Modelling the Thermodynamics of Maggot Masses during Decomposition

by

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Abstract

Estimating the minimum PMI (mPMI) based on larval age involves identifying the species, reconstructing the thermal history at a crime scene, and modelling the rate of development. However, few studies take into consideration the mass-generated heat produced by larvae co-existing in an aggregation. These localized increases in temperature are often highlighted in the literature as having an influence on larval development, but there are ongoing difficulties with incorporating this concept into mPMI estimates. This is mostly due to a lack of research on the topic, particularly with controlled laboratory experiments or in natural conditions simulations. The aim of this research was to determine whether heat generation varied in different sized aggregations and, if so, did it influence larval development and behaviour.

Various sized aggregations (50-2500 larvae) composed solely of *Lucilia sericata* (Meigen, 1826)(Diptera: Calliphoridae) larvae were reared in the laboratory at a constant ambient temperature of 22 °C (±1 °C). Data loggers and a thermal imaging camera were used to record mass temperatures throughout the feeding stage of development. Larvae were sampled from these different sized aggregations at set times and had their instar determined and/or their lengths and fresh weights recorded so that developmental rates could be monitored. To investigate the movement of larvae as they fed in an aggregation, individuals were tagged with a fluorescing elastomer. These larvae were easily distinguished from the rest of the cohort, which allowed their positions within the mass to be recorded at regular intervals.

The results showed a strong positive relationship between mass size and the amount of heat generated by the aggregation ($p<0.001$), with temperatures rising as masses increased in size. A minimum mass size of 1200 larvae was required for the local temperature to increase significantly above ambient, with aggregations of 2500 larvae producing temperatures that exceeded ambient by up to 14 °C (± 1.2 °C). Larvae sampled from increasingly large masses showed an accelerated rate of development during the 2nd and 3rd instar. This coincided with when masses were at their warmest. These faster growth rates resulted in larger aggregations entering the post-feeding phase of development an average of 13 hours earlier than smaller, cooler masses. Physical measurements taken from larvae at 70 hours development demonstrated that individuals sampled from larger masses were significantly longer and heavier than those sampled from smaller aggregations ($p<0.001$). This provided further evidence of faster growth rates. However, when compared to solitary larvae, all mass-reared larvae, regardless of the size of the aggregation, appeared to benefit from a faster rate of development, reduced mortality and larger body sizes at dispersal. Larvae were observed to be in a constant state of motion and continually repositioned themselves within the mass, rotating between the periphery and the centre where they presumably fed.

The thesis highlights the need to incorporate mass temperatures into forensic casework when using larval development to estimate the time of death. Larvae sampled from large masses, particularly during the 3rd instar, could appear older than they actually are due to the accelerated rates of development experienced under warmer conditions. If this isn't taken into consideration then it could result in an overestimation of the mPMI. Future research should focus on identifying

how other variables influence heat generation in masses, as well as finding ways to estimate the size of a mass, and hence its thermal history, at a crime scene.

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Abbreviations

ADD	Accumulated degree days
ADH	Accumulated degree hours
CTRL	Control sample
hsp	Heat shock protein
LDTT	Lower developmental threshold temperature
mPMI	Minimum Post-Mortem interval
PMI	Post-Mortem Interval
RH	Relative humidity
VIE	Visible Implant Elastomer

1 Introduction

The purpose of this research project was to investigate heat generation in aggregations of necrophagous blowfly larvae (Diptera: Calliphoridae) and its effect on development and behaviour. Maggot masses are defined in the literature as an assemblage of feeding larvae in which the metabolic heat raises the temperature of the immediate surroundings above ambient (Huntington *et al*., 2007). It has been proposed on numerous occasions that the temperature of a mass is related to its size, or volume, with masses composed of several thousand larvae reaching highs of 45-50 °C (Charabidze *et al*., 2011; Deonier, 1940; Gallagher *et al*., 2010; Marchenko, 2001; Richards & Goff, 1997; Richards *et al*., 2009; Slone & Gruner, 2007). Despite the fact that these aggregations are often reported to be several degrees warmer than ambient, their influence on larval development and behaviour is rarely studied in any detail. Blowflies are poikilothermic, which means their development is temperature dependent. Therefore, one might assume that if the temperature of the aggregation differs significantly from ambient, then the developmental rates experienced by larvae in that mass might be different from those predicted using the ambient temperature alone (Williams, 1984). For example, if the mass increased the local temperature to an optimum level, then the rate of larval development would also increase. With individuals becoming larger sooner, entomologists might mistake them for older larvae, which could lead to an overestimation of the post-mortem interval (PMI).

1.1 The Importance of this Research

Using inappropriate or unsubstantiated data to produce potentially inaccurate PMI estimates could have major implications for forensic casework. The Daubert standard provides a rule of evidence regarding the admissibility of expert witnesses' testimony during United States federal legal proceedings (Tarone & Foran, 2008). Therefore, assessing the validity, and thus admissibility, of scientific evidence has become common practice for forensic cases. In 2002, the well-publicized kidnapping and murder case of seven-year old Danielle Van Dam illustrated the need for validation studies of PMI estimates based on entomological evidence. At the trial of David Westerfield (People v. Westerfield) four renowned forensic entomologists all provided minimum PMI estimates based on slightly different methods that either overlapped or came within four days of overlapping (VanLaerhoven, 2008). This suggests that despite forensic entomology being accepted in courts, there is a growing need to develop standard operating procedures for casework, which would increase precision in mPMI estimates and mitigate against criticism, as well as reducing the risk of miscarriages of justice.

To be able to increase accuracy in mPMI estimates, it is imperative that the conditions at a crime scene, including the thermal history experienced by developing larvae, are accurately replicated in controlled conditions (Pai *et al*., 2007). The presence of a maggot mass is indicative of a variable thermal history. However, even though mass-generated heat is often highlighted as having an influence on the larval development rates (Amendt *et al*., 2011; Campobasso *et al*., 2001; Catts, 1992; Charabidze *et al*., 2011; Goodbrod & Goff, 1990; Huntington *et al*., 2007; Ireland & Turner, 2006; Marchenko, 2001; Slone & Gruner, 2007), there are on-going difficulties with incorporating this concept into PMI estimates.

Expert witness reports and published case studies often mention larval aggregations and might ask the court to bear mass-generated heat in mind (Amendt *et al*., 2000; Anderson, 1999; Arnaldos *et al*., 2005; Benecke, 1998; Introna *et al*., 1998; Pai *et al*., 2007; Vanin *et al*., 2008). But worryingly, some research papers acknowledge there is a difference between mass and ambient temperatures yet continue to report results with only the ambient temperatures included in calculations, claiming that mass temperatures before the discovery of the carcass are not available to law enforcement (Michaud & Moreau, 2009). This brings into question the reliability of the results.

1.2 The Current Research Project

Despite awareness increasing, there still remains a distinct lack of research that models the thermodynamics of maggot masses, particularly with controlled laboratory experiments. The few papers that have been published on the subject have successfully recorded the temperatures of masses both out in the field (Cianci & Sheldon, 1990; Deonier, 1940; Richards *et al*., 2009; Slone & Gruner, 2007) and in smaller scale laboratory experiments (Charabidze *et al*., 2011; Greenberg, 1991; Huntington *et al*., 2007). But, whilst there is something to learn from each of these studies, many of them neglected to investigate whether this mass-generated heat influenced larval development or behaviour. This research project will help bridge that gap by tracking temperatures in various sized aggregations whilst monitoring larval development rates, body sizes and feeding behaviour. This will be achieved by running a series of controlled experiments in the laboratory using single species masses and acquiring quantitative data that can be tested statistically to identify any significant differences. These experiments were designed after an extensive

review of the literature, which helped identify appropriate techniques whilst flagging unsuitable ones. Original elements were conceived to address the specific aims of the research, and experiments were designed to be reproducible so researchers could use them to study other species of forensic importance in future studies. If successful, this research could aid casework by highlighting instances where mass temperatures should be factored into mPMI estimates in order to increase their accuracy.

1.3 Aim and Specific Objectives

The aim of this research project is to determine whether heat generation varies significantly between different sized maggot masses and, if so, does it influence larval development and behaviour. It is hypothesized that as the size of the mass increases, the localized temperature will also increase. These larger, warmer masses might cause larvae to develop at an accelerated rate, allowing them to reach the post-feeding stage of development earlier in comparison to individuals reared in smaller, and hence cooler, aggregations. By conducting a series of controlled experiments, each one with a specific objective, it should be possible to further our understanding on the topic of mass thermodynamics. Specific objectives are:

- To record temperatures in different sized maggot masses during the feeding phase of larval development
- To determine whether the amount of heat generated by the mass influences the rate of larval development

- To investigate whether mass size, and hence temperature, influences the size of larvae
- To document the development of larvae feeding in an isolated environment
- To investigate the movement of larvae through a mass

1.4 Outline of the Thesis

Following this introduction, a comprehensive review of the literature will be detailed. This will describe how time of death is estimated using larval development and what factors should be considered when making these calculations. The chapter then goes on to focus on the cause and effect of maggot masses, detailing what they are, the benefits for larvae feeding in them, and what variables might influence the temperatures they produce. The subsequent chapter discusses the experimental design and methodology that is common to all or the majority of the experiments. It outlines the equipment used, the rearing conditions in the laboratory, how masses were set up, and provides information regarding the life history of the studied blowfly species, *Lucilia sericata*. Following the methodology, six chapters will be presented, each one focusing on an individual experiment and formatted to include a separate introduction, methodology, results and discussion section specific to that experiment. The final chapter will offer a general discussion, which will sum up the outcome of the research, the implications of the findings and make suggestions for future research projects.

2 Aspects of Forensic Entomology and Larval Masses

Forensic entomology is the application and study of insect evidence and other arthropod material to forensic matters. Whilst the discipline only truly gained momentum during the 1960's, it is by no means a new science. The use of insects in forensic investigations can be traced back to 13th century China, and the publishing of a training manual titled *The Washing Away of Wrongs* (Benecke, 2001). The book, written in 1247 by a high ranking judicial administrator named Sung Tz'u, relates a murder in a rural village where a male victim was found with numerous slash wounds to his head. By lining up the men of the village with their farming tools, Sung Tz'u was able to identify the perpetrator as the owner of the sickle attracting flies.

Subsequently there were few reports of arthropods being utilised in criminal cases until the mid 19th century. In 1848, Orfila, a European pathologist, listed 30 insects and other arthropods that were observed to oviposit and feed on a corpse. He reported that flies were the first to arrive at carrion and were later succeeded by beetles, bringing to light the process of succession (Benecke, 2001). Shortly afterwards in 1855, the French physician Bergeret d'Arbois became the first person to apply forensic entomology a case. He successfully used his knowledge of insect succession to determine the post-mortem interval of mummified juvenile remains discovered behind a mantelpiece in a house in Paris. His findings directed the criminal investigation away from the French couple who owned the property at the time, and toward the previous tenants, who were eventually found guilty of murder (Catts & Goff, 1992). In 1881, Hermann Reinhard, a medical doctor from Germany, carried out the first systematic study in forensic entomology. He

exhumed a number of dead bodies in East Germany and demonstrated that the development of different species of insects was related to the condition of the remains. But it wasn't until the late 19th century, after Megnin observed specific insects were associated with different stages of decomposition, that the concept of forensic entomology became established. Jean Pierre Megnin (1828-1905) was an army veterinarian who reported that not only were flies were the first insects to colonise a body, but exposed corpses were subject to successional waves of faunal colonisation (Catts & Goff, 1992). His published work described how exposed corpses were subjected to eight successional waves of insect colonisation, whilst buried bodies only experienced two waves.

Nowadays, insect evidence collected from a crime scene has the potential to link suspects to a victim or location (Catts & Goff, 1992), indicate whether or not a body has been moved (Goff, 1991), provide specimens for toxicology (Greenberg & Kunich, 2002), identify a missing body (Marchetti *et al*., 2013), signpost potential cases of neglect (Benecke, 1998; Benecke & Lessig, 2001; Benecke *et al*., 2004) and assist in apprehending poachers (Anderson, 1999). However, forensic entomology is most commonly applied to cases involving a deceased individual, be it murder, suicide, accidental or sudden death, where its primary purpose is to estimate or strengthen the post-mortem interval (PMI) (Anderson, 1997; Arnaldos *et al*., 2005; Benecke, 1998; Catts & Goff, 1992; Goff & Flynn, 1991; Pai *et al*., 2007; Pohjoismäki *et al*., 2010; Pujol-Luz *et al.,* 2006; Vanin *et al*., 2008).

2.1 Determining the Post-Mortem Interval

An accurate estimation of the post-mortem interval is of primary importance in many death investigations, since it focuses the investigation on the correct time frame, providing evidence that can support or refute an alibi (Introna *et al*., 1998). In forensic entomology the minimum post-mortem interval (mPMI) is estimated using insect evidence as opposed to the PMI. The mPMI is different to the PMI since it only accounts for the period of time eggs or larvae were present on the corpse and not the interval between death and initial colonization, which can be influenced by various factors to be discussed later in this chapter. For the first 72 hours following death, regular methods such as the decline in body temperature or the extent of rigor mortis, are of equal or greater accuracy in determining the PMI (Amendt *et al*., 2011). After that initial three-day window, however, insect evidence becomes the most accurate and sometimes the only method of determining time since death (Amendt *et al*., 2011; Martinez *et al*., 2007; Sharma *et al*., 2013). When insects arrive at a corpse, often within minutes of death, a biological clock is started which provides the basis for estimating the mPMI. Time of death can be estimated to some degree of accuracy by using one of two methods outlined below.

2.1.1 mPMI based on insect succession

For cases where an individual is recovered during the later stages of decomposition, a minimum PMI can be estimated using the concept of insect succession (Amendt *et al*., 2000; Catts & Goff, 1992; Goff & Flynn, 1991; Martinez *et al*., 2007; Sharma *et al*., 2013). Carrion is a temporary and rapidly changing ecological resource, which is capable of supporting a large, dynamic arthropod

assemblage, yet one which puts limitations and restrictions on colonizing larvae (Grassberger & Frank, 2004; Putman, 1977; Richards & Goff, 1997; Shiao & Yeh, 2008). As this microhabitat decomposes, it undergoes a series of biological, chemical and physical changes, which provide a progressively changing food source to a wide variety of organisms (Early & Goff, 1986; Lopes de Carvalho & Linhares, 2001; Rodriguez & Bass, 1983; Segura *et al*., 2009). Since different insect species have ecological and biological preferences, they colonize carrion in waves, with each species being associated with a specific stage of decomposition (Anderson & VanLaerhoven, 1996; Campobasso *et al*., 2001; Cianci & Sheldon, 1990; Early & Goff, 1986; Leclercq, 1978; Martinez *et al*., 2007; Matuszewski *et al*., 2008; Megnin, 1894; Payne, 1965; Rodriguez & Bass, 1983). Therefore, by having an understanding of insect successional patterns, it is possible to use the presence or absence of a particular insect species as an indicator of time of death.

2.1.2 mPMI based on insect development

The second method involves considering the degree of development exhibited by insects collected from a body. Whilst insect succession is useful in estimating the mPMI during the later stages of decomposition, it is the aging of individual insect larvae that can yield more accurate estimates during the initial three to four weeks following death (Catts & Goff, 1992; Introna *et al.,* 1998; Sharma *et al*., 2013; VanLaerhoven, 2008). Since this research project focuses on how larval masses influence development, emphasis will placed on this method rather than insect succession. A detailed knowledge of blowfly development under various environmental conditions and temperature regimes is imperative if Calliphoridae are to be accurately aged and used effectively as tools in estimating the mPMI. The following sections will therefore describe the blowfly life cycle, paying particular

attention to the larval stage. Whist numerous factors have been shown to influence larval development (e.g. photoperiod, food availability) this thesis will concentrate on discussing the importance of temperature, and how the two (thermal history and larval development) are incorporated in time of death estimates.

2.2 An Introduction to the Biology of Calliphoridae

Calliphoridae, or blowflies, are recognised amongst forensic entomologists as a valuable source of evidence in forensic investigations owing to their relatively predictable behaviour and worldwide distribution (Campobasso *et al*., 2001; Kashyap & Pillay, 1989). Since carrion represents a temporary and changing food source, blowflies have adapted to enhance their success on a discrete and exhaustible resource. This includes having a short life cycle and large reproductive potential (Levot *et al*., 1979; Putman, 1977). For example, at temperatures of 21.8 °C *Lucilia illustris* can complete the larval stage of development in as little as 136 hours (Anderson 2000) whilst Byrd and Allen (2001) demonstrated that *Phormia regina* required as few as 8.8 days (211 hours) at 35 °C for adult emergence. Females will typically lay up to ten egg batches during their lifetime, with each batch composed of 150-250 eggs dependent on the species.

