Comparison and Selection of Saprophagous Diptera Species for Poultry Manure Conversion

by

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ABSTRACT

COMPARISON AND SELECTION OF SAPROPHAGOUS DIPTERA SPECIES FOR POULTRY MANURE CONVERSION

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Efficient disposal methods are needed to manage manure produced by industrial animal production. Saprophagous fly larvae could potentially convert manure into fertilizer and produce protein; however, the process is not well studied. Musca domestica, Hydrotaea aenescens, and Coproica hirtula were investigated to determine the most suitable species and conditions that facilitate efficient poultry manure conversion. The objectives were to (1) develop laboratory protocols and timelines for fly production; (2) identify environmental conditions that affect conversion; and (3) determine the ideal manure moisture content, depth, and fly egg-to-manure ratio for manure conversion and protein production. Mass-production was possible for every species and timelines were established for all species except C. hirtula. The most promising species for use in a conversion system was M. domestica and the presence of C. hirtula facilitated complete conversion. When using these species simultaneously the ideal initial conditions were: 77.5% moisture, 2.9cm deep and 0.82g eggs/kg manure.
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Chapter 1: Introduction and literature review

1.1 Issues with manure disposal

Animal production systems have evolved substantially in the last century. In the past, farms were small, family-run operations, in which manure produced by livestock was usually used to fertilize the fields immediately surrounding the farm. The manure was critical to the system’s success as it restored nutrients that had been removed from the soil by harvesting crops. These traditional farming techniques were sustainable and caused little environmental damage because they mimicked natural decomposition cycles and allowed nutrients to be recycled between trophic levels. Over the past five decades farming practices have changed drastically and livestock are now produced in high-density farms focused only on animal production in order to improve efficiency and increase profits. As animals are held at higher densities and rural land is increasingly used for non-agricultural practices, the spreading of manure on fields has become a less acceptable option for manure disposal; increasing the need for alternative disposal methods (Ostrander, 1963; Lapping et al., 1983). Common manure treatments include: composting, sanitary landfill, dehydration, incineration and lagooning; however, these approaches are costly and labour intensive (Ostrander, 1963; Collins et al., 1999). The efficiency of manure-handling systems has been improved through newly developed equipment, such as storage containers, pumps and spreaders, but this increase in efficiency does not always justify the increased costs involved and no treatment method offers an ideal means of manure disposal (Collins et al., 1999).

A layer chicken will produce between 0.1 and 0.2kg of manure every day; therefore, an average-sized farm of 100,000 birds will generate 10 to 20 tons of manure daily (El Boushy and van der Poel, 2000; Collins et al., 1999; Tao and Mancl, 2008). Fresh poultry manure is approximately 75% moisture and cannot be easily transported unless first processed because of its consistency, mass and odour (Miller, 1969; Collins et al., 1999). Manure must be removed
frequently from animal holding facilities because it creates an unsanitary environment and attracts manure-inhabiting flies that can spread pathogens to humans and animals. For an average-sized farm, manure is often physically removed with machinery two to four times a day, and manure handling constitutes much of the daily work (Collins et al., 1999). Mechanical breakdowns cause manure to accumulate rapidly and extra effort is required to quickly and safely recover. Excess manure must be properly stored to prevent environmental damage, decrease the odour and mitigate fly breeding (Ostrander, 1963; Collins et al., 1999).

The nitrogen in animal manures that could be used to improve soil conditions is currently wasted because there are limited applications for fresh or processed manure (Calvert, 1979). Farmers often have a difficult time marketing manure to crop-growers because regulations limit the amount and frequency that manure can be spread over fields, and supply is always far greater than demand (Ostrander, 1963; Hilborn, 2002). High processing, handling and startup costs outweigh the potential profit that could be made by selling converted manure to the public; as of yet, there is no disposal system that can quickly, easily and economically transform fresh poultry manure into a stabilized, dry, high-value and safe product (Collins et al., 1999). If such a system were to be developed it could potentially solve many problems associated with manure disposal and transform the worldwide view and treatment of manure (Calvert et al., 1969; Miller, 1969; Teotia and Miller, 1973).

A novel approach to solving many of the issues with industrial-scale manure disposal is to use saprophagous organisms that convert wastes into useable nutrients. Organisms that feed on decaying or rotting organic matter (such as bacteria and insects) play an important role in all ecological systems. Saprophages recycle the otherwise unusable nutrients and resources from waste products, making energy available to autotrophic producers (Mason, 1976; Putman, 1983). Without the decomposition process unusable material in carrion and fecal matter would
accumulate, nutrients would quickly be depleted from the soil and the cycling of resources would stop (Putman, 1983).

The use of naturally occurring organisms and processes to convert poultry manure may provide an efficient method to safely dispose of the copious manure that is an inevitable byproduct of today’s industrial livestock production. In this thesis research, poultry manure conversion was investigated using saprophages fly larvae at a laboratory scale and provided preliminary information that will aid in the future development of an industrial-scale system. Several fly species were tested and their manure conversion ability was compared in order to determine the species most likely to be successful at converting poultry manure at an industrial scale. For this species, the environmental factors and the appropriate conditions that optimize conversion were determined. The work presented here is divided into two themes: initial qualitative exploratory research to identify potential issues and to develop preliminary hypotheses relating to small-scale, fly larvae-mediated conversion (Chapters 2 and 3); and quantitative experimentation based on these preliminary hypotheses (Chapter 4). The results presented in this thesis further the understanding of the novel concept of using larvae to efficiently convert manure into value-added products, such as fertilizer and pupal protein. Future large-scale research can be developed on the basis of this information.

1.2 Systems using saprophagous fly larvae

1.2.1 Manure conversion

Synanthropic flies associated with waste materials are often considered pests, and are sometimes referred to as “filth flies” (this term is defined as such throughout this thesis). Many fly species are involved with the natural decomposition of the material they inhabit as larvae. Adult female filth flies deposit their eggs on decaying waste material that will sustain larval development. The actual decomposition of the waste is performed by bacteria that break down the compounds found in waste materials into smaller molecules that can be used by other
organisms (Mason, 1976). The larvae filter-feed on these bacteria, breaking up and aerating the substrate as they move (Schmidtmann and Martin, 1992). Aeration accelerates decomposition by allowing more oxygen to enter and stimulating the growth of bacteria beneficial to manure conversion (Putman, 1983). The bodily secretions of microbe-grazing larvae also promote the growth of beneficial bacteria and inhibit the growth of moulds and fungus that could hinder manure conversion (Rohlfs and Churchill, 2011).

A system using larvae to convert manure could sustainably and efficiently turn what is currently considered an inevitable downside and cost of raising animals into an additional source of revenue (through the sale of the fertilizer and pupal protein that are byproducts of conversion). A conversion system using larvae could be used worldwide in any size of farm to increase revenue, increase supply of low-cost animal feed, help combat deteriorating soil conditions and decrease fly populations. In locations where urban areas are encroaching onto farmlands this approach could also help render manure less offensive and more marketable. The technology even has potential applications in space exploration as it would enable astronauts to produce fertilizer from wastes (M. Dixon, pers. comm.). Fly-mediated manure conversion seems ideal for this application as flies add little mass, conversion occurs quickly and the system does not require bulky equipment or long-term storage.

Cost-efficient systems using *Hermetica illucens* (Linnaeus 1758) are already in use to reduce the accumulation of municipal organic waste and to produce biodiesel (Diener et al., 2009; Li et al., 2011). More research into the biology of saprophagous flies, with the emphasis on manure conversion, needs to be undertaken before an industrial-scale poultry manure conversion system can be developed and successfully implemented. With the worldwide increase in industrial farming it is necessary to investigate alternative methods for treatment and disposal of manure.
1.2.2 Larval protein production for animal feed

Insect protein is a suitable substitute for the soy protein currently used in poultry diets and, unlike soy, would not compete with human food production (Calvert, 1974; Sheppard and Newton, 2000). Studies have shown that animals fed insect protein develop as well or better than their grain-fed counterparts and eating insects may even increase the palatability of the meat produced (Sheppard et al., 1994). The use of insect protein could ease the pressure to grow food for livestock, which saves valuable grains for human consumption and potentially reduces the cost of animal production. Currently the demand for insect protein is low because animal producers do not see the benefit of feeding their livestock insect-based protein; therefore, there is no incentive for producing insect-meal and it is not cost-effective (Ravindran, 2010). If an efficient system for mass-producing puparia and transforming them into a protein feed can be developed and the meal can be effectively marketed to farmers, the use of insect protein in animal production should increase.

Coprophagous insects feed on bacteria that are able to extract the protein found in manure. The protein is incorporated into the body tissues of the insect and becomes available to higher levels of the food chain (Calvert, 1979). Some microorganisms like yeasts, algae and bacteria are able to convert the inorganic nitrogen in manure into protein, but are not suitable as a food source for livestock (Calvert, 1974). Using only these organisms in a conversion system does not allow for the collection and sale of protein, contained in the larvae, that could be used for livestock feed. Insects feed on microorganisms and promote manure conversion, allowing for the efficient production of two products: soil-conditioner and insect protein for livestock feed.

1.2.3 Milinator system

EcoSpace Engineering Ltd. has developed the technology and machinery (i.e. The Milinator) to process large amounts of poultry manure using saprophagous fly larvae. Collaboration with the University of Guelph has led to further testing of the technology, optimization of the process and
generation of scientific data (some of which has been presented in this thesis). If the Milinator is successful, it will provide a solution for poultry waste management, while producing valuable animal feed (protein-rich larvae) and organic fertilizer, thereby closing the gap in nutrient cycling created by current livestock production.

The prototype Milinator processing plant consists of a stack of five modified conveyor belts that hold the larvae and manure while it is being converted (Figure 1). Every 24 hours, fresh manure and fly eggs are added to the top layer, and the previous day’s manure is moved by conveyor to the next level; manure moves down one level each day until it is completely converted. Larvae are collected underneath the machinery as they migrate out of the manure and off the sides of the conveyor belts to pupate.

The Milinator has a unique method of ventilation that is vital to the success of the system. The whole system is double enclosed and the air is circulated between the processing area and between the enclosures. The air is taken from inside the enclosures and pushed across the surface of the manure from opposite sides during processing. This movement of air is conducive to the conversion process because it removes the accumulating ammonia, moisture and other gasses from the manure and immediate area.

A prototype of the Milinator system has been built and is operational at Arkell Research Station, University of Guelph.

1.3 Saprophagous fly species
1.3.1 Ideal species

It has been said that for every biological problem there is a species best suited for studying and understanding the problem, and perhaps a species that has evolved a solution to the problem. If this is true there should be a species that can be exploited and used in an industrial manure conversion system to help solve the serious environmental problems that industrial farming has
created. There are many species of Diptera that inhabit manure as larvae but differences in their conversion ability, biology, pest status and ecology affect their value to the system. All aspects of potential candidate species will have to be investigated and considered before a conversion system can be designed to most effectively utilize their natural abilities; however a “perfect” fly is unlikely to be found, so some compromises will have to be made.

Figure 1: Simplified Sketch of the Milinator “Processing Block” (Provided by EcoSpace Engineering Ltd. 2011). Top diagram depicts a side view the Milinator and the bottom is a front view of the side away from the holding tank.
1.3.1.1 Colony stabilization

A stabilized colony is important for the development and management of an industrial-scale manure conversion system. A colony is stabilized by only allowing a select part of the colony (i.e. that undergo events at the same time) to go on to the next life stage (J. Hogsette, pers. comm.). For example, only eggs that are laid at nearly the same time are seeded in the larval medium, larvae that pupate too early (or too late) are discarded and only flies that emerge from puparia within a certain time span are used for breeding (J. Hogsette, pers. comm.). A few generations of this selection will bring the timing of these lifecycle events into synchrony, allowing large-scale production to be planned around the predictable schedule of a stabilized colony.

When investigating the development of organisms in response to different environmental and physical factors it is important to know when they will undergo key lifecycle events. For flies, these events include the initiation of egg production, subsequent oviposition events, pupation and emergence. If the timing of these events is not known, planning of experiments becomes difficult because any time-sensitive preparations cannot be completed in advance. Timing becomes particularly important when studies are conducted to determine the larval response to varying environmental conditions. Controlling the natural larval variation for these kinds of studies requires preparation several days before oviposition in order to ensure an appropriate number of eggs with minimal age variation are collected (J. Hogsette, pers. comm.). If the eggs are not deposited at the expected time or too few are available, the experiment may be delayed or cancelled.

Timing becomes even more important when conversion of poultry manure is performed at an industrial scale. A stabilized colony is essential for producing the number of eggs needed on a regular basis for an industrial operation, which is several orders of magnitude greater than what is needed for laboratory experiments. A system will depend on the reliability of a colony to
produce enough eggs and larvae to convert the manure. If insufficient eggs are available, the manure produced at the farm will accumulate or the manure will not be completely converted and additional processing will be necessary for disposal or sale of the manure residue. A stabilized colony may also reduce the number of eggs (larvae) needed for conversion as the larvae in the colony will be adapted for particular manure conditions. The manure produced by an individual farm is likely to have consistent characteristics and, if flies are provided with only this particular manure over several generations they will become specialized for those specific conditions. Over time they may become more efficient and fewer eggs should be required for conversion.

1.3.1.2 Characteristics important for rearing

A species with a short lifecycle would be advantageous to an industrial-scale system because the colony will stabilize quickly and allow the traits desired for manure conversion (such as large puparia) to be emphasized. A stabilized colony that follows a precise protocol will be easier than an unstablized colony for a farmer or farm employees (without entomological backgrounds) to successfully maintain, ensuring the manure conversion system runs efficiently. An industrial manure conversion system will have to be located very close to or on the farm itself, thus avoiding the costs and risks associated with transporting fresh manure. The egg-producing colony will also have to be close to the farm because eggs typically hatch within a few hours of being laid and require manure immediately; this makes it impossible to transport the fly eggs very far.

The ideal species will regularly produce many viable eggs over an individual adult’s lifetime. To facilitate stabilization and bulk egg production, eggs should be deposited synchronously by all individuals in a population, ideally into a simple oviposition (egg collection) device.
For an industrial-scale conversion system to be successful it is desirable that a few additional rearing requirements be met:

- The maintenance required should be low;
- Adults should not require supplementary food sources. (Alternatively, if adults do require supplements, they should be made from non-perishable, inexpensive ingredients that can be easily prepared in advance. For example, females that require a blood meal to produce eggs would be far too costly and labour intensive to mass-produce); and
- All life stages should be able to survive at high population densities.

Additional characteristics of the species to be used in a manure management system that are desired but not required, include:

- The species should not be a pest;
- Females should produce many eggs with few males in colonies; and
- There should be no associated health concerns for people or livestock, as large colonies will have to be maintained on site.

**1.3.1.3 Characteristics important for a conversion system**

The food that manure-inhabiting larvae utilize (microorganisms, other insects) may affect their ability to convert the manure and therefore, their usefulness in a conversion system. For example, all manure-inhabiting larvae aerate the manure, but only microbial-grazers promote the growth of the beneficial bacteria that promote decomposition (Brookes and Fraenkel, 1958; Beard and Sands, 1973). Species that are facultative predators may promote beneficial bacteria like microbial-grazers, but may not be able to achieve the high population density necessary for efficient conversion. At high densities many species will begin to cannibalize younger or smaller
larvae when the bacteria they normally feed on become a limiting resource. Any reduction of the population will decrease the number of larvae in the system (decreasing the efficiency of the conversion process) and reduce the number of puparia that can be harvested. Therefore, a species that is solely a microbial grazer would likely be the best choice for a conversion system.

The following characteristics are desirable for the species that will be selected for the conversion system:

- Larvae and puparia should be large enough to collect and be of sufficient nutritional quality to transform into a protein supplement for livestock;

- Flies should be able to survive cold temperatures without going into diapause;

- The species should be a specialist in poultry manure (or alternatively, have attributes that increase manure-converting ability that can be selected for in a stabilized colony);

- A short development time would mean that larvae would grow quickly, require more food and likely convert more manure (per unit of time) than a species that develops slowly; and

- Puparia should be easily separated from manure residue so that both can be sold separately. Ideally larvae will self-harvest by migrating out of the manure to pupate.

### 1.3.2 *Hermetia illucens* (Linnaeus 1758)

*Hermetia illucens* (family Stratiomyidae), also known as the Black Soldier Fly, is native to the southern United States but has spread to many tropical and subtropical areas, including Australia, Asia, Central and South America (Kim et al., 2008; Diener et al., 2009). This species can be found in animal production facilities and occasionally occurs at high densities, especially in poultry and swine operations (Axtell, 1986). The adults are 2cm in length, non-feeding and not usually considered a pest in poultry facilities because they do not transmit disease or bother
animals and workers (Axtell, 1986; Sheppard et al., 1994; Diener et al., 2009). Eggs are laid between 27.5°C and 37.5°C in masses of 500 to 900 in dried crevices, and larvae can develop in a wide variety of organic substrates such as animal manures, municipal solid wastes, coffee, young plants and carrion (Booth and Sheppard, 1984; Axtell, 1986; Diener et al., 2009). Larvae are 2cm in length and are voracious eaters so that they can build up appropriate fat stores to maintain themselves as adults. Pupation occurs in as few as two weeks, but this period can be extended to up to four months by an inadequate food supply or low temperatures (Sheppard et al., 1994; Axtell, 1999; Diener et al., 2009). The pupal period is also variable and ranges between two weeks and five months depending on the ambient temperature (Sheppard et al., 1994). At 29°C, 38 days are required for development from egg to adult; development is extended to 60 days at 20°C (Axtell, 1986).

Oviposition occurs throughout the year given proper environmental conditions, which promotes large larval populations. Unlike other fly species, a large population of *H. illucens* is not necessarily harmful in an animal production facility because pest species (such as *M. domestica*) do not oviposit in manure when even small populations of *H. illucens* larvae are present (Bradley and Sheppard, 1984). Larval *H. illucens* decrease filthfly populations by preying on other species and outcompeting them for larval habitat (Sheppard et al., 1994).

### 1.3.2.1 Conversion systems using *Hermetia illucens*

The general principles of conversion systems using *H. illucens* are similar regardless of the organic waste being processed. Farms must first be populated with laboratory-raised *H. illucens* when the system is being established. After start-up, a wild breeding population is maintained in a suitable habitat (forested area) near the farm by releasing 10% of the puparia harvested; adult females return to the farm after mating to oviposit (Sheppard et al., 1994). Systems using *H. illucens* focus on the production and collection of protein, and the subsequent creation of manure residue is viewed as positive side benefit. Pre-pupae, the first non-feeding stage,
contain the highest amount of protein and are ideal for transformation into a valuable high-protein meal suitable to feed to various commercially produced animals (Sheppard and Newton, 2000).

In many conventional farms, manure from laying hens is left to accumulate underneath cages in small basins that are sloped to facilitate manure removal. When a conversion system using *H. illucens* is implemented, these basins are also sloped towards the outside of the building and end in a 15cm diameter pipe containing a small slit (Sheppard et al., 1994). This pipe attracts the pre-pupae as they search out a dry location for pupation, allowing them to self-harvest to a holding container where puparia are easily collected. In Florida, puparia can be collected every day from May to December at an average rate of 0.90g of puparia per chicken; however, larval mass is positively correlated with population size and more protein may be collected per bird at larger facilities because more larvae are inhabiting the manure (Sheppard et al., 1994). The production of protein is highly dependent on environmental temperatures; no puparia are collected when temperatures fall below 10°C and few are collected when average temperatures are below 15°C (Sheppard et al., 1994). The dependence on temperature and a wild adult population would cause the system to fail in cooler regions, including most of Canada.

Manure residue, which is reduced in mass by 50%, is moved into a slump at the end of the building twice a year (February and May) by a modified tractor designed to scrape the basins (Sheppard et al., 1994; Diener et al., 2009). Manure residue is liquefied, through the addition of water, to facilitate spreading with conventional fertilizing machinery and practices. Ideally manure should not be removed often because removal has a negative impact on the developing larvae and results in a reduced protein harvest in the following weeks. An annual removal of manure residue that coincides with the typical springtime application of fertilizer to fields would be most advantageous because manure can be used immediately and the larval population would only be disturbed once per year (Sheppard et al., 1994).
Although *H. illucens* has been successfully used to decompose manure and produce protein elsewhere, this species is of limited potential for processing manure in the Canadian climate. This species is inappropriate for the goals of this thesis because a wild population of adult flies (not mass-produced by personnel) must be present on or near the farm, which is impossible throughout most of Canada because it is too cold for *H. illucens* to survive year round. Females would not be ovipositing for most of the year, which leaves the manure unconverted and furthermore, the cold temperatures would prevent the collection of protein (Sheppard et al., 1994). For these reason, *H. illucens* was not considered to be a candidate species to use in a poultry manure conversion system and was not included in the investigations in this thesis.

1.3.3 *Musca domestica* (Linnaeus 1758)

*Musca domestica*, the House Fly, is a common, cosmopolitan, synanthropic fly of the family Muscidae. This generalist species is able to use a variety of moist organic substances for larval development, which brings it into close contact with humans (Skidmore, 1985). Adult *M. domestica* are typically 6 to 7mm in length, but size is dependent on larval conditions (Axtell, 1986). Temperatures from 7°C to 43°C are suitable, but *M. domestica* are most active at about 33°C (Schoof, 1964). Females have a longer lifespan than males. On average, at 26°C a female will survive 30 days as an adult, while a male will survive for 17 days (Schoof, 1964; Spiller, 1966). Ambient temperature affects the longevity of *M. domestica*; at 20°C flies can be expected to reach adulthood in just over 22 days, but at 35°C the same transformation from egg to adult will occur in only about nine days (Keiding and Arevad, 1964). A short life cycle and high fecundity allow populations of *M. domestica* to build up rapidly, and such populations often become problematic in livestock production. The manure produced on these farms attracts the flies and is perfect for sustaining a large population of developing larvae, making this species the most common pest on farms (Axtell, 1986). A large population of *M. domestica* can be
hazardous to both animals and farm workers as the mechanical transmission of several pathogens has been linked to this fly (Greenberg, 1973; Graczyk et al., 2001; Moon, 2002).

