentrations of 17α-ethynylestradiol affect the pupation time, emergence rate, and sex ratio of the species of blowfly *P. regina*?

1.5 Aims and Objectives

The aims of this research are the following:

- To understand the process of maintaining *P. regina* colonies

- To determine if the LD50 of 17α-ethynylestradiol for rats influences survival of *P. regina*
- To determine if different concentrations of 17α-ethynylestradiol influence the number of eggs laid on a feed
- To determine if different concentrations of 17α-ethynylestradiol affect the pupation time, emergence time, and sex ratio of *P. regina*

To accomplish these aims the following objectives will be utilized:

- Follow previous maintenance protocols to learn the maintenance of *P. regina* colonies
- Observe the response of the colonies to maintenance protocols and adapt to maintain a heathier environment
- Introduce 17α-ethynylestradiol into a blowfly colony and measure the survival of flies against a Control colony
- Count number of eggs laid on feeds containing different concentrations of 17α-ethynylestradiol
- Record pupation and emergence times of colonies introduced to different concentrations of 17α-ethynylestradiol
- Record sex ratios of colonies before feed containing 17α-ethynylestradiol and the colonies that emerge

Chapter Two:

Materials and Methods

2.0 Materials & Methods

The following are the materials used in this research and the methods that were used to maintain colonies and introduce the estrogen into the colonies.

2.1 Materials

This section outlines where materials were obtained, such as the fly species and the estrogen used, as well as the set-up of a colony cage and rearing dish.

2.1.1 *Phormia regina* colonies

The *Phormia regina* (Meigen, 1826) flies used in this experiment were housed in the colony room within Dr. R. Christopher O'Brien's laboratory at the University of New Haven in West Haven, Connecticut. The species was raised of colonies from pupae received from Dr. Christine Picard's laboratory at Indiana University-Purdue University Indianapolis. This species was chosen due to its high presence in the Connecticut region.

The colony room was temperature Controlled, but the room temperature would fluctuate slightly with the changing weather of the Connecticut region. A space heater was therefore utilized in the colder months to keep the room temperature warm enough for the fly development, and a humidifier was also utilized in the colony room to keep the room moist for a healthier environment for the colonies, as maggots prefer higher heat and higher humidity when developing. The colony room was daylight regulated, at a 16:8 hour (light:dark) photoperiod (Heaton, Moffatt, & Simmons, 2014).

The room consisted of two metal racks used for colony maintenance, breading and rearing. One rack (Figure 2.1-1-a) held the active fly colonies on the lower three shelves and supplies on the top shelf. The second rack (Figure 2.1-1-b) held the rearing

dishes and supplies. When space was limited on the fly colony rack, one of the supply shelves from the rearing dish rack was used as an overflow colony shelf.

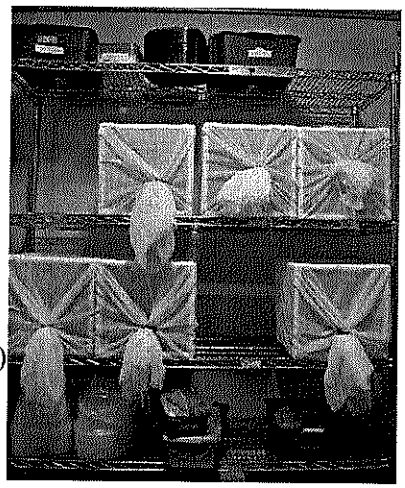
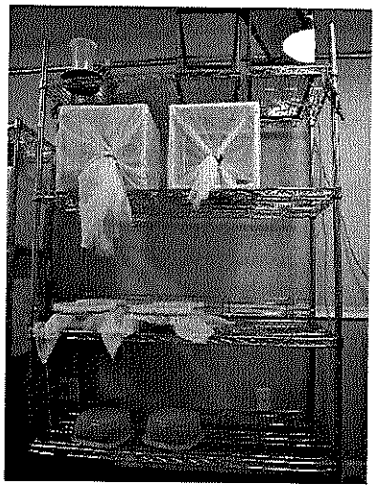
a)  b) 

Figure 2.1-1-a-b Racks in colony room for Rearing and Maintenance. a) First rack primarily used for active colonies. b) Second rack used for rearing and overflow (Photographs by Bethany Hoschar)

2.1.2 17 α-ethynylestradiol

The synthetic estrogen 17α-ethynylestradiol was obtained from Sigma Aldrich by the University of New Haven Forensic Science Department. Two grams of the chemical obtained were ≥98% purity ("17α-Ethynylestradiol ≥98% | Sigma-Aldrich," n.d.).

The estrogen was handled using Nitrile gloves. The bottles were placed in a small bubble wrap bag and placed in a closed plastic container stored in the temperature-Controlled colony room.

The estrogen 17α-ethynylestradiol and any contaminated sugar or protein feeds were disposed of according to proper protocols outlined in the Safety Data Sheet (SDS) for the chemical ("17α-Ethynylestradiol ≥98% | Sigma-Aldrich," n.d.).

2.1.3 Colony Cage Set-Up

A fly colony cage was a 1' x 1' x 1' cube built out of ½" PVC pipe and glued together (Figure 2.1-2-a). Two sides of mesh fabric were sewn together to cover the cages (Figure 2.1-2-b). The back end of the mesh was closed with a rubber band and the front end of the mesh was closed with a clip that can be removed easily for ease of feeding and maintenance.

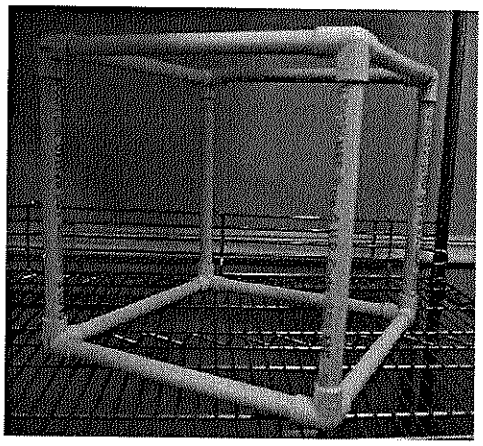
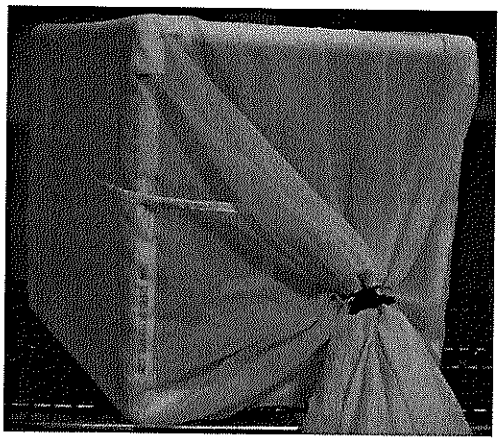
a)  b) 

Figure 2.1-2-a-b Pictures of PVC cage. a) PVC cage. b) Cage with mesh and clip (Photographs by Bethany Hoschar)

Every fly colony contained a sugar dish and a water cup. The sugar dish was a petri dish filled with sugar cubes and the water cup was a urinalysis cup with a hole punched through the lid. A piece of cotton fabric was placed through the hole in the cup, so that the water could be wicked upwards for the flies to drink from (Figure 2.1-3).

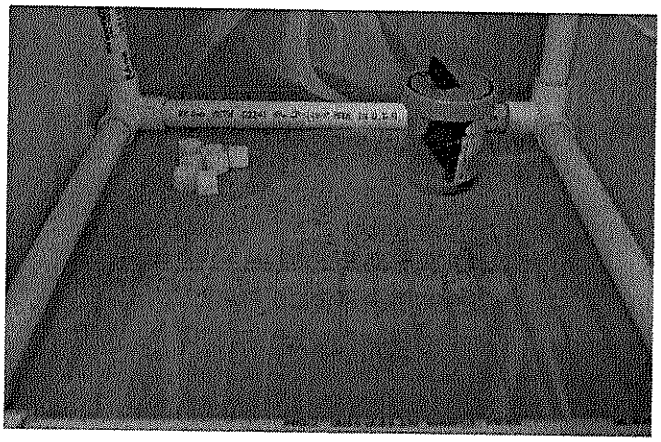


Figure 2.1-3 Picture of a cage set up (Photograph by Bethany Hoschar)

2.1.4 Rearing Dish Set-Up

A rearing dish was used to assist maggots develop to adult flies. To set up a rearing dish, a Three (3) liter plastic container was filled about a third of the way with sieved sand. A plate or bowl containing a protein source was placed on top of the sand. The protein source used for the experimental runs in this research was horse (*Equus caballus*) meat which was cut into cubic inch pieces and covered with 1-2 mL of horse blood. The fly eggs laid by the *P. regina* in colony were placed on top of the horse meat.

A square piece of mesh was placed over top of the dish and secured by a large rubber band. Each rearing dish was labeled and daily maintenance was recorded in an associated log (Figure 2.1-4).

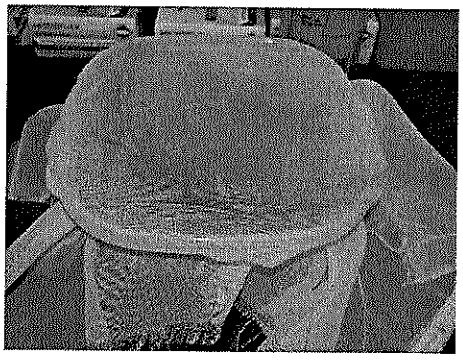
a)  b) 

Figure 2.1-4-a-b Rearing Dish Set Up Example. a) dish with horse meat and egg rafts on sand. b) dish covered with mesh (Photographs by Bethany Hoschar)

2.2 Methods

This section outlines the protocols that were followed for maintenance of the colonies and rearing dishes.

2.2.1 Maintenance of fly colonies and rearing dishes

Protocol for the maintenance of fly colonies and rearing dishes was developed in 2018 by Megan Descalzi (Appendix A) and adapted for the purposes of this research.

2.2.1.1 Fly colonies

To start a fly colony, a new cage was set up and *P. regina* pupae were placed in the colony. Some pupae from previous dishes would be kept in the fridge to slow the process of emergence so fly colonies could be started at a later date. If the pupae were coming from the fridge, the pupae would be placed in a bowl, which was then placed in the cage. If the pupae were coming from a completed rearing dish, the pupae would be

sieved out of the sand and placed in a bowl, which was then placed in the cage. The cage was closed to wait for the flies to emerge.