2.2.1 Locating carrion and oviposition

The general consensus is that blowflies are amongst the first insects to arrive at carrion. Under the certain conditions (i.e., warm temperatures, exposure to sunlight, unrestricted access to the body) species such as *Calliphora vicina*, *Calliphora vomitoria* and *Lucilia sericata* will arrive at a corpse within minutes of

its exposure (Campobasso *et al*., 2001; Cianci & Sheldon, 1990; Grassberger & Frank, 2004; Introna *et al*., 1998; Levot, 2003; Lopes de Carvalho & Linhares, 2001; Mann *et al*., 1990; Marchenko, 2001; Martinez *et al*., 2007; Morton & Lord, 2006; Nabity *et al*., 2006; Payne, 1965; Reed, 1958; Richards & Goff, 1997; Rodriguez & Bass, 1983; Shean *et al*., 1993; Slone & Gruner, 2007; Turner & Howard, 1992; VanLaerhoven, 2008). In rural north-central Florida, adult Calliphorids have even been observed on pig carcasses within 30 seconds of their placement outdoors (Gruner *et al*., 2007). Blowflies will remain dominant during the early stages of decomposition, with large numbers of necrophagous larvae present during the bloated and early decay stages (Bharti & Singh, 2003; Byrd & Castner, 2010; Goff, 1991; Grassberger & Frank, 2004; Grassberger & Reiter, 2002b; Introna *et al*., 1998; Levot, 2003; Lopes de Carvalho & Linhares, 2001; Martinez *et al*., 2007; Morton & Lord, 2006; Nabity *et al*., 2006; Payne, 1965; Reed, 1958; Richards & Goff, 1997; Segura *et al*., 2009; Sharanowski *et al*., 2008; Shean *et al*., 1993; VanLaerhoven, 2008).

Female flies have been reported to greatly outnumber males on carrion, with 8.8 females being recorded for every male (Reed, 1958). A study carried out by Ashworth and Wall (1994) on the responses of *Lucilia sericata* (Diptera: Calliphoridae) to odour reported that 75% of *L. sericata* caught on carrion baited traps were identified as female, with gravid females being four times more likely to be present than virgin females. These adult flies locate carrion using the appropriate chemical (volatile sulphur and ammonia compounds – a product of animal decomposition) and environmental cues as detected by the sense organs found primarily on the antenna (Ashworth & Wall, 1994). This then sets off a chain of behaviours, which ultimately leads to the fly landing and ovipositing on

the corpse (Ashworth & Wall, 1994; Byrd & Castner, 2010; Cragg & Thurston, 1950). The timing of oviposition depends on whether the female landing on carrion is a gravid female. Females may locate a corpse within minutes, but unless they have recently consumed a protein meal, which is required for their ovaries to develop, it could be an extra week until they are able to oviposit (Greenberg & Kunich, 2002; Mackerras, 1933). The timing of oviposition also varies between and within species (O'Flynn, 1983). For example, several *Calliphora* and *Lucilia* species have been reported ovipositing within 24 hours of corpse exposure, whilst others such as *Lucilia illustris* require less than an hour (Greenberg & Kunich, 2002). *Chrysoma rufufacies* have been observed ovipositing on fresh carcasses on some occasions, but at other times they will delay egg laying until the carrion is in a more advanced stage of putrefaction (O'Flynn & Moorhouse, 1979).

The females of some species (e.g. *Lucilia sericata, Lucilia cuprina*) are capable of laying up to 200-300 eggs per batch (depending on adult size, nutritional status and species), repeating this process three or four times during their lifetime (Ashworth & Wall, 1994; Greenberg, 1991; Greenberg & Kunich, 2002). Large mounds of eggs are often observed on carcasses exposed outdoors, a result of female flies having a tendency to oviposit near other females, regardless of the species (Barton Browne *et al*., 1969). These gravid females prefer to oviposit at moist sites and will target areas with low light intensity and easy access to the interior of the corpse, such as natural bodily orifices like the ears, nose, throat, eyes and anus, as well as open wounds (Amendt *et al.,* 2011; Campobasso *et al*., 2001; Cross & Simmons, 2010; Deonier, 1940; Lopes de Carvalho & Linhares, 2001; Putman, 1977).

2.2.2 Larval feeding

Eggs will hatch after approximately 6-40 hours, depending on the species and the ambient temperature (Campobasso *et al*., 2001), with larvae from the same batches hatching within minutes of one another (Greenberg & Kunich, 2002). The pointed anterior and blunt posterior ends provide larvae with a body shape that makes them efficient burrowers and able to penetrate carcasses within minutes of hatching (Greenberg, 1991). By burrowing into the underlying tissues and cavities of the carcass, larvae are afforded protection from desiccation, temperature fluctuations, predators, parasites and any sudden and heavy downpours (Greenberg & Kunich, 2002).

Since soft tissues on carrion are only available for a matter of days, larvae have adapted to feed efficiently and develop rapidly (Hückesfeld *et al*., 2010; Levot *et al*., 1979). This is reflected in the rapid decompositional weight loss of insect colonized cadavers (Greenberg & Kunich, 2002; Putman, 1977). For example, a pig carcass colonised by insect larvae can lose 90% of its original weight within six days whilst a carcass with restricted insect access requires 100 days for 80% weight loss (Payne, 1965). Starved larvae are able to fill their crops within two to three minutes as they use a highly variable feeding cycle. This consists of periods of vigorous feeding followed by less intensive cycles that are occasionally interrupted by "probing" behaviours (Schoofs *et al.,* 2009). Feeding consists of two phases. The first involves external digestion, where tissues are liquefied with mechanical maceration by the mouthparts and externally secreted digestive enzymes (Goodbrod & Goff, 1990; Green *et al*., 2003; Hobson, 1932; Schoofs *et al*., 2009). The second phase is internal digestion, which involves the hydrolysis and

absorption of digested food, aided by the rhythmic movements of the pharynx and oesophagous (Schoofs *et al.,* 2009).

2.2.3 Larval growth and metamorphosis

Larvae will commence this feeding behaviour whilst progressing through three larval instars, each one separated by a moult of the outer cuticle that enables further growth. The plasticity of the cuticle accommodates a ten-fold increase in size, with larvae growing from approximately 2-4 mm in the 1st instar to 15-22 mm in the 3rd larval instar (Byrd & Castner, 2010; Greenberg, 1991). It has been suggested that larvae feed voraciously during all three instars until they reach maximum length during the 3rd larval instar (Greenberg & Kunich, 2002; Sharma *et al*., 2013). The amount of time taken for 3rd instar larvae to reach this maximum size varies among species and is heavily influenced by the temperature and conditions such as food type, photoperiod, humidity and overcrowding (Levot *et al*., 1979). Once larvae reach this maximum size they cease feeding and disperse away from the carrion in order to pupate, their movement coordinated by a wave of telescopic segment contractions travelling along the larval body in a posterior to anterior direction (Hückesfeld *et al.,* 2011).

2.2.4 Dispersal and pupariation

The migratory stage of development varies between species, with some, like *Calliphora vomitoria* and *Lucilia caesar,* dispersing exclusively at night; a behavioural advantage that could minimise interactions with predators (Kocarek, 2001). Some species of post-feeding larvae can wander for several days in search of a suitable place to pupate, covering distances of up 34.5 m (Greenberg & Kunich,

2002), whilst others complete the process on or very close to the carcass on which they fed. As they wander, their length decreases, as the crop slowly discharges its contents into the digestive tract. The extent of this decrease is related to the species and the distance travelled (Greenberg & Kunich, 2002).

Migrating larvae use temperature and light sensitive receptors to locate a suitable site for pupariation, usually in cool and shaded areas. At this point they will cease wandering and contract their anterior and posterior ends. The cuticle then hardens into a rigid dark puparium, which houses the individual for several days whilst it metamorphoses into an adult fly. Depending on the temperature flies will emerge after approximately seven to ten days. The adult fly will push the cap (operculum) off the puparium using the ptilinum, which resembles an inflated airbag located on the insect's head. The adult is then able to push its way out of the puparial case, emerging with a tiny, dull coloured abdomen, shrivelled wings and spidery legs. A few hours after emergence the wings and abdomen expand whilst the cuticle hardens and its colour sets (Greenberg, 1991). The number of adults emerging from carcasses is estimated to be as low as 17.5% of the initial larval population (Putman, 1977).

2.3 Blowfly Development and Temperature

A key observation regarding insect development is that its timing depends on temperature (Higley & Haskell, 2010). Blowflies in all of their developmental stages are poikilothermic, meaning body temperature is governed by the ambient temperature, and the warmer the conditions, the faster the metabolic rate and hence the faster the rate of development (Ames & Turner, 2003; Anderson, 2000;

Byrd & Allen, 2001; Byrd & Butler, 1997; Catts, 1992; Cianci & Sheldon, 1990; Davidson, 1944; Grassberger & Reiter, 2002a; Grassberger & Reiter, 2002b; Hückesfeld *et al*., 2011; Nabity *et al*., 2006; Niederegger *et al*., 2010; Richards *et al*., 2008; Slone & Gruner, 2007). This temperature dependent growth rate in blowfly larvae is the driving force behind this research on heat generation in larval aggregations. Understanding the effect temperature has on development will help explain how the presence of a mass might influence any mPMI estimates based on larval age.

Larvae possess temperature receptors on their cephalic lobes, which are involved in thermal stimulation (Hückesfeld *et al*., 2011). Temperature sensation allows animals to detect thermal changes and alter their behaviour accordingly. In larvae, it can affect their behaviour in two ways. In the short-term it causes a reflex type reaction, which allows individuals to avoid coming into contact with extreme temperatures (Hückesfeld *et al*., 2011). In the long-term it promotes faster development by increasing the metabolic rate, which in turn encourages larvae to increase their food intake (Hückesfeld *et al.,* 2011; Rivers *et al*., 2010). Greenberg and Kunich (2002) reported that temperature influenced the rate at which food was observed to move through the larval gut. At 23 °C they found that gut motility ran at 1-2 mm/min with a food transit time of 65 minutes from mouth to anus. But at 31 °C motility rate increased threefold, with food taking only 20 minutes to move through the digestive system.

The relationship between the rate of development and temperature is described by the temperature development curve, which shows the relationship to be curvilinear at high and low temperatures and linear between (Figure 2.1) (Higley

& Haskell, 2010). This development curve exists for all insect species, although the specifics of the curve will vary among species. Within this temperature range constricted by the upper and lower temperature limits, the duration of development decreases in a linear manner with increasing temperatures (Ames & Turner, 2003; Anderson, 2000; Byrd & Allen, 2001; Davidson, 1944; Nabity *et al.,* 2006; Richards *et al*., 2009; Saunders & Hayward, 1998).

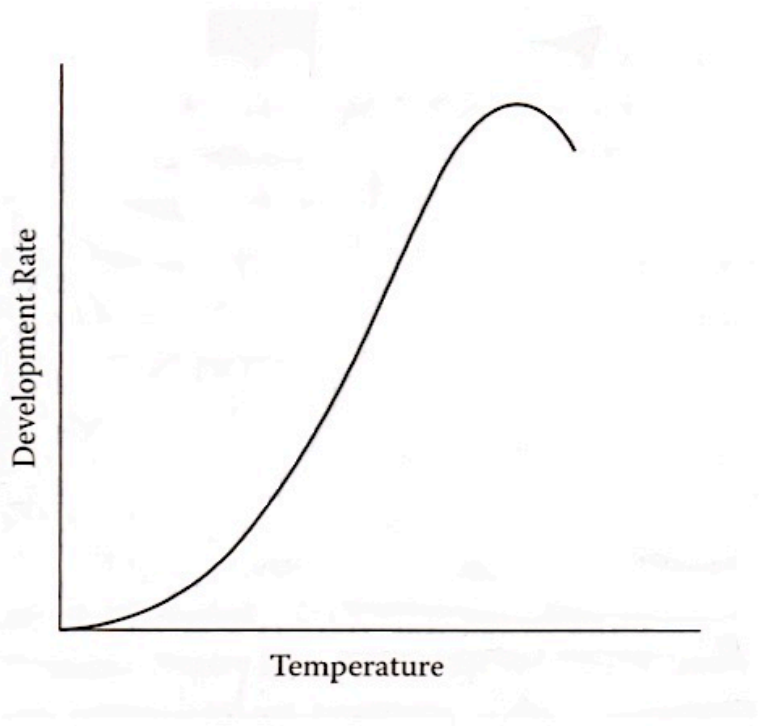


Figure 2.1. The thermal development curve – the generalized relationship between temperature and rate of development (From Byrd & Castner, 2010; pp. 391)

Upper and lower temperature thresholds are species and population specific. The base temperature, or the lower developmental threshold (LDTT), is the lowermost temperature limit for larval development. If temperature drops below this minimum threshold then development may cease and mortality rates increase (Marchenko, 2001). For example, *Calliphora vicina* eggs fail to hatch at temperatures below 4 °C, but larvae of the same species are able to complete

development at 5 °C (but at a very slow rate) (Faucherre *et al*., 1999). Another forensically important species, *Phormia regina*, requires a minimum temperature of 11 °C for eggs to hatch, 12.2 °C for larval development, and 14 °C for adults to emerge from pupariation (Nabity *et al*., 2006). These results suggest that each phase of development has its own stage-specific lower temperature threshold and development rate.

As the temperature increases above the lower limit, developmental rates progressively increase, with faster rates of development being reported at the higher temperatures. However, despite growth rates being at a maximum at elevated temperatures, survivorship is reportedly better at lower temperatures since the upper threshold typically lies near the upper lethal temperature for many species (Higley & Haskell, 2010; Richards *et al*., 2008). Increasing levels of physiological stress at higher temperatures are expressed in failed pupariation and adult eclosion, smaller mature sizes and increased mortality rates (Davidson, 1944; Niederegger *et al*., 2010; O'Flynn, 1983; Richards *et al*., 2008; Rivers *et al*., 2010; Tarone *et al*., 2011). Richards and colleagues (2008) reported developmental rates for *Chrysomya albiceps* at 13 different constant temperatures. They observed that at 17.5 °C larvae required 22.5 days to complete development but only 5.5 days when reared at 30 °C, with the largest larval lengths being recorded at 25 °C (16.2 mm). However, outside of the temperature range 20-40 °C, development suffered, with no larvae surviving to reach pupariation at 15 °C or 45 °C, and no adults emerging at 17.5 °C or 40.5 °C. A species-specific upper temperature threshold may influence species distribution and contribute to resource partitioning on carrion, or permit species with a high upper lethal limit dominate in interspecific competition (Richards *et al*., 2009).

The general trend appears to be that species with cooler lower/upper lethal temperature limits originate from northern temperate zones or higher altitudes (Richards *et al.,* 2009). These cold adapted, or psychrophilic species, are more likely to be found on smaller carcasses, which are only able to support small masses and lower levels of heat, or at the cooler periphery of larger masses. Meanwhile, species with a high lower/upper threshold originate from warmer climes and are more likely to be attracted to medium or large carcasses where they might experience intense inter/intraspecific competition (Richards *et al*., 2009).

2.4 Estimating the mPMI using Larval Development

Estimating the mPMI based on larval age is a three-step process (Amendt *et al.,* 2007; Nabity *et al*., 2006; Sharma *et al*., 2013; Williams, 1984), which involves:

1. Identifying the species
2. Reconstructing the thermal history
3. Modelling the rate of development

An accurate identification of the insects sampled from a body is the first priority in a forensic analysis of the evidence, since species will differ in terms of arrival time, growth rates and overall body size. Researchers can then estimate how far development has progressed in an individual larva by examining the posterior spiracles (to determine instar) and measuring body length. For feeding larvae size is a function of age. Recently hatched individuals measuring 1-2 mm in length will begin to feed shortly after they emerge, gradually increasing in size as they progress through the three feeding instars (Figure 2.2). Maximum body size is attained just prior to the start of dispersal and is species specific.