1.3.3.1 Conversion systems using *Musca domestica*

Research into the possibility of rearing *M. domestica* larvae in chicken manure began in the late 1960's with preliminary but promising results for both manure conversion and protein production (Miller and Shaw, 1969; Calvert et al., 1970; Miller et al., 1974). The first detailed description of a production system included methods and equipment that allowed 4.5 to 5.5 kg of manure to be broken down into a soil-conditioner in as few as eight days while larval protein was produced (Calvert et al., 1970; Morgan et al., 1970). The number of eggs seeding the manure affects larval development and the final properties of the substrate. Larger puparia can be collected from manure that has a lower larval density and maintains its mass and moisture content (Miller et al, 1974; Barnard et al, 1998). Therefore, the conversion is improved by using more eggs to seed the manure but the puparia are less suitable as a feed resource. Calvert (1970) selected a seeding rate of 3 eggs per gram of manure, which produced pupal protein at a rate of 3.2% of the mass of fresh manure; however, the moisture content of the manure was only reduced by 50% and required further processing to be converted into fertilizer (Calvert et al., 1970; Morgan et al., 1970). Similarly, Miller et al. (1974) concluded that the ideal seeding rate was 0.5 to 1.0g of *M. domestica* eggs per kg of manure (11-22 eggs per gram), which produced pupal protein and reduced manure moisture content by 58%; however, the amount of larval protein produced wasn't measured. Under field conditions, Bernard et al. (1998) were able to reduce the moisture content of the manure by 90% when 1200 larvae converted 100g of manure (0.54g of eggs per kg of manure). At this rate, larvae had the lowest survival (17%) and had the lowest individual mass (5.7 mg). The total mass of puparia produced was only 1.2% of the initial manure mass (Bernard et al., 1998). The differences are likely a result of not addressing other factors (such as scale) and not having a clear definition of “converted manure”. For example Miller (1974) used
4kg of manure for each experimental unit while Bernard et al. (1998) only used 100g, and neither investigated the effect of environmental factors such as initial moisture content or depth. Few studies have specifically defined converted manure. Manure was considered converted after a specific amount of larval activity but no final properties of the residue were used to define conversion (Bernard et al., 1998). The conclusions made (for example the correct number of eggs to seed manure with) were often very different between studies and may differ because some studies focused on protein production while others focused on manure degradation. Differences in the experimental scales, measurements, and definitions have made comparisons between studies difficult.

The technology developed by Calvert et al. (1974) provided a simple method of separating larvae from manure residue. The device consisted of two stacked, shallow wooden boxes separated with screen mesh and a bright light above. Fresh manure and eggs were added to the top box, where conversion occurred. After development, the negatively phototactic larvae self-harvested by migrating out of the manure through the screen down into the box below for pupation (Calvert et al., 1973). This separation of third instar larvae, rather than puparia, decreased the time the larvae spent tunneling through and processing the manure, which produced a less converted product (Putman, 1983). Calvert et al. (1974) claim that this device would be useful in industrial chicken farms to dispose of the copious manure produced, though it seems more appropriate for protein-production.

Other drawbacks to this device are related to the scale of industrial farming. An average laying-hen operation produces upwards of 12.5 to 20 ton of manure daily but the device was only designed to convert a few kilograms of manure (Morgan et al., 1970). At this rate, over 2400 of these devices (as they are described) would be needed to handle the manure produced by only one farm. The size of the device could be increased to improve efficiency; however, the maximum size would be limited because M. domestica larvae are not able to survive at depths
greater than about 7cm (Miller et al., 1974). Miller et al. (1974) have calculated that the manure from 100 000 hens could be converted and the system would produce up to 450kg of puparia daily; this seems unlikely with the proposed technology alone. The system would likely require too much space and too much daily maintenance to be profitable.

Other proposed systems using *M. domestica* focus solely on creating an inexpensive feedstuff for poultry. El Boushy and van der Poel (2000) addressed the possibility of using degraded poultry manure containing fly larvae as an ingredient in poultry feed. Fresh manure is unacceptable as a feed ingredient because of its high moisture content and low nutrient availability. Non-protein nitrogen (NPN), such as ammonia, creatinine, and uric acid are mildly toxic chemicals that cannot be digested by poultry, but make up more than half of the nitrogen found in poultry manure (El Boushy and van der Poel, 2000). When the manure is converted via digestion by a saprophagous invertebrate (such as *M. domestica*) or fermented by microorganisms, the manure residue becomes more suitable as an alternative protein source. Using converted poultry waste as a protein source reduces the accumulation of manure and provides farmers with an environmentally safe method of disposal, while producing an inexpensive and protein feed (El Boushy and van der Poel, 2000). Unlike other production systems, the insects were not removed from the manure residue and the final product contained the dried manure and insect protein, which is an acceptable substitute for meat, fish, or soybean meal in the diet of starter chicks. The insect protein contains most of the nutrients despite it being the minor proportion of the feed (Teotia and Miller, 1974). Separation of the manure residue and the flies would allow two products to be produced; protein meal and soil fertilizer; however, there is currently no satisfactory means by which that could be done at an industrial-scale (El Boushy, 1991).

In the production system described by El Boushy and van der Poel (2000), manure was scraped into a pit underneath the poultry cages (deep pit accumulation) once a week and was moved via
conveyer belts to a separate building where manure was stored and spread evenly on the floor. Once a week, *M. domestica* eggs were added at a rate of 0.75g of eggs per kg of fresh manure. Development from egg to puparia was completed in about seven days. Once the storage building was full, the manure residue (containing fly larvae and puparia) was removed, dried and sterilized by conventional means.

As described, El Boushy and van der Poel (2000) claimed the cost to run a system was equivalent to the drying systems that are currently used by many poultry farms, but their estimates did not take into account the cost of maintaining the large colony of *M. domestica* that would supply an appropriate number of eggs (El Boushy and van der Poel, 2000). Seeding manure with 0.75g of eggs per kg of manure would require at least 52.5kg of eggs weekly at an average-sized industrial farm. Assuming each female will lay 500 usable eggs, a colony of over 5 million adult flies would be required to produce a sufficient number of eggs. Several fly colonies and full time specialists would be required to regularly produce this enormous number of *M. domestica* eggs (El Boushy and van der Poel, 2000).

Another important issue that needs to be addressed is the possibility of the local area becoming infested by escaping flies (Beard and Sands, 1973). If the manure storage building is only cleared when it is full, flies have ample time to emerge from puparia and become a nuisance. El Boushy and van der Poel (2000) suggested that a hot air cannon be installed in the manure storage building so that any emerging flies would be killed before they could become a problem (El Boushy and van der Poel, 2000). Sealing the manure storage building to prevent flies from leaving would be difficult, as proper ventilation is required to disperse the ammonia emitted from the manure. An infestation would be likely even if only a small percentage of adult flies survive and escape because *M. domestica* is able to reproduce in a wide variety of moist decaying materials (Moon, 2002). A system that does not let flies reach the adult stage would be less likely to cause problems.
1.3.4 *Hydrotaea aenescens* (Wiedemann 1830)

*Hydrotaea aenescens*, (previously *Ophyra aenescens*), a member of the family Muscidae, is native to North America but has become established across Europe and is commonly encountered in poultry operations on both continents (Hogsette and Washington, 1995). These flies are about two thirds the size of *M. domestica* and have a similar, but slightly longer, lifecycle (Axtell, 1986). The larvae inhabit poultry manure where they are facultative predators of other manure-inhabiting larvae. Most notably, *H. aenescens* prey on *M. domestica* larvae in large numbers (15 to 20 larvae daily) and reduce their populations in poultry and swine farms (Turner and Carter, 1990; Hogsette and Jacobs, 1999). This tendency has prompted commercial sale and release of laboratory-reared *H. aenescens* as a biological control agent for *M. domestica*. Greater control can be accomplished by reducing *M. domestica* populations through pesticide application before a large number of *H. aenescens* are released (Hogsette and Jacobs, 1999). The population of *H. aenescens* will quickly establish and become the dominant species; any *M. domestica* that try to re-establish are outcompeted and cannot build up large pestilent populations.

*Hydrotaea aenescens* are considered pests because they are able to mechanically transmit several pathogens to humans and animals. The justification for mass release of this species for *M. domestica* control is that *H. aenescens* adults prefer dark areas and avoid buildings, humans and animals, making them less pestilent (Nolan and Kissam, 1987; Hogsette and Jacobs, 1999; Hogsette et al., 2002).

It is not known whether *H. aenescens* larvae have the capacity to decompose poultry manure in the same manner as *M. domestica* and *H. illucens*. The occurrence of *H. aenescens* in poultry manure has been well documented and some important environmental factors that influence survival have been identified, including moisture content, temperature, pH and age of manure (Hogsette and Washington, 1995; Farkas et al., 1998). Since *H. aenescens* is a facultative
predator, it may not be able to convert manure at the rate that is required in an industrial poultry manure conversion system; however, it is not cannibalistic even at high population densities and mass-production of this species is possible (Hogsette and Washington, 1995).

*Hydrotaea aenescens* may prove to be a good choice for use in a poultry manure conversion system because the species is unlikely to become a pest to farms or the surrounding communities, even when large populations are present. A large population of *H. aenescens* may even be beneficial because it decreases *M. domestica* populations without any additional cost or treatment. The decomposing ability of this species will have to be investigated to determine its usefulness in an industrial system.

1.3.5 *Coproica hirtula* (Rondani 1880)

*Coproica hirtula* (Rondani, 1880) are small (1.4 to 2.0mm in length) sphaerocerids (Sphaeroceridae, Diptera) that are commonly associated with manure (Papp, 1975; Bergeron et al., 2012). Although *C. hirtula* is synanthropic and found worldwide, little is known about its specific biology. Other synanthropic *Coproica* are normally located on the manure surface and rarely leave the immediate vicinity of the manure where they breed (Papp, 1975; Lachmann 1990; Bergeron et al., 2012). Females show a preference for fresh manure, but will oviposit in older manure as well (Hafez, 1939; 1949). In closely related species, eggs hatch about eight hours after they are laid and the microbial-grazing larvae inhabit the manure for three to four days (Hafez, 1949).

All life stages of *C. hirtula* are associated with bird manure and can build up large populations in poultry farms, but this species is rarely of economic importance (Bergeron et al., 2012). It may be possible for them to mechanically transmit some diseases because of their habitat; however, their habit of remaining close to manure makes transmission unlikely. *Coproica* spp. are often the first to arrive at the manure and can be seen one to two minutes after manure is deposited.
In caged layer houses, *C. hirtula* are among the first arthropods to become established (Stoffolano and Geden, 1987) and are considered a constant feature of the environment in many poultry farms (Beard and Sands, 1973).

This species may be useful as a poultry manure converter because its larvae are microbial grazers and ubiquitous on poultry farms; however, its small size may limit its capacity for pupal protein production and industrial-scale manure conversion.

1.4 Laboratory colonies

A farm that uses a larvae-mediated conversion system will have to produce several kg of fly eggs daily to ensure that all the manure is completely converted. Colonies will have to be maintained on or very near the poultry farm because of the difficulties, environmental risks and costs of transporting fresh manure (El Boushy and van der Poel, 2000). Maintaining this colony will likely constitute most of the work required for a fly-mediated conversion system. A species that can easily be maintained in a high-density colony is desired because it would decrease the daily work and input required for the conversion system.

Some species, like *M. domestica*, have been well studied and many protocols for mass-production have been published. The economic importance of *H. aenescens* has led to the development of protocols, but specific details are considered proprietary and have not been made publicly available. Other species, like *C. hirtula*, have not been the subject of any published protocols and little is known about their biology. The following is a summary of the published mass-production protocols that were used as references to initiate laboratory colonies of *M. domestica* and *H. aenescens* for this thesis.

1.4.1 *Musca domestica*

Laboratory rearing of *M. domestica* for entomological research began in the early 20th century (West, 1951). The qualities that make *M. domestica* a pest also allows this species to be easily
mass-produced in laboratories (Schoof, 1964). A large number of flies can be regularly produced quickly and cheaply without any special equipment, making *M. domestica* a convenient choice for testing the effectiveness of pesticides and other control methods that may be later adapted to other pest species. The regular use of *M. domestica* for these types of experiments has led to the development of a variety of culture methods for mass-production (Spiller, 1966; Pastor et al., 2000).

**1.4.1.1 Adults**

All methods of cultivation have similar instructions for adult care and focus on maintaining an environment conducive to egg production (Sawicki and Holbrook, 1961). Cages containing adults have wire or mesh portions to allow air flow into the colony, and an opening with a sleeve keeps flies in the cage during maintenance. Overcrowding of flies can result in physiological differences as resting and oviposition space become limiting, so cages vary in size depending on the number of flies needed and how often eggs are required (Louw, 1964). Less than 16cm$^3$ of space per fly reduces the number of eggs produced per female and decreases the longevity of the colony (Schoof, 1964). When flies were given 56.8cm$^3$ per fly, each female laid an average of 446.6 eggs and the colony produced 3.93 eggs per cm$^3$ (Pastor et al. 2011). In the most productive colony flies were only given 14.2cm$^3$ of space per fly and each female produced only 263 eggs, but the colony produced 9.26 eggs per cm$^3$ (Pastor et al. 2001). Adults are typically kept in a room with a temperature of about 25°C, an artificial long-day photoperiod (16h L: 8h D) and a relative humidity of 60-70%, which is low enough to limit fungal populations but high enough to prevent desiccation of eggs (Keiding and Arevad, 1964; Louw, 1964). An inverse correlation between ambient temperature and lifespan has been used to manipulate colonies. Keeping the flies used for breeding in a warmer environment produces eggs faster, and flies used for experiments are kept cooler so that their lifespan will increase (Keiding and Arevad, 1964).
1.4.1.2 Diet

Many non-biting muscid flies, like *M. domestica*, ingest nutrients by secreting liquefying digestive enzymes (present in their vomit and saliva) onto solid food sources. The resulting liquid is then drawn in using a sponge-like proboscis. These specialized enzymes and mouthparts, used by *M. domestica* and other higher Diptera, allow them to utilize both solid and liquid food sources.

Many different diets have been used to rear adult *M. domestica* in laboratory colonies. Table 1 lists a variety of adult and larval diets used by researchers to mass-produce *M. domestica* for entomological research. All diets contain both sugar and protein but the source of these nutrients varies. Early methods for culturing flies often used liquid milk-based diets that had to be tended daily to keep them suitable for flies (Spiller, 1966). Cotton or paper was used to soak up the food to prevent flies from drowning in the pool of liquid. This method was laborious as this material had to be replaced daily to maintain a constant supply of acceptable protein for egg production (Shipp and Osborn, 1967).

Solid diets are more commonly used to mass-produce *M. domestica* in modern laboratories that regularly produce thousands of flies. Solid diets require less maintenance and are less expensive because food can be prepared in large batches and stored. A food dish with a surface area of 90cm$^2$ supplies up to 2000 flies with nutrients *ad libitum*; limiting feeding space will result in starvation and fly mortality (Louw, 1964). Solid diets also require less maintenance than liquid diets because food dishes that present solid food to flies do not need to be refilled daily. Flies do become reluctant to feed when the surface of the solid food becomes excessively soiled and they may eventually die of starvation (Basden, 1947). Depending on the food and the number of flies, dishes may only need to be replaced a few times over the lifespan of the colony (Basden, 1947; Louw, 1964).
Table 1: Adult and larval diets for laboratory cultures of *Musca domestica*

<table>
<thead>
<tr>
<th>Source</th>
<th>Adult diet</th>
<th>Larval medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagleson, 1943</td>
<td>agar, banana, sugar, skimmed milk, formol, gelatin and water</td>
<td>-solution of 5% spray-dried non-fat milk with solids and 2% sugar</td>
</tr>
<tr>
<td>Soap Blue Book, 1960</td>
<td>-dry powdered milk</td>
<td></td>
</tr>
<tr>
<td>Sawicki and Holbrook, 1961</td>
<td>6 parts granulated sugar, 6 parts powdered milk, 1 part powdered egg</td>
<td>equal weights of whole-cream dried milk and flocked paper supplemented with 5% (by weight) of dried yeast and 2.1ml of water per gram of mix</td>
</tr>
<tr>
<td>Gahan, 1963, from Schoof, 1964</td>
<td>-20 g daily in feeding cups</td>
<td>-1000 flies are produced from 63g of mix and 135ml of water</td>
</tr>
<tr>
<td>Keiding and Arevad, 1964</td>
<td>-whole cream dried milk and cane sugar (1:1) supplemented with 10% autolyzed yeast and 0.1% cholesterol</td>
<td>-400g of wheat bran</td>
</tr>
<tr>
<td></td>
<td>-20 g daily in feeding cups</td>
<td>-200g of alfalfa meal</td>
</tr>
<tr>
<td></td>
<td>-flies not used for egg production are fed only dry cane sugar</td>
<td>-10g of baker's yeast</td>
</tr>
<tr>
<td></td>
<td>-equal weights of whole-cream dried milk and flocked paper supplemented with 5% (by weight) of dried yeast and 2.1ml of water per gram of mix</td>
<td>-15ml of Diamalt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1000ml of water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-4L jar</td>
</tr>
<tr>
<td>Louw, 1964</td>
<td>-cane sugar and water ad libitum</td>
<td>-400g of wheat bran</td>
</tr>
<tr>
<td></td>
<td>-diluted whole milk (1:1) starting 2-3 days after emergence</td>
<td>-200g of alfalfa meal</td>
</tr>
<tr>
<td></td>
<td>-undiluted milk to induce oviposition</td>
<td>-10g of baker's yeast</td>
</tr>
<tr>
<td></td>
<td>-whole milk powder, icing sugar and yeast (100:100:2)</td>
<td>-15ml of Diamalt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1000ml of water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-4L jar</td>
</tr>
<tr>
<td>Unpublished memorandum of the Communicable Disease Center Laboratories, Savannah, GA. from Schoof, 1964</td>
<td>-sugar cubes and water</td>
<td>-mixture of 60g dried brewer's yeast, 60g malt extract, 2400ml of water, 960g of sterilized sifted wheat bran and 440g sterilized alfalfa meal</td>
</tr>
<tr>
<td>Schoof, 1964</td>
<td>-solid sugar and a mixture of equal parts milk and water in separate containers with 1: 2000 parts of formol as a preservative</td>
<td>-transparent round containers 20cm high and diameter of 19.3cm</td>
</tr>
<tr>
<td></td>
<td>-milk powder and sugar</td>
<td></td>
</tr>
<tr>
<td>Spiller, 1966</td>
<td>-granulated sugar, liquid milk 10% sucrose</td>
<td>-feces of: cow, nursing calf, horse, swine, goat,</td>
</tr>
</tbody>
</table>
Iniguez-Covarrubias et al., 1994

- milk powder, yeast, granulated sugar (23:5:1)
- water

- composted swine, chicken manures and dog, for experiments

Larrain and Salas, 2008

- milk powder, yeast and glucose (23:5:1)
- water

- swine feces for experiments

A diet that increases fecundity reduces the size of colony that is needed to produce eggs for experimentation and is therefore highly sought after in mass-production (Louw, 1964). A change of diet can cause disruptions and temporarily decrease fecundity until the colony has adapted to its new nutrient source (Spiller, 1966). Colonies require time to adapt to a new diet, although the number of generations necessary was not reported. Studies comparing diets (Schoof, 1964; Shipp et al., 1967) have not mentioned stabilizing the colony prior to experimentation, and their results may be misleading.

Water is necessary for fly colonies that rely on a solid diet. Flies are able to survive for periods of time without food as long as they have ample water, but they will die without water when only dry food is available (Sawicki and Holbrook, 1961). An inverted beaker filled with water in a dish lined with paper towel can supply a colony with fresh water for several days (Sawicki, 1964). An absorbent material (such as paper towel or cotton) must be used when presenting water to flies to prevent drowning (Teotia and Miller, 1973). Spiller (1966) suggests that tap water is unacceptable to flies and advises using a 2.5% sugar water solution; however, in all other diets tap water is used. There is no experimental evidence to suggest that sugar water is superior to tap water for *M. domestica* production.

### 1.4.1.3 Eggs

Female *M. domestica* generally begin oviposition three or four days after emergence and deposit several batches of eggs in their lifetime, although this can vary with environmental conditions.
conditions (Teotia and Miller, 1973). Fly colonies produce the most eggs when collection occurs every other day (Spiller, 1966). The mass and number of eggs collected is influenced by environmental conditions (Shipp and Osborn, 1967). Each egg weighs 45 to 74µg (Miller et al., 1974; McIntyre and Gooding, 2000) and 500 to 750 eggs will displace 0.1ml of water (Keiding and Arevad, 1964; Miller et al., 1974). The number of eggs deposited by a female in one bout of oviposition depends on the population density of the colony (explained previously) and the strain of flies. Wild females can be expected to deposit batches of about 150 eggs (Louw, 1964; Schoof, 1964), while some laboratory strains have achieved 500 to 800 eggs per female in a single oviposition event (Moon, 2002). The number of eggs in the batch is positively correlated to the hatchability of the eggs and survival of 1st instar larvae; however, no chemical interaction between newly hatched larvae and eggs has been detected (Shipp and Osborn, 1967). The mechanism of this relationship appears to be that newly hatched larvae increase the temperature and moisture in the area surrounding the eggs, decreasing the incubation period. The environment becomes increasingly more suitable for young larvae and the bacteria they feed on as more eggs hatch, further increasing survivorship (Bryant, 1970, Schmidtmann and Martin, 1992; Lam et al., 2009). Hatchability and larval survival differ between batches of 80 and 640 eggs (Bryant, 1970). Laboratory experiments tend to be done at small scales and, therefore, few eggs and larvae are present in the medium. The results and behavior of the flies when 50 eggs are deposited are not necessarily applicable to a scenario where 20 000 eggs are added to the substrate, even if the same egg to substrate ratio is used. Despite this, many researchers have used less than 100 eggs or larvae per experimental unit, which is less than one female would typically deposit. Using a few eggs does not reproduce natural conditions because multiple females will lay eggs in the same area.

An egg collection device should attract flies and stimulate oviposition so that eggs can be easily collected for experiments. Fresh manure (Bryant, 1970), cotton soaked with a solution of milk
powder, malt extract and yeast (Miller et al., 1974), cotton soaked in milk and honey water (Louw, 1964), fermenting dog biscuits (Schoof, 1964) and many other fermenting organic materials have been used as oviposition media. All these media cater to the preference of the flies to lay eggs on a fermenting material with a rough surface that provides ample surface area; however, eggs are often soiled with substrate and difficult to remove (Schoof, 1964). Removal of eggs is aided by the tendency of many females to deposit eggs in the same location. A flat spatula blade or paintbrush is often used to move the delicate eggs from the substrate for quantification (Miller et al., 1974). An alternative method for collecting eggs solves this problem by wrapping the decaying substrate in a black cloth. The eggs are laid on the cloth and can be gently rinsed off, never directly contacting the substrate (Louw, 1964; Iniguez-Covarrubias et al., 1994).