Once the flies emerged, various maintenance on the colony occurred, including protein feeds, water changes, and sugar changes.

A protein feed consisted of horse meat placed inside a bowl and covered with blood. This protein feed was then placed in the colony and removed the following day. Any eggs laid would be transferred to a rearing dish. A protein feed was placed inside each fly colony a minimum of once a week. If a colony was not laying eggs, additional protein feeds would be prepared to assist oviposition.

The water in each was changed once a week but would be monitored daily to ensure the colony had a continual supply of water. To change the water, the water cup would be removed, and the old water dumped out. The lid of the water cup was cleaned under warm water, and the cup filled with cold water. The water cup was then placed back into the colony.

The sugar in each colony was changed about once a month but was also monitored daily to ensure the colony had a continual source of sugar. Once the sugar cubes started to crumble and fall apart, the sugar dish would be changed. To change the sugar, the sugar dish was removed and cleaned out. New sugar cubes were placed on the sugar dish and the dish was placed back into the colony.

When a colony would need to be collected, it would be placed in a freezer for at least an hour. The colony would be removed from the freezer and the flies would be

collected in a urinalysis cup for any further information needed, such as the sex ratio of the colony or total number of flies present in the colony.

To clean a colony, the mesh would be removed and soaked in warm, soapy water. After soaking for at least a day, the mesh would be rinsed off and hung to dry. Sanitizing wipes were used to clean PVC cages.

2.2.1.2 Rearing dishes

Once a rearing dish was set up, the horse meat would be watered twice a day through the mesh with a spray bottle. Once the maggots started to wander and begin to pupate, the meat would be removed. After all the maggots pupated, the pupae would be sieved out of the sand and placed back into the colony. If the colony was active and thriving and did not need pupae, the pupae would be placed in a urinalysis cup with a mesh-covered hole on the lid, and labeled with species, initials, and date. This cup would then be placed in the fridge at 4°C.

To clean a rearing dish, excess sand would be placed back into the container of sand. The plastic container would be rinsed with warm water and scrubbed with soap if needed. Old meat would be thrown away once it was removed from the dish, and the bowl or plate would be scrubbed with soap and warm water.

2.2.1.3 Record keeping

To assist in maintenance of colonies and rearing dishes, a record keeping system was developed by Megan Descalzi in 2018 (Appendix A) and adapted to this project to track what maintenance was done and when. A maintenance binder was present in the

colony room containing the following sections: Colony Sheets, Rearing Dish Sheets, Old Colony Sheets, Old Rearing Dish Sheets, and Experimental.

All colonies would have a colony sheet for them. This sheet included the label on the colony, such as "Colony #1," the species, and the date the colony was started. Underneath this was a table with the following columns: Date, What Was Done, Initials, Signature, Comments. Whenever maintenance was done on a colony, it would be recorded on the colony sheet using specific codes (Appendix B).

These colony sheets were placed in the "Colony Sheets" section of the Maintenance Binder. When a colony was closed, the colony sheet was moved to the "Old Colony Sheets" section of the Maintenance Binder.

All rearing dishes would have a rearing dish sheet. This sheet included the label on the rearing dish, the date the dish was started and the date the dish ended. Underneath this was a table with the following columns: Date, What Was Done, Initial, and Comment. The label on a rearing dish would be the number of the colony it came from, and a letter, starting with A. For example, the first rearing dish prepared for Colony 1 would be 1A, and the second dish prepared for Colony 1 would be 1B if 1A was still active. These rearing dish sheets were in the section "Rearing Dish Sheets" in the Maintenance Binder. Once a dish was done and the pupae were removed, the sheet was placed in the "Old Rearing Dish Sheets" section of the binder.

The rearing dish sheet for regular rearing dishes varied slightly from the rearing dish sheet for experimental rearing dishes. For experimental dishes the information included Colony, Species, Dish Number, Date Started, and Date in Colony. The table

below this information contained the following columns: Date, What Was Done, Number of Pupation, Number of Emergence, Initial, and Comment. These rearing dish sheets were in the "Experimental" section of the Maintenance Binder.

2.3 Experimental Set-Up

The methods used for maintenance of fly colonies and rearing dishes were modified slightly to contaminate protein feeds and dishes with estrogen and monitor the development more closely. Two phases were completed. The first phase involved contaminating the sugar in the colony – this phase consisted of two colonies, a Control and an LD100, to determine if the flies were deterred from the estrogen. The second phase consisted of two trials. Trial 1 involved three colonies with contaminated protein feeds and rearing dishes, a Control, an LD100, and an LD50, in which the pupation rate and emergence rate were closely monitored. Trial 2 of the second phase involved two colonies, a Control and an LD25, in which the pupation rate was closely monitored.

2.3.1 Concentration Calculations of the Estrogen in Feeds

The LD values used in this research were values calculated from the known lethality of 17α-ethynylestradiol to rats, as said in literature. The doses given to feeds are not true lethal doses for flies. The LD100 (Lethal Dose 100%) value of 17α-ethynylestradiol is 1920 mg of estrogen per kg of a rat ("17α-Ethynylestradiol ≥98% | Sigma-Aldrich," n.d.), meaning that concentration of the estrogen is expected to be lethal to 100% of a population of rats. Therefore, the LD50 value of 17α-ethynylestradiol is half of this value – 960 mg/kg. The LD25 value of 17α-ethynylestradiol would be 480

mg/kg (Table 2.2-1). The 'kg' in the LD values referred to the substrate the estrogen contaminated. In this research, these substrates included the sugar and the horse meat.

Table 2.2-1 Table of Contamination values of the estrogen 17α-ethynylestradiol and their specific concentrations

Contamination value	Concentration of Estrogen (mg/kg)
Control	0
LD25	480
LD50	960
LD100	1920

To make the calculations to determine how much estrogen to add to an experimental feed, some conversion factors had to be used to get the values in the correct units (Table 2.2-2).

Table 2.2-2 Table of Conversion Factors Used to Convert between Units of the Value Obtained and Units Needed

Conversion Factor	First value	Second value
g to kg	1000 g	1 kg
g to mg	1 g	1000 mg

The amount of estrogen needed for each trial was calculated using the desired concentration of estrogen for each contamination (LD) value and the weight of the sugar or meat substrate (Equation 2.3-1).

$$Estrogen\ needed = Concentration\ desired\ \left(\frac{mg}{kg}\right) * amount\ of\ substrate\ (kg)$$

Equation 2.3-1 Amount of Estrogen Needed to Contaminate a Feed

The actual concentration of estrogen was then calculated using the amount of estrogen weighed out and the weight of the sugar or meat substrate (Equation 2.3-2).

$$Actual\ concentration = \frac{Amount\ estrogen\ used\ (mg)}{Mass\ of\ substrate\ (kg)}$$

Equation 2.3-2 Actual Concentration of the Contaminated Feed

The weigh by difference technique was used to weigh the amounts of substrate and estrogen. To use this technique, the container used for measuring the amount of substrate is weighed with the substrate. Once the substrate is transferred to another container, the container used for measuring the amount is re-weighed, and the difference between the two weight measurements is the true amount of substrate that was used. Equation 2.3-3 shows how the weigh by difference technique was used to calculate the true mass of substrate.

$$True\ mass\ substrate = (Mass\ substrate\ with\ container) - (Mass\ container\ after\ transfer)$$

Equation 2.3-3 True mass substrate from weigh by difference technique

2.3.2 Contamination Of Sugar

To preliminarily study if there is an effect of 17α-ethynylestradiol on *P. regina* adult flies, the estrogen was placed in the sugar of an active colony.

Two active colonies, Control and LD100, were used for this initial study. Previously dead flies were removed from the colonies to prevent them from interfering with the numbers of dead flies counted during every observance. The sugar cubes on the

petri dishes in the Control and LD100 colony were removed and replaced with granulated sugar. The granulated sugar for the LD100 colony was contaminated with the amount of estrogen calculated for this contamination value, while the granulated sugar in the Control colony remained uncontaminated. The estrogen and sugar were homogenized within a Magic Bullet blender before placing in the LD100 colony (Figure 2.3-1).

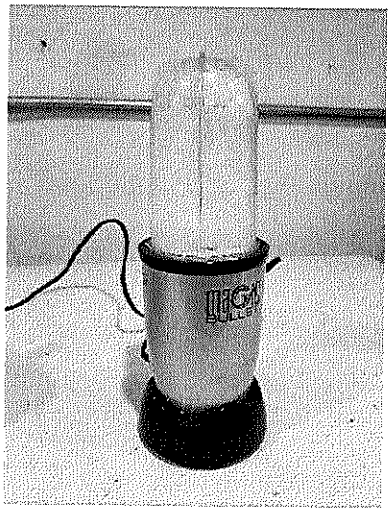


Figure 2.3-1 Blender used to homogenize the sugar and estrogen (Photography by Bethany Hoschar)

The colonies were observed every day for a week and the number of fly deaths were recorded. After observances suggested flies may not have been feeding on the contaminated sugar, a camera was set up on the colony with the contaminated sugar for a few days (Figure 2.3-2).

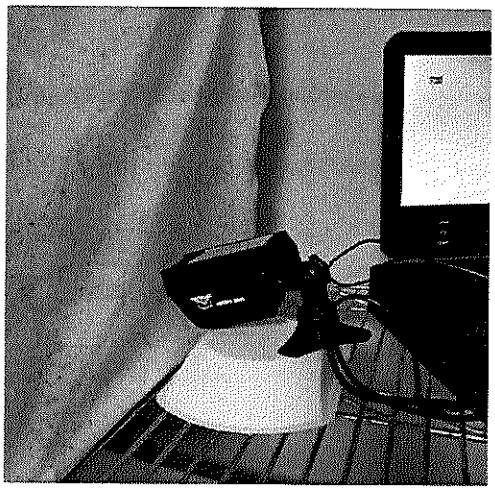


Figure 2.3-2 Night Owl Camera set up to observe sugar feeding (Photograph by Bethany Hoschar)

The mesh on the left is the mesh covering the colony containing the contaminated sugar dish. The camera is set-up in the middle on a weigh boat, and the computer recording the video is stationed to the right of the camera.