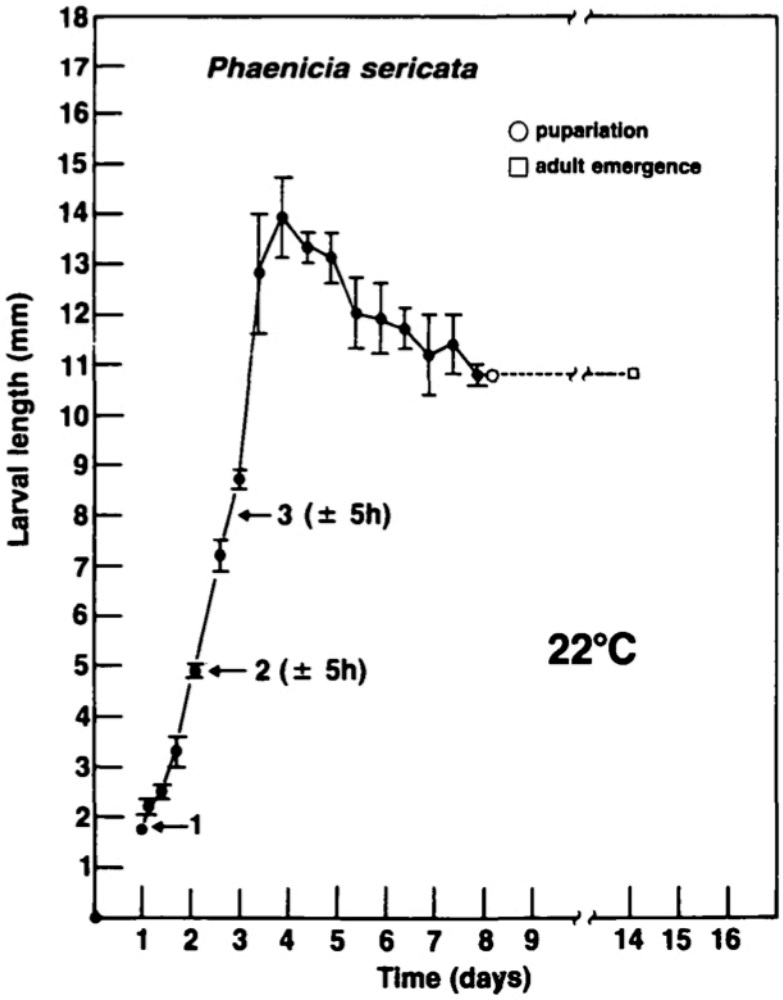


Figure 2.2. Development of *L. sericata* from oviposition to eclosion at 22 °C where 1= start of 1st larval instar, 2= start of 2nd instar and 3= start of 3rd instar (from Greenberg & Kunich 2002; pp. 125)

Whilst length is the most common measurement used to estimate age, various other methods related to body size have been proposed, such as larval width (at the junction of the fifth and sixth abdominal segments) (Day & Wallman, 2006), weight (Williams 1984) and crop length (Greenberg & Kunich, 2002). Care should be taken though when using physical measurements to estimate larval age, since they can be influenced by a number of factors including the type and availability of food (Clark *et al*., 2006; Green *et al*., 2003). By referencing laboratory studies that have monitored larval growth rates at controlled temperatures, entomologists are then able to predict the age of larvae collected from a corpse based on these

physical measurements and their thermal history. Using retrospective temperature data and thermal summation models, it should be possible to backtrack from the observed degree of development to the time of oviposition. This converts insect age into a time estimate, or the minimum post-mortem interval (mPMI) (Amendt *et al*., 2011; Anderson, 2000; Byrd & Allen, 2001; Campobasso & Introna, 2001; Donovan *et al*., 2006; Grassberger & Reiter, 2001; Grassberger & Reiter, 2002a; Introna *et al*., 1998; Marchenko, 2001; Nabity *et al*., 2006; Wells & Lamotte, 2010).

2.4.1 Thermal summation models

Larval development can be modelled using a process known as thermal summation. This is based on the temperature-dependent rate of development of immature insects and models their growth in terms of accumulated degree hours (ADH) or accumulated degree days (ADD) (Richards *et al*., 2008; Wells & Lamotte, 2010). The insect age can be estimated by calculating the ADD/ADH required for larvae to reach a particular stage of development (as represented by larval instar and physical measurements), which is a measure of the thermal time taken to reach a developmental event (Catts & Goff, 1992; Higley & Haskell, 2010; Ireland & Turner, 2006). However, despite thermal summation models having their uses, there is a growing need to assess whether they should be reworked to account for the effect of the microenvironment (Rivers *et al*., 2010). It should also be mentioned that using ADD/ADH models is more accurate during the earlier stages of development (egg, 1st and 2nd instar), since these are the shortest developmental stages (Tarone & Foran, 2008). As larval development progresses, error rates increase and confidence intervals become wider, becoming most pronounced towards the end of feeding and during the post-feeding and pupae stages. This can

be accounted for by giving large age ranges to post-feeding larvae (Tarone & Foran, 2008). ADH will be discussed in more detail in the following chapter.

2.4.2 Isomegalen- and isomorphen- diagrams

Another method designed to facilitate a quick and precise estimate of the mPMI, even in the hands of an inexperienced investigator, is the use of isomegalen- and isomorphen-diagrams (Grassberger & Reiter, 2001). These plots relate the length of a larva with the time of development under various temperature conditions. Using the length of a larva sampled from a death scene, investigators are able to read an age or "time from hatching" directly off the graph. However, the diagrams are based on constant temperatures, which renders them inappropriate for outdoor crime scenes where ambient temperatures fluctuate. Grassberger and Reiter (2001) suggested that for these outdoor crime scenes, age could be estimated from between points (based on the maximum and minimum recorded temperatures) to give an age range for larvae. Yet despite their uses, neither of these models gives any indication of the amount of error in the estimates.

2.5 Problems when Estimating the mPMI

When estimating time of death, it is imperative that the conditions at the crime scene be carefully considered. The closer the match between these conditions and those used to generate reference data, the smaller the margin of error when estimating the entomologically-based portion of the PMI (Wells & Lamotte, 2010). There are numerous factors reported to influence blowfly development and activity, which will be discussed in this section of the literature review. Identifying these factors and determining how they affect larval development is an active area of research in Forensic Entomology. In order to identify any influence mass

generated heat may have on larval development, it is vital that researchers are able to rule out any outside influences that could skew results or have an affect on the formation or size of the mass. Recognizing these factors in the early stages of the research also helps determine what should be controlled for in the laboratory or considered in the methodology to reduce the risk of error (e.g. food type, photoperiod, study species, killing and preservation techniques).

2.5.1 Population and genetic variation

Genetic variation in developmental time and forensically important phenotypes, such as larval body size, has been observed in fly species but little mention of it is made in the forensic entomology literature (Tarone *et al*., 2011; Tomberlin *et al*., 2011). This could result in inaccuracies when predicting the mPMI with blowfly evidence. In individual cohorts, it is common practice to observe an inherent variability in growth rates, with some individuals growing at a slower rate than others (Donovan *et al*., 2006). Random variation in natural populations is normal. Larvae of equal age reared under the same conditions will display a range of sizes, including a number of individuals that are significantly smaller or larger than the sample mean.

Various studies have provided evidence that regional variation in developmental times exists between populations of the same species, with fly strains adapting to their local environment and climate (Gallagher *et al*., 2010). A recent study by Tarone and colleagues (2011) was carried out to determine if there were any differences in the minimum time of development and pupal sizes of three populations of *Lucilia sericata* (California, Michigan and West Virginia) at two

different temperatures (20 and 35.5 °C). Results showed that the developmental times differed significantly between strains and temperatures. At 20 °C they reported no significant difference in minimum development time for all three strains, but at 35.5 °C the Californian strain showed a slower rate of development compared to the Michigan and West Virginia strains (Tarone *et al*., 2011). Another study using the same species was carried out to investigate variation in the developmental rates of populations from Sacramento CA, San Diego CA and Easton MA at three different temperature regimes (16, 26 and 36 °C) (Gallagher *et al*., 2010). Again, it was observed that the distribution of developmental times differed significantly between the three populations in each temperature treatment.

It is therefore important to evaluate entomological evidence on a regional scale, which will account for genetic differences in development. Genetic variation may be due to random drift among populations or differences in selection pressures (Tarone *et al*., 2011). Ecological factors such as elevation, photoperiod, temperature, moisture and the presence of different species will vary among regions and could contribute to the evolution of traits (Gallagher *et al*., 2010; Tarone *et al*., 2011). If larval age is estimated using developmental data from the wrong population, or if minimum development times vary among populations, then age estimates, and hence mPMI estimates, may differ significantly (Tarone *et al*., 2011; Tomberlin *et al*., 2011).

2.5.2 Delayed oviposition

The circumstances surrounding the death scene can affect the timing of initial insect colonisation on a corpse. They might also restrict the size or movement of a

mass, or prevent its formation all together. If these factors are not taken into consideration by investigators, then they could potentially lead to discrepancies between the entomologically estimated mPMI and the actual time of death.

2.5.2.1 Weather

Weather is an important determinant of fly activity, with strong winds, cloudy conditions, heavy rain and cool temperatures effectively grounding adults and delaying oviposition for several days (Campobasso *et al*., 2001; Deonier, 1940; Introna *et al*., 1991; Mahat *et al*., 2009). Aside from influencing adult activity, rainfall may help maintain moisture in soft tissues, preventing the carcass from drying out and encouraging larval feeding (Archer, 2004; Bharti & Singh, 2003). It has been reported that if rainfall is heavy and prolonged, then eggs and 1st instar larvae will be washed away (Cross & Simmons, 2010). However, several papers refute this claim, reporting that severe rain is largely unsuccessful at washing away eggs and larvae will continue feeding by moving into body cavities or to the underside of the carcass (Grassberger & Frank, 2004; Mann *et al*., 1990; Reed, 1958; Reibe & Madea, 2010; Slone & Gruner, 2007).

2.5.2.2 Nocturnal Oviposition

For a long time blowflies were considered to be inactive at night. With many murders being committed after dark, it was always assumed therefore, that for these cases there was potentially a 10-12 hour time frame (depending on the time of the murder) where insect access was inhibited. Yet there have been numerous reports published over recent years that contradict this theory (Amendt *et al.,* 2008; Greenberg, 1990; Pritam & Jayaprakash, 2009; Singh & Bharti, 2001). Greenberg (1990) reported nocturnal and low light oviposition in three forensically important blowfly species, *Calliphora vicina*, *Lucilia sericata* and

Phormia regina, with adults actively entering dark environments to oviposit. However, Singh and Bharti (2001) claimed Greenberg's experiment was flawed since the bait was placed on the ground amongst bushes, providing resting flies an opportunity to climb onto the meat and oviposit as opposed to having to take to the air. They tested this by exposing meat baits on wooden platforms positioned 6 ft off the ground and away from any artificial lights between the hours of 10 pm and 3 am. Results showed oviposition in 5 of their 14 trials, but with greatly reduced egg numbers in comparison to daylight trials. The same results were reported by Pritam and Jayaprakash (2009), who also observed a reduced number of larvae emerging from nocturnal/twilight bait traps several days after their exposure. These findings imply that nocturnal oviposition is a possibility but could result in a limited numbers of eggs and a developmental delay of two to three days. The authors claimed this was due to reduced fly activity during twilight and nocturnal hours.

2.5.2.3 Concealment of body

Concealment of the body, e.g. when the remains are wrapped, buried, hidden or placed in a sealed container, may prevent or limit exposure to arthropods, delay oviposition and ultimately restrict the formation and movement of a maggot mass (Campobasso *et al*., 2001; Voss *et al*., 2008). This subject was investigated by Goff (1992) who monitored insect colonization in pig carcasses wrapped in two layers of blankets. He noted that flies were able to penetrate the blankets through folds around the head of the pig, but only 2.5 days after initial exposure. Longer delays of four to five days were reported for pigs covered in plastic sheeting and up to seven days for remains covered with a thin layer of soil (Goff, 1992). But despite Goff's observations, delays in oviposition have not been reported in other studies. An investigation was carried out in South Africa where six pigs were divided into

three groups (clothed, wrapped and unwrapped) and closely monitored for insect activity (Kelly *et al*., 2009). Results showed that oviposition occurred simultaneously on all six carcasses during the first day of exposure, with all pigs progressing through decomposition at the same rate regardless of their treatment (Kelly *et al.,* 2009). Other studies have supported this claim (Marchenko, 2001; Voss *et al*., 2011). In fact, wrappings may even benefit insect colonization and mass formation since, when soaked with blood and body fluids, they provide more sites for oviposition, shelter for feeding larvae and retain moisture, which prevents soft tissues drying out (Grassberger & Frank, 2004; Greenberg, 1991; Kelly *et al*., 2009; Voss *et al*., 2011).

Larval development rates have been shown to be the same on both indoor and outdoor corpses, but adult blowflies have been shown to require extra time to access indoor bodies, which delayed colonization from 24 hours up to five days (Anderson, 2011; Pohjoismäki *et al*., 2010; Reibe & Madea, 2010).

2.5.3 Tissue type for feeding

The type of tissue on which larvae feed, as well as the species from which the tissue was sourced, might influence larval growth rates and overall size. Clark and colleagues (2006) monitored the development of *Lucilia sericata* larvae fed on a range of tissue types obtained from pigs. They reported that growth rates varied significantly, depending on whether larvae were reared on lung, kidney, heart, brain or liver, with larvae showing a developmental lag of two days when fed on the latter (Clark *et al*., 2006). These results were mirrored in an earlier study carried out by Kaneshrajah and Turner (2004). As well as growing significantly faster (completing feeding 31 hours earlier), larvae reared on lung tissue grew 2

mm longer and gave rise to larger adults, in comparison to larvae reared on liver (Clark *et al*., 2006). Day and Wallman (2006a) supported this claim when they published results that *Calliphora augur* and *Lucilia cuprina* reared on sheep brain and soft tissue reached their maximum length two days earlier than larvae reared on liver. Studies have also implied that it is not just the tissue type that makes a difference, but the animal from which that tissue was sourced, with larvae reared on pork developing faster as well as being significantly larger than larvae reared on beef (Clark *et al*., 2006).

Given that different tissue types vary considerably in fat/protein ratios as well as carbohydrate levels, it would be fair to assume that the nutrient content of these tissues will affect developmental times and larval size in different ways. For example, one study showed that *Calliphora vomitoria* larvae fed on liver showed a faster rate of development when compared to the same species reared on brain, which was deemed less nourishing due to its low protein levels, softer consistency and high water content (Ireland & Turner, 2006). Experiments altering the nutrient content of food given to *Phormia regina* demonstrated that when protein levels were increased, mortality rates decreased and developmental rates increased (Green *et al*., 2003); in contrast, increasing carbohydrate in the diet resulted in an extended larval period, increased mortality and reduced larval weights as the metabolism was put under stress trying to remove excess carbohydrate from the haemolymph (Green *et al*., 2003). Poor larval development on liver could be attributed to toxins in the organ and/or its lower lipid content in comparison to other organs or tissues, with larvae having to expend more energy to liberate lipids, which comes at the expense of growth (Day & Wallman, 2006a).

These results emphasize the need for consistency in what entomologists feed larvae in laboratory-based experiments. Using pigs in decomposition studies may also be affecting results, as there are potential differences between human and pig tissue (Clark *et al*., 2006). If smaller, poorly developed larvae are mistaken for younger larvae, then this could result in an underestimation of the mPMI of up to two days (Day & Wallman, 2006a).

2.5.4 Overcrowding and competition

Since carrion is an ephemeral resource, it is only able to support a limited population for a short period of time. When the number of feeding larvae exceeds its carrying capacity overcrowding occurs. This produces a competitive feeding environment. Larvae feeding in these conditions might experience a faster rate of development but at the expense of size, producing smaller larvae that pupate early and give rise to smaller than average adults (Ireland & Turner, 2006; Shiao & Yeh, 2008). The effect of overcrowding varies among species depending on how well adapted each is to a competitive environment. Shiao and Yeh (2008) demonstrated this by monitoring development in *Chrysomya rufifacies* and *Chrysomya megacephala* cultures of increasing density. They reported that *C. megacephala* experienced accelerated development once density exceeded 160 larvae/60 g meat. But *C. rufifacies* larvae were able to withstand a higher degree of overcrowding and only began to demonstrate the same accelerated development once density exceeded 600 larvae/60 g meat.

2.5.5 Drugs and toxins

There are numerous chemical-based products, drugs and toxins that have been shown to influence larval development if ingested shortly before death by the deceased or applied externally to the body (Goff & Lord, 2010). Various household products such as mosquito repellent, petroleum and perfume have a repellent effect on several species of fly attracted to carrion, as do clothes stained with chemicals and combustibles (Charabidze *et al*., 2009; Mann *et al*., 1990; Marchenko, 2001). The effect of the repellent could depend on the weather and cadaver exposure, the quantity of the repellent and its location on the corpse. A recent study from Malaysia showed that Malathion, a drug commonly used in suicides, could delay oviposition on carcasses by as much as one to three days (Mahat *et al*., 2009). Larvae feeding on tissues contaminated with cocaine experience an accelerated rate of development, especially during the 2nd and 3rd larval instars, by 12-18 hours (Catts, 1992; Greenberg & Kunich, 2002). Meanwhile, some poisons like mercury have an adverse effect on development, slowing growth rates and reducing larval size by up to 60% (Benecke, 1998; Catts, 1992). However, the exact effects drugs and toxins have on various species remains hard to determine and quantify, with some studies demonstrating variability between species (Charabidze *et al.,* 2009).

2.5.6 Techniques for killing and preserving larvae

How larvae are killed and preserved after collection can have a significant effect on their size and any subsequent mPMI estimates. It is vital that researchers have a thorough understanding of the effects these procedures might have on larval size to ensure experiments are designed appropriately. This would reduce the risk of any false or inaccurate results being reported. One paper, authored by Tantawi

and Greenberg (1993), described the effect of killing and immersion in various preservatives on the larval lengths of *Protophormia terraenovae* and *Calliphora vicina* of known ages. They observed larval shrinkage in all 15 of the preservative solutions tested. But the amount of shrinkage varied depending on species and larval age, with young 3rd instar larvae undergoing a greater reduction in size compared to older larvae (Tantawi & Greenberg, 1993). The type of preservative used also has a significant effect on larval length and age estimates. Greatest shrinkage was observed in San Veino and formalin preservatives, both of which are used to wash the body at autopsy. When placed in San Veino solution, 3rd instar *Protophormia terraenovae* larvae were observed to shrink from 15.6 mm to 11.2 mm, effectively reducing an estimate of their age by 11 hours (Greenberg & Kunich, 2002). Some species, like *Calliphora augur*, show a degree of shrinkage that correlates with the concentration of the preservative; their greatest reduction in size occurring when placed in 100% ethanol (Day & Wallman, 2008).