1.4.1.4 Maternal age effect

Reproductive capacity is maximized when eggs are laid by “middle aged” flies that are about one week post-emergence (Hogsette and Washington, 1995). In order to harvest the greatest number of viable eggs it has been suggested that egg collection should begin four to five days after emergence when flies are held at 25°C (Callahan, 1962; Louw, 1964; McIntyre and Gooding, 2000) and eggs with little hatching variability can then be collected every other day for about one week (Louw, 1964; Spiller, 1966; Shipp and Osborn, 1967; Beard and Sands, 1973). Eggs from females more than 18 days postemergence are often not viable, high percentages of these eggs will not hatch and the eggs that do hatch may never pupate; however, if pupation does occur, then the duration of the developmental stages is not affected (Louw, 1964). The offspring of young females are more tolerant of unfavorable conditions, and are more likely to survive than the offspring of older females (Callahan, 1962). The maternal age effect is an important factor that can affect the population density and the susceptibility of flies to adverse environmental conditions (Callahan, 1962). In order to avoid misleading results, all M.
domestica eggs used for experiments should be collected in the shortest time frame possible, from flies less than one week post-emergence.

1.4.2 Hydrotaea aenescens

Hydrotaea aenescens are commercially produced for biological control of M. domestica on poultry and swine farms; however, protocols are often not published because many details are considered proprietary information. Published protocols for rearing H. aenescens are very similar to production methods used for M. domestica. In studies examining both species they are reared under the same environmental conditions and often fed the same diet (Callahan, 1962). The previous section describes the protocols for mass-producing M. domestica in detail and many of the same features apply for H. aenescens; because of this, the following section will focus on the differences in the protocol necessary for rearing H. aenescens.

1.4.2.1 Adults

The same type of cage is used to house both H. aenescens and M. domestica colonies. A cage with the dimensions of 46cm x 38cm x 38cm can comfortably hold about 6000 adults (Farkas et al., 1998). This is a slightly higher population density (about 11cm$^3$ per fly) than is acceptable for M. domestica (Hogsette and Washington, 1995). Swarming behavior has been observed for H. aenescens and flies typically cluster in corners with close contact between flies, which may be the reason less space is necessary for this species (Schoof, 1964).

The diets proposed for H. aenescens are very similar or identical to M. domestica because they have similar natural diets and mouthparts; however, one protocol for H. aenescens requires fish or bone meal to be sprinkled over food as a protein supplement (Hogsette and Washington, 1995). The extra protein may be unnecessary as many protocols, even by the same authors, have omitted this step (Hogsette et al., 2002).
1.4.2.2 Eggs

_Hydrotæa aenesæns_ that are five to ten days postemergence will only lay about 74 eggs per female in 24 hours, compared to 120-150 eggs in three to four hours for each female _M. domestica_ (Farkas et al., 1998). Females are also more selective of oviposition sites, and require dark conditions to lay eggs, increasing the difficulty of mass-producing these flies and collecting eggs of equal age for experimentation.

Many production systems for _H. aenesæns_ have not included methods to quantify egg production. Most protocols leave the larval medium in adult cages for a specific amount of time and estimate egg production from the number of adults that eventually emerge (Johnson and Venard, 1957; Turner and Carter, 1990; Hogsette and Washington, 1995). A collection device was invented by Hogsette and Washington (1995) that encouraged females to oviposit in unison and allowed for easy collection and quantification of eggs. This device consisted of two disposable squat cups that have been painted black, with one overturned onto the other and a small hole cut into the top to allow flies to enter. The oviposition medium (grain-based medium that has supported one cycle of _M. domestica_ larvae) is wrapped in black cloth and placed inside the cups. Eggs are easily washed from the black cloth and can be measured volumetrically; 10 000 eggs will occupy about 1ml (Hogsette and Washington, 1995). This device effectively meets the conditions _H. aenesæns_ requires to oviposit and 30 000 eggs can be collected from 12 000 adults in 24 hours, or five eggs per female (Hogsette and Washington, 1995). This device is an improvement over other methods, but egg production is very low when compared with _M. domestica_ and is a major constraint of working with _H. aenesæns_ (Hogsette and Washington, 1995).

1.4.2.3 Larval conditions

Many different larval media have been suggested for rearing _H. aenesæns_, including agar (Hogsette and Washington, 1995), clay (Roddy, 1955) and various grain media suitable for _M.
domestica larvae (Muller, 1982). Additional steps are required when using grain-based media developed for M. domestica, which must be fortified with protein such as fish or bone meal (Turner and Carter, 1990; Hogsette and Washington, 1995). Larvae can also develop in various livestock manures that are 50% to 80% moisture; but under these conditions they never achieve larval masses equivalent to those on grain-based larval mediums (Farkas et al., 1998). In swine manure, survival and pupal mass were not greatly affected by the moisture content; however, the largest puparia were recovered from poultry manure at 70% moisture (Farkas et al., 1998; Hogsette and Jacobs, 1999; Hogsette et al., 2002). In all media the survivorship of H. aenescens larvae is generally lower than for M. domestica larvae (Farkas et al., 1998). Colonies of H. aenescens are difficult to stabilize, requiring three years of rearing before colonies are considered stabilized (J. Hogsette, pers. comm.), which may account for the lower survival and mass when reared on manure. Flies are generally reared on a grain-based diet and only moved to manure for experimentation.

Environmental conditions for H. aenescens are the same as for M. domestica (Hogsette and Washington, 1995; Farkas et al., 1998). To increase the number of puparia produced, eggs should be added to the medium in a single mass at 20 000 eggs per 6000ml of modified M. domestica larval medium (Hogsette and Washington, 1995; Farkas et al., 1998). If a higher or lower population density is used, the percentage of eclosing larvae will decrease. Larvae also require increased temperature and humidity for the first three days of development; this is achieved by covering the larval containers with a black cloth for this period (Hogsette and Washington, 1995). After eleven days puparia are separated from the medium by flotation (Hogsette and Washington, 1995).
1.5 Objectives

This thesis sets forth to accomplish the following objectives:

1. Determine if mass-production is possible for *Musca domestica*, *Hydrotaea aenescens*, and *Coproica hirtula* and develop rearing protocols and techniques for mass-production using poultry manure as the sole larval medium.

2. Compare mass-production, manure conversion ability and biology of mass-produced species to determine the most appropriate species for manure conversion and to identify the environmental factors that influence the efficiency of manure conversion.

3. Determine the optimal manure moisture content, manure depth and fly egg-to-manure ratio for efficient manure conversion and larval protein production using the most promising fly species.
Chapter 2: Development of mass-production protocols and timelines for *Musca domestica*, *Hydrotaea aenescens*, and *Coproica hirtula* for laboratory-scale production and beyond

2.1 Introduction

The capacity to mass-produce flies is essential for the success of an industrial poultry manure conversion system. If manure were to be seeded at a rate of one gram of eggs per kilogram, an averaged-sized farm would require more than ten kilograms of eggs a day to efficiently process the manure. To obtain this considerable mass of eggs a large stabilized colony adapted to living in poultry manure is required. Egg production must be regular and predictable to reliably convert manure. If flies do not produce enough eggs, manure will quickly accumulate and become a serious problem.

Fly colonies will have to be maintained on site and a simple protocol that can be followed by farm workers without experience in insect rearing is necessary if the system is to go beyond the pilot stage. *Musca domestica*, *Hydrotaea aenescens*, and *Coproica hirtula* have potential as manure converters and their potential for mass-rearing is compared in this chapter. *Fannia canicularis* (Linnaeus 1758) is another species with larvae that develop in poultry manure and are commonly found in poultry facilities. This species was discovered by chance in manure that was to be used for rearing another species and was preliminarily evaluated for mass production at a much smaller scale than the previously mentioned species. Limitations with *F. canicularis* prevented further investigation of this species in this thesis. Laboratory-production protocols have been published for every species except *C. hirtula* (Schoof, 1964; Hogsette, 1992a; Hogsette and Washington, 1995); however, these protocols are not appropriate for a manure conversion system as they do not allow flies to adapt to manure conditions. In these protocols larvae are reared on artificial or grain-based media, and manure is only used for experiments. Protocols for a manure conversion system will be similar to the published protocols, but will have to be refined to promote efficiency and survival of the larvae in poultry manure.
Not all manure-inhabiting flies lend themselves to mass-production, and some species will be easier to cultivate than others. A straightforward mass-production protocol must be designed and tested at a smaller laboratory-scale to evaluate the potential of each species before full-scale cultivation begins. Important lifecycle events (such as oviposition) require special attention and species-specific solutions, which are simple enough to be adapted and applied at an industrial scale. The objective of this chapter is to evaluate the mass-production potential of each species and to develop laboratory protocols and techniques that can be adapted to an industrial-scale system. The exploratory investigations in this chapter identify features of mass-production protocols that will be pertinent to fly-mediated manure conversion (such as egg collection, stirring of the manure, and separation of puparia and manure residue) to be identified and for the generation of preliminary hypotheses; however, quantification of these features are required prior to them being implemented into a full-scale system.

2.2 Methods

2.2.1 Initial manure moisture content

Fresh poultry manure was obtained from the Arkell Research Station (Guelph, Ontario, Canada) from egg-laying hens that were fed a standard diet. Manure was collected within 24 hours of deposition on belts located underneath cages, when it had a moisture content of about 75%. The moisture content of fresh manure was measured by mixing the manure thoroughly with a shovel then drying three samples of known mass in a 60°C oven overnight, then reweighing; any change in mass was a result of water loss. This same method was used to determine the moisture content of manure when larvae did not survive. The following equation was used:

\[ \text{% moisture} = \left( \frac{m_1 - m_2}{m_1} \right) \times 100 \]

\[ m_1 = \text{fresh manure (g)} \quad m_2 = \text{dried manure (g)} \]

[1]
2.2.2 Development of protocols and timelines

To determine the elements that were important to rearing, several generations of each fly species (M. domestica, H. aenescens, and C. hirtula) were observed. Adult behaviors, oviposition, larval development and pupation were monitored as protocols and techniques were initially developed through trial and error, using published protocols as a guide. Techniques described in published protocols (Basden, 1947; Sawicki, 1964; Schoof, 1964; Hogsette and Washington, 1995), or by other entomologists (J. Hogsette, pers. comm.), which were described in Chapter 1, were applied and their impact on mass-production was observed through qualitative assessment of the number of eggs produced, the reliability of egg production, larval survival, and pupation. The changes that were deemed beneficial to the mass-production process or facilitated efficient evaluation were added to the protocol. To maintain a sufficient population of each species, any changes that decreased the population would not be repeated in the next generation and would be replaced with techniques that were previously successful. When populations recovered, techniques that would be more efficient at an industrial-scale were applied and evaluated.

Timelines were constructed for the species with the most information gathered (M. domestica and H. aenescens) by recording the days on which important events occurred (oviposition, pupation, etc). The time when these events occurred for the mode of the population was used in the timeline, the variability of which decreased as the colony stabilized.

After the successful initial protocols and timelines were determined through trial and error for each species (results not discussed), aspects of the protocols that would affect the manure conversion system (egg collection, timing of stirring, and separation of puparia and manure residue) were deemed most important and selected for further investigation.
### 2.2.2.1 Oviposition devices

Several oviposition devices were qualitatively tested for *M. domestica* and *H. aenescens*. An oviposition device provided females with a place to oviposit and was designed on the basis of up to seven versions of four variables (oviposition medium, container, material and configuration) (Table 2). Oviposition devices were compared based on:

- whether the oviposition devices were attractive to ovipositing females (if females were attracted, eggs were laid in the oviposition device and not the water dish);
- whether the eggs could be easily collected (eggs were easily collected if females laid eggs in a communal mass rather than distributing them over the oviposition medium); and
- whether the egg masses were easily measurable (eggs were most easily measured if they were unsoiled by oviposition medium).

Ammonium carbonate was also tested to see if it could be used to attract flies to a specific location for oviposition, as suggested by some protocols (Spiller, 1966; Barnard et al., 1998). Several methods were used to incorporate ammonium carbonate powder into the oviposition substrate: 1) mixing the powder into the fresh manure; 2) sprinkling the powder over the intended oviposition site (Schoof, 1964) or 3) soaking a paper towel or cloth that covered the substrate with a solution made of equal parts deionized water and ammonium carbonate powder (Spiller 1966).

Variations in the size and productivity of the adult colonies made it difficult to compare the numbers of eggs collected in different devices. Quantification of the egg mass was also not always possible because in some collection devices eggs could not be separated from the oviposition medium.
Table 2: Configurations and components that were tested as oviposition devices for *M. domestica* and *H. aenescens*. Oviposition devices were tested with combinations of variables (configuration, oviposition medium, container and material). Complete devices are described by a combination of four numbers representing the each of four variables according to this table. Not all combinations were tested; those combinations that were physically possible are given in Table 3 and the subset of combinations that were used is given in Table 4.

<table>
<thead>
<tr>
<th>Variation number</th>
<th>Configuration (Conf)</th>
<th>Oviposition medium (O)</th>
<th>Container (Cont)</th>
<th>Material (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>oviposition medium in container (Basden, 1946)</td>
<td>none (water)</td>
<td>in water dish</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>material soaked in liquid oviposition medium in container (Keiding and Arevad, 1964; Schoof, 1964; Spiller, 1966)</td>
<td>poultry manure (fresh) (Beard and Sands, 1973)</td>
<td>yellow pan trap dish (18cm top diameter by 9cm bottom diameter by 3.5cm deep)</td>
<td>paper towel</td>
</tr>
<tr>
<td>3</td>
<td>material (approx. 6cm ball (Hogsette and Washington, 1995)) tightly wrapping oviposition medium with no folds in material and closed with elastic</td>
<td>fresh poultry manure and water (above 90% moisture) (Miller et al., 1974)</td>
<td>yellow pan trap (as above) painted black (acrylic paint)</td>
<td>black cotton cloth (Hogsette and Washington, 1995)</td>
</tr>
<tr>
<td>4</td>
<td>material (approx. 6cm ball (Hogsette and Washington, 1995)) loosely wrapping oviposition medium with folds in material</td>
<td>poultry manure residue (rewetted to ~75% moisture)</td>
<td>small hexagonal weighing dish (7.5cm top length by 5cm bottom length by 2cm deep)</td>
<td>white muslin</td>
</tr>
<tr>
<td>5</td>
<td>material acts as a wick soaking up oviposition medium and fits through lid of container 7</td>
<td>grain based (Hogsette, 1992b)</td>
<td>large hexagonal weighing dish (15cm top length by 10.5cm bottom length by 2.5cm deep)</td>
<td>sponge</td>
</tr>
<tr>
<td>6</td>
<td>one container was filled with substrate and an identical container inverted over the first leaving a small space for flies to enter (Miller et al., 1973; Teotia and Miller, 1973; Hogsette and Washington, 1995)</td>
<td>milk solution (Keiding and Arevad, 1964; Spiller, 1966)</td>
<td>disposable plastic food container (11cm top diameter by 9cm bottom diameter by 8cm deep)</td>
<td></td>
</tr>
</tbody>
</table>
Oviposition devices, made from different combinations of versions (1 to 7) of each variable (oviposition medium, container, material and configuration), were qualitatively assessed to determine their effect based on the criteria above and were scored based on their relative success. It was not possible to test every combination and some configurations could only be used with a subset of the other variables. Table 3 shows the possible combinations and Table 4 shows which combinations were assessed for the *M. domestica* colony. Pretrial experiments suggested that the oviposition media and containers (Figure 2) used in the oviposition device did not affect the cleanliness or collectability of eggs. Scores for the oviposition media and container were based on their attractiveness to ovipositing females and the degree to which they led to oviposition in the device rather than the water dish. Each version was given a score from 0 to 4 from least effective to most effective. Materials and configurations, which did affect the cleanliness or collectability of eggs, were each scored out of a possible 6 points based on three qualitative questions:

- Is the material/configuration conducive to communal egg laying?
- Can eggs be collected clean and free of oviposition media?
- Are flies attracted to the material/configuration?
Table 3: Versions of oviposition medium (O), container (Cont) and material (M) that could be used together for each configuration (Conf).

<table>
<thead>
<tr>
<th>Configuration</th>
<th>Oviposition medium</th>
<th>Container</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1, 2, 3, 4, 6</td>
<td>1, 2, 3, 4, 5, 6</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1, 3, 6</td>
<td>2, 3, 4, 5, 6</td>
<td>1, 2, 3, 4, 5</td>
</tr>
<tr>
<td>3</td>
<td>2, 4, 5</td>
<td>2, 3, 4, 5, 6</td>
<td>2, 3, 4</td>
</tr>
<tr>
<td>4</td>
<td>2, 4, 5</td>
<td>2, 3, 4, 5, 6</td>
<td>2, 3, 4</td>
</tr>
<tr>
<td>5</td>
<td>1, 3, 6</td>
<td>7</td>
<td>2, 3, 4, 5</td>
</tr>
<tr>
<td>6</td>
<td>2, 4, 5</td>
<td>2, 3, 4, 5, 6</td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td>7</td>
<td>1, 3, 6</td>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4: Different oviposition media (O), containers (Cont) and material (M) assessed for *M. domestica* oviposition and egg collection.

<table>
<thead>
<tr>
<th>Configuration</th>
<th>Oviposition medium</th>
<th>Container</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2, 3, 4, 5, 6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2</td>
<td>4, 5</td>
</tr>
<tr>
<td>4</td>
<td>2, 3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Questions could be answered with “yes” (2 points), “no” (0 points) or “depends on other variables” (1 point). The higher the score (out of the possible 20 points), the more useful it was likely to be for collecting eggs for *M. domestica* as long as it was one of the possible combinations (Table 3). The three most successful devices for use with *M. domestica*, some described with multiple variations of variables, were chosen and the situations where they are most advantageous (when they should be used in the protocol) were outlined. The same
selection process, using the same variables was attempted with *H. aenescens*; however, because it was more selective, the variations were not scored.

2.2.2 Timing of stirring

Preliminary observations of larval-mediated manure conversion revealed that manure often became stratified, with some areas being more extensively converted (usually near the surface) than other moister areas (near the bottom). Other researchers have suggested that aeration was an important part of the manure conversion process, but have not offered a means to aerate manure effectively (Beard and Sands, 1973; Miller et al., 1974). Stirring the manure during the conversion process constituted a low-tech, intuitive method that counteracted stratification and increased the aeration of the manure. Surprisingly, stirring had not been utilized in previous culture methods for *M. domestica* and some authors have explicitly
recommended that the medium be left undisturbed during larval development (Basden, 1947). Pre-trial experiments for this thesis suggested that stirring the manure was essential to the conversion process; however, the optimal day to begin stirring and at what frequency manure should be stirred were unknown. To determine the start time and frequency of stirring that promoted manure conversion and larval development, manure was stirred once per day, less than once per day, or multiple times per day, at various stages of development (when eggs were added to manure, or when larvae were in their first, second or third instar). The effect of this stirring was then qualitatively assessed by observing the relative survival of the larvae based on the mass of the 3rd instar and the percent of puparia eclosing from the eggs used to seed the manure (Table 5). Moisture content, density, and temperature (Haupt and Busvine, 1968; Black and Krafsur, 1987) all potentially affected larval mass and eclosion, but were not always controlled. Controlled experimentation was not possible at this time because of previously explained differences in the unstabilized adult colonies and manure conditions. Results and conclusions were, therefore, based on qualitative, relative observations over several generations. Even though the results are not quantitative they allowed preliminary hypotheses to be developed.

Table 5: Definition of decreased and normal survival in assessing the effectiveness of manure stirring at different developmental stages of the flies. Numbers were generated through preliminary research (results not shown) and are averages of typical larval responses for several generations.

<table>
<thead>
<tr>
<th></th>
<th>Decreased survival</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larvae</td>
<td>less than 0.01g (average mass was often 0.002 to 0.004g)</td>
<td>greater than 0.01g (average mass was often 0.02 to 0.03g)</td>
</tr>
<tr>
<td>Eclosure</td>
<td>less than 1% of eggs survive to become puparia</td>
<td>greater than about 10% of eggs survive to become puparia (can be greater than 30%)</td>
</tr>
</tbody>
</table>
2.2.2.3 Separation of puparia and manure residue

It has been suggested that there is no satisfactory means to separate puparia and manure residue at an industrial scale (El Boushy, 1991). The flotation method (Basden, 1946; Keiding and Arevad, 1964; Teotia and Miller, 1973) and the screening method (Louw, 1964; Schoof, 1964; Beard and Sands, 1973) were often used in rearing protocols to separate puparia from the larval medium. These two methods were qualitatively investigated at a laboratory scale to determine if, and in what situations, they could be used. If neither method was appropriate, puparia were separated by hand using hard forceps.

2.2.3 Fly production

2.2.3.1 Fly origins

*Musca domestica* colonies were established from puparia obtained from cultures maintained by Ecospace Engineering Ltd. at Arkell Research Station. Colonies of *Hydrotaea aenescens* were started from puparia obtained from a commercial supplier (Natural Insect Control, Stevensville, Ontario, Canada). Both *C. hirtula* and *F. canicularis* colonies were established from wild flies that were found in manure from Arkell Research Station. Only about 60-70 larvae of *F. canicularis* were recovered from the manure, which limited the investigation possible for this species. The investigation of this species was performed because opportunity arose. Less time and effort were applied to rearing *F. canicularis* compared to the other species that were investigated. All other colonies were started with at least 1000 flies. Voucher specimens for all species are available in the University of Guelph Insect Collection.

2.2.3.2 Initial protocol for *Musca domestica, Hydrotaea aenescens* and *Fannia canicularis*

Techniques and methods that were originally developed and used for *M. domestica* were used for *H. aenescens* and *F. canicularis*. Other researchers have suggested that the protocols for these species were similar, with only a few minor differences (Schoof, 1964; Hogsette and
Washington, 1995). The original protocol was customized based on each species’ specific requirements as discrepancies were discovered.

**Environmental conditions and colony cages**

The colony room was maintained at 27°C ± 1°C, 60% relative humidity, with an artificially long 16h light: 8h dark photoperiod. Adult flies (*M. domestica, H. aenescens* and *F. canicularis*) were held in rectangular screened plastic (0.08m³, 47cm X 35cm X 47cm) or wooden (0.03m³, 31cm X 33cm X 31cm) cages with a tied-off cloth access sleeve. When flies were no longer useful (because their eggs were no longer viable) they were euthanized by freezing at -18°C for several hours. Cages and all containers within were then cleaned with dish soap, thoroughly rinsed with hot tap water and left to dry overnight.