2.3.3 Contamination by Estrogen Of Protein Feeds And Rearing Dishes

Horse meat was separated into bowls for each experimental protein feed. For Trial 1, three colonies were used: Control, LD100, and LD50. For Trial 2, two colonies were used: Control, and LD25.

The meat for all contaminated dishes was weighed out. Calculations were completed to determine the amount of estrogen needed for the desired contamination value. For each experimental feed, once the desired amount of estrogen was added, the bowl was stirred with a wooden rod to evenly mix the estrogen.

The protein feeds were all placed in an active *P. regina* colony after all previously dead flies had been removed. The dead flies had been removed to avoid interference in

the true sex ratio of the colony when the protein feed was inserted into the colony. The protein feeds were then removed the following day.

To keep the protein source at the same contamination value, rearing dishes were also contaminated with the same value that the protein feed had been. Using the same calculations, rearing dishes were created for the protein feeds. Due to the significant amount of eggs laid in all the protein feeds, two dishes were created for each protein feed to split the eggs between, to prevent any interference from crowding within the rearing dish. Therefore, the eggs laid on the protein feed removed from the LD100 colony were split into two rearing dishes labeled LD100 (1) and LD100 (2). This split was done for the Control, LD50, and LD25 colony as well.

The same procedure for contaminating a protein feed was used for contaminating the rearing dishes. The Control rearing dishes only contained horse meat with no estrogen introduced.

2.3.4 Collection of data

Protein feeds with varying concentrations of 17α-ethynylestradiol were placed into active colonies on the same day. After removing the protein feed the following day, the eggs laid from each colony were split between two experimental rearing dishes of the same concentration of the colony the eggs came from, in order to produce replicates. This split was done with all the colonies given an experimental protein feed. After the rearing dishes were created, the colonies that had been given the experimental protein feeds were killed off using a freezer, and the flies were collected in a urinalysis cup for future sex identification.

Once an experimental rearing dish was set up, it was observed twice a day. When the meat was still present in the dish, the dish was watered during every observation. Once the maggots began to pupate, the observation included recording the number of pupae present in the dish. If the maggots pupated within the meat itself, they were removed from the meat using tweezers. After the meat was removed and all the maggots pupated, the pupae were sieved from the sand and placed in a bowl. This bowl was then placed into a new cage.

The new experimental cage continued to be observed twice a day, this time recording number of emergences. If the number of flies present in the colony became too many to count, the bowl containing the pupae would be removed during the observance. Any pupae still needing to emerge or in the process of emerging would be noted during the observance and the bowl containing the pupae would be placed back into the colony. After all the pupae emerged, the colony was killed off using a freezer, and the flies were collected in a urinalysis cup for future sex identification.

For Trial 1, the pupation rate, emergence rate, and sex ratio were all obtained. Due to time restrictions, only the pupation rate for Trial 2 was obtained.

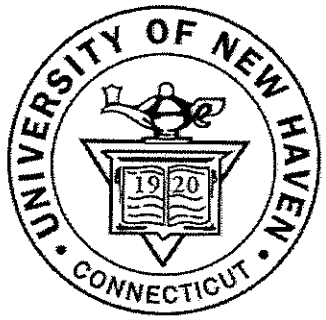
2.3.5 Analysis of data

The record keeping binder was used for data storage. Observations of number of pupated maggots or number of emerged flies at specific times were recorded using this binder and compared between difference concentrations of the estrogen. The sex ratio of colonies before and after an experimental feed were also compared between the differing concentrations of the estrogen.

Statistical analysis was conducted using the VSN International GenStat ® 19th edition statistical package. Statistical tests such as analysis of variance (ANOVA) were conducted to determine relationships and differences between various factors in this research.

Chapter Three:

Results



3.0 Results

The following sections detail results found in the experimental studies conducted, in addition to statistical results obtained.

3.1 Fly colony maintenance and rearing

Once a new cage was set up and a lot of pupae was placed in the cage, the pupae would take a week or two to emerge, dependent on where the pupae were received from. Pupae from an active rearing dish would emerge faster than pupae removed from the fridge.

After the colony emerged, the colony would take two to three weeks to begin to lay eggs. During this time, protein feeds were still given to the colonies to promote oviposition. Once a colony had laid two weeks in a row, they were considered an active colony and could be used in an experimental trial.

During the development of the experimental protocol, maintaining of non-experimental colonies and dishes assisted with understanding of the optimum conditions for development. Temperature, humidity, and light were regulated to keep the environment consistent. Some observations were made of overcrowding of maggots, so maintenance of the dishes assisted with learning how to prevent overcrowding by splitting the total amount of eggs laid by a colony among multiple dishes.

3.2 Conditions in the Colony Room

During the experimental phase of Trial 1, the temperature in the room was about 25 °C, however varied slightly between the values of 22 °C and 27 °C. The humidity in

the room was around the value of 40 %, ranging between 30 % and 50 %. The daylight in the room remained regulated throughout the duration of the experiment, at the 16:8 hour (light:dark) photoperiod.

During the experimental phase of Trial 2, the temperature in the room was about 25 °C, however varied slightly between 23 °C and 27 °C. The temperature of the room dropped to 18 °C one night due to a power surge, however, the temperature was maintained back to around 25 °C. The humidity of the room stayed consistent between 25 % and 30 %. The daylight in the room remained regulated throughout the duration of the experiment, at the 16:8 hour (light:dark) photoperiod.

3.3 Contamination with 17α-ethynylestradiol

17α-ethynylestradiol was used to contaminate sugar dishes, protein feeds, and rearing dishes. The following outlines the results of the contamination.

3.3.1 Sugar contamination with 17α-ethynylestradiol

The contaminated sugar dish in the LD100 colony contained 39.773 g of granulated sugar and 77 mg of 17α-ethynylestradiol, calculated using the concentration calculations, to make a concentration slightly higher than an LD100 concentration (Figure 3.3-1).

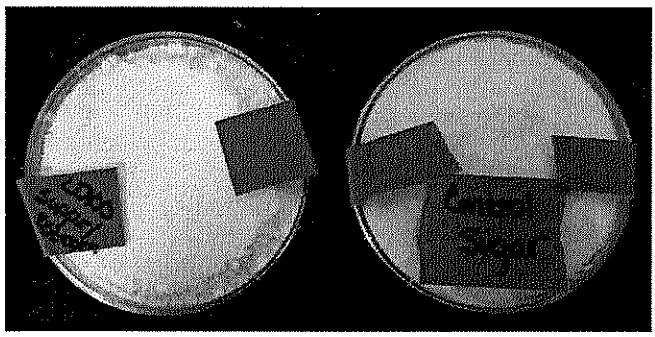


Figure 3.3 -1 Sugar in Petri dishes before placing in colony. Lid was removed before placement. (Photography by Bethany Hoschar)

Over the week of observations, the number of fly deaths of both the Control colony and LD100 colony were recorded. In addition, observations of the fly interaction with the sugar dish were noted. During most observation times, there were no flies observed on the LD100 sugar dish, however, many were observed on the Control sugar dish (Figure 3.3.2-a-b).

a) b)

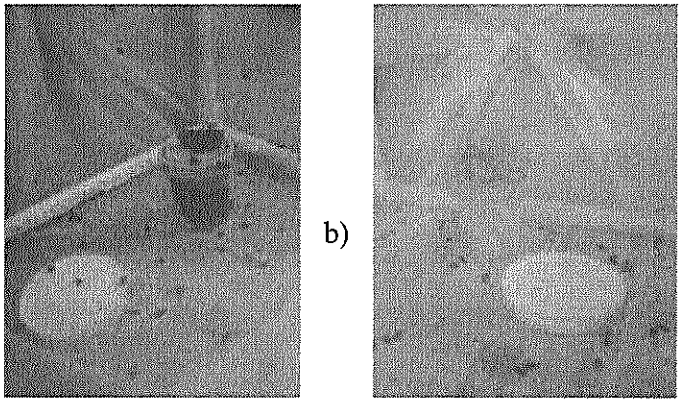


Figure 3.3-2-a-b Flies present on sugar on Day 2 of Sugar Contamination Phase. a) Control colony. b) LD100 colony. (Photographs by Bethany Hoschar)

The night owl camera was set up to record video to determine if the flies were deterred from the LD100 sugar dish.

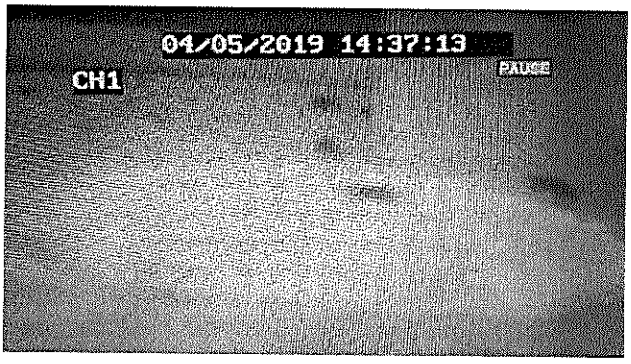


Figure 3.3-3 Snapshot of video showing feeding on sugar of the LD100 colony

While there was decreased feeding on the contaminated sugar, video (Figure 3.3-3) confirmed that the flies were not deterred and would still eat the contaminated sugar. The largest amount of fly deaths occurred during the first day of observation for both colonies. The day after the sugar dishes were placed there was 46 recorded dead flies in the experimental colony and only 16 recorded dead flies in the Control colony. The number of deaths per day for each colony decreases after day one. The average amount of fly deaths per day for the experimental colony from day one to day six was 16 deaths, while the average amount of fly deaths per day for the Control colony was 8 deaths (Table 3.3-1).

Table 3.3 -1 Fly deaths for Experimental and Control colonies

Day	Date	Experimental Colony # dead flies	Deaths in the past day	Control Colony # dead flies	Deaths in the past day
0	3-Apr-19	0	0	0	0
1	4-Apr-19	46	46	16	16
2	5-Apr-19	54	8	25	9
3	6-Apr-19	68	14	33	8
4	7-Apr-19	72	4	41	8
5	8-Apr-19	78	6	47	6
6	9-Apr-19	78	0	49	2

3.3.2 Protein Feed Contamination with 17α-ethynylestradiol

For Trial 1, three colonies were used: Control, LD100, and LD50. The Control colony was given 115 g of meat. The meat separated for the LD100 colony was weighed to be 110 g and the amount of estrogen needed to be added was calculated to be 211 mg. 217 mg was added to the dish, making the concentration over an LD100 concentration. The meat separated for the LD50 colony was weighed to be 95 g and the amount of estrogen needed to be added was calculated to be 90 mg. 91 mg was added to the dish, making the concentration close to an LD50 value.