To avoid any reduction in size, larvae should first be killed in boiling water before being placed in a preservative (Amendt *et al.* 2007; Tantawi & Greenberg, 1993). Heating lessens autolysis by denaturing proteins and destroying digestive enzymes and gut flora, as well as altering the permeability of the cuticle, which allows larva to remain extended and rigid (Day & Wallman, 2008). But a study by Adams and Hall (2003) investigating killing and preservation techniques on post-mortem larval length, found that whilst water temperature did not affect the size, the amount of time they were submerged did affect length. Shrinkage resulting from inappropriate killing and preservative techniques can make larvae appear more than half a day younger than they actually are (Tantawi & Greenberg, 1993) with age estimates ranging from 2-28% younger than reality (Adams & Hall, 2003).

This implies there is a need to standardize maggot treatments for lengths to be valid for mPMI estimates (Tantawi & Greenberg, 1993). The proposed method of immersing larvae in 80 °C water for 30 seconds before preserving in 80% ethanol appears to result in a minimal change in length (Adams & Hall, 2003; Amendt *et al.,* 2007; Byrd *et al*., 2010; Day & Wallman, 2008).

2.5.7 Thermal history

The thermal history of blowfly larvae colonising a corpse can be extremely complex, but an accurate record of conditions during the post-mortem interval is key in determining the age of associated larvae (Catts & Goff, 1992). Making assumptions regarding the thermal history can lead to substantial errors, with some entomologists going as far to suggest that the temperature data used in degree-day calculations is likely to be the most significant single source of error in PMI estimates (Higley & Haskell, 2010; Williams, 1984). Researchers should be aware that there might be significant differences between the temperatures recorded at the crime scene and readings taken from the nearest weather station (Dabbs, 2010). Rarely are the two in close proximity to one another. To determine if weather station data are suitable for mPMI estimates, ambient temperatures should be recorded at the crime scene for several days and then compared with weather station temperatures (Amendt *et al*., 2011; Catts & Goff, 1992; Grassberger & Reiter, 2002b). Investigators also need to consider swings in diurnal temperature and whether temperatures are constant or fluctuating, the latter being more likely at outdoor crime scenes (Greenberg, 1991; Niederegger *et al*., 2010). This is important since some species like *Sarcophaga argyrostoma* and *Lucilia illustris* develop more rapidly under fluctuating temperatures, whilst others

(e.g. *Calliphora vicina* and *Calliphora vomitoria*) appear to take longer (Niederegger *et al*., 2010).

The rate of development only partly depends on the ambient temperature. In fact, temperature records from local weather stations or measurements taken at the scene could differ considerably from the temperatures experienced by larvae on or in a cadaver (Turner & Howard, 1992; Williams, 1984). It is possible that larval development is influenced by the heat loading of a corpse in the sun, microbial action and the exothermic nature of the maggot mass (Greenberg & Kunich, 2002). For a reliable mPMI estimate it is essential that multiple temperature readings are taken from both the body and the mass, if present.

2.5.7.1 Heat loading

The size of the carcass, the nature of the ground it is laid on and its exposure to sunlight can all have a major effect on heat loading. Experiments comparing decomposition in direct sunlight with that of shaded carcasses have recorded ambient temperatures at sun exposed sites to be consistently 11 °C warmer than their shaded counterparts, which can result in ADH estimates being 28-48% higher in in the sun (Greenberg & Kunich, 2002). It is also important to consider the nature of the ground (how much heat does it absorb/radiate) as well as the carcass size, with large carcasses taking longer to heat up due to their smaller surface-to-volume ratio. Heat loading and exposure to direct sunlight can also influence heat generation in larval masses, a subject that will be discussed later in the chapter.

2.5.7.2 Microbial action

In his 1965 study which monitored the decomposition of pig carcasses, Payne noted that carrion became considerably warmer than the surrounding

environment, particularly during the active and advanced stages of decomposition. He recorded the carcass reaching highs of 37.7 °C whilst the ambient and soil temperature reached 22 °C (a difference of 15.7 °C) and proposed it was due to the high metabolic rates of bacteria and larval activity (Payne, 1965). Some researchers have suggested that the bacterial elevation of carcass temperature is negligible (Deonier, 1940; Greenberg & Kunich, 2002). Whilst monitoring temperatures in un-infested sheep carcasses, Deonier (1940) claimed that heat from bacterial decomposition alone was not perceptible. But a recent study carried out by Johnson and colleagues (2013) contradicts this idea. They investigated the heat associated with decay and the types of bacteria present during the decomposition of fifteen pig carcasses. All of these cadavers, whether fresh, frozen or infested with maggots, reportedly reached an average maximum temperature of 32 °C whilst ambient remained at 23 °C. These results suggest that carcasses have the potential to generate heat in the absence of maggot masses, solar radiation and other external heat sources, and bacterial metabolism plays a significant role in carcass thermogenesis (Johnson *et al*., 2013).

2.5.7.3 Mass-generated heat

Another factor influencing the carcass microclimate is the heat generated by larval aggregations commonly observed on carrion. The literature defines a maggot mass as an assemblage of feeding larvae in which metabolic heat raises the localised temperature above ambient, often reaching temperatures of 27-35 °C (Campobasso *et al*., 2001; Haskell *et al*., 1997; Higley & Haskell, 2010; Joy *et al*., 2002; Kelly *et al*., 2009; Reed, 1958; Rivers *et al*., 2010; Shean *et al*., 1993). Yet despite experts in the field being in agreement that it exists and could pose a problem for mPMI estimates, heat generation in larval aggregations is rarely

investigated or quantitatively described. What is known about the subject will be discussed in the remainder of this chapter.

2.6 Larval Aggregations and Heat Generation

A maggot mass originates in the collective oviposition frenzy of female blowflies and can exist as either a single or mixed species aggregation (Campobasso *et al*., 2001; Cianci & Sheldon, 1990; Ives, 1991). A recent study by Boulay and colleagues (2013) demonstrated that blowfly larvae actively aggregate and masses are not solely attributable to the clustering of egg batches. Using young 3rd instar *Lucilia sericata*, they showed that aggregations became established in less than three hours, with larvae using a contact and/or odour mediated signal to attract individuals to a specific area (Boulay *et al*., 2013). In the field large maggot masses are commonly observed during the decay stage of decomposition, by which point larvae that emerged from eggs oviposited on fresh or bloated carcasses have reached their 2nd larval instar (Anderson & VanLaerhoven, 1996; Campobasso *et al.*, 2001; Grassberger & Frank, 2004; Richards & Goff, 1997) (Figure 2.3).

Figure 2.3. Image of a maggot mass feeding under the front left leg of a pig decomposing out in the field during June 2012

2.6.1 Recorded mass temperatures

Metabolic heat generated by a feeding aggregation was first reported in 1869 by Girard, who measured a temperature in excess of 32 °C in a box full of *Lucilia caesar* larvae (Greenberg & Kunich, 2002). In the laboratory, Greenberg (1991) packed a human skull with 1400 g of ground beef and 10,000 *Phormia regina* eggs. Using a temperature probe inserted through the frontal bone, he recorded temperatures several times a day, noting that very little heat was produced during 1st larval instar. But by the time larvae reached 2nd and 3rd instar, the mass was producing a significant amount of thermal energy, reaching highs of 41 °C by late 3rd instar (18 °C above ambient) (Greenberg, 1991).

Field studies utilising large carcasses, which are able to support greater numbers of larvae, have provided researchers an opportunity to observe just how hot these aggregations can get, with some masses peaking at temperatures in excess of 50 °C (whilst ambient ranges from 9-24 °C). One of the earliest published reports was by Deonier (1940), who monitored 33 sheep carcasses in Texas and another 40 in Arizona during the mid-1930s. Temperatures were recorded to peak at 42.2-48.9 °C and it was reported that these large aggregations remained active even when ambient temperatures dropped to lows of -4 °C (Deonier, 1940). However, it is important to bear in mind that this study did use unshorn sheep and the woollen fleece could easily have contributed to heat retention and insulation. Fifty years later, Cianci and Sheldon (1990) examined the endothermic nature of developing maggot masses on pig carcasses. Field studies using pig models give results that are more applicable to cases involving human cadavers. Using four exposed carcasses, each weighing approximately 20 kg, they took daily temperature recordings of the maggot mass, core body and ambient air. They found that mass temperatures remained comparable to air and core body temperatures until day six of exposure, at which point the temperature of the mass rose sharply. This correlated with the appearance of 3rd instar larvae. These temperatures were recorded peaking at 41 °C, where they stabilized until day 12 when larvae began to disperse (Cianci & Sheldon, 1990).

During recent years field studies in Hawaii using pig carcasses have recorded internal carcass temperatures reaching highs of 50 °C or more, 26 °C above ambient (Richards & Goff, 1997) whilst dog carcasses have supported masses producing temperatures ranging from 30-49 °C (Marchenko, 2001). In southern Africa decomposing rhino carcasses were reported to be covered with a 2 cm layer

of feeding larvae, which yielded temperatures ranging from 40.9-49.3 °C whilst ambient temperatures ranged from 10.3-38 °C (Richards *et al*., 2009). Slone and Gruner (2007) recorded internal and mass temperatures daily from 80 pig carcasses exposed to insect activity outdoors in Florida and Indiana. On numerous occasions they reported temperatures taken from the centre of larval aggregations peaking close to 50 °C (ambient = 30 °C) and on one occasion reaching 50.7 °C. Even small carcasses, such as rats and rabbits, have shown considerable temperature increases despite only being able to support small larval aggregations (Tomberlin & Adler, 1998; Turner & Howard, 1992). For example, temperatures in excess of 40 °C were maintained for up to 10 hours in rabbit carrion left exposed during summer months in the UK (Turner & Howard, 1992). High temperatures in maggot masses have been recorded to peak during the 3rd instar approximately 18 hours before maximum larval length is attained (Goodbrod & Goff, 1990), after which temperatures begin to drop (usually during the advanced stage of decay) as larvae migrate away from the carrion and enter the post-feeding stage of development (Campobasso *et al*., 2001).

2.6.2 The benefits of feeding in a mass

Various reasons have been proposed to explain why larvae might have evolved to form feeding masses. One such explanation for this gregarious behaviour is that it maximises their feeding efficiency by producing sufficient proteolytic enzymes for tissue breakdown (Cianci & Sheldon, 1990; Goodbrod & Goff, 1990; Green *et al*., 2003; Hobson, 1932; Ireland & Turner, 2006; Richards *et al*., 2009; Schoofs *et al*., 2009). A single larva will struggle to penetrate food, whilst the collective effort of larvae feeding in a mass and secreting excess enzymes helps facilitate access to soft tissues via liquefaction and ingestion (Charabidze *et al*., 2013; Greenberg &

Kunich, 2002). Mass feeding larvae are therefore able to extract more nutrients, resulting in a faster rate of development. This notion is supported by comparing single larvae reared in isolation with group reared larvae, the former demonstrating stunted growth, lighter larval and pupal weights and a slower rate of development (Green *et al*., 2003; Richards *et al*., 2008).

By maintaining a stable microclimate at raised temperatures, larvae are also protected against any chilling or cold shock injury brought on by sudden and temporary drops in ambient temperature (Campobasso *et al*., 2001; Deonier, 1940; Mann *et al*., 1990). This is important since studies have shown that insects do not recover properly after a period of cooling, regardless of the duration of the cooling period or larval age (Myskowiak & Doums, 2002). Deonier (1940) reported that if the heat energy generated by larvae was added to that absorbed by the sun, then carcasses could maintain their temperatures at a level high enough for continued larval development, even when the minimum atmospheric temperature dropped below 10 °C. This assertion has also been supported by Huntington and colleagues (2007) after they investigated the effects of cold storage on the temperature of larval masses. Human and pig cadavers colonized with blowfly larvae were placed in body bags and immediately stored in a morgue cooler set to run at temperatures ranging from -1 °C to 4 °C. Despite these low ambient temperatures, readings taken at ten-minute intervals from inside the body bag remained significantly higher than ambient (Huntington *et al.,* 2007). In fact, the average temperature inside the body bag was 14.1 °C compared to the mean ambient temperature of 6.3 °C. Even when stored in a cool environment, temperatures inside the body bag remained at a sufficient level for feeding and development to continue. This ultimately led to cadavers experiencing a substantial loss of tissue whilst in storage

awaiting post-mortem examination, which might result in the loss of evidence. The same paper reported that error rates as high as 8.6-12.8% could occur in mPMI estimates if this continued development in cold storage was not taken into consideration (Huntington *et al*., 2007).

Other evolutionary advantages to elevated mass temperatures may include a species-specific competitive edge in food acquisition as well as the avoidance of predation and parasitism (Cianci & Sheldon, 1990; Rivers *et al*., 2010). Some species with a high heat tolerance may use mass heat to outcompete others with a lower threshold when on a limited food resource like carrion (Cianci & Sheldon, 1990; Richards *et al*., 2009). This interspecific competition can lead to spatial partitioning. Mass-generated heat may also aid in reducing larval predation by other arthropods (rove beetles, carrion beetles etc.) by shortening the length of the juvenile period (Cianci & Sheldon, 1990). If larvae can accelerate their growth rate, then they could reduce their window of vulnerability.

2.6.3 The disadvantages of feeding in a mass

Despite the aforementioned advantages, there are also numerous disadvantages resulting from this gregarious behaviour, both from a human and larval standpoint. From an investigators point of view, large maggot masses have the potential to destroy evidence, consuming the majority of the soft tissue within a matter of days. For this reason heavily colonized corpses should be prioritised for autopsy rather than being kept for days in the morgue cooler awaiting examination (Campobasso & Introna, 2001; Huntington *et al*., 2007).

For larvae, there is a risk that the faster rates of development associated with warmer temperatures come at the expense of larval size. Since development is dependent on metabolism and the mechanical acquisition of food, it is possible that high mass temperatures could give rise to feeding rates that cannot keep pace with the faster metabolism (Tarone *et al*., 2011). This would result in smaller individuals. Rivers and colleagues (2010) suggested that this altered development was a consequence of heat stress conditions as evidenced in the expression of heat shock proteins in larval brains. They found that the levels of heat shock proteins (hsp) in *Sarcophaga bullata* (Parker, 1916) (Diptera: Sarcophagidae) and *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) increased in conjunction with the amount of time spent feeding (age) and the size of the mass (temperature). Temperatures greater than 32 °C are considered mild heat shock conditions and can induce the expression of several hsps as part of the heat shock response (Rivers *et al*., 2010). Depending on the severity of the heat, the heat shock response can be active in synthesizing stress proteins, which will occur at the expense of normal protein production, resulting in a decrease in pupal weights as well as eclosion rates (Rivers *et al*., 2010).

There is still much uncertainty regarding the upper lethal temperature threshold for many blowfly species of forensic significance, with some papers reporting limits of 39-45 °C whilst others estimate them to be higher (Greenberg & Kunich, 2002). Some species have been shown to have an upper lethal temperature limit, which falls several degree lower then the temperatures recorded in some large maggot masses (Byrd & Allen, 2001; Donovan *et al*., 2006; Nabity *et al*., 2006). Under these conditions, one would expect to observe an increase in larval mortality, and at the very least, inhibited development. For example, *Phormia*

regina has been shown to have its development adversely affected at temperatures over 35 °C, with larval mortality increasing dramatically at 40 °C (Byrd & Allen, 2001). Yet they can still be recovered from heavily infested carcasses approaching highs of 50 °C. A summer study carried out in South Africa monitoring insect colonization and decomposition in wrapped, unwrapped and clothed pig carcasses reported a high larval mortality rate (Kelly *et al*., 2009). This was attributed to high daytime ambient temperatures of approximately 35 °C pushing internal carcass temperatures to highs of 44-50 °C, with one reading peaking at 56 °C. Aside from being well in excess of the upper lethal limit for many species, the researchers hypothesized that the excessive heat caused by the sun and the maggot mass trapped under a wet sheet created an unfavourable environment and possible build up of noxious gases (Kelly *et al.,* 2009). Slone and Gruner (2007) also reported observing lifeless larvae on the periphery of large masses where temperatures were recorded as high as 50.7 °C. But since many larvae survive these high temperatures it is fair to assume the mass must have a way of regulating temperature to avoid complete overheating, possibly by way of migration or evaporative cooling (Amendt *et al*., 2011; Charabidze *et al*., 2011; Kelly *et al*., 2009; Sharanowski *et al*., 2008).

2.6.4 Avoiding thermal stress

Larvae exposed to high temperatures show behavioural adaptations, which could allow them to manage thermal stress (Charabidze *et al*., 2008). One such behaviour is thermotaxis, where individuals will crawl away from unfavourable temperatures (Hückesfeld *et al*., 2011). On pig carcasses exposed to the sun in Saskatchewan, Canada, undersized larvae were observed migrating away from the corpse and into the surrounding grass once mass temperatures became too hot

(Sharanowski *et al*., 2008). In one laboratory based experiment, *Lucilia sericata* larvae were monitored migrating away from meat once temperatures reached highs of 35 °C. By reducing the size of the aggregation, its temperature never exceeded 42 °C (a lethal high temperature for *L. sericata*) even when ambient temperatures were set at 33 °C and more than 15000 larvae were used (Charabidze *et al*., 2011). Larvae are extremely sensitive to numerous parameters of their surroundings and continually orientate themselves to adapt. A video tracking system, used to follow larval movements and estimate speed at five different temperatures, recorded larvae crawling at faster rates when exposed to increasingly warmer temperatures (Charabidze *et al*., 2008).