A minimum space per fly was maintained for *M. domestica* (16cm³) and *H. aenescens* (11cm³) by adding the appropriate number of puparia to cages; lower population densities were used when there were not enough puparia produced in the poultry manure. The population density for *F. canicularis* was not measured because no oviposition took place and only 60 to 70 flies were observed.

**Feeding**

Flies were fed a solid diet of a mixture of granulated sugar, 2% milk powder and yeast at a ratio of 45:45:10, presented *ad libitum* in a plastic container with a surface area of 95cm². Food was stirred weekly to prevent the top layer from becoming unsuitable for flies and was replaced when it became too soiled with dead flies and digestive by-products.

Water was provided *ad libitum* to flies in a container filled with crumpled paper towel. Colonies were observed once a day to ensure that there was an appropriate amount of clean water available.
Addition of eggs to manure

As described by Teotia and Miller (1973), a small groove was created in the surface of the manure and eggs were washed in with a small amount of water to mimic how females deposit eggs in crevices. The eggs were then gently covered by manure to prevent desiccation.

2.2.3.3 Initial protocol for Coproica hirtula

Published protocols were not available for C. hirtula. Initially, the same methods used in protocols for other species were used to develop a protocol for C. hirtula. Cages were not used for C. hirtula colonies (because this species did not leave the manure surface) and flies were held inside a walk-in environmental chamber (25°C ±1°C, 60% RH and 16h L: 8h D) regularly supplied with fresh manure. The population density was not calculated for this species because they were not confined to a cage. The surface of the manure was fully covered by adults, and the walls of the larval container would be almost covered by puparia within a few days; however, it was impractical to physically count them or collect them for population estimation due to their small sizes.

2.2.4 Measurement techniques

Manure and puparia

During larval development the total mass of the manure, larvae and any puparia that were present was measured daily (Sartorius LC 1200 P, 2220g; Bradford, MA, USA). Puparia were removed (through various methods previously explained) from the manure and weighed with a balance (Mettler AE 1000; Mississauga, Ontario, Canada). The number of puparia was calculated by dividing the total pupal mass by the average pupal mass, estimated by weighing 15 individual puparia. To start the next generation of the colony, puparia were collected and added to adult cages. The number and mass of puparia were determined and Pearson’s
correlation coefficient between these two observations was determined for 18 larval trays that were seeded with between 0.1 and 1.0g of eggs.

**Temperature**

The temperature of the substrate (in this case manure) and the temperature of the maggot mass are commonly used by forensic entomologists to estimate larval activity (Haskell et al., 2001). Each site was measured daily (at approximately the same time) with two Barnant 100 thermocouple thermometers (Barrington, IL, USA) and averaged. To ensure accurate readings, temperatures were measured prior to stirring. The maggot mass was manually located and thermocouples were inserted into the center of the mass and into an area not containing larvae. Temperatures were taken from 20 larval trays over the first eight days of manure conversion. Variables such as seeding rates, initial moisture content, etc. were not controlled and only temperatures were statistically compared. Differences between the average maggot mass and manure temperatures were compared with a paired t-test (two-tailed) for each day.

2.3 Results and discussion

2.3.1 Important techniques developed

2.3.1.2 Egg collection devices

Each species responded to egg collection devices differently. No collection device was successful for *F. canicularis* and eggs were never collected from this species. It is possible that the flies needed a larger adult population than was observed (approximately 60 to 70 individuals) or additional steps were required to induce reproduction. Published protocols suggest that soaking cotton pads in protein hydrolysate can induce oviposition on a normally unsuitable substrate, but that chicken manure was the substrate of choice (Calvert et al., 1973; Hogsette, 1992a). No special egg collection devices were suggested in the literature.
The rearing methods used for *C. hirtula* did not require the use of collection devices because adults were able to oviposit directly into the manure the larvae would inhabit. Egg masses could not be weighed because of their small size, large number and absence of communal egg masses. It has been suggested that other members of *Coproica* force eggs under the surface of the manure during oviposition (Hafez, 1939, 1949), which is consistent with the observations made in this study.

Female *M. domestica* often laid eggs on the crumpled paper towel used to supply water to the colony even when a seemingly more suitable substrate was available. If eggs were laid in the water dish it was assumed that the device or the oviposition medium was not attractive to flies and therefore should not be used in the protocol. It had been suggested that ammonium carbonate would draw the flies to a specific spot for oviposition (Spiller, 1966; Barnard et al., 1998); however, no difference was observed in site selection when ammonium carbonate was used and flies continued to lay eggs in their water source despite the presence of ammonium carbonate (results not shown). Therefore, ammonium carbonate was not used as an oviposition stimulant in the final protocol.

Observations and the resulting scores for each version (1-7) for each variable (oviposition medium, container, material and configuration) are shown in Table 6. Grain-based larval media (O:5), milk-based media (O:6) and rehydrated manure residue (O:4) were each tested as an oviposition medium for all species with limited success. Rehydrated poultry residue was the least attractive and failed to deflect females from ovipositing in the water dish. Fresh poultry manure (O:2) and poultry manure solution (O:3) attracted flies to the oviposition device better than the other oviposition media. Using poultry manure may have helped adapt the colony to the conditions in the poultry manure (on which the larvae were reared), and it decreased the colony maintenance because it required no advance preparation. For these reasons, when a solid
Table 6: Observation and scoring (following the observation in parentheses) for the variations 1 - 7 (described in Table 2) of the variables (oviposition medium, container, material and configuration) that make up the oviposition devices when used to collect eggs from *M. domestica*. Oviposition media and containers were scored on a scale from 0 to 4 points based on their relative attractiveness to ovipositing females (based on whether or not females continued to lay eggs in the water dish). Materials and configurations were based on three questions (see Methods section) with a possible total of 6 points each. A high score corresponded to conditions beneficial to egg collection and a configuration likely to be useful in an oviposition device.

<table>
<thead>
<tr>
<th>Variation number</th>
<th>Configuration (Conf)</th>
<th>Oviposition medium (O)</th>
<th>Container (Cont)</th>
<th>Material (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Q1 – yes</td>
<td>eggs laid in water dish</td>
<td>eggs laid in water dish</td>
<td>Q1 – no</td>
</tr>
<tr>
<td></td>
<td>Q2 – no</td>
<td></td>
<td></td>
<td>Q2 – no</td>
</tr>
<tr>
<td></td>
<td>Q3 – yes</td>
<td></td>
<td></td>
<td>Q3 – no</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td></td>
<td>(0)</td>
</tr>
<tr>
<td>2</td>
<td>Q1 – yes</td>
<td>flies were attracted and readily laid eggs</td>
<td>some eggs still laid in water</td>
<td>Q1 – yes</td>
</tr>
<tr>
<td></td>
<td>Q2 – no</td>
<td></td>
<td>dish but attractive enough for</td>
<td>Q2 – depends</td>
</tr>
<tr>
<td></td>
<td>Q3 – depends</td>
<td></td>
<td>egg collection</td>
<td>Q3 – depends</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>3</td>
<td>Q1 – yes</td>
<td>attractive to flies but unless soil surface</td>
<td>some eggs still laid in water</td>
<td>Q1 – yes</td>
</tr>
<tr>
<td></td>
<td>Q2 – yes</td>
<td>is available they will not oviposit</td>
<td>dish but attractive enough for</td>
<td>Q2 – depends</td>
</tr>
<tr>
<td></td>
<td>Q3 – depends</td>
<td></td>
<td>egg collection</td>
<td>Q3 – yes</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>(3)</td>
<td>(5)</td>
</tr>
<tr>
<td>4</td>
<td>Q1 – no</td>
<td>some would oviposit but many laid eggs in</td>
<td>too small to collect more than</td>
<td>Q1 – yes</td>
</tr>
<tr>
<td></td>
<td>Q2 – depends</td>
<td>water dish</td>
<td>about 1g of eggs and many eggs</td>
<td>Q2 – no</td>
</tr>
<tr>
<td></td>
<td>Q3 – yes</td>
<td></td>
<td>laid in water dish</td>
<td>Q3 – no</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
<td>(1)</td>
<td>(4)</td>
</tr>
<tr>
<td>5</td>
<td>Q1 – yes</td>
<td>attractive but preparation is needed and</td>
<td>some eggs still laid in water</td>
<td>Q1 – no</td>
</tr>
<tr>
<td></td>
<td>Q2 – yes</td>
<td>flies are not exposed to manure</td>
<td>dish but attractive enough for</td>
<td>Q2 – no</td>
</tr>
<tr>
<td></td>
<td>Q3 – yes</td>
<td></td>
<td>egg collection</td>
<td>Q3 – no</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td></td>
<td>(3)</td>
<td>(0)</td>
</tr>
<tr>
<td>6</td>
<td>Q1 – yes</td>
<td>attractive but preparation is needed and</td>
<td>not as attractive as shallower</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q2 – depends</td>
<td>flies are not exposed to manure</td>
<td>containers and eggs laid in</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q3 – yes</td>
<td></td>
<td>water dish</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td></td>
<td>(2)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Q1 – yes</td>
<td>complicated set up but attractive</td>
<td>some eggs never touch material</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q2 – yes</td>
<td>and eggs never touch material</td>
<td>(4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q3 – no</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
oviposition medium was needed fresh poultry manure (O:2) was used, and when a liquid medium was required the poultry manure solution (O:3) was used in the *M. domestica* rearing protocol.

The type of container did not affect the attractiveness of the oviposition device; however, small containers, such as container 4, were not large enough to collect enough eggs for quantitative testing on larvae. When container 4 was used, the oviposition device yielded no more than 1g of eggs. The flies seemed to perform better in shallower containers; a greater proportion of flies oviposited in their water dish when container 6 was presented than when containers 3, 4, or 5 were available. Container 7 allowed for the easiest collection as the eggs were never in contact with the manure; however, it was also the most complicated to set up. It was also somewhat less attractive to flies, especially when a wick was not used, and flies continued to lay eggs in their water dish. It was given the highest score because it allowed for the easiest and most accurate egg collection. The use of the container 7, however, would require a larger colony (relative to the amount of eggs desired) to ensure enough eggs could be collected despite the loss of eggs in the water dish.

The material used in the oviposition device did affect the cleanliness and collectability of the eggs as well as the attractiveness of the device. Using any material seemed to decrease the attractiveness of the device relative to uncovered manure (Conf:1) because eggs were often laid in the water dish when a material was used. Eggs were never deposited on sponge (M:5). Females deposited eggs in communal masses on paper towel (M:2), black cotton (M:3) and white muslin (M:4); however, the cleanliness of the eggs was affected by the material used. The muslin was too loosely woven and eggs were often deposited through the fabric and were soiled with manure. This also occurred, but to a lesser extent, with the paper towel and black cotton. The cotton, which seemed to be the most attractive material, also shielded most of the eggs from the manure, allowing a greater number of eggs to be collected and measured.
The configuration of the oviposition device did appear to affect its attractiveness to flies. All configurations allowed eggs to be collected in one communal mass except configuration 4. The folds that occurred from only loosely wrapping the material provided flies with many surfaces on which to deposit eggs and females seemed to oviposit away from other females, which made collection more difficult, especially when eggs were soiled with manure. More comparisons between similar configurations are described in the following discussions of the most successful devices.

The three most successful devices for *M. domestica* and the conditions in which they were most appropriate for use were:

**Device one (Conf:1; O:2; Cont:2, 3, 5; or 6 M:1; = 10 to 11 points)**

A ball of fresh material (manure) was placed in a plastic container; the type of container did not make a noticeable difference. This simple design worked well for stimulating egg-laying and females would oviposit in a communal mass; however, the egg mass was heavily soiled by the manure. It was often not possible to weigh eggs accurately because not all of the manure could be removed from the egg mass. If eggs did not need to be weighed accurately, for example when eggs were only needed for starting the next generation of the colony (not for experimentation), this method was used.

**Device two (Conf:3, 4 or 7; O:2; Cont:2, 3, 5 or 6; M:2, 3 or 4; = 14 to 16 points)**

A ball of fresh manure wrapped in moistened paper towel or dark cotton cloth was placed in a plastic container. Flies were attracted to the device for oviposition and the eggs were washed or gently scraped from the surface without being soiled by the manure. Difficulties arose because flies often laid their eggs through the material and many eggs were soiled with manure. Fewer eggs were soiled when the black cotton cloth wrapped the oviposition medium. The covered configuration (Conf:7) could also be used but it did not seem to affect the oviposition of *M.*
domestica. This device was used only when few eggs were needed; eggs that were soiled with manure were not used for experiments because they could not be weighed accurately.

**Device three (Conf:5; O:2; Cont:7; M:2; = 18 points)**

A mixture of fresh manure and water was placed inside a sealable plastic container. Small holes were cut in the lid of the container and a wick of moistened material was positioned through one of the holes. Manure would attract flies to the device but the holes were too small for flies to fit through. The paper towel connected the manure to the outside of the container where flies would deposit their eggs. This device prevented the eggs from being soiled by the substrate and allowed for the easiest collection; however, this device was not as attractive to flies and, although some flies used the device, many still continued to lay eggs in their water source. This method of collection was used to collect eggs for the response surface experiment in Chapter 4. Configuration 7 was very similar to this device; the only difference was that configuration 7 did not use a material wick and relied on the oviposition medium in the container to attract the flies. Configuration 7 is similar to the devise Shipp and Osborn (1965) used, but is not as useful as configuration 5 because it is not as attractive to flies.

**Modified Hogsette and Washington (1995) device (Conf:6; O:2; Cont:3; M:2 or 3; = 16 - 17 for M. domestica; not scored for H. aenescens)**

It was quickly observed that the devices that were used for M. domestica could not be used for H. aenescens, which were very selective about where they would oviposit. Oviposition only occurred in configuration 6, which was recreated from a device designed by Hogsette and Washington (1995) (Figure 3). Instead of grain-based larval medium, a ball of fresh manure was wrapped in black cotton or paper towel and placed inside plastic dishes painted black for the flies to oviposit on. Another black plastic dish was then overturned and covered the first, as described by Hogsette and Washington (1995).
Figure 3: Egg collection device (Conf:5; O:2; Cont:3; M:2) for H. aenescens. Collection device modeled after a device designed by Hogsette and Washington (1995).

### 2.3.1.3 Timing of stirring

It was determined that daily stirring should begin when larvae were in their second instar (Table 7). Stirring the manure too soon (when eggs or 1\textsuperscript{st} instar larvae were present) resulted in low larval mass and eclosion because it broke up the larval or egg mass, which negatively affected development and, in turn, manure conversion. When the egg mass and initial maggot mass were left intact in the manure, it appeared to promote larval development. It has been observed in calliphorids that hatching rate and larval survival are positively correlated to maggot mass size and that incubation time is negatively correlated to maggot mass size, but the mechanisms behind these effects are unknown (Turner and Howard, 1992; Slone and Gruner, 2007). When second and third instar larvae made up the majority of the population (day three for \textit{M. domestica} and day five for \textit{H. aenescens}), the manure was stirred daily to ensure that all areas were inhabited by larvae. At this stage larvae still spent some of their time in a mass, but began
to actively travel through the manure, which naturally broke up the larval mass; because the larvae already spent time away from the maggot mass, stirring had no obvious negative effects.

Manure that had a moisture content greater than about 60% formed a crust on its surface when it was not stirred daily. Larvae were not able to convert the crust, which likely decreased the food availability and reduced the number of larvae that survived. The hard, dry crust remained if the manure was stirred after the crust formed, and was never converted, despite conversion having occurred in other areas of the larval tray. Stirring the manure once a day after the larvae had reached the second instar did not negatively affect larval survival and prevented the formation of this crust. As larvae reached the third instar, they actively moved through the manure and no negative effects were seen when manure was stirred daily or multiple times per day. No negative effects were observed when the manure was stirred less than once per day as long as the moisture content of the manure was less than about 60%. When manure was not stirred appropriately, the finished product resembled the manure residue obtained when insufficient eggs were used to seed manure.

Table 7: Observations on the survival of *M. domestica* larvae (defined in Table 5) when the manure they inhabited was stirred at different stages of larval development.

<table>
<thead>
<tr>
<th></th>
<th>Stirred less than once a day</th>
<th>Stirred once a day</th>
<th>Stirred more than once a day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Egg</strong></td>
<td>decreased if stirred at all</td>
<td>decreased</td>
<td>decreased</td>
</tr>
<tr>
<td><strong>1st instar</strong></td>
<td>decreased if stirred at all</td>
<td>decreased</td>
<td>decreased</td>
</tr>
<tr>
<td><strong>2nd instar</strong></td>
<td>decreased (surface of manure formed a crust that larvae would not convert)</td>
<td>normal</td>
<td>decreased</td>
</tr>
<tr>
<td><strong>3rd instar</strong></td>
<td>normal (if manure had a low enough moisture content to prevent the surface from crusting over (about 60%))</td>
<td>normal</td>
<td>normal</td>
</tr>
</tbody>
</table>
2.3.1.1 Separation of puparia from manure residue

Different methods were used to separate manure residue from puparia depending on the amount of manure and the extent of conversion. Other methods are described in the literature but were not appropriate for use here, as additional equipment and a more complicated set-up would have been required (Calvert et al., 1973; Beard and Sands, 1973). If industrial conversion via flies is to take place, a separation method to quickly remove large numbers of puparia needs to be developed because the flotation and screening methods were not appropriate in all circumstances. A description of the advantages and disadvantages for each method is provided below. Separation of puparia and manure residue is one of the major obstacles in developing a functional industrial-scale poultry manure conversion system. El Boushy (1991) stated that separation of the two products cannot be efficiently done at an industrial-scale. He therefore focused on creating poultry feed that contains both the manure residue and puparia.

Removal by screening

The screening method has been used in laboratory protocols where sand or sawdust was added to the larval medium to encourage pupation (Louw, 1964; Schoof, 1964). The sand or sawdust is easily sifted away from the puparia because the particles are much smaller than the puparia. When larvae were reared in manure, puparia were considerably larger than the particles of the dry, converted manure residue and a screen of 2 to 3mm was sufficient for separation; however, this method could only be used when the manure was completely converted (see Chapter 3 for definition). Unconverted manure was moist and formed sticky particles that were of equal size or larger than puparia and was not easily sifted out. Large chunks of unconverted manure often contained puparia and required a different method of separation. Basden (1947) advised against sifting puparia from the larval medium as it caused injury to developing flies and resulted in damaged, unusable wings. Flightless flies may be a benefit to an industrial-scale system because the possibility of flies escaping would be
decrease; however, no damage to wings was observed when puparia were sifted from the manure residue in this study.

**Flotation method**

Puparia floated to the surface when submerged in water, while manure residue settled to the bottom. This was the fastest and easiest separation method for manure that had not completed conversion and still had high moisture content; however, flotation rehydrated the manure residue and changed its physical properties. In an industrial conversion system, manure that was separated from puparia using the flotation method would have to undergo additional processing to remove the water again. This method was used when a large number of puparia had to be separated from the manure quickly and the manure residue was not needed for evaluation.

**Removal by hand**

Puparia that were removed for observation before conversion was complete, or when few puparia were present, were picked out using forceps. This method was also used in manure that was too wet to be screened and when the flotation method was not appropriate (i.e. rewetting was unwanted). Removal by hand was time-consuming, as even at the laboratory scale there were often several thousand puparia present. This method also often injured puparia; therefore, this method was only appropriate when other methods were not feasible.

**2.3.2 Important measurements and observations**

**2.3.2.1 Temperature**

The maggot mass was warmer than the surrounding manure, which indicated that the larvae increased the temperature of the manure during conversion (Figure 4). Average temperatures of the maggot mass were higher than the temperature of the surrounding manure during the first
seven days of conversion when larvae were in their first or second instar. On average, the maggot mass temperature was 0.63°C higher than the manure temperature. There was no difference between the two temperature readings by the eighth day when most larvae had reached their third instar. After the eighth day of conversion, many larvae had pupated and the third instar larvae that remained no longer formed a discrete maggot mass, therefore temperature difference could not be measured. Remaining larvae continued to develop in the manure until pupation, but the day that pupation occurred varied based on other environmental conditions not measured.

Figure 4: Manure and maggot mass temperatures (°C) over eight days of conversion using *M. domestica*. The temperatures of the maggot mass and unoccupied manure were measured for 20 larvae trays using one kilogram of manure and seeded with between 0.1 and 1.0 grams of eggs. Environmental conditions were consistent with the suggested protocol. Means followed by different letters are significantly different P<0.05. Data are mean ± standard deviation (n =20).
2.3.2.3 Larvae

Larval appearance and behaviour were used to estimate when pupation would occur and to assess larval survival in poultry manure. Healthy larvae that developed according to the expected timeline were cream in colour, plump and active. Unhealthy larvae were less active, discoloured, smaller than expected for their age and often failed to pupate. When pupation did occur adults often did not emerge. The most likely cause of unhealthy larvae appeared to be either overpopulated manure or unfavourable moisture content. Healthy *H. aenescens* larvae were often seen on the surface of the manure in the second and third instar; healthy *M. domestica* larvae were not seen unless the manure was stirred. The larvae of *M. domestica* were only seen on the surface when they began to search out another food source and attempted to leave the larval tray; however, after this was observed, care was taken to select larval rearing containers that would prevent larval escape.

Larval behaviour and developmental stage of *M. domestica* and *H. aenescens* were used to estimate when the larvae would stop feeding and therefore, the end of conversion. First instar larvae were very small, easily recognizable and concentrated in a dense maggot mass close to where eggs were deposited. Second instars were larger with easily visible mouthparts, and remained in the maggot mass for the majority of time, which agreed with descriptions by Axtell (1986). As development continued, larvae migrated from the maggot mass and moved through the manure. This behaviour has also been observed with other fly species and may be related to thermoregulation (Schoof, 1964). Third instar larvae had a dark center (digestive tract filled with manure) surrounded by cream-coloured tissue and moved through the manure, while the remainder of the maggot mass settled to the bottom of the larval tray. Before pupation, larvae ceased feeding, lost their dark center and searched for a drier place to pupate (such as dry clumps in the manure), which also agreed with Axtell (1986).
2.3.2.4 Puparia

Puparia were recovered from manure as long as there was sufficient moisture content (approximately 60%) throughout development. When moisture content dropped excessively early in development (below approximately 60%) larvae died in the first or second instar and did not pupate. Larvae remained in their first two instars for a prolonged period if the manure was not moist and death occurred between day 2 and day 6. The average pupal mass, a measure of fly size, and number of puparia collected were negatively correlated (Figure 5). Pearson’s correlation coefficient for the 18 larval trays with an average mass greater than 10mg was -0.70 (p≤0.05). The two larval trays (with puparia below 10mg) that were removed from the analysis did not complete conversion and most larvae died early in development. Other researchers have noticed the same correlation between the number and mass of puparia (Slone and Gruner, 2007). The relationship between size and number of puparia will have to be considered when the system is increased to industrial-scale. Insect protein production could add considerable revenue to a farm, but it is unknown whether larval size or number is more important for the transformation into a food source for livestock. The relationship between the number of puparia and the level of manure conversion will also have to be considered.