For Trial 2, two colonies were used: Control, and LD25. The Control colony was given about 100 g of meat. The meat separated for the LD25 colony was weighed out to be about 95 g, needing 46 mg of estrogen. 53 mg of estrogen was added to the dish, making the concentration slightly higher than an LD25 value (Table 3.3-2).

Table 3.3 -2 Concentration values of 17α-ethynylestradiol for protein feeds

Colony	g of meat	mg of estrogen	True Concentration (mg/kg)
Control (Trial 1)	115.427	0	0
LD50	109.952	217	1973
LD100	94.242	91	965
Control (Trial 2)	99.845	0	0
LD25	95.237	53	556

The day after the protein feeds were inserted into the colony, they were removed and the eggs laid on the feeds were photographed (Figure3.3-4-a-b-c).

a) b) c)

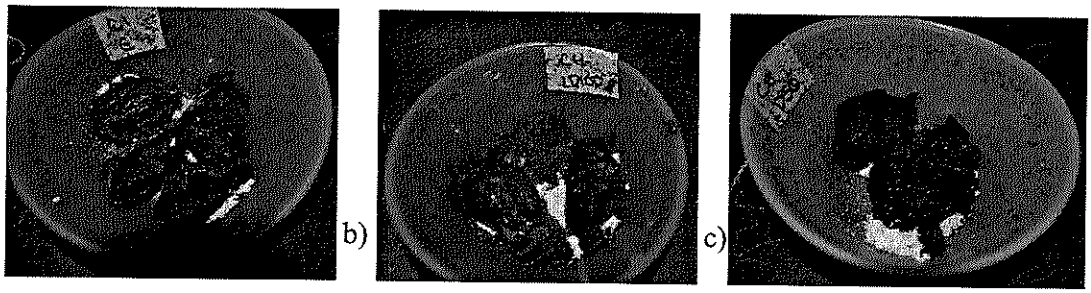


Figure 3.3-4-a-b-c Experimental Protein feeds after removal from colonies a) Control, b) LD100, c) LD50

Due to the large amount of eggs present on each protein feed, the eggs were split between two replicates for each concentration.

3.3.3 Rearing Dish Contamination with 17α-ethynylestradiol

The rearing dishes were also contaminated to keep the protein source at the same contamination value (Table 3.3-3).

Table 3.3 -3 Rearing dish label and the amount of 17α-ethynylestradiol added

Rearing Dish	Amount of meat (g)	Amount of estrogen added (mg)	Calculated concentration (mg/kg)
LD100 (1)	81.333	161	1979
LD100 (2)	78.77	150	1904
LD50 (1)	85.334	81	949
LD50 (2)	87.026	72	827
LD25 (1)	96.775	41	423
LD25 (2)	107.499	57	530

All rearing dishes were contaminated using the same weighing and stirring method (Figure 3.3-5).

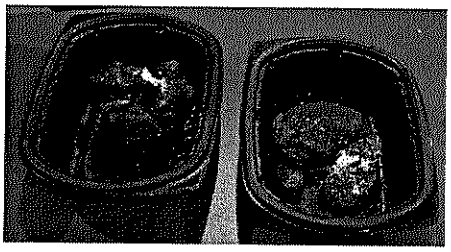
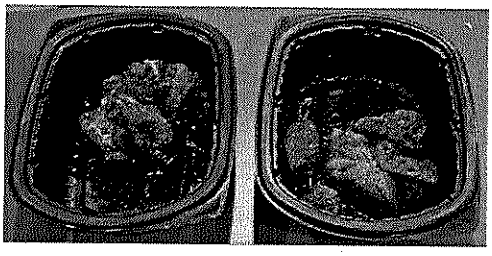
a)  b) 

Figure 3.3-5-a-b Rearing dishes before and after contamination with estrogen, a) LD25 (1), b) LD25 (2). (Photographs by Bethany Hoschar)

Once the rearing dishes were contaminated with estrogen, eggs from the protein feed of the same concentration were placed on the contaminated dish.

3.4 Rate of Development of Maggots after Experimental Feed

The rates of development were calculated by hours since the rearing dish was started. For Trial 1, the rearing dishes were started at 12:00 on 11 October 2019. For Trial 2, the rearing dishes were started at 1000 on 5 November 2019.

3.4.1 Pupation Rate of Maggots after Experimental Feed

The rearing dishes pupated at varying rates (Table 3.4-1). The elapsed hours calculated are since the experimental feed was removed from the colony. The first dish that began to pupate was an LD50 dish at 194.5 hours, and this was one of the first dishes to finish pupating, along with an LD100 dish. One LD100 dish did not pupate, limiting the LD100 dose down to one replicate. All maggots that did pupate pupated between the elapsed hours of 194 hours and 332 hours.

Table 3.4 -1 Pupation range of rearing dishes

Trial	Rearing Dish	Elapsed Hours at First Pupation	Elapsed Hours at Last Pupation
1	Control (1)	239	332.5
1	Control (2)	211.5	309.5
1	LD100 (1)	DID NOT PUPATE	
1	LD100 (2)	211.5	285
1	LD50 (1)	239.5	310
1	LD50 (2)	194.5	285.5

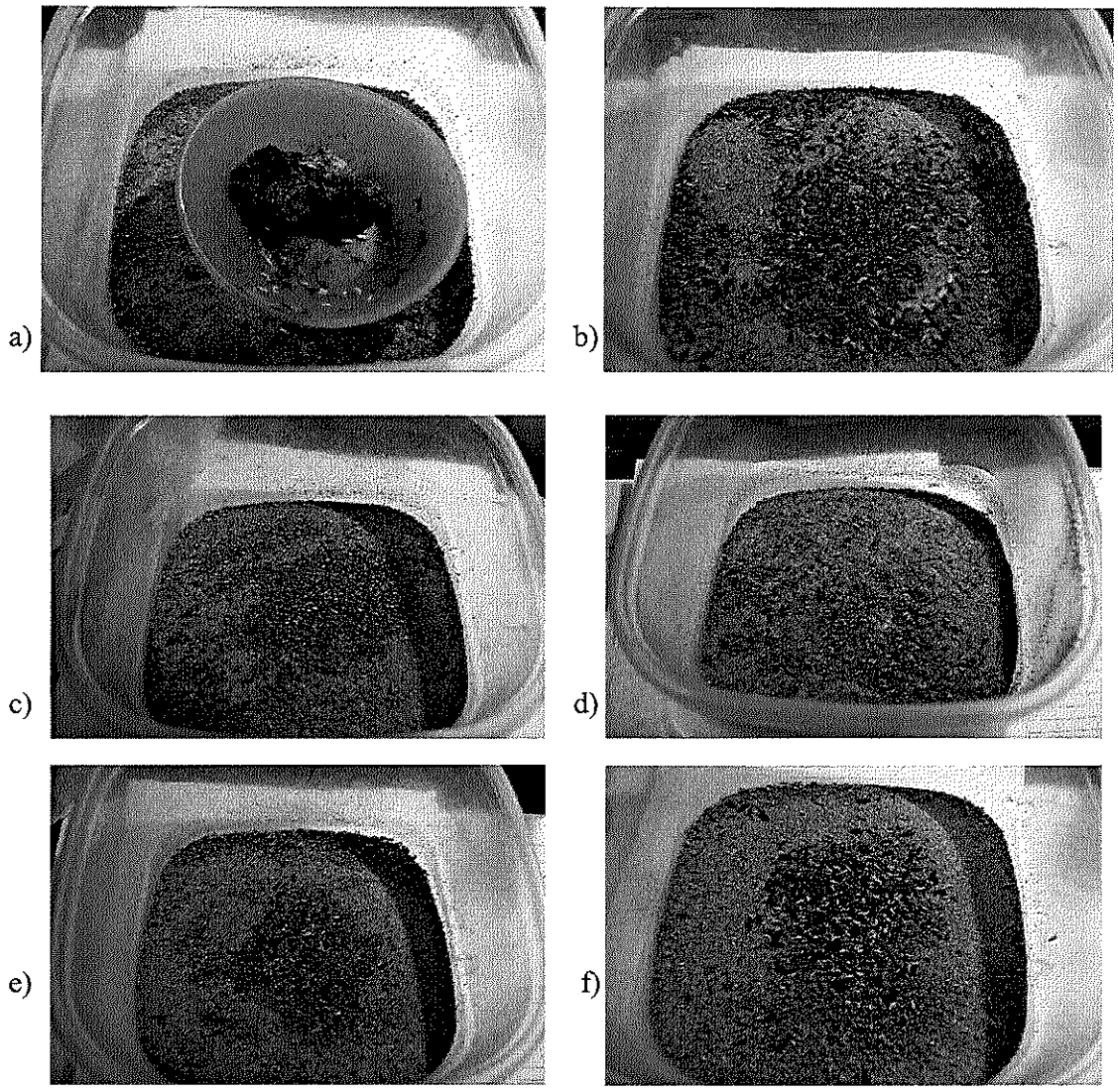


Figure 3.4-1 Pupae present in dishes at 285 hours in Trial 1, a) Control (1), b) Control (2), c) LD100 (1), d) LD100 (2), e) LD50 (1), f) LD50 (2). (Photographs by Bethany Hoschar)

At 285 hours, the dishes LD100 (2) and LD50 (2) dishes had completed their pupation, and the LD100 (1) dish still did not have any pupae present. The Control dishes seem to contain more pupae than the dishes that had been contaminated (Figure 3.4-1).

The hours of pupation for each larva that reached the pupae stage were documented in an Excel spreadsheet. Data collected as multiple replicates were combined

into one variate. An Analysis of Variance (ANOVA) test was run on the data collected for pupation time (Figure 3.4-2).