2.6.5 Movement in a mass

Larval dynamics within a mass are complex. Recent studies have proposed that larvae do not feed continuously whilst in an aggregation and instead divide their time between three identifiable activities, which combined help explain the mass structure (Morvan *et al*., 2008):

1. ***Feeding*** – larvae immobile, vertically aggregated and in direct contact with the substrate
2. ***Searching for food*** – larvae with an empty crop try and reach the centre of the mass for feeding
3. ***Digestion*** – larvae with a full crop move around the mass searching for optimum temperature

These three activities make up what has been described as “foraging behaviour” (Charabidze *et al*., 2013). This behaviour creates a permanent movement inside larval masses, also referred to as “scramble behaviour”, which may be linked to the larval mass effect and could be responsible for enhancing metabolic heat emission.

Larvae may be able to survive the extreme temperatures of large aggregations by using a feedback loop that involves heat exchanges and physiological and behavioural thermoregulation processes (Charabidze *et al*., 2011). Research carried out by Byrd and Butler (1996, 1997, 1998) using temperature gradients demonstrated that larvae have a species-specific preferred temperature for optimal rates of development and will actively reposition themselves to regulate their temperature for accelerated development. It has been proposed that by alternating between the hot feeding centre of the mass and the cooler periphery, larvae are able to avoid overheating by experiencing evaporative cooling (Ames & Turner, 2003; Catts, 1992; Charabidze *et al*., 2011; Hückesfeld *et al*., 2011; Slone & Gruner, 2007). This thermotactic behaviour might be essential for larvae to escape the lethal high temperatures detected in large masses by causing the average temperature experienced by each larva to be less than the hottest temperature in the mass (Slone & Gruner, 2007). It could also explain the increased activity and movement observed in larger masses. However, at present, there is a lack of experimental evidence supporting or refuting this hypothesis.

2.6.6 Factors influencing the thermal output of larval masses

There are numerous factors that should be considered whilst attempting to model the thermodynamics of larval aggregations. By considering these factors and the possible effect they might have on heat generation, investigators can more accurately estimate developmental rates and, hence the mPMI.

2.6.6.1 Mass size

The amount of heat generated by a mass depends largely on the size of the aggregation, the general rule being that the greater the number of larvae, the greater the amount of heat produced (Charabidze *et al*., 2011; Gallagher *et al*., 2010; Slone & Gruner, 2007). It has been suggested that these raised mass temperatures can only be maintained if aggregations exceed a minimum size. Yet aggregations containing as few as 25 individuals have been reported to generate a level of heat higher than ambient (Greenberg & Kunich, 2002). Using 3rd instar *Chrysomya albiceps,* one study recorded mass temperatures 3-3.8 °C above ambient in aggregations comprised of 25-75 larvae, 6.4-10 °C increases with 100-150 larvae and 13.6-19.5 °C increases when aggregations contained 200-780 individuals (Greenberg & Kunich, 2002). Other reports have claimed that masses weighing 50 g or less (approximately 1000-2000 larvae) produce temperatures equal to or slightly higher than ambient, whilst 300 g masses (10,000-15,000 larvae) are able to generate highs of 42 °C (whilst ambient is 33 °C) (Charabidze *et al*., 2011).

A large-scale field study utilising 80 pig carcasses provided its investigators the opportunity to estimate mass volume and compare it with the thermal output (Slone & Gruner, 2007). Estimating mass volume out in the field was found to be problematic, with some aggregations having an average depth of 2-5 cm whilst others measured more than 10 cm deep in parts. The results of this study indicated that mass volume did have a strong influence on heat generation but was a poor predictor of the difference between mass temperature and ambient (Slone & Gruner, 2007). For small masses with a volume of less than 20 cm^3, the ambient temperature influenced internal temperatures. But larger masses exceeding 20

cm^3 had a strongly regulated internal temperature determined by the mass volume and not the ambient temperature (Slone & Gruner, 2007). In fact, aggregations with a volume of 20-50 cm^3 have been shown to be capable of producing temperatures in the range of 30-35 °C, which for many species is the optimum for larval development (Slone & Gruner, 2007). The same paper also presented evidence of a temperature plateau in plots that illustrated the relationship between mass volume (cm^3) and aggregation temperature (C°). As aggregations approached a volume of 1000 cm^3, heat emission began to level out at approximately 45-50 °C. Charabidze and colleagues (2011) also described a "slow down" of mass-temperature increase in large aggregations. When plotting the effect of mass size and ambient temperature on heat generation in aggregations, they found that the slope of the graph became shallower as masses approached temperatures of 40-45 °C. If the thermal output of a mass were to increase above the upper lethal temperature limit, then larvae would be effectively killing themselves with thermal stress.

Rivers and colleagues (2010) carried out a similar experiment on a smaller scale in the laboratory, investigating the development of two different fly species in different sized maggot masses. Using *Sarcophaga bullata* and *Protophormia terraenovae*, masses containing 25-2000 individuals were reared on beef liver whilst stored in an incubator at 25 °C. Mass temperatures were recorded daily from the centre of the mass. Results showed that both species produced elevated temperatures whilst feeding in a mass, which was dependent on its size, or the number of larvae present (Rivers *et al*., 2010). And whilst all masses were capable of generating temperatures that exceeded ambient, including those containing only 25 larvae, the highest were recorded in aggregations containing 500 or more. This

same study also went on to show that faster rates of development were linked to these increasing mass sizes, with the amount of time required to complete 1st, 2nd and 3rd instar being significantly shortened as mass size, and hence temperature, increased.

2.6.6.2 Mass density

Several studies have proposed that heat emission is strongly affected by mass density, with higher temperatures being recorded in denser, more tightly packed aggregations, which are able to retain heat better (Charabidze *et al*., 2011; Goodbrod & Goff, 1990). Rearing larvae at a density of 1-2 larvae/g meat is considered un-crowded whilst 7 larvae/g is classified as "severely crowded" (Charabidze *et al*., 2013; Saunders *et al*., 1999). Maximum mass temperatures have been recorded for populations at densities of 20 and 40 larvae/g meat (Catts, 1992; Charabidze *et al*., 2011; Goodbrod & Goff, 1990).

Charabidze and colleagues (2011) altered the quantity of food fed to masses of a fixed size and observed that aggregations of varying density gave rise to different levels of heat emission. They recorded maximum temperatures for masses reared on 50-100% of their weight in meat but thereafter, the peak temperature decreased as the quantity of food increased. For example, 3000 larvae (weight = 100 g) reared on 100 g of meat (density = 30 larvae/g) produced temperatures that were approximately 8 °C warmer than masses composed of the same number of larvae but reared on 400 g of meat (density = 7.5 larvae/g). The authors suggested these denser aggregations generated higher temperatures as a result of reduced evaporative cooling as well as displacement of competing larvae and an increase in muscular-activity related heat emission (Charabidze *et al*., 2011).

2.6.6.3 Species differences

Different species may not share the same aggregating or thermal properties. A recent study investigating the development of two species of fly reared in different sized masses reported that masses comprised of the flesh fly *Sarcophaga bullata* consistently produced higher temperatures than same sized masses of *Protophormia terraenovae*, a feature that was attributed to the larger body size of *S. bullata* (Rivers *et al*., 2010). Since larger maggots consume more food they would be expected to yield more metabolic heat. This idea is supported by research that has demonstrated a relationship between heat generation and larval age, with 3rd instar masses being shown to generate more heat than 2nd instar masses (Charabidze *et al*., 2011; Cianci & Sheldon, 1990; Rivers *et al*., 2010). Charabidze and colleagues (2009) reported that masses containing a fixed number of 2nd instar larvae have been shown to produce temperatures 2-4 °C lower than 3rd instar masses containing the same number of individuals. The warmer microclimate created by the larger *S. bullata* larvae resulted in them experiencing an accelerated rate of development in comparison to larvae in the *P. terraenovae* masses. By generating more heat, *S. bullata* were observed completing each individual larval stage in less time than *P. terraenovae*, reducing their overall developmental time by 20-30 hours (Rivers *et al*., 2010). It was suggested that this accelerated development allows them to outcompete other species, which is an advantage when feeding on an ephemeral resource like carrion. However, Slone and Gruner's 2007 pig study contradicted this idea by reporting that mass temperatures remained the same regardless of the species composition and whether aggregations were mixed masses or monocultures.

2.6.6.4 Competition

Interspecific competition in mixed-species masses could also influence thermal output and researchers conducting outdoor studies involving natural colonization should be aware of its effects. These include spatial partitioning and species with a high upper lethal temperature raising the mass temperature to outcompete species with lower thresholds (Richards *et al*., 2009). "Heat balling" is a form of interspecific competition seen in Asian honey bees, where bees overpower individual wasps by swarming around them and actively raising the temperature inside the swarm above the wasps upper lethal limit (bee upper limit = 50 °C; wasp upper limit = 46 °C), effectively killing them (Richards *et al*., 2009). The same type of behaviour could occur in larval aggregations. For example, whilst investigating the thermal ecophysiology of seven carrion breeding blowflies in Africa, Richards and colleagues (2009) were able to make comparisons between species and their temperature thresholds, as well as monitoring their distribution on carcasses. They found that species with a high upper lethal limit dominated in interspecific competition by pushing the temperature of the microclimate to a level several degrees warmer than the upper limit of other blowfly species. Of the seven species studied, *Chrysomya marginalis* had the highest upper lethal temperatures at 50.1 °C (where 50% died) whilst *Calliphora croceipalpis* had the lowest at 42.9 °C. On rhino carcasses with temperatures as high as 47 °C, *Chrysomya albiceps* with its upper lethal limit of 48.8 °C, was observed forming a single species mass on the periphery with a lower average temperature of 38 °C (Richards *et al*., 2009). On other carcasses where temperatures approached 50 °C, all the larvae sampled were *C. marginalis*. This species with its high upper lethal limit was able to dominate since no other species could cope with such high temperatures (Richards *et al*., 2009).

2.6.6.5 Ambient temperature and sun exposure

Environmental conditions such as the ambient temperature and exposure to sunlight might also influence the microclimate of an aggregation. Using the same number of larvae, a hotter ambient temperature could result in masses producing increased levels of heat (Charabidze *et al*., 2011). Given enough larvae (approximately 300 g), masses reared at an ambient of 10 °C can produce temperatures up to 14.5 °C higher (Charabidze *et al*., 2011). But as the ambient temperature is increased to 28-33 °C, heat emission within the mass becomes less important. The optimum ambient temperatures for heat generation in masses was found to range from 22-25 °C, which resulted in maximum heat emission per larva (Charabidze *et al*., 2011).

Temperatures recorded in sunlit masses showed a strong positive correlation with ambient temperatures in comparison to shaded aggregations (Joy *et al*., 2006). In Washington state, exposed pig carcasses were recorded to have a microclimate 16.7 °C warmer than ambient, whilst shaded pigs had a mass temperature 11.4 °C above ambient (Shean *et al*., 1993). The same trends were observed on pig carcasses in Saskatchewan, Canada, where the average internal temperature of sun exposed pigs was 26 °C whilst at shaded sites temperatures averaged only 18.2 °C (Sharanowski *et al*., 2008). These results contradicted an earlier study investigating larval activity on sunlit versus shaded racoon carcasses, which reported that mass temperatures remained the same regardless of whether they were shaded or sunlit (Joy *et al*., 2002). However, the carcasses used in this study were only able to sustain small masses due to their size (3.2 kg and 5.8 kg) and, with data collected every three hours, carcasses experienced much disturbance.

A few years later the same investigators ran a similar study but substituted racoons with larger pig carcasses (23 kg and 24 kg) (Joy *et al*., 2006). Joy and colleagues reported that maggot masses did not always exhibit stable temperatures and that this may have been a function of the ambient temperatures. At both the sunlit and the shaded sites, not only were ambient temperatures the same, but mass temperatures were significantly higher than ambient on both pigs (Joy *et al*., 2006). However, both the 2002 and 2006 studies only used two carcasses (one in each condition) and researchers failed to replicate the experiment, which would have reduced variability, removed the risk of error and hence strengthened their results. The results of a single experiment applied to such a small number of subjects should not be accepted without question.

Mass temperatures on sunlit carcasses do positively correlate with ambient temperatures, a trend that has not been observed in shaded carcasses. These sun-exposed carcasses were also observed decomposing at faster rates than their shaded counterparts, possibly as a result of this warmer microclimate and more extreme temperature fluctuations (Catts, 1992; Joy *et al*., 2006; Shean *et al*., 1993). Slone and Gruner (2007) further investigated the subject and claimed that the effect ambient temperature had on mass temperature depended on the size of the mass. They suggested that it was only in small masses with a volume of less than 20 cm^3 that heat emission had a strong dependence on ambient, whilst larger aggregations with a volume exceeding 20 cm^3 displayed behavioural thermoregulation and produced temperatures fully independent of ambient.

2.7 Summary

The duration of blowfly development is largely dependent on the species and the temperatures experienced during the juvenile stage, with warmer temperatures resulting in a faster rate of development. This temperature-dependent rate of development is the basis for time of death estimates. Entomologists are able to estimate larval age, and hence mPMI, using their size, stage of development and thermal history. However, there are numerous factors to be considered when interpreting entomological evidence, which can influence both larval body size and growth rates. These include population variation caused by geographical and genetic differences, interactions with the surrounding environment, and the effect of mass-generated heat. The latter is a result of the gregarious behavior of blowfly larvae, with individuals aggregating into feeding masses during the 2nd instar. By 3rd instar these masses are well established and have been recorded to produce temperatures as high of 50 °C, 25 °C warmer than ambient and well above the proposed upper lethal temperature limit for many species. It has been demonstrated that these high temperatures can only be maintained if aggregations exceed a minimum size, with thermal output increasing as masses become larger. However, there are other factors that should also be considered when modelling mass temperatures, including species composition, ambient temperature, sun exposure and mass density. Yet despite the fact that this mass-generated heat is often highlighted in the literature as having an effect on larval development, there are ongoing difficulties with incorporating this concept into mPMI estimates. Whilst thermal summation models use ambient temperatures to determine growth rates and, hence, larval age, there is no equivalent model for mass-generated heat. This is due to a lack of controlled research on the subject, with very few papers reporting the temperatures of different sized masses or determining whether they

have an effect on larval growth rates and behavior. Having established the background of larval development and heat generation in maggot masses, as well as the aims of the project, this thesis now will now detail the methods used to conduct the research.

3 Experimental Design and Development

This research project was divided into six experiments, each with a specific focus and set of objectives. All six experiments shared some common methods and techniques and all were carried out in a controlled laboratory environment, which permitted a level of control over variables not possible in field studies. This chapter contains information concerning the methodological commonalities regarding experimental design, how colonies were developed and maintained in the laboratory, incubator settings, and the techniques used to setup and monitor masses. It also describes how ADH was calculated and discusses the blowfly species studied. There have been numerous laboratory based experiments carried out in the past that have investigated larval development, but the techniques and rearing conditions described vary slightly from paper to paper. Whilst planning the experimental design for this research, careful consideration was given to each.

3.1 Blowfly Species – *Lucilia sericata*

The use of laboratory reared blowfly colonies that require controlled conditions is typical in entomological research. For this research project a single species was used. There were two reasons for this decision. Firstly, it was to account for the fact that different blowfly species have demonstrated different aggregating or thermal properties (Rivers *et al.,* 2010). Secondly, by focusing on a single species, additional time was made available to do more treatment levels and replicates. Had a second species been introduced into the research there would have been a trade-off between the number of treatment levels, replicates and species. The species used in all six experiments was *Lucilia sericata* (Meigen)(Diptera:

Calliphoridae), commonly known as the "greenbottle" (Figure 3.1). *Lucilia sericata* was selected for the research since it has a cosmopolitan distribution, a well-studied lifecycle and is a synanthropic species frequently recovered from both indoor and outdoor crime scenes in urban areas. These factors have resulted in it being recognised as a species of forensic importance (Anderson, 2011; Grassberger & Frank, 2004; Levot, 2003; Marchetti *et al.*, 2013; Pohjoismäki *et al.*, 2010; Vanin *et al.*, 2008).

Figure 3.1. Adult *Lucilia sericata* (Meigen) (Diptera: Calliphoridae)

Females of the species typically lay 150-200 eggs per batch and have been observed to favour fresher carrion for oviposition, in particular those exposed to direct sunlight with a warm surface temperature (Pohjoismäki *et al.*, 2010; Smith & Wall, 1997). Pre-adult mortality in the wild has been demonstrated to be high in *L. sericata,* accounting for losses of up to 93.3% in each generation with zero egg hatch occurring if relative humidity drops below 50% (Wall *et al.*, 2001). Several studies have observed the species to exhibit intense activity and dispersal behaviour during the migratory phase, with less than 1% remaining on the food to pupate (Anderson, 2000; Greenberg & Kunich, 2002; Mackerras, 1933).

3.1.1 Temperatures of significance

Using a well-studied species like *L. sericata* is beneficial since there have already been numerous experiments that have modelled their growth and development at different temperature regimes. Like many other blowfly species, the duration of their development is shortened as the temperatures they are exposed to increase. For example, larvae reared at 15.8 °C have been observed to take up to twice as long to develop in comparison to those reared at 23.3 °C (Anderson, 2000). Zhang and colleagues (2008) reported that the ambient temperature also influenced the duration of the egg stage as well as the larval stages, with warmer temperatures reducing the amount of time required for egg eclosion.