2.3.3 Species-specific observations

2.3.3.1 *Musca domestica*

Flies started to lay eggs about four days after emergence and continued until they were euthanized (about 3 weeks after emergence). Eggs were collected every two to three days after egg production began. About 20 days after emergence, egg fertility varied and they were no longer be used for experiments. An unstabilized colony was not able to produce the several grams of eggs required for experiments, because eggs were not laid in a short enough timeframe. In a stabilized colony, flies simultaneously produced eggs at expected intervals and allowed egg collection to take place in the shortest time frame possible; ideally in less than six
hours. When more than twelve hours were required for egg production, the earliest laid eggs hatched and these larvae immediately began conversion when added to manure, potentially affecting results. When larvae were present in the egg mass, care was taken not to add them to larval trays used for experiments. Separating eggs and first instar larvae was a laborious practice that would not be acceptable for an industrial system.

Figure 5: Mass of *M. domestica* puparia (mg) by total number of larvae collected from 20 larval trays at the end of conversion. From each tray 15 puparia were randomly selected after all larvae had pupated and individually measured. Manure was seeded with between 0.1 and 1.0g of eggs and environmental conditions followed the protocol. ( ) were removed from analysis.

Ensuring that flies had adequate food and fresh water was a particular concern during fly production. In smaller colonies (of a few hundred flies) food was left for the duration of the generation without issue, but food needed to be replaced about every two weeks for larger
colonies (greater than 1000 flies). When the ingredients of the dry food were presented separately, they fused into hard clumps in a matter of days and flies did not feed. This problem was avoided by thoroughly mixing all components of the food together. Water was replaced every three days for most colonies and once a day in colonies with higher populations, as well as directly following emergence. The same time frame was also used for *H. aenescens*. Other researchers have suggested that water can be left for one week or longer in colonies of about 600 flies (Keiding and Arevad, 1964); however, in the work presented in this thesis, water either evaporated or became too soiled to be used by flies after a few days.

When 2.5% sugar water was presented to flies no observable difference in oviposition was seen. Sugar water was not investigated any further. Tap water was equally acceptable and did not become as readily soiled; therefore it was used in the protocol because it decreased the maintenance required for the colony.

Rearing various sized colonies over several generations revealed that larger colonies were more difficult to maintain and required more daily maintenance than smaller colonies. In an industrial-scale production system it may be easier to maintain several smaller colonies over one very large colony. The ideal colony size will need to be determined.

**2.3.3.2 *Hydrotaea aenescens***

The same adult conditions and many of the techniques developed for rearing *M. domestica* were successfully used for *H. aenescens* colonies. There were a few key differences between rearing protocols that allowed for greater survival and fecundity of this species. The main differences between these species are related to egg production, as *H. aenescens* required special conditions.

Egg production began about four days after *H. aenescens* emerged from puparia. Eggs that were to be used for experiments (Chapter 4) were collected between five and ten days after fly
emergence; eggs produced after seventeen days were used to maintain a large colony. Specific conditions are required in order for *H. aenescens* to produce eggs. Swarming behavior, necessary for mating in this species, was encouraged by providing dark areas in the colony cage. Flies often clustered in the dark corners or in the shadows of the various food and water containers. This behaviour was also seen by Hogsette and Washington (1995) and Nolan and Kissam (1987).

In nature, *H. aenescens* occurs at high densities and was therefore reared at higher population densities than *M. domestica*. Colonies of over 5000 individuals provided adequate eggs for small experiments (about 1g of eggs within six hours) with minimal maintenance. Larger colonies may be more desirable; however, at a laboratory scale, poultry manure did not support the development of enough puparia for this to be investigated. Smaller colonies of less than 1000 flies resulted in fewer eggs per female being produced (no controlled experiments completed), which contributed to further reduction in the size of the colony. Hogsette and Washington (1995) also observed limited egg production with *H. aenescens*, and claimed that 5 eggs per female in the colony were adequate for their needs; however, this is much less than the several hundred eggs per female produced by *M. domestica*. At an industrial scale, colonies of *H. aenescens* would have to be several times larger than a *M. domestica* colony to produce the same number of eggs.

This species required more attention and maintenance than *M. domestica* to ensure enough adult flies were available to continue the colony. Starting with a larger colony may reduce the risk of colony collapse, which will likely occur in smaller populations; however, this may always be a risk at an industrial-scale regardless of the starting colony size.
2.3.3.3 *Coproica hirtula*

The rearing methods developed for *C. hirtula* were very different than the protocols used for the previous two species. The short lifecycle of this species made separation of life stages difficult, if not impossible, but a very large population was built up in less than a week. The same environmental conditions were used for both adults and larvae, and they were reared together in the same environmental chamber. Manure was used as the only adult food source to supply the flies with both nutrition and water. All life stages of this species were able to survive in manure that was between 50% to 85% moisture, which is a wider range than what was acceptable for the other species investigated.

The adults seldom traveled more than a few centimeters from the manure so containment was not necessary for *C. hirtula*. Adults flew freely in the environmental chamber and larval populations were maintained by allowing adults to continuously emerge and oviposit in the manure. This species could not be stabilized because the life stages could not be separated and all flies invested were from an unstabilized colony. Eggs were laid one day post adult emergence and the entire life cycle was completed in about six days.

Compared to the other species tested, *C. hirtula* colonies required the least amount of maintenance because only the moisture content of the manure had to be monitored and maintained. Adults and larvae had the same environmental requirements and very little regular maintenance was necessary. An unexpected observation was made when a colony was left undisturbed in a large sealed plastic container for about five weeks. When the container was opened, approximately the same number of *C. hirtula* that was present before sealing the container remained, and individuals were healthy and continued reproducing. The population of flies could not be quantified because of the very large number present.
Fresh manure collected from Arkell Research Station often contained *C. hirtula*, which suggested that flies were present within the poultry facility and colonized the manure within a few hours after it was deposited. Despite being present in large numbers they were not pests and there were no complaints from farm workers. In the laboratory these flies were not noticed until they emerged as adults, but closer inspection revealed the presence of the very small larvae and puparia in manure samples.

**2.3.3.4 Fannia canicularis**

Larvae were discovered in poultry manure when they were at least a few days old and within five days they had all pupated. Emergence started nine days after pupation and 50 to 60 adults emerged over four days. This timeframe is slightly longer than that of the other species being investigated (*M. domestica* emerge from puparia after 5 days at the same temperature) and is supported by other research (Monroe, 1960; Lachance et al., 1977; Axtell, 1986). Only one generation of *F. canicularis* was studied. Flies never oviposited even after several attempts with various egg collection devices that were successfully used for other species.

**2.4 Conclusions**

*Musca domestica* and *Hydrotaea aenescens* both showed potential for mass production and use in an industrial poultry manure conversion system. Protocols and timelines were developed for the maintenance of all life stages and practices were identified to allow for close monitoring of the colonies. Techniques for measuring temperature and collecting eggs were developed, and important trends were identified for further research.

Mass-production of *M. domestica* was easier than of *H. aenescens*, because *M. domestica* has a shorter lifespan and requires less maintenance to produce eggs. Increasing the size of the *H. aenescens* colony may increase the efficiency of egg production for this species. Conversely, it is easier to maintain smaller colonies of *M. domestica* than larger colonies, which may be an
issue when mass-producing these flies at an industrial scale. Developed protocols used simple techniques that can be applied to a much larger production system. Both species will be further investigated in the next chapter because preliminary work suggests that they are easily stabilized, and mass rearing and regular production of eggs may be possible at an industrial-scale.

Mass production was also possible with *C. hirtula*; however, its size and short life cycle made the separation of life stages nearly impossible, which may be required if this species is used in a poultry manure conversion system. Juveniles and adults shared the same environmental requirements and made this species the easiest to mass-produce in the laboratory; however, there was no evidence that *C. hirtula* colonies could be stabilized. Despite its limitations, the species did show enough promise for use in a poultry manure conversion system to justify further study. If this species is to be used in an industrial system, measurement and management techniques will have to be developed; specifically, a method to collect and quantify eggs is needed. Currently, environmental factors cannot be properly investigated because of the limitations imposed by this species, such as their small size, short life cycle, and un stabilized colonies. It is possible that this fly could also be used in conjunction with *M. domestica* as the two species were able to co-exist in manure. The simultaneously use of two species is further explored in Chapter 4.

It may be possible to use *F. canicularis* to convert poultry manure; however, it has many of the same undesirable qualities as *M. domestica* (both are pests and potential disease vectors). The biology of *F. canicularis* may also make it less effective than *M. domestica* in a conversion system. Most notably, *F. canicularis* live in sparser populations, reproduce at a slower rate and are smaller than *M. domestica*, which may make them less efficient to mass produce and less suitable as a protein feed for livestock (Anderson and Poorbaugh, 1964; Axtell, 1986).

Preliminary work in this thesis suggested that the larvae of *F. canicularis* are able to survive in
poultry manure, but because it was not possible to mass-produce *F. canicularis* in the laboratory this species was not investigated further.

### 2.4.1 Protocols and timelines for mass production

Timelines, presented below, were constructed for laboratory colonies of *M. domestica* and *H. aenescens*. Maintenance schedules based on similar timelines could be used by farm employees to simplify fly rearing. These timelines are designed to work at a laboratory-scale to produce eggs for experiments and would have to be re-evaluated and adapted to produce eggs for an industrial conversion system. The protocol used for *C. hirtula* is briefly described but a timeline was not constructed.

#### 2.4.1.1 Protocol and timeline for *Musca domestica* colony

For the timeline to be accurate the temperature should be 27°C, relative humidity should be 70%, and the moisture content of the poultry manure should be about 75%.

**Day 0** – Determine the moisture content of the manure (as described earlier in this chapter).
- Add eggs to fresh poultry manure.

**Day 1 and 2** – Larvae begin to hatch and convert the substrate.
- Record the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion each day (as described earlier in this chapter).
- Do not stir the manure.

**Day 3** – Most larvae should be second instar.
- Record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion (as described earlier in this chapter)
- Stir manure to ensure it will be converted.

**Day 4** - Most larvae should be second instar, some will be third instar.
- Record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion (as described earlier in this chapter)
- Stir manure to ensure it will be converted.

**Day 5** - Most larvae should be third instar, some will still be second instar.
- Record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion (as described earlier in this chapter)
- Stir manure to ensure it will be converted.

**Day 6 and 7** – Third instar larvae begin to migrate to drier areas and begin pupation. Manure should be completely converted if egg-to-manure ratio is correct.
- Record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion each day (as described earlier in this chapter)
- Continue to stir manure daily if it is not converted.

**Day 8 and 9** – Most larvae should have pupated.
- If manure is not converted, record (before stirring) the manure mass, manure temperature, manure depth, maggot mass temperature, and describe the larvae and ongoing conversion (as described earlier in this chapter) and stir manure each day. Any puparia collected will emerge in about 5 days and the timeline should be adjusted accordingly.
- Separate puparia (by screening if manure is converted), weigh and find the average mass and number of puparia (as described earlier in this chapter).
- Add puparia to cages, ensuring no less than 16cm$^3$ of space per puparium

**Day 10** – Puparia should begin to emerge. Adults feed on water and dry food (granulated sugar, 2% milk powder and yeast at a ratio of 45:45:10) *ad libitum*.

**Day 11** – Most adults have emerged.
- Replace water.

**Day 12 to 14** – Mating will occur during this time.
- Stir food when needed.
- Replace water daily.

**Day 15** – All adults should have emerged. Egg production should begin.
- Place the most appropriate oviposition device in cage (as described earlier in this chapter).
- Remove the oviposition device after six hours (to ensure eggs are of approximately the same age) and determine the mass of eggs. Gently remove eggs with a spatula and separate them from the manure used in the oviposition device. Eggs can then be added to fresh manure at an appropriate rate.
- Stir food when needed.
- Replace water.

**Day 16 +** – Collect eggs every two to three days. Place the most appropriate oviposition device in cage (described earlier in this chapter).
- Remove the oviposition device after six hours (to ensure eggs are of approximately the same age) and determine the mass of eggs. Gently remove eggs with a spatula and separate them from the manure used in the oviposition device. Eggs can then be added to fresh manure at an appropriate rate.
- Stir food when needed (about once per week).
- Replace water daily.

**Day 30** – Euthanize flies by freezing (viability of eggs is reduced at this point).

### 2.4.1.2 Protocol and timeline for *Hydrotaea aenescens* colony

For the timeline to be accurate the temperature should be 27°C, relative humidity should be 70%, and the moisture content of the poultry manure should be 75%.

**Day 0** – Determine the moisture content of the manure (as described earlier in this chapter).
- Add eggs to fresh poultry manure.

**Day 1 to 4** – Larvae begin to hatch and convert the substrate.
- Record the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion each day (as described earlier in this chapter).
- Do not stir manure

**Day 5 and 6** – Most larvae should be second instar.
- Record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion each day (as described earlier in this chapter).
- Stir manure daily to ensure it will be converted.

**Day 7 and 8** - Most larvae should be second instar, some will be third instar.
- Record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion each day (as described earlier in this chapter).
- Stir manure daily to ensure it will be converted.

**Day 9** - Most larvae should be third instar, some will still be second instar.
- Record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion (as described earlier in this chapter).
- Stir manure to ensure it will be converted.

**Day 10 and 11** – Third instar larvae begin to migrate to drier areas and begin pupation. Manure should be completely converted if egg-to-manure ratio is correct.
- Record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion each day (as described earlier in this chapter).
- Continue to stir manure daily if it is not converted.

**Day 12 to 14**—Most larvae should have pupated.

- Separate puparia (by screening if manure is converted), weigh, and find the average mass and number of puparia (as described earlier in this chapter).
- Add puparia to adult habitat, ensuring no less than 11cm³ of space per puparum.
- If manure is not converted, continue to record (before stirring) the manure mass, manure temperature, manure depth and maggot mass temperature and describe the larvae and ongoing conversion (as described earlier in this chapter) and stir manure each day. Any puparia collected will emerge in about 6 days and the timeline should be adjusted accordingly.

**Day 16**—Puparia should begin to emerge. Adults feed on water and dry food (granulated sugar, 2% milk powder and yeast at a ratio of 45:45:10) *ad libitum*.

**Day 17**—Most adults have emerged.

- Replace water.

**Day 18 to 20**—Mating will occur during this time.

- Stir food when needed.
- Replace water daily.

**Day 21 and 22**—All adults should have emerged. Egg production should begin.

- Place the covered oviposition device (Hogsette and Washington, 1995) in cage (as described earlier in this chapter).
- Remove the oviposition device after twelve hours (to ensure eggs are of approximately the same age) and determine the mass of eggs. Gently remove eggs with a spatula and separate them from the manure used in the oviposition device. Eggs can then be added to fresh manure at an appropriate rate.
- Stir food when needed.
- Replace water daily.

**Day 23 +**—Collect eggs every two to three days. Place the covered oviposition device (Hogsette and Washington, 1995) in cage (as described earlier in this chapter).

- Remove the oviposition device after twelve hours (to ensure eggs are of approximately the same age) and determine the mass of eggs. Gently remove eggs with a spatula and separate them from the manure used in the oviposition device. Eggs can then be added to fresh manure at an appropriate rate.
- Stir food when needed (about once a week).
- Replace water daily.

**Day 40**—Euthanize flies (viability of eggs is reduced at this point).
2.4.1.3 Protocol for *Coproica hirtula*

All life stages of *C. hirtula* were maintained at 25°C ± 1°C, 60% RH with automatic lighting (16h L: 8h D) in a walk-in environmental chamber. Adults flew freely in the chamber, but never flew more than a few centimeters from the manure. Fresh manure (moisture content about 75%) presented to flies in uncovered plastic storage containers provided moisture and nutrition for all life stages. Adult flies quickly (several minutes to an hour) colonized the manure and completely covered its surface. It was assumed that mating and oviposition occurred at this time, when adults were about one or two days old. When manure was closely inspected, larvae were found on the day after colonization and on every day after. Puparia could be seen soon after. Larvae often crawled up the wall of the container and would pupate outside of the manure.

The moisture content of the manure did not need to be maintained. Manure of approximately 85% to 55% moisture was able to sustain a very large population and adult flies completely covered the manure surface. If moisture levels fell below 55%, or a thick crust started to form on the surface of the manure, the population of *C. hirtula* would decrease. The population could be quickly increased when water was added to the manure or the manure was thoroughly stirred. The manure did not need to be stirred daily.

The life stages of *C. hirtula* could not be efficiently separated. Their small size and large number made removing flies by hand too time-consuming and many would emerge or pupate as they were removed. Although a large number of these flies can be produced very quickly with minimal maintenance there is not, as of yet, a method to quantify the production.
Chapter 3: Investigations of the behaviour and conversion ability of saprophagous larvae reared in the laboratory (M. domestica, H. aenescens and C. hirtula) and the identification of important environmental factors affecting manure conversion

3.1 Introduction

It is necessary to understand the larval behaviour of each candidate species that could potentially be used in a poultry manure conversion system. The ability of the larvae to efficiently and completely convert fresh poultry manure at laboratory scale is a critical screening criterion for selecting the most appropriate species for an industrial-scale system. Laboratory-scale investigations allowed species to be compared in controlled environments to assess the larval responses to various environmental factors and the ability of the larvae to completely convert manure before pupation.

It is possible to mass-produce M. domestica, H. aenescens and C. hirtula in the laboratory with the protocols and techniques developed in the previous chapter. Other aspects of each species such as their biology and the maintenance necessary for mass production need to be considered when selecting the most appropriate species for an industrial poultry manure conversion system. All species investigated in this chapter are found in poultry manure; however, no studies have directly compared the larval development of various species in poultry manure, and only M. domestica has been used to convert poultry manure (Calvert et al, 1969; Barnard et al., 1998; El Boushy and van der Poel, 2000).

Most information required to design a properly controlled experiment is unavailable; environmental factors that affect conversion are unknown and no quantitative methods are available to assess manure conversion. Barnard et al. (1998) defined degraded manure as “the medium remaining after 28 days of larval feeding activity (at 26°C and 60%RH) when a starting density of 600 first instars [M. domestica larvae] per 100g of manure was used.” This definition is not appropriate because it focuses on the process of conversion, instead of the conditions of
the final manure residue. There have been no other published definitions of converted or
degraded manure. This chapter focuses on exploratory research to identify important aspects
affecting conversion (environmental factors), methods to control variables and a general
definition of converted manure (based on the properties of the remaining residue) that will aid in
future studies on fly-mediated manure conversion.

The objectives of this chapter were 1) to define and characterize the properties of larvae-
converted manure to; 2) to determine the important environmental factors that influence manure
conversion; and 3) directly compare the efficiency of each species when processing chicken
manure at a laboratory scale.

3.2 Methods

3.2.1 Fly colonies
Colonies of *M. domestica*, *H. aenesens* and *C. hirtula* were mass-produced in the laboratory
following the protocols developed in Chapter 2. Each species was maintained separately for at
least five generations before experiments commenced. The colonies were not completely
stabilized because of the short time frame of the project. The process of initially stabilizing a
colony decreases the population size as many flies are excluded from breeding. Since a large
number of eggs were required for experiments, limiting the adult population was avoided when
possible. Larvae that pupated either very early or late were often not included in cultures, but
puparia collected over several days were used to establish the adult population for all species,
which may have slowed colony stabilization.

3.2.2 Assessment of manure conversion
Fresh manure was collected and treated as described in Chapter 2. In order to compare the
effects of environmental factors and the ability of each species to process manure, a consistent
method to assess the conversion of the manure was developed. Quantitative and qualitative
characteristics of the manure residue and larvae were assessed as *M. domestica* larvae inhabited and converted the manure. Stages based on the ranges, averages and descriptions of these characteristics were defined. These same stages were also used to define manure conversion when *H. aenescens* and *C. hirtula* larvae were present. Completely converted manure needed to be fine in texture, homogeneous and dry, ensuring that puparia were easily removed. Conversion of the manure ended when all larvae either died or pupated, whether or not manure was completely converted.

### 3.2.3 Daily observations

All larval colonies were observed daily to determine any changes in the larvae or manure substrate. The following parameters were assessed and recorded daily:

- Manure mass;
- Manure temperature;
- Manure depth;
- Stage of conversion and description of the manure;
- Larval behaviour (maggot mass);
- Larval stage (instar);
- If possible, the number and the mass of puparia present; and
- Any changes to the protocol that had to be made to maintain conversion or larval development.

Measurement techniques were performed as they were described in protocols in the previous chapter.

During manure conversion, when the conditions allowed, the opportunity was taken to observe changes to the system when extra manure, water or eggs were added to the larval tray during the conversion process. Extra manure was added when complete conversion occurred before
pupation. Water was added when manure became too dry and dead larvae (first or second instar) were seen. Additional eggs were added when larvae pupated before conversion was completed. Additions of manure, water or eggs were made in an attempt to synchronize the occurrence of pupation and complete conversion and to ensure that puparia would be available to continue the next generation. These opportunistic investigations were not replicated, controlled or statistically evaluated; however, the preliminary information gathered was used to generate hypotheses for future investigations.

3.2.4 Identification of environmental factors and species selection

Larvae of each species were observed in poultry manure over several generations. Environmental attributes of the manure (initial moisture content and depth) and larval density were altered at various levels within the appropriate ranges for each species and the conversion process was observed. Environmental factors that caused variation in larval period, which in turn negatively impacted the conversion process (i.e. manure was not completely converted), were identified as the most important. Changes were not quantitatively evaluated because of variability within the fly colonies and the manure. The purpose of this chapter was to determine which environmental factors should be further investigated. The specific effects of these factors are further discussed in Chapter 4.

Each species shares many characteristics with the “ideal species” described in Chapter 1. Selection of the most promising species took all of these factors into consideration. The species selected must:

- Have an easy-to-follow mass rearing protocol with techniques that can be used at an industrial-scale;
- Efficiently and completely convert poultry manure at a laboratory-scale and pupate in the same timeframe; and
3.3 Results and discussion

3.3.1 Manure conditions – stages of conversion

In order to compare the manure conversion ability of the larvae, various stages of conversion had to be defined. Stages of manure conversion were defined based on larval behaviour and properties of the manure residue. Ranges and averages for each quantifiable characteristic, and descriptions for qualitative characteristics, for each conversion stage are presented in Table 8. Monitoring the transitions through the stages was a convenient way to compare environmental factors and the conversion ability of each species. A single characteristic could not be used to identify the stage of conversion that manure had achieved because there was too much variation within each stage and overlap between stages. To assess the stage of conversion, all aspects had to be considered including the behaviour of the larvae and other qualitative features. The following describes the stages of the conversion that were used in this thesis and examples are presented in Figure 6.