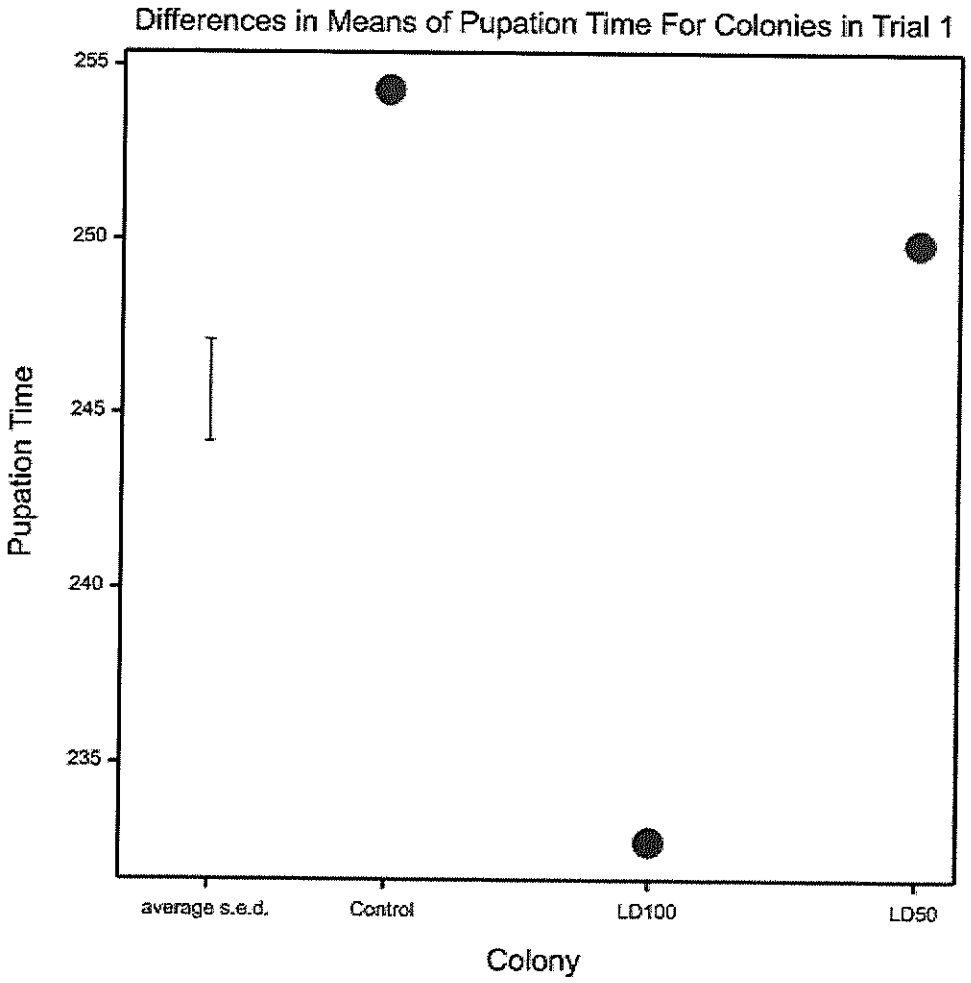


Figure 3.4-2 Differences in mean pupation time between Control, LD100, and LD50 colonies (ANOVA, $F_{(2,1016)} = 22.85$, $p < 0.001$, s.e.d = standard error of differences)

The least significant difference of this statistical test is 7 hours. Since the mean pupation time for the Control was 254 hours and the mean for the LD50 dose was 249 hours, the ANOVA above shows that there is no difference in the average pupation rates of the Control and LD50 colonies. However, the mean of the LD100 dose was 232 hours.

Therefore, the LD100 dish could potentially have pupated faster than the Control dishes and the LD50 dishes. Since the first LD100 dish did not pupate at all, only one replicate of the dish was completed as opposed to the two replicates each of the other doses received. More research would need to be conducted to understand if this is a statistical anomaly or a true result.

The data for Trial 2 were collected into Excel and analyzed using the same statistical test, an Analysis of Variance (ANOVA) (Figure 3.4-3).

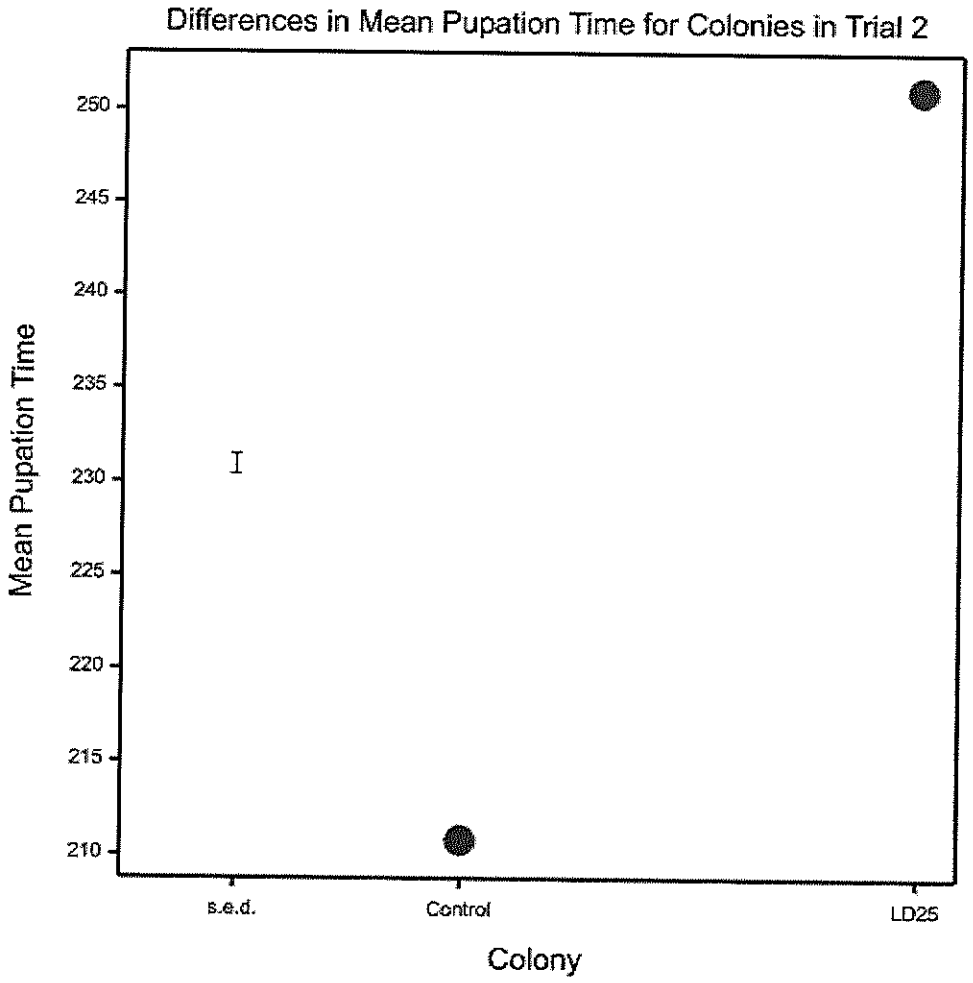


Figure 3.4-3 Differences in mean pupation time between Control and LD25 colonies in Trial 2

(ANOVA, $F_{(1,1548)} = 1469.05$, $p < 0.001$, s.e.d = standard error of differences)

The least significant difference of this test was 2.06 hours, so with the mean pupation time of the Control colony at 210 hours, and the mean pupation time of the LD25 colony at 251 hours, the LD25 colony was shown to pupate at a slower rate than the Control colony.

The data for the two trials were then combined to observe differences in pupation time over all concentrations (Figure 3.4-4).

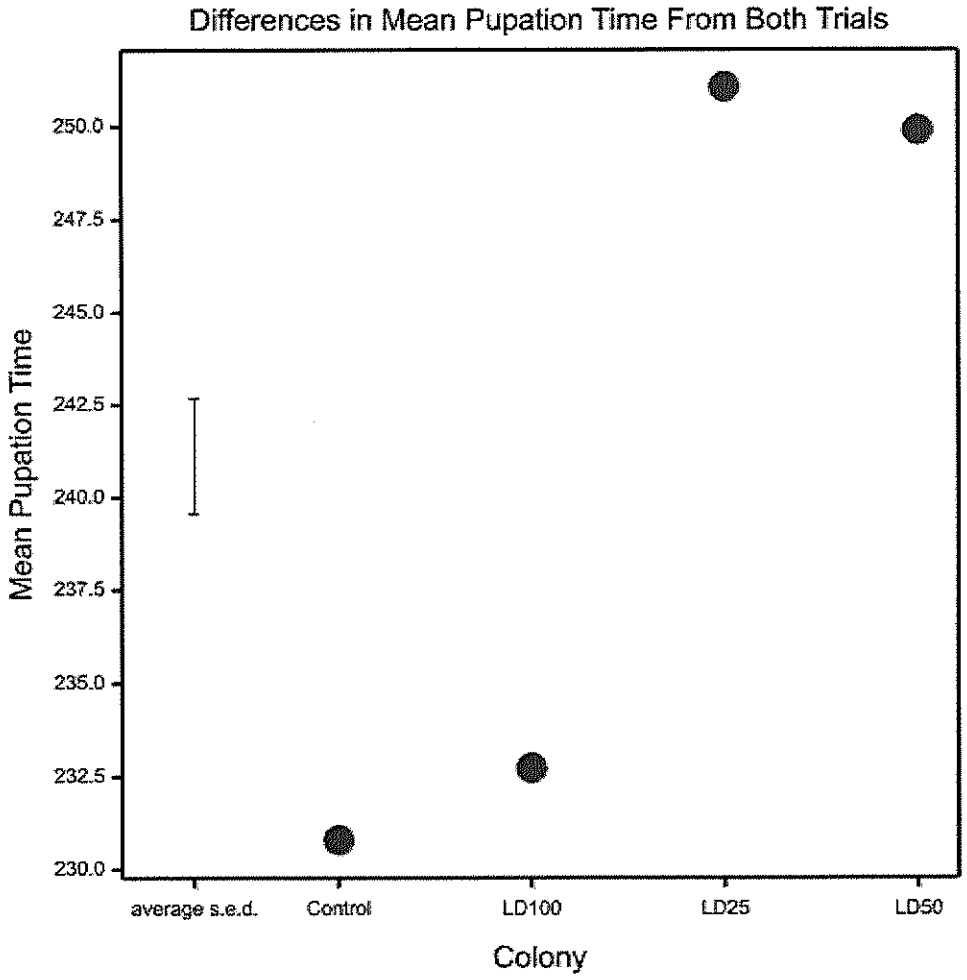


Figure 3.4-4 Differences in mean pupation rates from all concentrations (ANOVA, $F_{(3,2565)} = 96.59$, $p < 0.001$, s.e.d = standard error of differences)

The least significant difference was determined to be 2.5 hours for this statistical test (Figure 3.4-4). The mean pupations times for Control, LD100, LD50, and LD25 are 230, 232, 249, and 251 hours, respectively. Based on these numbers, there is not a difference in pupation rate between the first two colonies (Control and LD100) and the

second two colonies (LD50 and LD25). However, there is a difference between the two groups of colonies – Control and LD100 both pupated differently than LD50 and LD25.