Several publications have reported a variety of developmental durations for *L. sericata* larvae at different constant temperatures, ranging from 38 days at an ambient of 15.3 °C (Anderson, 2000) down to 10.8 days at 34 °C (Grassberger & Reiter, 2001) (Table 3.1). However, one must remain cautious since differences in these intervals could be influenced by other variables in the laboratory unrelated to temperature; these include humidity (Byrd et al., 2010), food type (Clark *et al*., 2006), photoperiod (Nabity *et al*., 2007) and mass size (Slone & Gruner, 2007), as well as genetic differences between populations (Gallagher *et al*., 2010; Tarone *et al*., 2011). Marchenko's (2001) developmental data for *L. sericata* is notably absent from Table 3.1, despite being reported in other publications (Amendt *et al*., 2007). There is much confusion concerning the methods Marchenko (2001) used to obtain this data. In his paper he describes laboratory studies that were conducted to determine periods of development for 17 fly species. Yet out of 300 experiments carried out, only one involved *L. sericata*. In a table he later uses to illustrate the number of tests carried out on each species under specified

temperature conditions, nothing is recorded for *L. sericata* (Marchenko, 2001). This questions the validity and accuracy of the data that has been presented for the species.

Table 3.1. Developmental periods in hours and days (in parentheses) from oviposition for *Lucilia sericata* calculated by three authors at different constant temperatures (°C) (* indicates where larvae pupated but failed to emerge as adults) (table adapted from Amendt *et al*., 2007)

	Source					
Temp (°C)	Greenberg (1991)		Grassberger & Reiter (2001)		Anderson (2000)	
	pupa	**adult**	**Pupa**	**adult**	**pupa**	**adult**
15			612 (25.5)	*		
15.8					382.3 (15.9)	775.0 – 917.2 (32.3 – 38.2)
17			400 (16.7)	842 (35.1)		
19	221 (9.2)	391 (16.3)	271 (11.3)	564 (23.5)		
20			242 (10.1)	451 (18.8)		
20.7					245.7–356.9 (10.2 – 14.9)	486.2 – 647.8 (20.3 – 27)
21			221 (9.2)	379 (15.8)		
22	197 (8.2)	341 (14.4)	202 (8.4)	339 (14.1)		
23.3					264 (11)	468.5 – 624.5 (19.5 – 26.0)
25			172 (7.2)	297 (12.4)		
28			155 (6.5)	275 (11.5)		
29	158 (6.6)	290 (12.1)				
30			149 (6.2)	268 (11.2)		
34			139 (5.8)	259 (10.8)		

The lower developmental threshold for *Lucilia sericata* is 9 °C (Marchenko, 2001; Wall *et al*., 1992; Wall *et al*., 2001). A recent report published by Shiravi and colleagues (2011) suggested that each developmental stage had its own lower temperature threshold. They estimated that the lower temperature threshold for *L. sericata* could be calculated by averaging the basal rates of all the stages (8 °C, 8.2 °C, 11.3 °C, 9 °C), which gave an average of 9.5 °C (Shiravi *et al*., 2011). However, it should be noted that these four temperatures do not differ significantly from 9 °C ($t= 0.165$, $df= 3$, $p= 0.879$). The upper temperature

threshold for the species has been less well defined, with some researchers proposing an upper lethal limit of 35 °C (Wall *et al*., 2001) whilst others claim it is closer to 47.5 °C (Richards *et al*., 2009).

3.1.2 Benefits of using Lucilia sericata *in the laboratory*

Practicalities are important when choosing a model species for any population study, and blowflies are no exception. *Lucilia sericata* is a suitable species for controlled studies since it has been shown to reproduce readily in the laboratory and exhibits no delays in development after handling (Anderson, 2000). A study carried out to compare life cycles of *L. sericata* reared in the field with those reared in the laboratory showed that there were no differences between the two (Introna *et al*., 1989). In captivity adults will die after approximately seven weeks, with females surviving longer than males (Mackerras, 1933). Perhaps most importantly though, the species has shown no evidence of inbreeding, which could negatively affect the stock (Mackerras, 1933).

3.2 Rearing/Maintaining Blowfly Colonies in a Laboratory

The *Lucilia sericata* colonies used in this research originated from larval stock purchased from a local fishing tackle outlet. The use of shop bought larvae over wild stock was more a necessity than a choice since research began in the early winter when adverse weather conditions and cold ambient temperatures resulted in there being little to no fly activity observed in the field. However, despite using shop bought stock to avoid a prolonged delay in the start of practical work, it was several weeks before viable adults emerged in the laboratory. During these weeks

multiple batches of larvae were purchased, which consistently pupated but failed to emerge as adults.

3.2.1 Housing blowfly colonies

Adult flies were maintained in cages that measured 32.5 x 32.5 x 32.5 cm BugDorm-43030 Insect Rearing Cage. The sides of the cage were composed of polyester netting (mesh count 96x26 per sq. inch), which allowed air to circulate and light to enter. The front panel was composed of clear plastic with an 18 cm sleeve opening for the addition and removal of food and water, which otherwise remained knotted to prevent escape. Care was taken to limit fly numbers in each cage so as to reduce the risk of overcrowding and injury. Adults were provided with a constant supply of water and sugar. Porcine liver was also placed in the cage shortly after adult emergence to provide nutrients for ovary development. This liver was removed after two to three days so that eggs were not laid before they were required for experiments.

3.2.2 Incubator settings

Fly cages were housed in a certified and calibrated walk-in Coolwell incubator, which allowed light and temperature conditions to be pre-programmed (Figure 3.2). All six experiments were carried out in this same incubator, which measured 285 x 165 x 236 cm. Throughout the entire research period the incubator settings remained constant. Temperature was set at 22 °C since this has been demonstrated to be within the optimum range for *Lucilia sericata* where maximum heat emission is promoted (Charabidze *et al*., 2011). This constant temperature was confirmed by placing an EL-USB-1 temperature data logger (resolution 0.5 °C,

accuracy ± 1 °C) inside the incubator set to record ambient temperature every five minutes for the experiment duration. The light source was supplied by standard 40 W fluorescent tubes, which emitted warm white light programmed for a 16:8 hour (light:dark) photoperiod. A 16:8 hour photoperiod was used since previous research has demonstrated that this day length has minimal influence on the life cycle (Gennard, 2007). Whilst other studies have been carried out under a 12:12 photoperiod, this was not considered an issue for the current research project since comparisons were not going to be made between these experiments. A humidifier was kept in the incubator and set to maintain humidity at 65-70%. Maintaining humidity at this level was important since eggs and early 1st instar larvae were prone to desiccation if the atmosphere became too dry.

Figure 3.2. The layout of the walk-in incubator taken from the doorway with shelves along two of the four walls and fly cages located to the right of the image

3.2.3 Harvesting eggs and larvae for experiments

Eggs were harvested by introducing pork liver into cages housing mature, gravid females to stimulate oviposition. Pieces of liver measuring approximately 5 cm across were held in small plastic pots lined with damp paper towels to prevent the meat from drying out. These baits were left in the fly cages for three to four hours and then removed before the liver became overcrowded with eggs. A vented lid was placed on these pots covered with a fine netting, which allowed air to circulate but prevented hatched larvae from escaping. Pots that contained liver and eggs were then placed on a shelf inside the incubator where they remained undisturbed until 1st instar larvae emerged. Early trials proved problematic since eggs and newly emerged larvae were prone to desiccation. To prevent this the contents of the pots were misted twice daily with water to keep them moist, once in the morning and again in the evening.

Various studies have suggested that the growth rate of some blowfly species fed on liver is reduced in comparison to larvae fed on other tissues (Clark *et al*., 2006; Kaneshrajah & Turner 2004). Clark and colleagues (2006) reported that *Lucilia sericata* larvae fed on liver sourced from pigs showed a developmental lag of up to two days compared to larvae fed on other tissues (Clark *et al*., 2006). However, liver was deemed acceptable for this research since it was only being used as a bait and site for oviposition. Shortly after hatching 1st instar larvae were transferred to muscle tissue for the duration of feeding.

3.3 Setting Up and Running Experimental Masses

Each of the six experiments carried out as part of this research project involved rearing different sized maggot masses whilst monitoring their thermal output and/or development. Whilst different experiments utilised different mass sizes, the basic experimental design and methods used for set-up remained the same. All experiments used the same equipment and laboratory settings in order to reduce variation as much as possible and to facilitate comparisons. These methods are described below in detail.

3.3.1 Counting out 1st instar larvae

Different sized larval masses were prepared by counting out 1st instar larvae as opposed to individual eggs. This method was favoured for two reasons:

1. Early trials in the laboratory demonstrated that eggs suffered from a high mortality and were susceptible to desiccation. Since mass size was an important variable being investigated, it was vital that masses were composed of a specific number of individuals. Given that "dead" eggs cannot be readily distinguished from "living" eggs, it was possible that masses would be consistently smaller than expected due to unaccounted for egg mortality
2. Eggs were observed to stick to one another, making it hard to count out the exact number. Although there have been methods described on how to separate and handle these tiny specimens, there was concern that in doing so they might become damaged and mortality rates would further increase.

Harvested eggs were monitored at hourly intervals and 1st instar larvae were observed to hatch after approximately 20 hours (+/- 1 hr) whilst stored at an ambient temperature of 22 °C. These 1st instar larvae were then left to feed for a further 14-16 hours, allowing them to increase slightly in size and robustness, which made them less susceptible to damage whilst handling. These minute larvae were then counted out and randomly allocated to different sized masses. Depending on the experiment, the mass sizes used ranged from 50-2500 individuals. To avoid damaging fragile 1st instar larvae, individuals were separated and counted out using a fine, moistened, artists paintbrush (Amendt *et al*., 2007).

3.3.2 Transferring larvae to a food source

These masses composed solely of individuals in their 1st larval instar were then placed directly onto lamb breasts. A lamb breast is part of the forequarter and belly of the animal and contains a few ribs. The cut is oblong in shape measuring approximately 20 inches in length, seven inches wide and two inches thick. The flesh, which was still attached, was less than 1 cm thick all over and constituted less than 10% of the total weight of the food source. Lamb breasts were used since they were readily available from a local butcher at low cost and weighed between 1000 g – 1500 g each, which provided ample food for even the largest experimental masses. A large quantity of meat such as this ensured that larvae could complete development without experiencing competition, which can reportedly influence mass temperature (Campobasso *et al*., 2001; Campobasso & Introna, 2001; Shean *et al*., 1993). The meat and larvae were then stored in 10 litre plastic containers that measured 27 x 27 x 16 cm. To ventilate the containers a panel was removed from the lid and a fine netting was secured in its place to prevent larval escape (Figure 3.3). In the early trials it appeared as if larvae

experienced a period of disturbance immediately after being transferred to the lamb breast. Individuals were observed attempting to migrate away from the meat and out of the container whilst others aggregated to form non-feeding masses in the corner of the container. After approximately two hours, larvae were observed to return to the meat where they formed a single feeding aggregation. Since feeding larvae made no further attempt to leave the container, the lid would then be removed.

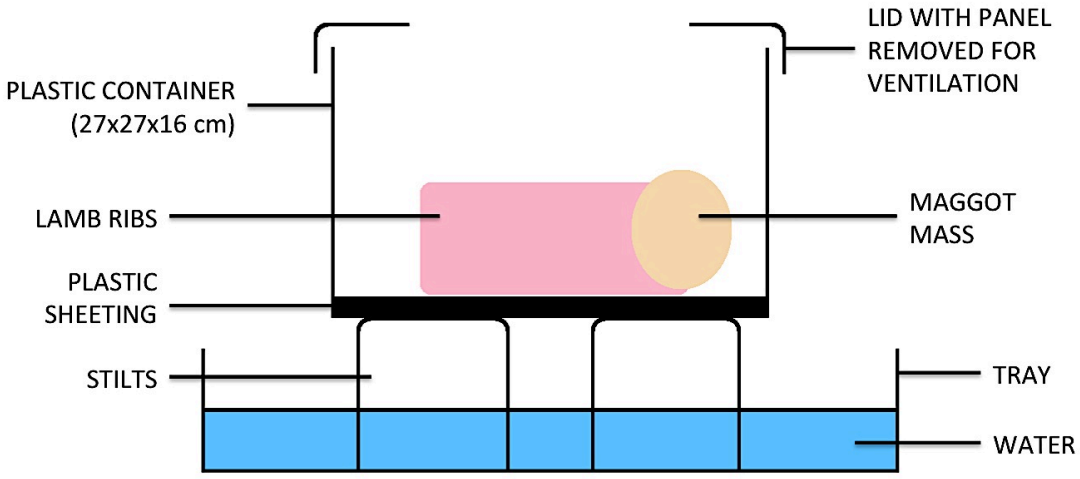


Figure 3.3. Diagram showing the experimental setup for maggot masses in the incubator, with the meat and mass held in a 10 litre plastic container suspended over trays filled with water to contain dispersing larvae. Note the vented lid is removed two hours after the start of the experiment once larvae have recovered from the initial disturbance of setup

3.3.3 Identifying the Start of dispersal

Larvae continued to feed in the mass until they reached the post-feeding stage of development. At this point individuals ceased feeding and began to migrate away from the aggregation in search of a suitable site for pupariation. *L. sericata* larvae displayed strong dispersal behaviour and on several occasions were observed escaping tightly sealed containers. For the majority of the experiments, the start of

dispersal marked the end of the data collection and was identified as the point at which 10% of the larvae had migrated away from the food. This figure of 10% has been used in previous studies as a marker for specific developmental points (Clarkson *et al*., 2004; Grassberger & Reiter, 2001). Therefore, an important part of the experimental design was that it had to be possible to accurately record the number of larvae completing feeding and migrating away from the food source. But since the lid had been removed to accommodate this dispersal, there was the risk that larvae might overrun the incubator and laboratory if their movements were not restricted once outside of the container.

Trials in the laboratory provided an opportunity to test various methods for capturing and containing wandering larvae. One such method involved placing trays filled with sawdust and wood shavings underneath the 10 litre plastic containers. It was assumed that migrating larvae would exit the container, land in the trays and pupate in the wood shavings. Instead the larvae continued to migrate out of these trays and into other areas of the laboratory. Another method, which showed greater success, involved replacing the wood shavings with water approximately 1.5 cm deep. As larvae crawled out of the container they landed in the underlying trays filled with water, but were unable to climb out. This made counting their numbers easy (as opposed to searching for larvae amongst wood shavings) and since no data was required from larvae that had already migrated (e.g. lengths and/or weights) it was of no consequence to the experiment if they drowned. It is important to note that since water is a poor absorber of heat and often remains several degrees cooler than ambient (specific heat capacity of water: 4181 J/kg °C), the container holding the meat and larvae was raised out of the

water on upturned pots or “stilts” to avoid any cooling effect it might have (Figure 3.3).

3.3.4 Aging larvae

The first two experiments required larvae to be sampled periodically so that their instar could be determined. Aging larvae to their instar made it possible to monitor development and record the duration of each of the three feeding instars. Ten larvae were sampled from all parts of the mass (centre and periphery) at regular hourly intervals for the duration of the feeding stage of development. Their posterior spiracles were examined under a Leica M80 stereomicroscope and individuals were identified as being in either 1st, 2nd or 3rd larval instar (Figure 3.4). All sampled larvae were immediately returned to the aggregation to avoid an overall reduction in mass size.

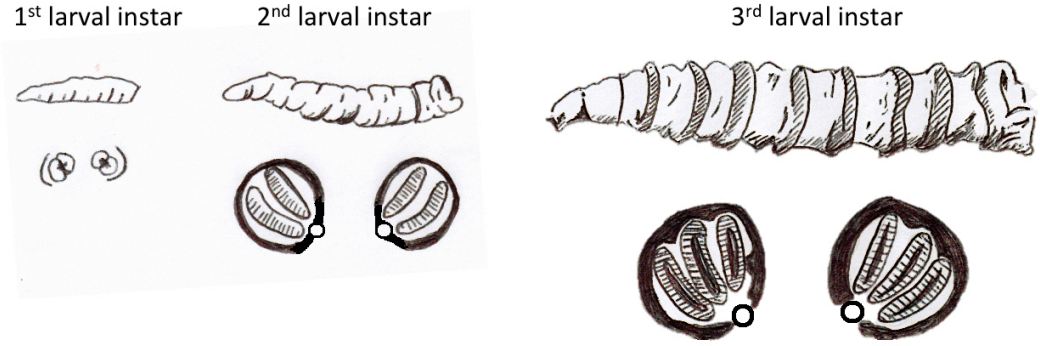


Figure 3.4. Diagrams showing 1st, 2nd and 3rd larval instars for Calliphoridae with the posterior spiracles drawn underneath for the benefit of aging (image hand drawn based on descriptions provided in Goff 2001)

3.3.5 Killing larvae and recording length/weight

For Experiments 3, 4 and 6 the lengths and weights of larvae reared in different sized masses were measured at specific points in development. To ensure these measurements were as accurate as possible, care was taken to select a technique for killing larvae that would result in minimal shrinkage. The general consensus among entomologists is that larvae are best killed by immersion in very hot water that is close to boiling. Heating lessens autolysis by destroying digestive enzymes and gut flora, as well as altering the permeability of the cuticle, which allows larva to remain extended and rigid (Day & Wallman, 2008). This makes it easier to record length. Because Adams and Hall (2003) proposed that larvae immersed in 80 °C water for 30 seconds experienced a minimal change in length, this was the technique applied throughout the current research project. Once larvae were killed they were left to dry for approximately ten to fifteen minutes on paper towels. This was the standard protocol for all larvae that were sampled and weighed. All larvae were dry at the time of measuring and weighing with any moisture either evaporating or being absorbed by the paper towels they were spread out on. Fresh weights were recorded as opposed to dry weights since there were concerns that the dry weights of individual larvae would be so small they would not register accurately on a standard laboratory balance. Larval length was then measured to the nearest 0.01 mm using a Mitutoyo Absolute Digimatic Calliper 0-200mm (accuracy ±0.02 mm) whilst larval weight was recorded to the nearest 1 mg with a Denver Pinnacle PI-403 weighing balance (accuracy ±1.0 mg).