**Fresh manure**

Fresh manure contained around 75% moisture, had a “mud-like” consistency, and maintained its shape and had a strong distinctive odour. At 80% moisture it ceased to hold its shape and became semi-liquid. If manure was not inoculated with fly eggs, it began to decompose and increased in moisture content when kept in a sealed container.

**Stage one**

As conversion began, the surface of the manure started to become level as second instar larvae moved away from the maggot mass and mixed the manure. At this stage, the manure was still identifiable, had a strong odour and the areas that did not contain larvae still resembled fresh
manure. Stirring the manure ensured that larvae and moisture were equally distributed for continued uniform conversion throughout the larval tray.

Table 8: Ranges and averages, or descriptions for characteristics of the manure residue and larvae when conversion stopped (larvae ceased feeding) and the stage of manure conversion associated with those characteristics. Each range was based on 40 conversion events using *M. domestica* larvae, except the percent of eggs that became puparia, which was based on 23 events.

<table>
<thead>
<tr>
<th>Stage of conversion</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (% of initial) at the end of conversion</td>
<td>N/A</td>
<td>25 - 37% average 30%</td>
<td>29 - 53% average 42%</td>
<td>27 - 50% average 38%</td>
<td>25 - 41% average 30%</td>
</tr>
<tr>
<td>Volume (% of initial) at the end of conversion</td>
<td>N/A</td>
<td>50 - 100% average 83%</td>
<td>57 - 90% average 68%</td>
<td>47 - 81% average 65%</td>
<td>33 - 58% average 41%</td>
</tr>
<tr>
<td>Day conversion ended</td>
<td>N/A</td>
<td>8 - 11 average 10</td>
<td>9 - 12 average 11</td>
<td>10 - 13 average 11</td>
<td>10 - 13 average 12</td>
</tr>
<tr>
<td>% of eggs that became puparia</td>
<td>0%</td>
<td>0 - 4% average 2%</td>
<td>0 - 18% average 9%</td>
<td>3 - 33% average 13%</td>
<td>8 - 9% average 9%</td>
</tr>
<tr>
<td>Life stage/behaviour</td>
<td>2nd instar larvae begin to move away from maggot mass</td>
<td>2nd instar larvae</td>
<td>2nd or 3rd instar larvae move to bottom of larval tray</td>
<td>3rd instar larvae move to surface and pupate</td>
<td>3rd instar or puparia</td>
</tr>
<tr>
<td>Is the manure residue able to be sifted?</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Is the manure residue homogenous?</td>
<td>no</td>
<td>no</td>
<td>potentially</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Is there an odour?</td>
<td>yes (ammonia)</td>
<td>yes (ammonia)</td>
<td>yes (earthy)</td>
<td>yes (earthy but mild)</td>
<td>no</td>
</tr>
</tbody>
</table>

Stage two

By this stage the surface of the manure was level, and the larvae re-leveled the manure within 1 to 2 hours after it was stirred. An appropriate seeding rate ensured all areas were broken up and were being converted; if too few larvae were present, or if manure was not stirred as
defined by the mass-production protocols, unconverted areas remained even when other parts of the manure were converting as expected. At the end of the second stage of conversion, manure consisted of sticky moist particles and the smell of the manure (ammonia) was evident but noticeably reduced.

Figure 6: Stages of manure conversion using *M. domestica* larvae. Examples of each stage of conversion (based on larval behaviour and the texture, mass, moisture content and volume of the manure residue), which occurred under various environmental conditions using *M. domestica* larvae that were reared under the protocol described in Chapter 2.
Stage three

At stage three the manure had a soil-like, fluffy texture, was well-aerated, and smelled like earthy compost. Third instar larvae were found throughout the manure, but the maggot mass that remained settled to the bottom of the larval tray, which was moister than the surface. Moisture and larvae needed to be evenly distributed throughout the manure by stirring to ensure complete conversion.

Stage four

Pupation began and conversion ended as the manure lost mass and became more granular. The few larvae that remained inhabited the moister areas of the manure, which were usually at the bottom corners of the larval tray. The manure residue resembled fine soil and was almost completely converted. If larvae were no longer present in the manure to complete its conversion further drying was necessary to prevent mould growth.

Stage five – complete

Conversion was completed and manure residue was dry, dusty, uniform and odourless. Manure residue was too dry to stick to puparia and no unconverted areas that could contain hidden puparia remained. All larvae had either died or pupated and could be easily removed by sifting with a 5mm mesh screen. This is the ideal final stage for manure residue in an industrial system because such manure residue does not need additional processing and puparia can easily removed.

These stages were also applied to conversion using *H. aenescens* and *C. hirtula*. Conversion stages for these species were based on qualitative characteristics because fewer instances of conversion were observed and there were difficulties quantifying the characteristics on which
the stages were based. The conversion stages still provided a good estimation of conversion for comparison of the ability of each species.

3.3.2 Larval development in manure

3.3.2.1 *Musca domestica*

The larvae of *M. domestica* began to pupate six days after inoculation and most larvae pupated on the seventh day. A minority of larvae continued to inhabit the manure after this time and pupation continued for several more days, depending on manure conditions. This variability would probably be reduced with a stabilized colony.

Manure conditions, larval density and the mass of manure that was used determined if manure could be completely converted. The acceptable range of one factor depended on the levels of the others. Complete conversion could only be achieved if all factors were within acceptable ranges. The trends between factors that were observed qualitatively are presented in Table 9. Anecdotal descriptions are presented below with examples of levels of factors that led to complete conversion.

The earliest complete conversion (stage five) was seen after nine days of larval activity when the initial manure mass was 1kg, initial moisture content was between 65% and 75% and manure was seeded with about 0.75g of eggs. Complete conversion was also seen after eleven days if the initial moisture content was higher (closer to 80% moisture) or population density was lower (less than 0.5g of eggs per kg of manure).

The number of eggs used to seed the manure affected the rate of larval development and the ability of the larvae to completely convert the manure. When 0.1g of eggs were used to seed 700g of manure, larvae were not seen in the substrate until day six, pupation occurred on day 12 and by day 14 the manure was dry and less than 30% of its initial mass, but had only reached the first or second stage of conversion. When too few eggs were used, or if the initial
moisture content was too high, all larvae pupated before the fifth stage of conversion was achieved. Conversion continued when more eggs were added after pupation or while larvae were still developing. At an initial moisture content above 80%, larvae remained in drier areas and pupated before the manure was completely converted. Larvae did not penetrate deeper than 7cm; when the manure was deeper than this, conversion failed to occur in the bottom of the tray.

Table 9: Summary of the trends that were identified by monitoring manure conversion, using *M. domestica* larvae, in various sized larval trays with various initial moisture contents, seeding rates and initial depths.

<table>
<thead>
<tr>
<th>Factor (range that allowed complete manure conversion)</th>
<th>Qualitative interaction observed between factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>less than approx. 0.5kg of manure</td>
</tr>
<tr>
<td><strong>Initial manure moisture content (approximately 60 to 80%)</strong></td>
<td>greater than approx. 75% moisture</td>
</tr>
<tr>
<td></td>
<td>*moisture levels at the higher end of the range required to completely convert manure.</td>
</tr>
<tr>
<td><strong>Mass of eggs added to manure (0.4 to more than 1.0g of eggs per kg of manure)</strong></td>
<td>a seeding rate of more than 1.0g of eggs per kg of manure is required.</td>
</tr>
<tr>
<td></td>
<td>*seeding rate at the higher end of the range is required to convert manure. Also seen in <em>H. illucens</em> (Sheppard et al. 1994)</td>
</tr>
<tr>
<td><strong>Initial depth of manure (approximately 3 to 7cm)</strong></td>
<td>not less than 5cm</td>
</tr>
<tr>
<td></td>
<td>*initial depth must be at the higher end of the range in order to maintain manure moisture, so larvae can survive to third instar and manure can be completely converted.</td>
</tr>
</tbody>
</table>
Conversion was often completed before pupation when the seeding rate was too high, manure had a lower initial moisture content, or the manure was too shallow. When roughly 500g of manure with a moisture content of about 70% and a depth of three centimeters was seeded with around 0.2g of eggs, conversion was reached by the seventh day but larvae were still developing. In one instance, this was corrected by adding additional fresh manure to the larval tray, which increased the lifespan of the larvae. Pupation began on the eleventh day and continued until day 28, at which time no larvae remained. In this particular case, fresh manure was also added on day 13 and water was added on day 22 so that the manure continued to support larval development.

At smaller scales, where the manure lost mass rapidly, conversion was often completed before pupation. When higher seeding rates (about 0.8g of eggs per kg of manure or higher) were used with small amounts of manure (less than about 300g) pupation and conversion were not completed. When about 0.1g of eggs were added to 100g of manure all larvae died by day four. Although the manure was dry, it remained in dry chunks and only reached the first or second stage of conversion. When the scale was increased to around one kilogram of manure, the same seeding rate was adequate for complete conversion and puparia production.

Data collected in this thesis are consistent with observations made by other researchers. Brookes and Fraenkel (1958) determined that the size and health of *M. domestica* larvae were dependent on larval conditions. Extremely dry conditions (less than 6ml of water for 2.2g of larval medium) prolonged the larval period and resulted in small puparia and too much moisture (more than 8.5ml of water for 2.2g of larval medium) also prolonged the larval period; and, resulted in the death of most larvae before pupation (Brookes and Fraenkel, 1958). Barnard et al. (1998) found that high population densities (initial density of more than six larvae per gram of manure) caused nutrients to become a limiting resource, many larvae died and smaller puparia were produced. It was observed by chance that when *M. domestica* and *C. hirtula* larvae were
both present in the manure the environmental conditions that led to conversion were different than if only *M. domestica* was present. It was often difficult to observe conversion trials without *C. hirtula* present because it almost always inhabited manure collected at Arkell Research Station and was impossible to remove from the manure. It was also difficult to completely remove the population of *C. hirtula* from the environmental chamber when manure was present. Both species inhabited the manure when larval trays, with a measured amount of *M. domestica* eggs, were placed in the environmental chamber. Adult *C. hirtula* landed on the fresh manure within minutes and oviposition occurred shortly thereafter. The larvae of both species were found in the manure from day 1 until about day 8. After day 8 only *M. domestica* larvae and puparia were found. By the time the manure reached the 5th conversion stage and *M. domestica* larvae had pupated, all *C. hirtula* adults had emerged and moved to other more suitable manure. It appeared that mass was lost faster and manure residue was finer than if *M. domestica* was converting the manure alone. Although observations were not quantified at this time, it appeared that complete conversion occurred in more larval trays when both species were present because excess moisture and high population densities had less of a negative impact than they do when only *M. domestica* was present. This hypothesis was expanded and quantitatively tested in Chapter 4.

3.3.2.2 *Hydrotaea aenesens*

*Hydrotaea aenesens* reacted to environmental conditions in much the same way as *M. domestica*, but on a different timeline. The range of environmental conditions that led to conversion was assumed to be narrower for *H. aenesens* than it was for *M. domestica* because conversion was often not completed. Conversion via *H. aenesens* was described by the same conversion stages as manure converted by *M. domestica*; however, only qualitative characteristics and the mass were used because conversion was not observed often enough to produce ranges for each stage of conversion. When conversion was completed, manure residue
was darker in colour than residue produced by *M. domestica*. Other characteristics were comparable to manure residue completely converted by *M. domestica*, and was about 30% of its initial mass, fine in texture, homogenous, odourless, and all larvae had either died or pupated.

Conversion was completed by day 15, but when a low seeding rate (less than about 0.5g of eggs per kg of manure) was used, conversion required 25 days. Pupation occurred as early as the fifth day; however, if the manure remained moist, the larvae were able to feed and pupation was often postponed until about day 17. Similar to *M. domestica*, the larval period of *H. aenesens* was lengthened by the addition of fresh manure (approximately 500g), when conversion was completed before all larvae had pupated. Conversion often occurred before pupation at smaller scales or when a population density was too high.

The same manure could not be inhabited by *H. aenesens* and *C. hirtula* simultaneously. When larval trays with *H. aenesens* were in the environmental chamber with freely flying *C. hirtula*, larvae of both species could be seen for the first three days. After three days, *C. hirtula* larvae or puparia were no longer seen, even though they were still present in other nearby manure that did not have *H. aenesens*. No obvious changes in the population of *H. aenesens* larvae were seen at any time. It appeared that *H. aenesens* and *C. hirtula* did not share the same complementary effect on conversion that *M. domestica* and *C. hirtula* had.

3.3.2.3 *Coproica hirtula*

*Coproica hirtula* was not able to convert the manure in the same manner as the previously mentioned species. Although difficult to measure, the lifecycle was completed in as little as six days, with the majority of time being spent as larvae. Puparia were too small and adult flies emerged too quickly for the puparia to be harvested and converted into a livestock feed. A high population density was achieved and all life stages were present simultaneously in the manure.
This species only penetrated about 2 cm into the manure and the manure formed a dry crust on the surface. Stirring broke up the crust but it always reappeared the next day. The crust caused chunks of unconverted manure to form that limited further processing but did not stop the development of the flies. The bulk of the manure remained in about stage one of conversion; however, small quantities of fine residue were often present when smaller-scale units were investigated. Moisture level was decreased (not quantified), and the mass was reduced to about 35% of initial after about 14 days; but complete conversion was never achieved by using this species even after it was given more than two months in a large-scale study using more than 15kg of manure.

3.3.3 Comparison to the ideal species

Each species was selected for investigation because it had some attributes of the “ideal species”. The following table (Table 10) compares *M. domestica, H. aenescens* and *C. hirtula* with regards to the characteristics that were important for mass production and poultry manure conversion. Information in the table is adapted from various sources, including new observations made during the current study. This information, as well as the performance of each species in poultry manure as described above, were used to identify the species most suitable for conversion of poultry manure at an industrial scale. Further quantitative data are discussed in Chapter 4.

3.4 Conclusions

Species followed different timelines while in poultry manure and had different conversion abilities. Environmental factors changed timelines by altering the development and behavior of the larvae, which affected manure conversion. Even though results were difficult to quantify for reasons previously discussed (such as inconsistencies with the adult colonies and manure), they allowed for the identification of trends in the conversion process for each species. Initial manure moisture, depth and population density all affected the conversion ability of the flies. A
Table 10: Comparison of the four species that could be mass produced to the “ideal species” based on characteristics important for mass production and for use in an industrial-scale poultry manure conversion system. References are as follows a: personal communication with J. Hogsette; b: Bernard et al., 1998; c: Teotia and Miller, 1974; d: Hogsette and Washington, 1995; e: Calvert et al., 1973; f: Basden, 1947; g: Bergeron et al., 2012; h: Hiroyoshi, 1964; i: Axtell, 1986; j: Farkas et al., 1998; k: Iniquez-Covarrubias et al., 1994; l: Larrain and Salas, 2008; m: from present thesis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ideal species</th>
<th>Musca domestica</th>
<th>Hydrotaea aenescens</th>
<th>Coproica hirtula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can be stabilized</td>
<td>yes</td>
<td>yes (a)</td>
<td>yes (but can take several years) (a)</td>
<td>unlikely (m)</td>
</tr>
<tr>
<td>Regularly produces many eggs</td>
<td>yes</td>
<td>yes (a)</td>
<td>yes (d)</td>
<td>yes (m)</td>
</tr>
<tr>
<td>Simultaneous oviposition</td>
<td>yes</td>
<td>yes (a)</td>
<td>yes (d)</td>
<td>no (m)</td>
</tr>
<tr>
<td>Minimal maintenance required</td>
<td>yes</td>
<td>yes (daily) (k, l, m)</td>
<td>yes(daily) (d, j, m)</td>
<td>yes (monthly) (m)</td>
</tr>
<tr>
<td>Adult food</td>
<td>none</td>
<td>sugar, milk powder, yeast (k, l)</td>
<td>sugar, milk powder, yeast (d, j)</td>
<td>manure (g)</td>
</tr>
<tr>
<td>Survives high population densities</td>
<td>yes</td>
<td>yes (b)</td>
<td>yes (d)</td>
<td>yes (g)</td>
</tr>
<tr>
<td>Pest species</td>
<td>no</td>
<td>very pestilent (i)</td>
<td>pestilent, but habits keep it from humans and animals (j)</td>
<td>unlikely, stays near manure but can build up large populations (g)</td>
</tr>
<tr>
<td>Few males required</td>
<td>yes</td>
<td>no (h)</td>
<td>unknown</td>
<td>unknown</td>
</tr>
<tr>
<td>Disease transmission</td>
<td>no</td>
<td>yes (i)</td>
<td>yes (i)</td>
<td>unlikely (g)</td>
</tr>
<tr>
<td>Detailed protocol with timeline</td>
<td>yes</td>
<td>yes (m)</td>
<td>yes (m)</td>
<td>no (m)</td>
</tr>
</tbody>
</table>

Characteristics important for a conversion system

<table>
<thead>
<tr>
<th>Larval food source</th>
<th>microbial grazer</th>
<th>microbial grazer (i)</th>
<th>facultative predator (i)</th>
<th>microbial grazer (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larvae suitable as a protein supplement for poultry</td>
<td>yes</td>
<td>yes (c, e)</td>
<td>unknown, but likely</td>
<td>unlikely (too small) (m)</td>
</tr>
<tr>
<td>Puparia can be separated from manure residue</td>
<td>yes</td>
<td>yes (e, f)</td>
<td>yes(m)</td>
<td>no (too small) (m)</td>
</tr>
</tbody>
</table>
Survive cool temperatures, no diapause

<table>
<thead>
<tr>
<th></th>
<th>yes</th>
<th>yes (i)</th>
<th>yes (i)</th>
<th>yes (g)</th>
</tr>
</thead>
</table>

Specialist in poultry manure

<table>
<thead>
<tr>
<th></th>
<th>yes</th>
<th>no (i)</th>
<th>no (i)</th>
<th>yes (g)</th>
</tr>
</thead>
</table>

Short larval development

<table>
<thead>
<tr>
<th></th>
<th>yes</th>
<th>yes (one week) (i)</th>
<th>yes (one or two weeks) (i)</th>
<th>yes (few days) (g)</th>
</tr>
</thead>
</table>

balance between all these factors (initial moisture content, initial depth and seeding rate) must be achieved if the manure is to be completely converted. Suboptimal levels of any one factor altered the larval period, which in turn caused larvae to either convert manure before their development was completed, pupate before conversion was completed or die in the early stages of development. The mass of manure that was used to investigate conversion was also important, and affected the levels of the previously mentioned factors. If conversion is to be conducted at an industrial-scale, it is important to investigate conversion at the largest scale possible so that results will be more generally applicable.

The conversion stages described in this chapter will allow for consistent comparisons between larval trays to determine the ideal environmental conditions. In the future, this scale could be made more precise by quantitatively analyzing the physical and chemical changes the manure undergoes during conversion. A narrow set of parameters could then be developed for each stage, which would allow for more precise comparisons between the manure residues converted by various species, which have qualities that could not currently be evaluated in detail.

The most efficient and successful manure conversion occurred when *M. domestica* larvae were present. Larval *H. aenescens* were able to convert the manure, but conversion took longer and was more affected by changes in the environmental conditions. Larvae of *M. domestica* remained throughout the manure, even at high larval densities, instead of congregating on the
surface as *H. aenescens* did. When larvae are on the surface they are not aerating and converting the manure, which may be a reason for less efficient conversion by *H. aenescens*. Manure was not converted by *C. hirtula* and puparia were not large enough to be transformed into a protein feed. After considering these factors it was decided that the conversion of poultry manure by *M. domestica* will be further investigated in the next chapter.

Conversion was more likely to be completed when both *M. domestica* and *C. hirtula* were present in the manure. Conversion occurred in a comparable time frame but environmental factors appeared to have less of an impact. The *M. domestica* larvae were less likely to die before conversion had been completed and the manure residue had a lower final moisture content; both conditions would be beneficial to an industrial-scale system. It may be that *C. hirtula* quickly dehydrated the manure from the surface while *M. domestica* larvae worked from the lower layers. This interaction between species was further investigated in Chapter 4.

All species shared many characteristics with the ideal species. The most efficient conversion occurred with *M. domestica* and a detailed mass production protocol has been established, but its biology is of the greatest concern. The most obvious issue is that *M. domestica* is one of the most problematic pests in poultry manure. Its high fecundity and ability to breed in many decomposing substrates allows large infestations to occur, if even a small number of flies escape. If this species is to be used in an industrial-scale conversion system, a reliable solution for preventing escape must be developed. Preventing the emergence of adult flies from puparia (for example with parasitoid wasps used for biological control of *M. domestica*) would only be acceptable if a breeding population can still be maintained. A better solution may be to develop a stable flightless strain of *M. domestica* that will not escape as easily (discussed in Chapter 5).
Chapter 4: Determination of the optimal initial manure moisture content, depth and fly egg seeding rate for poultry manure conversion using *Musca domestica* and *Coproica hirtula*

4.1 Introduction

Comparison among the candidate species (*Musca domestica, Hydrotaea aenesens, Coproica hirtula*) based on mass-rearing protocols and performance in poultry manure revealed that *M. domestica* showed the most promise for use in an industrial poultry manure conversion system. This species has a naturally high fecundity and requires little maintenance to quickly produce the large number of eggs required for quantitative experimentation and for use in an industrial-scale system. The larvae thrive in dense populations, develop quickly and have voracious appetites that enable them to convert large amounts of manure rapidly. Their short lifecycle also allows them to adapt to changing manure conditions in only a few generations, which would be important for a large-scale system.

The manure collected at the Arkell Research Station often contained *C. hirtula*. Other studies have described a small “ever-present Sphaeroceridae” that were a constant feature of the manure they collected (Beard and Sands, 1973). This fly was likely *C. hirtula*, as this species is ubiquitous in poultry manure and is the only *Coproica* species that has a known association with birds (Bergeron et al., 2012). This species was not considered a pest at the poultry facility from which manure was collected for the research in this thesis nor at the facility in Beard and Sands (1973), but large numbers of these flies were present. It is important to investigate conversion when both species are present because *C. hirtula* are likely to inhabit the manure that is to be converted because of its ubiquity. It may be more efficient to design a system that integrates both species, rather than attempting to eliminate *C. hirtula* from the manure.