3.4.2 Emergence Rate of Pupae after Experimental Feed

The dishes in trial 1 emerged at varying varying rates (Table 3.3-2). The emergence time was calculated from the time since experimental protein feeds were removed from the colony.

Table 3.4-2 Emergence of Experimental Rearing Dishes

Trial	Rearing Dish	Hours at First Emergence	Hours at Last Emergence
1	Control (1)	360	431
1	Control (2)	338	382
1	LD100 (1)	DID NOT PUPATE	
1	LD100 (2)	332.5	438
1	LD50 (1)	368	431
1	LD50 (2)	310	453

The hours to emergence for each larva that reached the adult stage were documented in an Excel spreadsheet. Data collected as multiple replicates were combined into one variate. An ANOVA test was run on the data collected for emergence time (Figure 3.4-5).

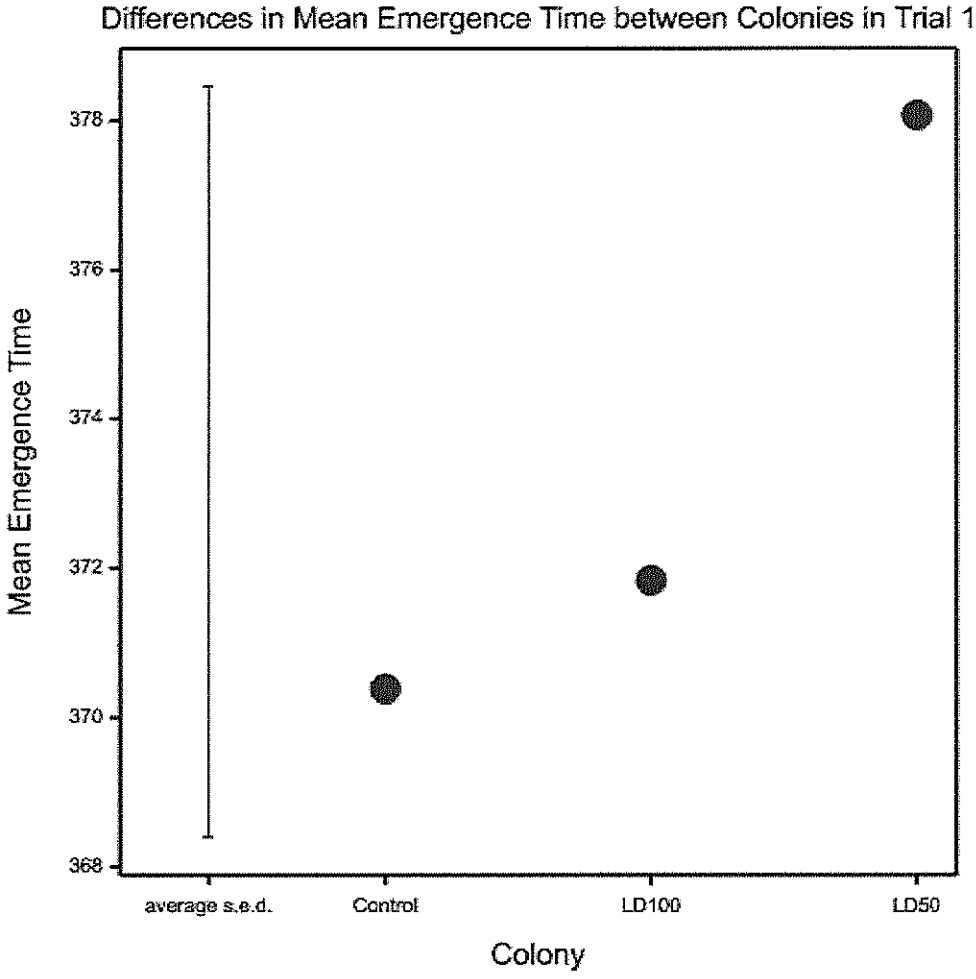


Figure 3.4-5 Differences in emergence time between Control, LD100, and LD50 colonies

(ANOVA, $F_{(2,323)} = 2.52$, $p = 0.082$, s.e.d = standard error of differences)

The minimum least significant difference for this ANOVA is 6.74 hours. The mean emergence time for Control, LD100, and LD50 were 370, 371, and 378 hours, respectively. Therefore, there is no significant difference in the emergence time between the Control and LD100 colonies, and a possible difference in the emergence time between the Control and LD50 colonies. Since the p value is larger than 0.05, the statistical test is not significant.

3.4.3 Percent Emergence of Pupae from Experimental Feeds in Trial 1

The total number of emerged flies and the total number of pupae were counted for each colony. These numbers were then used to calculate the percent of flies that emerged from the pupae stage into the adult stage (Table 3.4-3).

Table 3.4-3 Number of flies and pupae from colonies and the calculated percent emergence

Colony	Total emerged adults	# pupae	% emerged
Control 1	93	202	46.039604
Control 2	378	482	78.4232365
LD50 1	3	23	13.0434783
LD50 2	68	118	57.6271186
LD100 2	3	36	8.33333333
LD100 1	0	0	0

The percentages were then used to calculate the average percent emergence for each dose given in Trial 1 (Table 3.4-4).

Table 3.4-4 Average Percent Emergences of Pupae from Experimental Feeds for Trial 1

Dose	Avg percent emergence (%)
Control	62.23142
LD50	35.3353
LD100	4.166667

The percent emergence seems to decrease with an increase of dose of the estrogen 17α-ethynylestradiol (Figure 3.4-6).

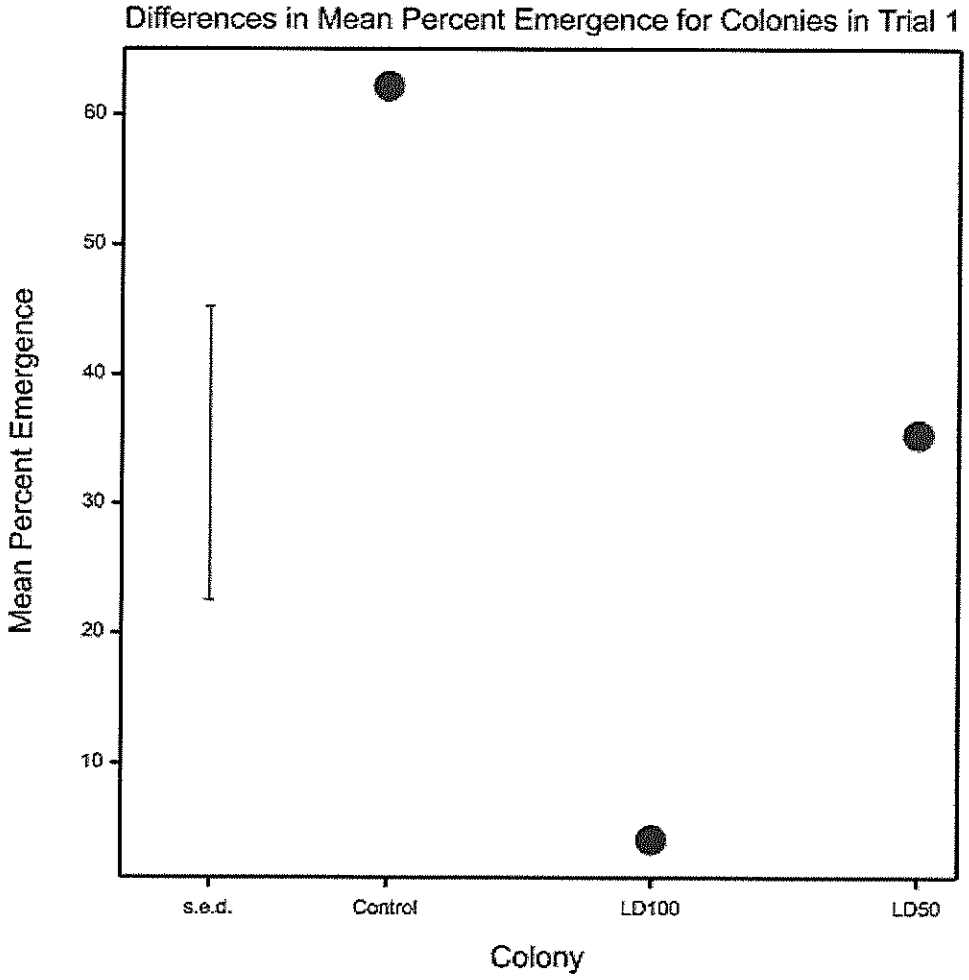


Figure 3.4-6 Differences in percent emergence between Control, LD100, and LD50 colonies

(ANOVA, $F_{(2,3)} = 3.26$, $p = 0.177$, s.e.d = standard error of differences)

The least significant difference in the means of the percent emergences was determined to be 72 % by the statistical software. Therefore, with the means of the three colonies falling between the values of 4 % and 62 %, the values have no significant

difference. The p value for this test is larger than 0.05, so more data would be needed to strengthen this result.

3.5 Sex Ratio of Colonies in Trial 1 before and after Experimental Feeds

The sex ratios of the three colonies that were given contaminated blood feeds in Trial 1 were determined. The eggs on the protein feeds were split into two for each colony. One LD100 dish died off, so five colonies emerged after the contaminated protein feeds. The sex ratios of these five colonies, considered the 'after contaminated feed' colonies, were also determined (Table 3.5-1). When the sex of a fly could not be determined, such as when the head was missing, or the fly was too degraded to be able to distinguish morphological features of the head, the fly was characterized as an unknown sex. The sex ratio was completed only for Trial 1 due to time constraints.