It is important to mention that at no point during this research project were any preservatives used on larvae. How best to preserve larvae is a contentious topic in the field of entomology. Tantawi and Greenberg (1993), observed larval shrinkage

in 15 preservative solutions tested, with young 3rd instar larvae undergoing a greater reduction in size compared to older larvae. For this reason, since some of the larvae would be sampled during early 3rd instar, larvae had their lengths and weights recorded immediately following death so the use of preservatives could be avoided altogether. Another reason for recording lengths and weights immediately after death became clear in several trials. In the absence of preservatives, larvae left in a refrigerator overnight, or even for a period of four to six hours, were observed to slightly alter their body shape. These changes included minor head curling and a "shrivelled" appearance to the body, both of which could have complicated measurements and increased error.

3.4 Calculating Accumulated Degree Hours (ADH)

A value of accumulated degree hours (ADH) was calculated for larvae developing in aggregations in the first two experiments. ADH was used instead of degree-days (ADD) since it provided greater sensitivity to the temperature fluctuations that might occur in feeding masses. As discussed in the literature review, ADH is a fixed quantity of metabolic activity, controlled by time and temperature, which is required to drive a biological process such as larval growth. Ames and Turner (2003) suggested that blowfly larvae needed to accumulate a specific number of degree hours to move from one stage of development to the next. Therefore, if there is a warm period (i.e., a maggot mass forms) the developmental time will be shorter but the ADH value will remain the same. This provides a more quantitative approach to estimating larval age and hence, the mPMI (Megyesi *et al*., 2005).

ADH was calculated in two ways. One was calculated using the ambient temperature alone, which remained a constant 22 °C. The second was estimated with mass temperatures, recorded at regular five-minute intervals using either an EL-USB-1 data logger or a FLIR T425 thermal imaging camera (to be discussed in more detail in the relevant chapters). These two ADH values could then be compared with each other at specific times during development or across a range of mass sizes.

Before calculating ADH, the *base temperature* for the species had to be determined. This is the minimum temperature below which growth and development will not occur. It is sometimes referred to as the lower developmental threshold temperature (LDTT) and can vary between species and geographic location (Gennard, 2007; Marchenko, 2001). If an inappropriate base temperature is used in ADH calculations then it might result in an overestimation of ADH, which could lead to a wrong mPMI. Previously published research has demonstrated that the base temperature for *L. sericata* is 9 °C (Marchenko, 2001; Wall *et al*., 1992). The method most frequently used to determine this species specific temperature is the x-intercept approach, in which developmental rates are measured in the low temperature range and results are fit in a linear regression (Higley & Haskell, 2010). The linear regression can then be extrapolated to the x-axis, where the rate of development is zero. But Higley and Haskell (2010) warned that if the base temperature was determined using the x-intercept method, then it has no biological meaning and is nothing more than a mathematical consequence of approximating the development rate through linear regression. This means that any base temperature determined in this manner might differ from the actual biological base temperature, which could result in inaccurate results. However,

until further research is carried out that either supports of refutes the proposed base temperature of 9 °C, it will continue to be used for this species.

The concept of degree hours is based on the assumption that between the upper and lower temperature thresholds, the relationship between the rate of insect development and temperature is linear (Gennard, 2007; Greenberg & Kunich, 2002). ADH can be represented as the area under a curve, for temperatures above the base temperature, in each one-hour period. Since ambient temperatures in the incubator remained a constant 22 °C for the duration of the research, the calculations used to estimate ambient ADH were straightforward and remained the same for each mass. This involved subtracting the base temperature from ambient and then multiplying this temperature by time. Gennard (2007) gave a simple formula for this:

$$\text{Time}_{(\text{hours})} \times (\text{temperature} - \text{base temperature}) = \text{ADH}$$

For example, to calculate ambient ADH after 25 hours the following sum was carried out:

$$25 \times (22 - 9) = 325 \text{ ADH}$$

However, estimating mass ADH for each of the experimental aggregations was slightly more complicated, since mass temperatures were observed to differ between aggregations and over time. By carrying out controlled experiments in the laboratory it was possible to record mass temperatures accurately at regular intervals, which successfully produced thermal units that could be summed for

ADH. For example, mass ADH was calculated for each separate aggregation using the following formula:

(Mean hourly temperature – base temperature) + previous hours ADH = ADH

E.g., Hour 1 (26.5 – 9) = 17.5 ADH

Hour 2 (27.2 – 9) + 17.5 = 35.7 ADH

Hour 3 (26.9 – 9) + 35.7 = 53.6 ADH

Hour 4 (27.5 – 9) + 53.6 = 72.1 ADH

Recording mass temperatures at regular five-minute intervals provided an accurate value for the mean hourly mass temperature since it would have accounted for any minor temperature fluctuations occurring within the aggregation. Temperatures recorded at five minute intervals were summed for each hour and then divided by the number of readings taken within that allotted time to provide an average hourly temperature. In reality though, at a crime scene, it is highly unlikely that such a complete thermal history would be available for degree-hour accumulations.

3.5 Tagging Larvae with Visible Implant Elastomer (VIE)

The aim of Experiment 5 was to investigate this movement of larvae in a feeding mass. In order to achieve this a number of individuals from laboratory-reared aggregations were sampled and tagged so they could be distinguished from the cohort. However, tagging individual larvae is problematic. In the past, various batch-marking methods have been used in population studies to monitor the

dispersal of adult insects. These have included mutilation, tagging, paints, dyes and dusts, radioactive tracers and visual genetic markers (Hagler, 1997; Hagler & Jackson, 2001). Selecting an appropriate tag or marker for a particular species depends on the size, life stage and habitat of the targeted insect. Ideally it should be durable, inexpensive, non-toxic, easily applied and clearly identifiable (Hagler & Jackson, 2001). It should also persist without influencing the insect's growth, development, behaviour or reproduction. However, the aforementioned techniques were not designed to distinguish between individuals of a cohort, especially those still in the larval stage.

Until recently, blowfly larvae may have been considered too small to be individually tagged or marked. Aside from the fact that paints and dyes risk blocking the posterior spiracles, the conditions inside the mass (constant friction with other larvae in a damp environment) make it likely that any external mark will quickly be transferred to other larvae and lost. In 2007, as part of her PhD project, Swiger attempted to evaluate the movement of a larva within a mass by coating individuals with Day-Glo fluorescing powder. However, she acknowledged that in the early stages of the experiment it became obvious that this method was unsuitable for marking larvae for any length of time. Within minutes of the dusted larva being returned to the aggregation it was lost as a result of powder transfer to other members of the cohort (Swiger, 2007).

Despite past failings, a recent publication by Moffatt (2013) has highlighted a technique that could potentially facilitate the tagging and identification of individual larvae in a mass. Developed by biologists at Northwest Marine Technology Inc. (Washington, United States of America), Visible Implant

Elastomer, or VIE, was first made available in the 1990s as a potential tag for fish and possible replacement for the more traditional external tags and fin chips (Bailey *et al*., 1998; Griffiths, 2002; Leblanc & Noakes, 2012; Olsen & Vollestad, 2001). VIE consists of a brightly coloured, bio-compatible, two part, elastomer material, which once mixed can be injected into translucent animal tissues. This then solidifies over time to form a pliable internal tag that is visible externally and retains its structural integrity as the animal grows. In 2009, Butt and colleagues assessed the use of VIE as a tag for earthworms. They reported that as well as remaining visible for up to 27 months, the elastomer had a limited effect on growth, reproduction and mortality rates. Last year Moffatt (2013) published a report that described in detail methods for using VIE as a tag for 3rd instar *Calliphora vicina* larvae. The techniques he used resulted in larval survival rates of 80%, with no subsequent differences in their rate of development (Moffatt, 2013). For these reasons, the methods detailed by Moffatt (2013) were used to tag early 3rd instar *Lucilia sericata* larvae for the purpose of monitoring their movement in a feeding mass. Given that 3rd instar *L. sericata* larvae are smaller than *C. vicina* of the same age, this would make them the smallest animal tagged to date with VIE.

3.5.1 Tagging larvae

Visible Implant Elastomer was prepared in the laboratory and loaded into a BD 0.3 cc insulin syringe with a 29 g needle for tagging. The tag was then injected at the 11th segment, dorsally in the midline between two tissue masses (Figure 3.5). Whilst being tagged, the larva was held between the thumb and forefinger against a bench with the posterior end exposed. Tagged individuals were then returned to the feeding mass and their movements monitored. To aid in the visualisation of tagged larvae, an ultra-violet light source was used. This torch radiated a deep

purple light with the peak of its spectrum at about 405 nm, which caused the tags to fluoresce and increased visibility considerably (Figure 3.6).

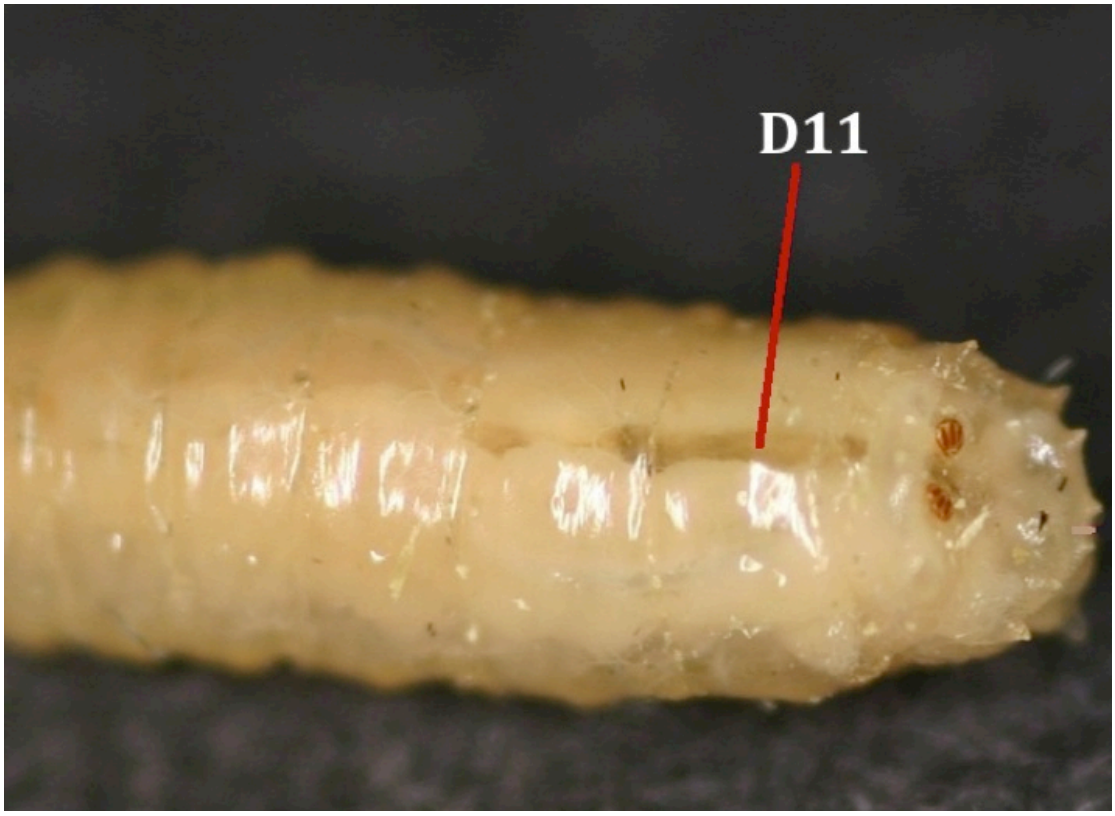


Figure 3.5. Posterior end of a 3rd instar blowfly larva showing the VIE injection site at the dorsum of segment 11 (D11) (Image courtesy of Moffatt, personal communication)

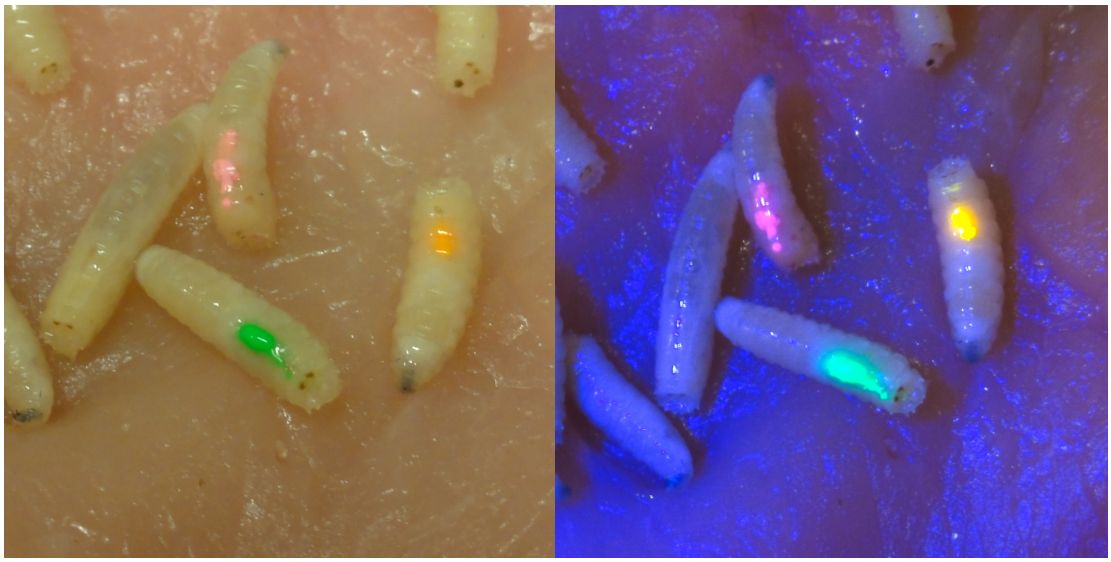


Figure 3.6. 3rd instar *Lucilia sericata* larvae tagged with pink, orange and green VIE as viewed under natural and ultraviolet light

3.5.2 Complications during tagging

Injecting larvae with VIE was not without its problems. Due to the small size of the larvae and their constant movement, injecting the tag in the correct location was difficult. As the needle was pushed through the integument there was a release of fluid from the injection site in every case. This same observation was made by Moffatt (2013), who reported that the loss of fluid did not appear to result in any subsequent deaths. However, larval death did occur if the needle was inserted too far. On several occasions this caused part of the larval gut to be extracted as the needle was withdrawn. But if not injected deep enough, the movement of the larval body was observed to push the tag back out via the wound. Tag losses such as this were reported in salmon where post-tagging handling caused the implant to be pushed out of the injection site (Bailey *et al*., 1998). Small "scatter tags" were also recorded, where a single tag was observed to separate into a collection of smaller tags after injection. Butt and colleagues (2009) also reported this separation of the elastomer and suggested it was due to the tagged animal being relatively small in proportion to the diameter of the needle (Butt *et al*., 2009).

4 EXPERIMENT 1 – Heat Generation in Different Sized Larval Masses[1]

The aim of this experiment was to quantify the relationship between the number of larvae in a maggot mass and the amount of heat generated locally. This was investigated by monitoring the thermal output of different sized larval masses reared under controlled laboratory conditions. The objective was to identify the minimum mass size that was capable of producing temperatures that were significantly warmer than ambient. This experiment also provided an opportunity to monitor peak temperatures in different sized aggregations as well as the timing of thermal changes. The results of this experiment should then help distinguish cases where mass-generated heat should be factored into mPMI estimates.

Following this introduction, the methods section will include details on the experimental set up and statistical analysis. The subsequent results section will include both quantitative and qualitative analysis, with graphs illustrating the thermal output of different sized aggregations during larval feeding and development. The results of pairwise comparisons between different sized aggregations are presented to illustrate which mass varied significantly in temperature. Bar charts will also highlight any differences between ADH calculated using ambient and mass temperatures. A brief discussion will follow explaining the findings.