An understanding of how environmental conditions affect the conversion process can facilitate the presentation of manure to the larvae in a way that will let them fully utilize the substrate. This
in turn, could facilitate a reduction in the number of eggs needed daily, making adult fly colonies smaller and more manageable. The success of a large-scale poultry manure conversion system will rely on a complete understanding of the species involved in order to increase breeding and conversion efficiency through manipulation of environmental and physical variables.

The explanatory variables that were selected for further investigation in a response surface experiment were larval population density, initial manure depth and initial manure moisture content. The variables were chosen through review of relevant literature, results of Chapter 3 and through personal communication with Ecospace Engineering (Guelph, Ont. Canada). These three variables were recognized as the most likely to affect the efficiency of conversion when using the Milinator system. The ranges of parameter values that were used in the response surface experiment came from existing literature and can be found in Table 11.

Table 11: Ranges for explanatory variables for the response surface experiment that were tested to identify the optimal levels of all three variables when using *M. domestica* in a poultry manure conversion system. The previous studies which outlined the limits of the species for each variable are given.

<table>
<thead>
<tr>
<th>Explanatory variable</th>
<th>Range</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Seeding rate (g of eggs/kg of poultry manure)</strong></td>
<td>0.13 to greater than 1.0g of eggs per kilogram of manure. Lower ratios are associated with studies focusing on protein production, not manure conversion.</td>
<td>Beard and Sands, 1973; Calvert, 1974; Barnard et al., 1998; el Boushy and van der Poel, 2000</td>
</tr>
<tr>
<td><strong>Initial manure moisture (%)</strong></td>
<td>60-80% very few larvae live outside of the range. Ideal for this study likely mid-range.</td>
<td>Miller et al., 1974; Calvert, 1974; Barnard et al., 1998; McIntyre and Gooding, 2000; Achiano and Giliomee, 2005; Larrain and Salas, 2008</td>
</tr>
<tr>
<td><strong>Initial manure depth (cm)</strong></td>
<td>&lt;7cm no larvae seen deeper. Ideal will likely be in upper portion of range to ensure efficiency. Minimum depth unknown.</td>
<td>Hogsette, 1996; Farkas et al., 1998; Hogsette and Jacobs, 1999</td>
</tr>
</tbody>
</table>
The first variable chosen was the seeding rate required to completely convert a given amount of manure. Previous research has focused on protein production and has not addressed the effect of population density on conversion. The population density and the final mass content of the manure do not have a linear relationship (Barnard et al., 1998). This may be the result of the scale used by Barnard et al. (1998), which was only 100g of manure per experimental unit. Population density has been shown to affect both the development of the (final mass of puparia) and the conversion of manure (final mass), but the optimal value that allows for complete conversion of the manure and puparia collection is unknown and needs to be investigated (Beard and Sands, 1973; Miller et al., 1974; Barnard and Geden, 1993).

The depth of manure that the larvae would penetrate and convert was of great interest to Ecospace Engineering, because it would help determine the optimum size of the conversion equipment. The conveyer belts in the Milinator machinery will have to be sufficiently wide and long, given the depth restriction of the larvae, to accommodate the manure that is being produced at the farm. Other studies have suggested that M. domestica will convert swine manure most efficiently at a depth of only 2.5cm (Barnard et al., 1998). There have been no previous investigations on the ideal depth of poultry manure for conversion via fly larvae, only that they can survive in a depth of up to 7cm (Hogsette, 1996).

Many studies have investigated the effect of moisture content on the development of M. domestica; however, the objective of most of these studies has been to discourage fly breeding by altering the moisture content of the manure (Achiano and Giliomee, 2005). This species has been recorded inhabiting manure with between 60% to 80% moisture (Iniguez-Covarrubias et al., 1994). It has been suggested that the optimum moisture content for larval survival and protein production is between 70% and 74% (Miller et al., 1974; Achiano and Giliomee, 2005); however, there are no published data on how initial moisture content affects conversion. In many studies moisture content is maintained by the addition of water at a rate equal to
evaporation. These results are not applicable to the work in this thesis because water is not added in a conversion system, and in fact, removal of moisture is one objective of a poultry manure conversion system. In Chapter 3 of this thesis, trends relating initial moisture content and manure conversion were identified, but the effects were not quantified.

Chapter 3 also indicated that environmental factors (now referred to as explanatory variables) are interdependent; for example, the ideal initial manure moisture content for conversion when a rate of 0.5g of eggs per kg of manure is used may not be the same as when 1.0g of eggs per kg of manure are used. Therefore, explanatory variables will have to be investigated simultaneously to determine the ideal levels of each explanatory variable. Previous studies on conversion or on the production of larval protein have only looked at environmental factors individually and their relationships to each other have not been studied; therefore, the ideal values that have been stated may not be valid for this system. When an industrial-scale conversion system is used, it is desirable to have all the variables that affect conversion at optimum levels. Deviation from ideal levels could result in incomplete conversion and manure would have to undergo further processing or unconverted manure would need to be stored.

In this study, multiple explanatory variables are investigated simultaneously using a response surface experiment. The response surface methodology, which is typically used in product development experiments in physics and chemistry, allows relationships among explanatory variables and their effect on the response variable to be determined. The combined and individual effects of the explanatory variables can be correlated to changes in the response variable (the final mass of the manure), quantified and modeled with a least squares regression equation (Box and Draper, 2007). This model, through statistical analysis, indicates the optimal level for each explanatory variable that will allow for the smallest final mass and therefore efficient manure conversion. Without a response surface design, many groups of experiments or a large full factorial experimental design would be required, each holding a single variable
constant and varying the others incrementally (Bowley, 2008). The ideal levels would have to be inferred from these results. With a response surface design results can be obtained from a single experiment with few experimental units. Egg production was determined to be a limiting factor and would establish the maximum size of the laboratory experiments, as flies would have to produce enough eggs for all experimental units in a short time period (about six hours). Using fewer experimental units than other designs meant that the scale of the experiment could be increased and the trends identified were more likely to be relevant at an industrial scale, because differences in conversion were observed (in Chapter 3) depending on the amount of manure used. For example, when more manure is used a lower egg seeding rate may be required. Statistical analyses allow trends to be identified and the ideal levels of each variable can be computed even if no experimental unit with this combination of variables was tested.

This type of experimental design is not typically used in entomological research; however, it is ideal for this project. Often in biological experiments, variables cannot be well controlled, results have stochastic influences and researchers often opt for using an approach that does not rely on complex statistical models. Other entomological research has shown that response surface designs are effective and results are valid even if the level of control is not as precise as in the physical sciences (Menke, 1973). The response surface method was chosen for this project because it allowed for explanatory variables and their interactions to be investigated with a relatively low number of large experimental units. The ideal values obtained could also be directly applied to further studies completed at an industrial scale.

4.2 Methods

Two trials using the response surface design were completed. The first trial investigated the response of *M. domestica* to the explanatory variables (initial moisture content, initial depth and egg seeding rate) and the second trial looked at the co-habitation and conversion of the manure
by *M. domestica* and *C. hirtula* and its response to these same variables. The final mass of the manure residue with puparia was used as the response variable in both trials.

The population of *C. hirtula* was not experimentally altered or quantified for the second trial because of difficulties monitoring this species. Large numbers of adult *C. hirtula* were free inside the environmental chamber and inhabited all experimental units in Trial 2. Manure that was not inhabited by *M. domestica* was also available to *C. hirtula* for oviposition and larval development, enabling them to select oviposition sites and maintain a large population. It was assumed that the larval populations would be approximately equal in all experimental units. Flies were able to oviposit in experimental units or other fresh manure made available that did not contain *M. domestica* larvae. No overcrowding of this species was evident in pre-trial experiments even when large adult populations were present. Puparia were too small to collect and exact population density could not be determined.

### 4.2.1 Statistical design and analysis

The experiment (Table 12) had a central composite circumscribed (CCC) design as described by Box and Draper (2007). Each of the three explanatory variables was investigated at five levels because their effects on the response variable were expected to be parabolic (Zhemchuzhina and Zvereva, 1989). As the design dictated, each explanatory variable was tested at two factorial points, two star points and a center point, which was replicated six times. On the coded scale (coded and original scales are given in Table 12), factorial points were -1 and +1, star points were -1.682 and +1.682 and center points were 0 for each variable, for a total of 20 experimental units. Each variable spanned the range that was known to be acceptable for *M. domestica* (Table 11). The initial moisture ranged from 60 to 80%, the initial depth ranged from 2 to 8 cm and the seeding rate varied from 0.1 to 1.0 g of eggs per kg of manure. Exact levels that were tested are given in Table 12. The locations of the experimental units were randomized for each trial using an online random number generator.
(http://www.random.org/sequences), and units were arranged in the chamber in four rows and five columns.

Table 12: Arrangement for rotatable central composite circumscribed (CCC) design for the three explanatory variables \(X^1=\text{mass of } M. \text{ domestica eggs (g)}, \ X^2=\text{initial moisture level (\%)}, \ X^3=\text{initial manure depth (cm)}\) at five levels using \(M. \text{ domestica}\) larvae (Trial 1) and \(M. \text{ domestica}\) and \(C. \text{ hirtula}\) larvae (Trial 2). As in all CCC designs, factorial points on the coded scale were -1 and +1, star points were -1.682 and +1.682 and the center points are 0 for each explanatory variable. Levels of each explanatory variable for each experimental unit are given in the coded (original) scale.

<table>
<thead>
<tr>
<th>Experimental unit</th>
<th>(X^1) (g)</th>
<th>(X^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1(0.18)</td>
<td>-1(64)</td>
</tr>
<tr>
<td>2</td>
<td>+1(0.72)</td>
<td>-1(64)</td>
</tr>
<tr>
<td>3</td>
<td>-1(0.18)</td>
<td>+1(76)</td>
</tr>
<tr>
<td>4</td>
<td>+1(0.72)</td>
<td>+1(76)</td>
</tr>
<tr>
<td>5</td>
<td>-1(0.18)</td>
<td>-1(64)</td>
</tr>
<tr>
<td>6</td>
<td>+1(0.72)</td>
<td>-1(64)</td>
</tr>
<tr>
<td>7</td>
<td>-1(0.18)</td>
<td>+1(76)</td>
</tr>
<tr>
<td>8</td>
<td>+1(0.72)</td>
<td>+1(76)</td>
</tr>
<tr>
<td>9</td>
<td>-1.682(0.1)</td>
<td>0(70)</td>
</tr>
<tr>
<td>10</td>
<td>+1.682(1.0)</td>
<td>0(70)</td>
</tr>
<tr>
<td>11</td>
<td>0(0.45)</td>
<td>-1.682(60)</td>
</tr>
<tr>
<td>12</td>
<td>0(0.45)</td>
<td>+1.682(80)</td>
</tr>
<tr>
<td>13</td>
<td>0(0.45)</td>
<td>0(70)</td>
</tr>
<tr>
<td>14</td>
<td>0(0.45)</td>
<td>0(70)</td>
</tr>
<tr>
<td>15</td>
<td>0(0.45)</td>
<td>0(70)</td>
</tr>
<tr>
<td>16</td>
<td>0(0.45)</td>
<td>0(70)</td>
</tr>
<tr>
<td>17</td>
<td>0(0.45)</td>
<td>0(70)</td>
</tr>
<tr>
<td>18</td>
<td>0(0.45)</td>
<td>0(70)</td>
</tr>
<tr>
<td>19</td>
<td>0(0.45)</td>
<td>0(70)</td>
</tr>
<tr>
<td>20</td>
<td>0(0.45)</td>
<td>0(70)</td>
</tr>
</tbody>
</table>

Analysis programs (proc rsreg) in SAS software (Version 9.0 SAS System for Windows XP Copyright © 2003; SAS Institute Inc., Cary, NC, USA) were used for the response surface and ridge analyses, which were conducted on the data from both trials to determine the value of each explanatory variable that reduced the final mass of the manure. Ridge analysis looked for the minimum response for the response variable (final manure mass) given ideal values for the explanatory variables. If a maximum or saddle point was found (instead of a minimum
response), there was no combination of explanatory variables that could produce a minimum value in the response variable. This indicated that the ranges of the explanatory variables would have to be altered in the direction indicated by the ridge analysis. If a minimum response was determined, a graphical representation was constructed with proc G3D when the least squares regression equation was determined with statistical analysis. The effects of two variables per graph were presented when the third variable was at its optimal level. The calculated, minimum value for the response variable (mass) was represented by the lowest point in the graph.

4.2.1 Experimental design

Each experimental unit consisted of 1kg of freshly collected chicken manure. The initial moisture content was determined as described in Chapter 2. In order to have manure that was collected at the same time to have different moisture contents some manure was left to air dry to approximately 60% moisture while other manure from the same batch was covered and maintained at around 75% moisture for three days. The exact moisture content was determined daily (as described in Chapter 2) for both. Moisture levels were achieved by misting a predetermined amount of distilled water to the manure followed by thorough mixing. The following formula was used to determine the amount of water necessary to raise the moisture content to the desired level:

\[ X = \frac{A(M1 - M2)}{100} \]

Where

- \( A = 100g \) of chicken manure
- \( M1 = \) target moisture content (%)  
- \( M2 = \) current moisture content (%)  
- \( X = \) the amount of water (ml) needed to increase moisture content
To determine the amount of water needed per experimental unit (1kg of manure) “X” was multiplied by 10, this amount was then multiplied by the number of experimental units at that moisture level. For example, there were four experimental units at 64% moisture (representing one moisture level); therefore, 4kg of manure was separated from the manure that had a moisture content of 60%. The equation above (Equation 2) was then used to determine the amount of water that would be needed to increase 100g of manure to the desired moisture content. This was multiplied by 10 (to get the amount of water needed for one experimental unit) and then by 4 (for the amount of water needed for four experimental units). The water was misted and mixed into the manure, which was then separated into the four experimental units. This process was repeated for each of the five moisture levels investigated.

Adult and larval flies were reared as explained in the protocols in Chapter 2. All physical techniques for taking measurements, egg collection, and treatment of flies were performed as previously described. Eggs were collected from a laboratory colony within a period of six hours and were added to the manure as specified by the statistical design. The experiment took place in a walk-in, controlled environment chamber set at 25°C ± 1°C, 60% RH and 16L: 8D cycle. Experimental units were lifted about 30 cm off the floor of the chamber with metal grates to prevent overheating. Conversion occurred in plastic, rectangular storage boxes of various sizes to allow the specific depths to be studied (Table 13, Figure 7). Several observations were made daily including:

- manure mass
- manure depth
- temperature (center of the maggot mass and manure with no larvae)
- manure conversion stage (as described in chapter 3)
- life stage of the fly (larval instar or puparium),
Table 13: Dimensions of the various plastic boxes used in the response surface experiments to hold larvae and converting manure. All experimental units started with the same mass of manure.

<table>
<thead>
<tr>
<th>Experimental units (EU)</th>
<th>Manure depth (cm)</th>
<th>Length (cm)</th>
<th>Width (cm)</th>
<th>Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>2</td>
<td>29.0</td>
<td>44.0</td>
<td>17.0</td>
</tr>
<tr>
<td>1- 4</td>
<td>3.5</td>
<td>34.2</td>
<td>20.9</td>
<td>11.8</td>
</tr>
<tr>
<td>9- 12, 15- 20</td>
<td>5</td>
<td>23.8</td>
<td>20.3</td>
<td>17.5</td>
</tr>
<tr>
<td>5- 8</td>
<td>6.5</td>
<td>20.3</td>
<td>16.9</td>
<td>9.9</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>14* (20.3)</td>
<td>16.9</td>
<td>9.9</td>
</tr>
</tbody>
</table>

*The box for experimental unit 14 was altered by fashioning a new side out of the box lid and hot gluing it into place, so that a depth of 8cm could be studied.

Figure 7: Storage boxes used to investigate manure conversion at five depths. Clockwise from top left: the boxes were used for 1) EU 13, 2) EU 1-4, 3) EU 14, 4) EU 9-12, 15-20 and 5) EU 5-8 and. Dimensions of each box are given in Table 13.
At the end of conversion, the puparia were removed and measured (as described in Chapter 2). The average final mass of the 20 experimental units was compared between trials with a two-tailed t-test assuming equal variances, to determine if using both *M. domestica* and *C. hirtula* together resulted in a different reduction of mass than using *M. domestica* alone.

Only the final mass was used in the response surface analysis. Other qualitative observations of importance to understanding how the flies were developing and converting the manure were recorded. The experiment ended for an experimental unit when no larvae remained to convert the manure because they had either pupated or died.

### 4.3 Results

#### 4.3.1 Daily changes of manure and larvae

The response surface experiment totaled 11 days for Trial 1 using only *M. domestica* and 13 days for Trial 2 using *M. domestica* and *C. hirtula* together. Once the larvae had either died or pupated, measurements were no longer recorded, as any change was not due to larval activity. Larval periods differed depending on the environmental conditions of the experimental units. The longest larval period was 13 days and occurred in experimental unit 12 in Trial 2, which had the highest initial moisture content (80%) of the trial (Table 14). A slightly shorter maximum larval period (11 days) was seen in Trial 1 in experimental units 5 to 8 and 14 which had the highest initial depth of manure (6.5 and 8cm respectively) (Table 14).

Only two experimental units reached the fifth stage of conversion, but nine reached the fourth stage. (Table 14) The manure in stage 4 still contained chunks of dry unconverted areas, which prevented experimental units from being scored as stage 5. Puparia were recovered from every experimental unit in Trial 1, but no puparia were recovered from experimental units 1, 2 and 13 in Trial 2 (Table 14). The experimental units that did not produce puparia also only reached the second or third conversion stage; however, experimental unit 3 only reached the third stage.
Table 14: Conversion stage that was reached and day conversion was completed for each experimental unit. The final conversion stage, as described in Chapter 3, is presented for each experimental unit (EU). The day that conversion was complete (when larval were no longer present in manure) is also presented. Trial 1 uses only *M. domestica*, and Trial 2 uses both *M. domestica* and *C. hirtula* to convert manure.

<table>
<thead>
<tr>
<th>EU</th>
<th>Initial conditions</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass of eggs (g)</td>
<td>Moisture content (%)</td>
<td>Depth (cm)</td>
</tr>
<tr>
<td>1</td>
<td>0.18</td>
<td>64</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>0.72</td>
<td>64</td>
<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td>0.18</td>
<td>76</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>0.72</td>
<td>76</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>0.18</td>
<td>64</td>
<td>6.5</td>
</tr>
<tr>
<td>6</td>
<td>0.72</td>
<td>64</td>
<td>6.5</td>
</tr>
<tr>
<td>7</td>
<td>0.18</td>
<td>76</td>
<td>6.5</td>
</tr>
<tr>
<td>8</td>
<td>0.72</td>
<td>76</td>
<td>6.5</td>
</tr>
<tr>
<td>9</td>
<td>0.1</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>0.45</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>0.45</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>0.45</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>0.45</td>
<td>70</td>
<td>8</td>
</tr>
<tr>
<td>15</td>
<td>0.45</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>0.45</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>0.45</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>0.45</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>19</td>
<td>0.45</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>0.45</td>
<td>70</td>
<td>5</td>
</tr>
</tbody>
</table>
conversion stage and puparia were recovered. Table 15 displays the total mass, average mass and estimated number of puparia that were recovered from Trial 1. Experimental unit 10 (which had the highest seeding rate) produced the greatest total mass (31.7g) and the most puparia (2224). Experimental unit 12 (which had the highest initial moisture content) produced the largest $M. domestica$ puparia (205mg). The fewest puparia (121) were collected from experimental unit 9, which had the lowest seeding rate. The smallest puparia (67mg) and the lowest total mass (1.66g) were collected from experimental unit 13, which had the lowest moisture content.

Trial 2 took an average of 11 days and 12 out of 20 experimental units reached the fifth stage of conversion (Table 14). The fourth stage of conversion was reached by 4 experimental units. Manure that was converted simultaneously by $M. domestica$ and $C. hirtula$ was finer in texture than manure residue produced by $M. domestica$ alone. The mass and number of puparia was not recorded for each experimental unit because of difficulties removing the puparia from the manure before emergence began.

Manure masses, as recorded daily, are presented in Figures 8 and 9. In Trial 1, experimental units 1 to 4 decreased in mass at a similar rate (Figure 8). The only constant exploratory variable (environmental factor) shared by these experimental units was an initial depth of 3.5cm. The next four experimental units (5 to 8), which had an initial depth of 6.5cm, also decreased in mass similarly, but at a slower rate and to a greater final mass than experimental units 1 to 4. Experimental units that started with a depth of 5cm (9 to 12, 15 to 20) also shared a similar rate of mass decrease. These experimental units had a faster rate of decrease than experimental units 5 to 8, but slower than 1 to 4, and also had an intermediate final mass. The mass of
Table 15: Total mass of puparia (g), average mass (g), and an estimated number of puparia that were recovered from each experimental unit (EU) in Trial 1. Initial conditions for each experimental unit are given.