Table 3.5 -1 Sex ratios of colonies before and after protein feeds

Before/After Contamination	Colony	# male	# female	# unknown*	Total
Before	LD100	235	237	51	523
Before	Control	268	246	14	528
Before	LD50	172	143	3	318
After	Control 1	41	50	2	93
After	Control 2	132	112	0	244
After	LD50 1	0	2	1	3
After	LD50 2	26	41	1	68
After	LD100 2	0	2	1	3

*A fly was given the designation of an unknown sex when the sex was unable to be determined, such as when the head was missing or damaged.

Replicates of Control colonies and LD50 colonies were combined, then the percent of female's present was calculated (Table 3.5-2).

Table 3.5-2 Percent of females present in colonies before and after contamination

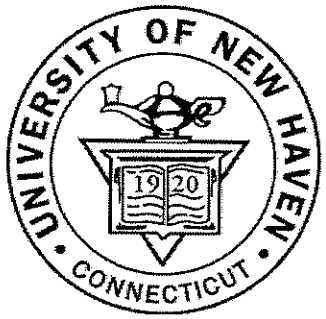
Colony	Sex	% before	% after
Control	Female	46.59091	48.07122
LD50	Female	44.96855	60.56338
LD100	Female	45.31549	66.66667

The table suggests an increase in percent of females present in colonies after contamination. However, the number of flies that emerged was less with the LD50

colony as opposed to the Control colony, and only 3 flies emerged from either of the LD100 colonies. This provided less replicates for a more accurate sex ratio for the LD50 and LD100 colonies.

Chapter Four:

Discussion



4.0 Discussion

After death, blowflies are attracted to decomposing remains for feeding and reproduction purposes. Since blowflies are usually the first species to colonize a set of remains, they are important to forensic scientists when estimating the time since death. Using the knowledge of the life cycle of a blowfly and any interferences of the environment that can affect the development, forensic entomologists can estimate the time since the arrival of blowflies, otherwise considered time since deposition. This research investigated how a specific estrogen present in pollutants affects the development time of blowflies, and therefore the time since death estimation.

4.1 *Diptera* in Entomotoxicological Research

Previous research in entomotoxicology more commonly involves using maggots or pupae to detect the presence of a drug from the set of remains the larva was feeding on, as opposed to studying the effects of a chemical on the development of the fly (Monthei & Paulson, 2009). Some research into chemical influences of the development of a blowfly has been conducted, but not on chemicals that are endocrine disruptors. Fruit flies, however, have been studied in depth for their oderant detection systems and their metabolisms, have been studied with regards to endocrine disrupting chemicals. Research has shown that fruit flies and blowflies are similar in how they react to certain conditions, such as larval crowding (Rivers & Dahlem, 2014). This study aligns some effects seen on fruit flies from exposure to 17α-ethynylestradiol to effects seen on the blowflies in this research.

4.2 Influences of the Environment in the Life Cycle of *Diptera*

The life cycle of a fly begins with oviposition, or the selecting of a suitable egg-laying site and the process of laying the eggs. Selecting the oviposition site involves visual or chemical cues (Lancaster et al., 2013). These chemical cues are likely due to the odor released from a set of decomposing remains, however, chemicals present in or on those remains could influence the odor released. This study first observed the Control and LD100 colonies after giving the experimental sugar dishes, recording the attraction of flies to the sugar and the death rate of the flies. The *P. regina* adult flies were attracted less to the contaminated sugar than to the non-contaminated sugar, confirming that blowflies were deterred slightly from a contaminated food source. However, there was not an obvious difference in the number of eggs that were laid between concentrations of 17α-ethynylestradiol, contrary to the previous research that showed chemicals present in a protein feed provided to a blowfly colony cause a deterrent effect (Monthei & Paulson, 2009). This suggests that the estrogen 17α-ethynylestradiol does not alter odor present during the decomposition to the point that would deter a fly, so only certain chemicals could create a deterrence effect.

4.2.1 Environmental Influences in the Life Cycle of *Diptera*

Insects are sensitive to changes in their environment, whether it be temperature, rain, sunlight, humidity, or other factors. A factor that plays a larger role in the development of an insect is temperature (Gasz, 2016). According to research previously done, the average total time a *P. regina* larva spends in the larval stages was a total time of 110-210 hours at approximately room temperature (Byrd & Allen, 2001). This was

confirmed during Trial 2, where the average time of the larvae present in the Control dish was a little over 210 hours, however, the larvae present in the Control dish in Trial 1 spent a longer time in the larval stages, with an average of 254 hours. The temperature between the two trials did not vary beyond a few degrees, so the research in this study confirms that temperature is not the only factor that can affect the development of maggots (Monthei & Paulson, 2009). Factors that may have led the maggots to take longer to develop include the humidity of the room, which had a larger variability, or larval crowding of the maggots.

Another method for estimating age of a larva is determining age based on length and weight, however the life cycle stage method is preferred due to interferences in development, such as diet and competition, that affect the size of a maggot (Gasz, 2016). This research confirms that diet of a maggot could limit the size of a maggot, as noted in visual observations during the developmental stages. The maggots feeding on the contaminated meat were visually smaller, suggesting they may have not had enough nutrients to develop properly or were slightly deterred from feeding, or that the development was slowed and the smaller maggots were still in an earlier instar.

4.2.2 Chemical (Entomotoxicological) Influences in the Life Cycle of *Diptera*

Qualitatively, entomotoxicology is generally used to detect drugs that would be present within the larva, assuming the larva had fed on contaminated remains. Previous research, done by Monthei and Paulson at the Virginia Polytechnic Institute, into detection of drugs in larvae and pupae have shown that qualitative results can be received

when analyzing larvae, but not when analyzing pupae. This suggests a metabolic process that eliminates drugs before the larva pupates (Monthei & Paulson, 2009). Another research article suggested a similar outcome, that drug concentrations in pupae were lower than drug concentration in larvae (Gosselin et al., 2011). If the drug is only present before the larva has pupated, the drug may have a larger effect on the development in the larval stages than in the pupation stage. After the data was analyzed, there was no significant difference in the emergence times, however, there was a difference in the pupation times, confirming that the drug may have had a larger effect on development times when in the larval stages. In the pupae stage, the drug had a larger effect on percent emergence, as less flies emerged the higher the concentration was.

4.2.3 Influence of Endocrine Disrupting Chemicals (EDCs) in the Life Cycle of *Diptera*

Chemicals have been shown to affect development of blowflies, however, there is little published research into the effect of endocrine disrupting chemicals on blowflies. This research aimed to study the effect of these endocrine disrupting chemicals on *P. regina.*

4.2.3.1 Process of Endocrine Disrupting Chemicals

In humans, the estrogen 17α-ethynylestradiol alters the normal functioning of the endocrine system. An overuse of these hormones could potentially produce overstimulation, also potentially altering the metabolism in the liver (Mnif et al., 2011). The LD100 value used in this study had an observed lethal effect on the flies, despite not to the level of an LD100 dose. This aligns with previous research into a different species

of fly, fruit flies, showing that the estrogen 17α-ethynylestradiol decreases survival time (Bovier et al., 2018). To humans, EDCs have been shown to disrupt reproductive and sexual development (Mnif et al., 2011). To flies, there were no signs of a difference in reproduction influenced by the EDC present, contrary to what is found in humans. However, a potential disruption in sexual development was not able to be determined in this research.

4.2.3.2 Demasculinization of Vertebrates with Endocrine Disrupting Chemicals

Previous research on demasculinization by endocrine disrupting chemicals has only been conducted on vertebrates. The previous research showed that effluent with 17α-ethynylestradiol present had demasculinizing effects on a specific species of fish, *Pimephales promelas* (Vajda et al., 2011). This study aimed to determine if there were any similar demasculinizing effects on invertebrates, however, the effect on sexual development was not determined.

4.2.3.3 Effect of Endocrine Disrupting Chemicals on Vertebrates

This study observed eggs laid on contaminated protein feeds and the time those eggs took to reach pupation and emergence. Trial 1 suggested the LD100 colony pupated faster than the Control and LD50 colonies. Trial 2 suggested the LD25 colony pupated slower than the Control colony. When the trials were combined, the data suggested the Control and LD100 colonies pupated faster than the LD50 and LD25 colonies.

Past studies have shown an accelerated emergence rate for fruit flies (*D. melanogaster*) exposed to the estrogen 17α-ethynylestradiol (Marcus & Fiumera, 2016), however this was not reciprocated in this current study with *P. regina*.

An accelerated emergence time could suggest accelerated development overall, possibly decreasing the pupation time as well. The higher concentration of the estrogen in the LD100 dose may have had enough effect on the flies to pupate faster. However, combined data suggests the Control and LD100 colonies did not differ in pupation time. The low amount of replicates for the LD100 colony could have led to skewed results. This would suggest concentration values of LD25 and LD50 would slow the pupation rate of colonies.

The proportion of fruit flies that pupated and emerged have been shown to reduce when fruit flies were exposed to endocrine disruptors (Marcus & Fiumera, 2016). This is similar to what was observed in the percent emergence of this study, however, the little number of replicates weakens this result.

4.3 Applicability to Forensic Science

A potential deterrent effect could influence PMI estimations if the estrogen was present on decomposing remains. While there is a deterrence of flies from their food source contaminated with the estrogen, PMI estimations likely will not be influenced due to deterrence because there were no signs of a deterrence of oviposition on contaminated feeds.

The lethal quality of 17α-ethynylestradiol would lead to deaths of flies in the environment if the estrogen was present on decomposing remains. An increase of presence of the estrogen on decomposing remains in the environment could influence the blowfly population. The effect may not be a decrease in number of blowflies, as blowflies

have a purpose in many biological systems, but a change in the balance of the population could occur, however, more research would need to be conducted to observe the effect on the environmental population.

There are cases where knowledge of the effect of endocrine disrupting chemicals on blowfly development for PMI estimations would be useful. A lake in New York, Lake Luzerne, was shown to have an increased number of male carp containing a hormone that indicates exposure to endocrine disrupting chemicals (USGS, 1998). 50% of all drowning deaths in New York occur in lakes and rivers, including Lake Luzerne ("Stats - End Drowning Now," n.d.). In 2010, a man was fishing in Lake Luzerne and fell into the lake after having a medical issue (Overit, 2016). Although the man was fishing with a relative and EMS was called shortly thereafter, if the man was fishing alone, PMI estimations would be helpful to determine when the man was on the water.

There have been signs of endocrine disruption in the Hudson River in New York (Baldigo et. al., 2006). On October 1st of 2019, the body of an unknown woman, about 35-55 years old, was found in the Hudson River (Cbs, 2019). In this case, PMI estimations could assist in determining when the woman had fallen into the river, and potentially when she had disappeared. While entomology may not have been used in this case, there is a potential to use time since deposition estimations through blowflies.

4.4 Limitations

One of the major limitations of this research was maintaining colonies that would be ready for experimental feeds at the same time. The location where the colonies were held did not support the maintenance of more than a few colonies. This school is not an

entomology-based school, and the research center is not solely focused on entomology, so the colony room is one small room with a few racks for storage of the colonies and supplies. For the sugar contamination phase, only two colonies needed to be active to begin the research at the same time, however, more colonies were needed for the protein feed contamination phase. Due to this limitation, the protein feed contamination phase was split into two parts, one with the Control, LD100, and LD50 doses, and the second with the Control and LD25 doses. If this research were to be conducted in a lab designed for entomology-based research, more replicates would be able to be produced.

In addition, a colony that was just begun would take 2-3 weeks to actively lay and be ready for experimental purposes, adding a time constraint to the process. A limited number of colonies were able to be maintained at the same time, so sometimes colonies for research were not able to be set-up until the previous experimental colonies were collected. Conducting this research in a larger lab designed for maintaining a larger amount of colonies would remove the impact of the time limitation seen during this study.

There was limited monitoring of experimental rearing dishes and colonies. This research was completed as an undergraduate honors thesis alongside of completing the undergraduate degree. Monitoring for number of pupae was only possible to occur at most twice a day, making the true pupation time for each pupa unable to be determined. A lab dedicated to research in entomology would have multiple individuals assisting in the monitoring of the research and would have more consistent monitoring. A lab that would be able to provide more consistent monitoring would less error in the pupation and emergence times. In addition, once most of the flies would emerge, monitoring the true

number of emergences was difficult. Counting the number of flies in an active colony becomes troublesome as the number of flies in the colony grows, and the flies will be constantly moving. A larger lab would have the means to more accurately record the number of emergences, either with instrumentation or a more developed method.

When determining the sex of collected experimental colonies, some flies were labeled as an 'unknown sex.' This was due to the head of the fly being damaged or missing, removing the ability to sex the fly based on distance between the eyes. This created a limitation in determining the true sex ratio of experimental colonies. To determine the sex of the unknown flies, the genitals could be pulled, or DNA could be run, however, the lab this research was conducted in did not have the capacity to complete either of these options.

4.5 Further Research

This study was not able to determine the effect of the estrogen on sexual development due to a low emergence, and this same low emergence skewed percent emergence results. Future research could provide more replicates, using the same protocols, to find effects of the estrogen on percent emergence and sexual development. In addition to more replicates, including the emergence rate and percent emergence of an LD25 dose would provide more information on effects that change dependent on dose.

The LD100 value used in this study was an LD100 value for rats. When the flies were given the LD100 dose, less than 100% of the colony died. Finding a concentration that would be a true LD100 dose for flies would provide more accurate effects of estrogen on development of blowflies. To find the true LD100 dose for flies, multiple

concentrations above the LD100 that was used in this study could be given to blowfly colonies and the mortality rate over the following week would be monitored.

Chapter Five:

Conclusions

5.0 Conclusions

The results of this research led to the following conclusions:

- The blowflies were deterred from eating the contaminated sugar as compared to the Control sugar
- The LD100 dose as reported for rats was not a true LD100 dose for *Phormia regina*
- There is an impact of the estrogen 17α-ethynylestradiol on pupation rate of *Phormia regina* larvae
    - The impact could not be determined to be a positive or a negative correlation due to limited replicates.
- Limitations in the research did not allow for a determination of impact on the emergence rate or sex ratio of the colonies
- Influences from the environment could have led to variations between the trials

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Table of Appendices

Appendix A: Protocol for fly maintenance developed by Megan Descalzi (2018)

Fly Colony Maintenance

- New cage set-up:
  - Place the previously cut mesh over the PVC cage.
  - Ensure that there is enough mesh on both sides so they can be closed. Close one side with a rubber band → this will be the back of the cage.
  - The other side can be closed with a clip → this will be the front of the cage.
  - **It is very important that you make sure both sides are closed completely so the flies don't escape!**
- Each fly colony will need:
  - A petri dish of **sugar cubes**. These cubes will need to be changed about once a month depending on the size of the colony. You will know they need to be changed when they are crumbling and falling apart.
  - **Water**. A hole should be made in the lid of the urine collection cup. Then a square from a T-shirt (previously cut in one of the baskets) should be put through the hole. Some of the shirt should be sticking out the top, while most should be sitting inside the cup. Fill the cup with water and ensure that the water is wicked up before placing inside the colony.
    - The water probably needs to be changed about once a week. Again, this changes depending on how large the colony is. I recommend checking this daily to ensure that they have water.
- To begin the colony:
  - If pupae are in refrigerator: put contents from urine collection cup on a petri dish and place in colony. Wait to emerge.
  - If using pupae directly from a rearing dish: remove meat and place dish (with sand and pupae) directly into colony. Wait to emerge.
  - If using a fly trap: place trapped flies into the colony

Blood Feed Instructions

- A blood feed should be done a minimum of once a week, sometimes twice depending on the colony. This can be based off of how many eggs are being laid. I would talk to Dr. O'Brien and come up with a game plan for blood feeds.
- Liver should be cut up into cubed form and placed in a large weigh boat (there is a sharp knife and cutting board to do this)
  - Use a pipette to transfer blood from the bottom of the liver container to the weighboat. Place a good amount of blood onto the liver, but do not saturate it as you do not want to drown the eggs. You can also spray the liver meat with a little bit of water, but also do not saturate it. There should not be a pool of liquid at the bottom.
- Place the weigh boat in the colony.
- Make sure to wash the knife, pipette and cutting board after finishing. When the blood dries it is difficult to wash off.

- Another thing to keep in mind is the amount of liver you have in the fridge. If you are running low, make sure to take out another liver far enough in advance so that it has time to thaw. You can stick the liver in a plastic container and place it into the fridge.

Rearing Dish Preparation and Maintenance

- The liver should be in the fly colony for about 24 hours. A rearing dish needs to be prepped the following day after the protein feed.
- If the flies did not lay, you can throw the weigh boat away. If the flies did lay, do the following, ○ Fill the plastic container with sand (about ½ full)
  - Place a good amount of ground beef on a plate or weigh boat. Place on top of the sand.
  - Similar to the protein feed, use a pipette to transfer some of the blood (from the liver container) on to the ground beef. Again, this is not to saturate it.
  - Place the liver cubes that have eggs laid on them on top of the ground beef. Try to get as many eggs as possible on to the beef.
  - Spray the beef with water. Hold the spray bottle about a foot away from the meat, and spray about 3 or 4 times. Again, we do not want to saturate them, just keep them moist!
  - Place a mesh square (already pre-cut) on top of the plastic container. Place a rubber band over the rearing mesh. You should make sure that there are no holes where wandering maggots can escape from. The rubber band should be tight and close to the rim of the container.
  - Place the rearing dish on the shelf and label with correct number for binder keeping. • The rearing dishes need to be sprayed **TWICE A DAY**
  - Make sure to take the mesh off and spray the meat directly (as directed above)
  - Again, make sure to put it on correctly so there are no holes for the maggots to escape
  - If the maggots are wandering to the outer rim of the plastic, be careful when taking the mesh off as the maggots will fall onto the table. At this point they are probably about to pupate so you can just leave the mesh on and just spray through the mesh.
- Once the maggots pupate, remove the meat and throw away. ○ Sieve the sand
  - Either put the pupae in a urine container and place in fridge (with species name, date and initials) or place directly into a fly colony
- Again, be aware of how much ground beef you have in the fridge. When you run low you need to take some out in advance so that it has time to thaw before you need it again
  - We are putting water crystals in the beef to keep it moist. When the meat you have taken out thaws completely, mix the water crystal beads with water. Wait for the beads to absorb the water and then mix in with the meat.

Other Important Info to Know

- You must fill out the binder each time you do something! This is so you and everyone else knows what has been done and which colony/dish is which.

- The binder consists of tabs for: Colony Sheets, Rearing Dish Sheets, Old Colony Sheets and Old Rearing Dish Sheets
- The codes for these sheets for what you have done are located on the wall next to the whiteboard
- On the top right of each sheet be sure to put which species is present in the colony/rearing dish (if known) • Numbering system for Rearing Dishes:
    - This will be based off of which colony the eggs came from and how many rearing dishes are going. ○ Number = cage number; Letter = how many rearing dishes are already going
    - For example, if you set up a rearing dish for the first eggs laid in your colony, which is cage #4, your rearing dish number would be 4A. However, if you do a few more blood feeds and you have two previous dishes from Cage #4 going, your new rearing dish number would be 4C.

Appendix B: Colony Sheet

Colony #: _____

Start Date: _____

Date	What was done (Code):	Initials	Signature	Comments

Appendix C: Blowfly colony Codes

Code	Description	Used for Colony/Dish
NC	New cage set up	Colony
PUP	Place lot of pupae	Colony
H2O	Change water or sprayed dish with water	Colony, Dish
SUG	Change sugar	Colony
BF	Blood/Protein feed placed in colony	Colony
LIV	Cut up liver or horse meat	Colony
PLIV	Pull liver or horse meat with egg rafts from colony	Colony, Dish
LBC	Place liver or horse heat in ice cream container	Dish
MWA	Monitor for wandering	Dish
SS	Sieve sand	Dish
PUA	Place pupae in urinalysis container	Dish
CC	Close Colony	Colony, Dish
RDF	Remove dead flies from colony	Colony
EXP BF	Experimental blood/protein feed placed in colony	Colony