<table>
<thead>
<tr>
<th>EU</th>
<th>Mass of eggs (g)</th>
<th>Initial moisture content (%)</th>
<th>Initial depth (cm)</th>
<th>Total mass of all puparia (g)</th>
<th>Average puparium mass (mg)</th>
<th>Estimated number of puparia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.18</td>
<td>64</td>
<td>3.5</td>
<td>12.49</td>
<td>94</td>
<td>1327</td>
</tr>
<tr>
<td>2</td>
<td>0.72</td>
<td>64</td>
<td>3.5</td>
<td>16.18</td>
<td>109</td>
<td>1490</td>
</tr>
<tr>
<td>3</td>
<td>0.18</td>
<td>76</td>
<td>3.5</td>
<td>6.73</td>
<td>158</td>
<td>427</td>
</tr>
<tr>
<td>4</td>
<td>0.72</td>
<td>76</td>
<td>3.5</td>
<td>17.09</td>
<td>132</td>
<td>1291</td>
</tr>
<tr>
<td>5</td>
<td>0.18</td>
<td>64</td>
<td>6.5</td>
<td>8.29</td>
<td>203</td>
<td>408</td>
</tr>
<tr>
<td>6</td>
<td>0.72</td>
<td>64</td>
<td>6.5</td>
<td>26.15</td>
<td>137</td>
<td>1909</td>
</tr>
<tr>
<td>7</td>
<td>0.18</td>
<td>76</td>
<td>6.5</td>
<td>9.40</td>
<td>199</td>
<td>471</td>
</tr>
<tr>
<td>8</td>
<td>0.72</td>
<td>76</td>
<td>6.5</td>
<td>23.80</td>
<td>158</td>
<td>1508</td>
</tr>
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<td>9</td>
<td>0.1</td>
<td>70</td>
<td>5</td>
<td>2.46</td>
<td>203</td>
<td>121</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>70</td>
<td>5</td>
<td>31.75</td>
<td>143</td>
<td>2224</td>
</tr>
<tr>
<td>11</td>
<td>0.45</td>
<td>60</td>
<td>5</td>
<td>31.65</td>
<td>170</td>
<td>1865</td>
</tr>
<tr>
<td>12</td>
<td>0.45</td>
<td>80</td>
<td>5</td>
<td>8.28</td>
<td>205</td>
<td>403</td>
</tr>
<tr>
<td>13</td>
<td>0.45</td>
<td>70</td>
<td>2</td>
<td>1.66</td>
<td>67</td>
<td>248</td>
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<tr>
<td>14</td>
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<td>26.41</td>
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<td>28.18</td>
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<td>1854</td>
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<td>16</td>
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<td>70</td>
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<td>5.81</td>
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<td>292</td>
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<tr>
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<td>70</td>
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<td>20.35</td>
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<td>1116</td>
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<tr>
<td>19</td>
<td>0.45</td>
<td>70</td>
<td>5</td>
<td>13.96</td>
<td>198</td>
<td>705</td>
</tr>
<tr>
<td>20</td>
<td>0.45</td>
<td>70</td>
<td>5</td>
<td>21.91</td>
<td>165</td>
<td>1324</td>
</tr>
</tbody>
</table>
Figure 8: Change in the mass of manure and inhabiting larvae (g) for 20 experimental units (EU) during Trial 1 using *M. domestica* larvae to convert manure. A five-level response surface experiment using initial moisture (markers), initial depth (line colour) and mass of eggs (line style) as explanatory variables was conducted and the mass of each experimental unit was measured daily. The combination of levels for each variable is presented in Table 12.
Figure 9: Change in the mass of manure and inhabiting larvae (g) for experimental units (EU) during Trial 2 using *M. domestica* and *C. hirtula* to convert manure. A five-level response surface experiment using initial moisture (markers), initial depth (line colour) and mass of eggs (line style) as explanatory variables was run and the mass of each experimental unit was measured daily. The combination of levels for each variable is presented in Table 12.
experimental unit 14 (the deepest investigated at 8cm) had the slowest rate of decrease and also had the greatest mass after conversion; conversely, the mass of experimental unit 13 (the shallowest investigated at 2cm) had the fastest rate of decrease and the smallest final mass. In Trial 2 (Figure 9) this same clustering pattern between depths was apparent but was not as distinct as it was in Trial 1. In Trial 2 the final mass was not related to the rate of decrease, as it was in Trial 1. In both trials, experimental units with greater initial manure depths maintained their mass longer than experimental units with shallower depths. Experimental unit 14 (with initial greatest initial depth of 8cm) had a final mass that was 52.8% and 41.9% of the initial mass, in Trial 1 and Trial 2 respectively. Experimental unit 13 (with the shallowest initial depth of 2cm) had a final mass that was 25.9% and 27.5% of the initial mass, in Trial 1 and Trial 2 respectively. This was not the smallest final mass for Trial 2. Experimental units (9, 12, 15 - 20), with larvae that survived 11 to 12 days (3 or 4 days longer than in experimental unit 13), had a final mass that was 25.4% of initial. The average manure mass lost by the 20 experimental units in Trial 2 was greater than the average loss in Trial 1. Average final mass was 39.5% and 31.8% of initial for Trial 1 and Trial 2 respectively (P<0.05).

4.3.2 Response surfaces
Response surface analysis of Trial 1 revealed a saddle point and no ideal combination of the variables, consequently the results and graph are not presented. The ideal values that were recovered were out of the range of the experiment and were therefore not valid points; because of this the least squares regression equation could not be used to predict a minimum value for the response variable. Trends that have been identified for this trial (presented above) are still valid and are compared to Trial 2. The ridge analysis of Trial 1 suggested that the ideal values for the explanatory variables could be found if the experiment were redesigned using similar ranges for initial moisture and seeding rate, but a lower initial depth (around 2cm).
The response surface analysis for Trial 2 revealed stationary points with minimum values and ideal values for each explanatory variable were determined. The minimum final mass (239.3g) could be achieved when the levels for initial moisture content, initial depth, and seeding rate were 77.5%, 2.9cm and 0.82g of eggs per kg of manure respectively. These response surfaces are presented in Figures 10 to 12 where ranges for two of the three explanatory variables are shown and the third is held at its ideal value.

The results of the response surface analysis revealed that both linear and quadratic regressions for initial moisture and initial depth were significant at 5%. The linear regression was significant for the number of eggs, but the quadratic regression was not. The cross-product term for mass of eggs by depth and the lack of fit for initial depth were not significant. All other cross-product terms, lack of fit variables and intercept were significant. The least squares regression equation was:

\[ \text{Final mass} = 3786.79 + (66.99 \times \text{Mass of eggs}) + (-86.78 \times \text{Initial moisture}) \]
\[ + (-144.49 \times \text{Initial depth}) + (69.60 \times \text{Mass of eggs} \times \text{Mass of eggs}) \]
\[ + (-2.59 \times \text{Initial moisture} \times \text{Mass of eggs}) \]
\[ + (0.55 \times \text{Initial moisture} \times \text{Initial moisture}) + (6.85 \times \text{Initial depth} \times \text{Mass of eggs}) \]
\[ + (1.15 \times \text{Initial depth} \times \text{Initial moisture}) + (8.56 \times \text{Initial depth} \times \text{Initial depth}) \]

When the ideal values are used in this equation the estimated minimum final mass would be 239.3g (reduced 76.07% from the initial).
Figure 10: Response surface graph showing minimum final manure mass determined by the least squares regression equation for Trial 2, in which both *M. domestica* and *C. hirtula* converted poultry manure. The graph shows the effects of the initial depth and mass of eggs used to seed the manure when the third variable (initial moisture) is fixed at its optimal value, 77.5%. The least squares regression equation that was used to create these graphs is presented in the Results section of this chapter. The calculated minimum mass is 239.3g (23.9% of initial) and is represented by the lowest point in the graph. The graphs displays ranges outside of what was tested to help show the trends; the response variable at these points is not valid.
Figure 11: Response surface graph showing minimum final manure mass determined by the least squares regression equation for Trial 2, in which both *M. domestica* and *C. hirtula* converted poultry manure. The graph shows the effects of the initial depth and initial moisture content when the third variable (seeding rate) is fixed at its optimal value, 0.82g. The least squares regression equation that was used to create these graphs is presented in the Results section of this chapter. The calculated minimum mass is 239.3g (23.9% of initial) and is represented by the lowest point in the graph. The graph displays ranges outside of what was tested to help show the trends; the response variable at these points is not valid.
Figure 12: Response surface graph showing minimum final manure mass determined by the least squares regression equation for Trial 2, in which both *M. domestica* and *C. hirtula* converted poultry manure. The graph shows the effects of the initial moisture content and mass of eggs used to seed the manure when the third variable (initial depth) is fixed at its optimal value, 2.9 cm. The least squares regression equation that was used to create these graphs is presented in the Results section of this Chapter. The calculated minimum mass is 239.3 g (23.9% of initial) and is represented by the lowest point in the graph. The graph displays ranges outside of what was tested to help show the trends; the response variable at these points is not valid.
4.4 Discussion

Poultry manure has been studied as a larval medium for *M. domestica* by several researchers; however, previous studies have focused on producing puparia for transformation into protein supplement in feed for livestock and the conversion of the manure was secondary. Other studies that have looked at environmental conditions in poultry manure have done so to develop systems to discourage fly breeding in poultry facilities (Axtell, 1986; Achiano and Giliomee, 2005). Most previous studies on poultry manure conversion using *M. domestica* have been small-scale, using around 100g of substrate or less, and have failed to consider multiple environmental factors simultaneously or interactions among these factors (Barnard et al., 1998). Some environmental factors, including initial depth, had not been previously investigated experimentally.

Previous studies of manure conversion have suggested that *M. domestica* larvae can reduce the mass of the poultry manure by 72% (Beard and Sands, 1973; Barnard et al., 1998) and 58% (Calvert et al., 1971). These experiments used various environmental conditions and many studies did not quantify the environmental factors that are shown here to affect the conversion process. The data presented in this thesis show that in experimental units with completely converted manure, the mass was reduced by at least 75% and was comparable to the best results found in some previous studies. Studies that found less of a decrease in mass were focused on larval protein production (not manure conversion) and resulted in heavier manure residue (likely from retention of moisture), and larger larvae and puparia (Beard and Sands, 1973). Larger larvae and puparia may be a result of higher moisture content or lower population density, which would likely hinder complete manure conversion as it is defined in this thesis (Chapter 3). Puparia were recovered from all experimental units when only *M. domestica* was used for conversion (Trial 1). When *M. domestica* and *C. hirtula* were used (Trial 2), no puparia were harvested from experimental units that ended before the tenth day. More experimental
units from Trial 2 reached the fifth stage of conversion than in Trial 1, and the manure in Trial 2 lost mass at a faster rate. The final manure residue from Trial 2 was also lighter and finer than it was in Trial 1. It appeared that higher final manure mass and lower larval population densities resulted in larger, healthier puparia, but interfered with manure conversion.

There has been no prior research into the conversion process using two species simultaneously. Complete conversion was more likely to reach the fifth stage of conversion when *M. domestica* and *C. hirtula* larvae both inhabited the manure. The efficiency of a conversion system is likely to be increased by utilizing both species and *C. hirtula* is usually present in the manure and cannot be easily removed (Bergeron et al., 2012). The environmental conditions that led to efficient conversion using *M. domestica* were different depending on whether *C. hirtula* was present or absent in the manure. If the eventual industrial-scale system ignored the presence of *C. hirtula*, and tried to convert the manure as if *C. hirtula* was not there, it is likely that the optimal conditions would not be used.

The efficiency of Trials 1 and 2 cannot be directly compared because the response surface analysis revealed a saddle point for Trial 1 (no unique minimum value was determined for the response variable) and the optimal values for environmental factors could not be determined. To determine the ideal values for the number of eggs, initial moisture and depth, the experiment would have to be redesigned using different ranges for the explanatory variables because the ridge analysis suggested that a shallower depth would be required to determine the ideal values. Since the experimental unit with the shallowest initial depth did not complete conversion, the current experimental design cannot be used to determine the ideal values for using only *M. domestica* for conversion. Analysis of Trial 2 revealed that the minimum final mass can be achieved by using levels of variables that are in the correct range for the combination of the two species.
The use of *M. domestica* and *C. hirtula* together in a conversion appears to be superior to *M. domestica* alone because the manure was more likely to be completely converted and a lower average final mass was obtained. An industrial-scale system needs to be reliable and not require additional processing after the larvae have pupated. It appears that *C. hirtula* facilitate a greater reduction of mass, possibly because the smaller larvae break the manure into finer pieces, which would increase the surface area and allow components which would make up the mass (moisture, gas, etc) to be removed faster. The nutrients in the manure available to *M. domestica* larvae might be decreased by the presence of *C. hirtula*; however, because *M. domestica* larvae are scavengers (Barnard et al., 1998), they may prey on *C. hirtula* larvae when food resources are low, allowing them to complete manure conversion. Further research on the interactions between these species is needed, including confirmation of whether *M. domestica* larvae are predators of *C. hirtula* larvae. More research is also needed on *C. hirtula* alone, as there is currently no method to evaluate the population density of the larvae (as discussed in Chapter 2).

### 4.5 Conclusion

The results of this study suggest that the explanatory variables influence larval development and manure conversion in complex ways and should be investigated together. The response surface methodology allowed quantification of the effects of the explanatory variables (initial moisture content, initial depth and mass of eggs used to seed the manure) on the response variable (final manure mass). The resultant model can be used to predict and approximate poultry manure conversion using both *M. domestica* and *C. hirtula* larvae together by predicting the final mass after conversion. Analysis revealed that using both species resulted in a valley (distinct minimum in the response variable), while using only *M. domestica* resulted in a saddle point (no distinct minimum value in the response variable). The lower final mass and more frequent complete conversion suggested that using *M. domestica* and *C. hirtula* together led to
a greater degree of manure conversion than using *M. domestica* alone. It was concluded for these reasons that poultry manure should be converted through the simultaneous use of both *M. domestica* and *C. hirtula*. The results of the response surface analysis revealed that the lowest final mass (239.3g, a reduction of 76%) could be achieved at initial moisture level of 77.5%, an initial depth of 2.9cm and an egg-seeding rate of 0.82g of eggs per kg of manure. Final mass should be at the lowest levels possible in converted manure; however, heavier manure will result in larger larvae and puparia. If these results were to be used in a conversion system, a compromise between these two goals (converted manure and amount of pupal protein) would have to be made because they would require different initial conditions.
Chapter 5: Summary

5.1 Summary

Four species (Musca domestica, Hydrotaea aenescens, Coproica hirtula and Fannia canicularis) were considered for use in an industrial-scale poultry manure conversion system. Any species used in such a system will have to produce a sufficient number of eggs daily in order to convert the large amount of manure that is produced at a commercial poultry farm. It is essential that the species can be easily mass-produced and maintained on site by farm workers with little experience rearing flies, so the techniques used to sustain the colony and collect eggs must be simple. A large stabilized colony is preferred for the system because the quantity and timing of egg production will be predictable, allowing the implementation of a routine based on the protocol used to rear flies.

Several generations of M. domestica, H. aenescens and C. hirtula were successfully maintained in large laboratory populations and protocols (based on published instructions) and timelines for mass-production were developed. The protocols for M. domestica and H. aenescens are similar and require daily maintenance and observation, and the protocols give specific details on what steps are required and when they need to be completed. Both these species can be stabilized, at which time egg production can be predicted. Colonies of C. hirtula are not as easily monitored because of their small size and short life cycles; however, colonies of C. hirtula required the least amount of maintenance. Colonies do not require additional food other than manure, and continue to produce eggs even after a month of neglect. The short lifecycle of this species (6 days) complicates its stabilization, as it was difficult to separate individuals of a given age. The difficulty of mass-producing F. canicularis in the laboratory using poultry manure as larval medium prevented further investigation of this species.

To confirm that M. domestica, H. aenescens and C. hirtula were able to convert poultry manure, observations were made on the larval development in manure. The time required for pupation
and for complete conversion of manure were determined for each species. Changes in the manure were observed and distinct stages of conversion were identified. There was previously no method of evaluating the conversion process so a subjective division of the process into “stages of conversion” was defined and utilized. Division into stages was based on the volume, mass and texture of the manure residue, as well as the behavior of the inhabiting larvae and the day conversion ended. Five stages, from fresh manure to complete conversion (stage 5) were used to assess the decomposing ability of the larvae.

Manure converted by *C. hirtula* larvae remained in the first or second stage of conversion even after being inhabited by several generations of larvae. The manure lost some mass, but true conversion, as defined by a fine loose texture, homologous composition and no remaining larvae, was never observed. Manure was completely converted by *M. domestica* and *H. aeneszens* larvae when environmental conditions allowed for it. The conversion process was slower for *H. aeneszens* and the manure residue produced was darker in colour and had a higher final mass than manure that was converted by *M. domestica*. The larval period of both species was altered by varying the environmental conditions. Changing the initial depth, moisture and population density affected larval development time and affected complete conversion of manure. Environmental conditions were interdependent and complete conversion was only achieved at a specific moisture content, if an appropriate number of eggs were used to seed manure at an appropriate depth. If manure was too deep, the manure would not lose enough mass to be considered completely converted; if manure was too shallow, mass was lost too fast and the larvae would not pupate. Many such interactions between initial moisture content, initial depth and the amount of eggs used must be considered in an industrial system.

The conversion process was also observed when more than one species inhabited the manure. The population of *C. hirtula* in the manure was extirpated in a few days; when *H. aeneszens* larvae were also present. On the other hand, the combination of larval *M. domestica* and *C.
*hirtula* had a synergistic effect on conversion; moisture was removed faster and the fifth stage of conversion was easier to achieve because imperfect environmental conditions had less of a negative effect. This combined effect is important as *C. hirtula* are often present in fresh manure and will either have to be removed or the conversion system will have to accommodate the presence of this species.

Other biological aspects for each species were also considered before more quantitative testing (response surface experimentation) was completed. Species were evaluated based on their biology, ease of mass-rearing and manure conversion ability. On the basis of these criteria *M. domestica* was confirmed as the best candidate for an industrial-scale system. It is the most efficient at converting manure and can be mass-produced and stabilized faster and with less effort than *H. aenescens*. The synergy with *C. hirtula* presents a potential benefit to an industrial conversion system.

A response surface experiment was employed to determine the optimal levels of the three explanatory variables: initial manure moisture content, depth of manure and the number of eggs needed for conversion. The experimental design allowed all three variables to be evaluated simultaneously at 5 levels each with the fewest experimental units as possible. The number of eggs the adult colony produced was a limiting factor and using fewer experimental units meant each individual unit could be larger. Two trials were conducted, one using only *M. domestica* (Trial 1) and the other using both *M. domestica* and *C. hirtula* together (Trial 2). More experimental units reached the fifth stage of conversion in Trial 2; however, more puparia were recovered from Trial 1 and conversion was completed sooner when it occurred. On average, more mass was lost in the experimental units of Trial 2, which might have led to premature larval death in units in which depth was too shallow. In experimental units where conversion was completed, many large puparia were recovered. Larvae only failed to pupate in experimental units that did not complete conversion, but not all experimental units that did not
complete conversion failed to produce puparia. To minimize the final mass of the manure when using *M. domestica* and *C. hirtula*, the least squares regression equation suggests that the initial moisture content, initial depth and seeding rate should be 77.5%, 2.9cm and 0.82g of eggs per kg of manure, respectively. No ideal values were determined for Trial 1 but the trends were consistent with the results from Trial 2. The experiment would have to be redesigned in order for ideal values to be determined for *M. domestica* alone, according to the ridge analysis that was performed.

### 5.1.1 Species best suited for industrial poultry manure conversion

Selection of the appropriate species for an industrial-scale poultry manure conversion system needs to be based on the consideration of several factors. The system using both *M. domestica* and *C. hirtula* to convert manure is superior to that of using *M. domestica* alone. Both species can be easily cultivated and eggs can be produced regularly. The greatest concern is that *M. domestica* is a notorious pest in poultry facilities and is known to spread a number of pathogens. This cannot be taken lightly as many commercial poultry facilities are located near rural subdivisions and other commercial and non-commercial farms where livestock are present (Lapping et al., 1983; Axtell, 1986). Farmers using *M. domestica* to convert manure could be blamed for health problems caused by naturally occurring populations of this ubiquitous pest or regarded as a source of flies, if the system were mismanaged (Beard and Sands, 1973).

The most promising species to use in a manure conversion system is *M. domestica*, with or without *C. hirtula*. Using *M. domestica*, manure could be quickly and completely converted into a dry, odourless fertilizer that can be marketed year round to the general public. The puparia that are also produced can be transformed into a nutritious protein supplement that can be fed to a variety of commercial animals. The short lifecycle of *M. domestica* can be easily predicted and events such as oviposition are easily stabilized after a few generations. Despite the advantages over other the species investigated, in order to use *M. domestica* in an industrial poultry manure
conversion system a method to eliminate escape must be developed. This could be accomplished by developing a stable flightless strain of the species that would perform in a similar manner as the wild-type (discussed further in section 5.2.2). Another solution would be to alter the puparia so that they will not emerge; however, any changes must only apply to the larvae used in conversion as the breeding population would have to survive to continue the next generation. Currently there is no method that would be appropriate at an industrial scale to limit emergence and a suitable flightless strain does not exist. More research on *M. domestica* should be conducted before this species is used as a poultry manure converter at an industrial scale.

### 5.2 Future considerations

#### 5.2.1 Response surface experiment with *Hydrotaea aenescens*

The use of *M. domestica* in a conversion system may put the farm and neighboring communities at risk of an infestation of this pest species. A solution would be to use a non-pest species that can be easily mass-produced and efficiently converts manure, such as *H. aenescens*. The lower efficiency and higher maintenance costs relative to *M. domestica* may be outweighed by the benign reputation of this species that rarely causes health or nuisance problems. Commercially produced *H. aenescens* are currently released in poultry facilities to reduce *M. domestica* populations, apparently without causing pest problems for farms or neighboring communities. Employing the same response surface experiment with explanatory variables (environmental factors) relevant to this species would allow a direct comparison between *H. aenescens* and the results reported here for a system involving *M. domestica* and *C. hirtula*.

#### 5.2.2 Flightless strain

A possible solution to the problems associated with *M. domestica* would be to produce a flightless strain of this species. Currently available flightless strains of *M. domestica* revert to the wild-type, are often nearly blind and are not as hardy as wild type strains (Springhalen,
Denmark, pers. comm.; Beard and Sands, 1973). These traits limit their use in a conversion system. Genetic modification and selective breeding of a wild type strain could produce a stable flightless strain, but that would require significant effort (A. Hilliker, pers. comm.). The uses for a flightless strain would not be limited to a conversion system. Any research using *M. domestica* would be simplified by the existence of a flightless strain that otherwise behaved like the wild type. Flies would be more easily handled and the possibility of escape would be minimized. A flightless strain would be useful to any animal breeders that use *M. domestica* as a food source. Captive animals, such as lizards, require live food; *M. domestica* is not a food of choice because it is difficult to handle and escapes before it can be eaten, but *M. domestica* are otherwise a nutritious food source. A flightless strain could be marketed to animal breeders so that they could rear them to use as feed, which would be another potential value-added product.

### 5.2.3 Conversion of other wastes

Once a poultry manure conversion system using *M. domestica* or another species is developed, the system could be altered to convert other organic wastes. The development of new systems could follow the same process that was implemented in this study to investigate poultry manure conversion. The largest change would be the use of a different species preadapted to deal with the kind of waste to be converted. Second to manure, the accumulation of dead birds is the largest problem on commercial poultry farms (Collins et al., 1999). Broiler chickens and chickens that die naturally cannot be sold for human consumption and their remains must be disposed of. A very similar disposal system could be designed using a species with larvae adapted to live in and decompose flesh, such as *Phormia regina* or other sarcosaprophagous calliphorids. As in the current system, a response surface experiment could be employed to test the conversion ability of the larvae and determine the ideal levels for the important environmental factors. These factors will depend on the material being converted and the species being used. Several other organic wastes that could be converted via fly larvae include
coffee grounds, plant matter that is left over after harvest stops, and food scraps from restaurants. All of these wastes can be quickly recycled into valuable products instead of ending up in landfills or being disposed of using slower conversion and composing technologies.
References


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