[1] The results of this experiment have recently been published and can be accessed online at onlinelibrary.wiley.com:

Heaton, V., Moffatt, C. & Simmons, T. (2014). Quantifying the Temperature of Maggot Masses and its Relationship to Decomposition. *Journal of Forensic Sciences*

4.1 Materials and Methods

Recently emerged 1st instar larvae were counted out and randomly allocated to different sized masses containing either 50, 100, 150, 200, 300, 600, 900, 1200, 1500, 1800 or 2500 individuals. Fifty was chosen to represent the smallest aggregation since it was felt that anything smaller would fail to generate a level of heat discernible from ambient (22 ±1 °C). From 50 up to 200, mass size was increased gradually in incremental steps of 50 larvae. This was to help identify the minimum mass size capable of producing temperatures warmer than ambient. Once masses exceeded 200 individuals, the size differences between successive masses became increasingly large. Practicalities were important in selecting 2500 as the maximum mass size. Anything larger was deemed unsuitable for the laboratory since it would have exceeded the carrying capacity of the equipment and food supply. This could have resulted in a build-up of noxious gases in the laboratory and the formation of a stressful environment that might have encouraged premature dispersal. It should also be mentioned that time constraints on large masses had to be considered, with 2500 larvae taking up to three hours to be set up. Anything larger would have been too time consuming and might have impacted data collection. For each of the eleven mass sizes there were three replicates, plus three controls, which gave a total of 36 sets of recorded data. Controls for the experiments consisted of the same type of meat held in the same type of box under the same incubator settings but with no larvae.

Masses composed solely of individuals in their 1st larval instar were placed directly onto lamb breasts held in the 10 litre plastic containers as described in the methodology chapter. Containers holding the meat and larvae were then placed back in the incubator at a constant temperature of 22 °C and with a 65% RH and

16:8 hour (light:dark) photoperiod. Larvae were sampled periodically throughout the experiment, their posterior spiracles examined under a Leica M80 stereomicroscope to help keep track of development. This was done at least four times daily every two to three hours until at least 50% of the sample had reached 3rd larval instar. All sampled larvae were returned to the aggregation to avoid an overall reduction in mass size.

4.1.1 Recording mass temperatures

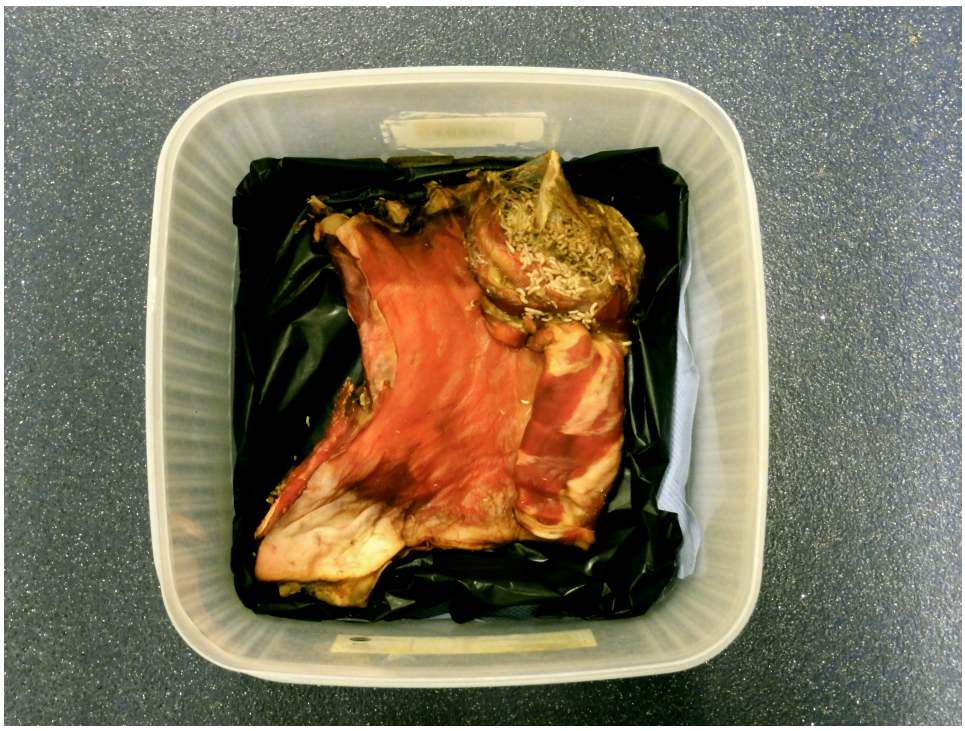
The objective of this experiment was to accurately record the temperatures inside different sized masses whilst they fed on lamb breasts and developed from 1st larval instar to 3rd instar post-feeding. Trials were carried out to test various techniques for recording these temperatures and highlighted a number of issues related to the structure of the mass and the behaviour of larvae. Preliminary experiments using temperature probes were found to be problematic since the movement of larvae resulted in the probes repeatedly being pushed out and away from the aggregation. The same was observed when using the Signatrol SL50 "button" data loggers, which were inserted into the centre only to reappear at the periphery several minutes later. Therefore, the slightly larger EL-USB-1 data loggers (minus probes) were used to record mass temperatures at regular five-minute intervals from the start of the experiment. It should be noted though that this method did not guarantee that every temperature recorded represented the maximum core temperature. It did however allow mass temperatures to be monitored at regular intervals over a period of time, which highlighted any patterns in heat emission.

4.1.2 FLIR T425 Thermal Imaging Camera

Temperature readings were verified periodically using a FLIR T425 thermal imaging camera (FLIR Systems Ltd (UK), 2 Kings Hill Avenue, West Malling, Kent, ME19 4AQ UK). This handheld camera has a temperature range of -20 – 1200 °C (accuracy= ±2% of reading) and came with features such as instant reports, periodic storage, and a laser pointer to pinpoint the hotspot on an infrared image. Infrared imaging is being used more frequently in scientific research due to its resolution, affordability and portability. Not only is the thermal imaging camera a non-invasive and non-destructive tool for measuring the thermal output of masses, it records temperatures over a wide area in a single reading, which makes it possible to locate several masses on a single corpse (Johnson & Wallman, 2014). However, it should be noted that infrared imaging records the surface temperature of an object and not the internal temperatures. In the field where large carrion is colonized by larval masses several centimeters deep (Slone & Gruner, 2007), it could be assumed that the temperatures recorded by the camera do not give a true representation of the actual temperature inside the mass. This was not considered to be an issue for laboratory experiments due to the much smaller mass sizes investigated (approximately 1 cm deep), which spent the majority of their time feeding at the surface. A recent study by Johnson and Wallman (2014) used small pig carcasses in the laboratory to test how effective thermal imaging cameras were at registering mass temperatures. They reported that the maximum temperature readings recorded by the camera had an almost perfect correlation with the temperatures recorded by data loggers located inside the aggregation (Johnson & Wallman, 2014).

Figure 4.1 shows a maggot mass containing 2500 3rd instar larvae feeding on a lamb breast (4.1a) with its associated thermal image (4.1b). The two images were taken at the same time. The thermal image (Figure 4.1b) illustrates variations in temperature, which allows warm objects, in this case the maggot mass, to stand out against a cooler background. A scale located on the right hand side of the image gives the temperature range (°C) for everything within the cameras field of view, with the maximum and minimum temperatures reported as the limits on the scale. For example, Figure 4.1b shows that the mass did not have a uniform temperature. Instead the aggregation had a hot centre (31.7 °C) with temperatures gradually decreasing out towards the periphery (approximately 25-27 °C). Mass temperatures were recorded with this camera at least four times daily prior to any larval sampling. Thermal images were recorded beforehand to account for any disturbance sampling might have had on the mass, which could have influenced the structure of the aggregation and hence its heat distribution.

a)



b)

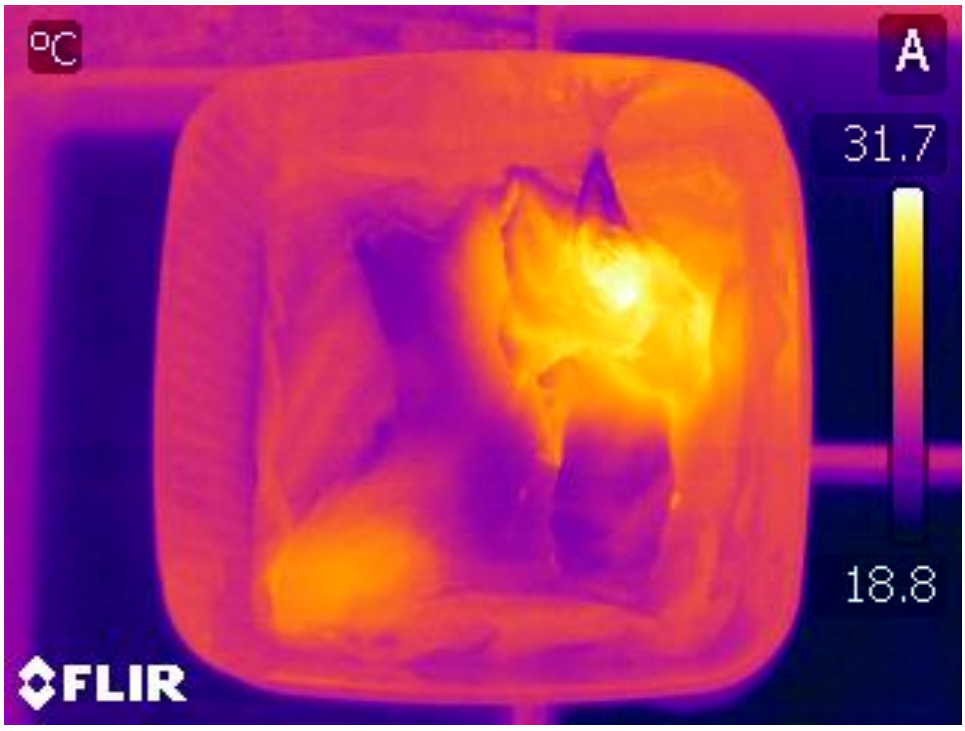


Figure 4.1. A maggot mass composed of 2500 3rd instar larvae feeding on a lamb breast as viewed normally (a). The same 2500 larval aggregation as viewed through a FLIR T425 thermal imaging camera (b)

Whilst the camera displayed a range of temperatures, it was the maximum reported temperature that was recorded since this represented the hottest part of mass. Temperature readings taken from the thermal imaging camera were later

compared with data logger readings made at the same time. Comparisons of these two readings were consistently within 1 °C of each other (Paired T-Test: t= 0.6759, df=120, p= 0.5119). An EL-USB-1 data logger was also used to take readings at five-minute intervals inside the incubator to ensure the ambient temperature remained constant. Once larvae entered the post feeding stage of development and began to migrate away from the food source dispersing the mass, data collection ceased.

4.1.3 Statistical analysis

For each of the 36 masses, the first two hours of temperature data were excluded from the analysis. This allowed time for both the refrigerated lamb breasts to adjust to ambient and for larvae to recover from any physical disturbance experienced in the initial stages of the experiment. It should be noted that whilst the food source was left in the incubator for some time prior to the start of the experiment in order for it to warm to ambient, the meat took longer than expected to heat up. On several occasions, if left for too long in the incubator, the soft tissue was observed to start drying out. Therefore, 1st instar larvae were added to the food source sooner than anticipated so that they could commence feeding before the food became too dry, which could have had a negative impact on their food consumption. The remaining data were then analysed using the statistical package R (version 2.12.1) (R Core Development Team, 2010). R was preferred over other statistical packages due to it being a multiplatform and open source, which enables easy collaborations and ensures the reproducibility of the analysis. In comparison to other packages such as SPSS, R also has improved graphics capabilities, is extremely versatile and available to download for free.

Diagnostic plots showed the data to be normally distributed and of uniform variance in every case. QQ-Norm plots were used to test for normality whilst a Residual vs. Fitted plot was used to assess the variances. A Mixed-Effects Model or Model III ANOVA (Zar, 2010) was carried out on the data to compare overall temperature in the different mass sizes between the times of 120 and 3000 minutes into the experiment. A Mixed-Effects Model is a class of ANOVA, which contains both fixed and random variables, taking each into account. Whilst identifying the response and explanatory variables, the model also takes into consideration the random variables, which in this case are the repeats. The results of this analysis were investigated further with a post-hoc multiple comparison of means using Tukey's test, which was used to identify which masses produced significantly different temperatures. A Tukey's test was used on the data since the test is designed to carry out multiple comparisons at one time. If separate tests were carried out for each pair then there would have been an increased risk of producing a low p-value purely at random. Therefore, rather than using multiple t-tests and correcting the p-value, it was deemed more appropriate to run a Tukey's test (since > 2 groups), which compared each pair and then produced a p-value that took into account the multiple comparisons. Peak temperatures recorded for the masses were examined using ordinary least squares linear regression. This determined whether there was a relationship between mass size and the maximum temperature produced and produced a regression equation, which could be used to estimate peak temperature based on mass size.

Accumulated Degree Hours (ADH)

Using the recorded temperature data and a base temperature of 9 °C, it was also possible to calculate accumulated degree hours (ADH) for each of the masses (as

described in the previous chapter). A simple one way ANOVA followed by post-hoc multiple comparison of means using Tukey's test were used on these data to identify which masses had significantly different ADH values at specific times during development. Again diagnostic plots showed the data to be normally distributed and of uniform variance in every case.

4.2 Results

4.2.1 Casual observations

Condensation was observed on the inside of the plastic containers housing the larger masses (1200+ individuals) along with a substantial amount of liquefied tissue accumulating in the vicinity of the aggregation. Larvae appeared more active in these larger masses, moving rapidly through the aggregation. On a number of occasions these large aggregations were observed to separate into two smaller units (Figure 4.2). Smaller masses remained whole and displayed less active behaviour, whilst the conditions produced inside their containers were considerably drier. Larvae began to move away from the meat after approximately 48-60 hours into the experiment (*c.* 96-108 hours after oviposition), with larger masses completing development earlier than smaller masses.

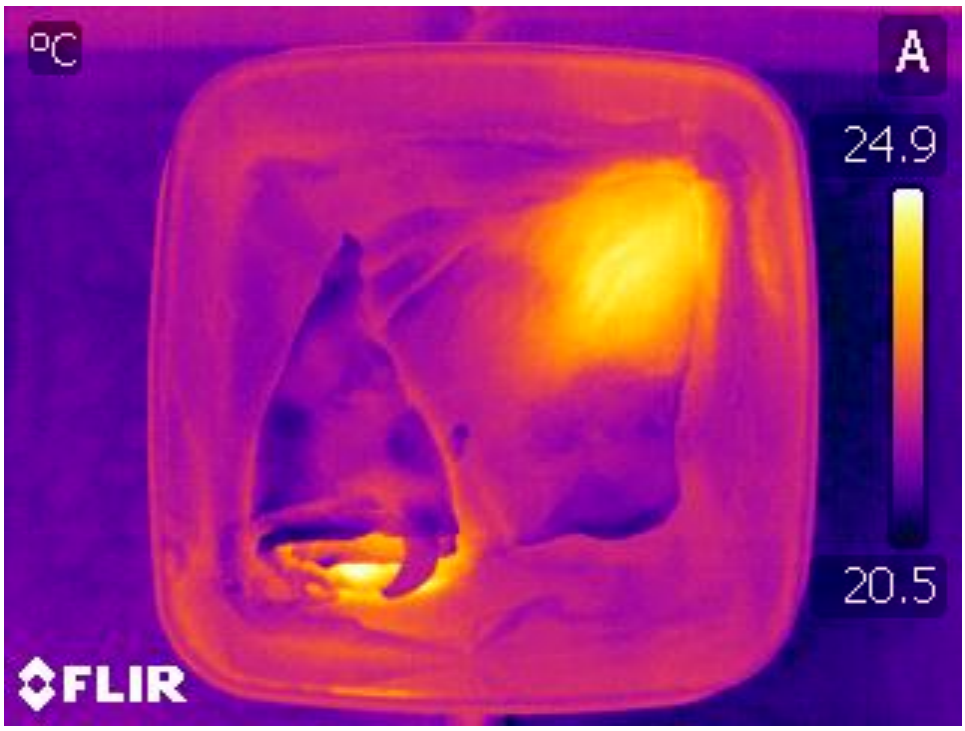


Figure 4.2. Thermal image of an 1800 larval mass observed to split and separate into two smaller aggregations positioned at opposite ends of the food source

4.2.2 Overall mass temperatures

Recorded temperatures taken from within the masses ranged from 20.5 °C (± 1.01) (1.5 °C below ambient) to 36 °C (± 3.74) (14 °C above ambient). Figure 4.3 clearly illustrates that as the larvae in the masses developed they were capable of generating heat greater than ambient, with temperature increasing in relation to mass size. Temperature changes were neither gradual nor constant and the highest temperatures were not recorded at the end of feeding when larvae were at their largest. Instead, temperatures were observed to rise and fall throughout development, producing two distinct temperature peaks. During the initial hours of development all masses, regardless of their size, showed temperatures that were not only comparable to one other, but also to ambient. For the first *ca*. 500 minutes, temperatures increased gradually before reaching a plateau where they remained constant for a further *ca.* 500 minutes. After this point mass temperatures became more variable. Larval masses became progressively warmer

as development advanced, with larger masses (1200+ larvae) showing more exaggerated increases. However, despite the 2500 masses peaking at approximately 36 °C, several degrees higher than the 1800 mass, their temperature curve was initially shallower in comparison. This indicated a slower rate of temperature increase. After peaking in the 3rd larval instar, temperatures dropped, but were maintained several degrees above ambient for the duration of the experiment until masses experienced a second, smaller peak just prior to the migratory stage of development.

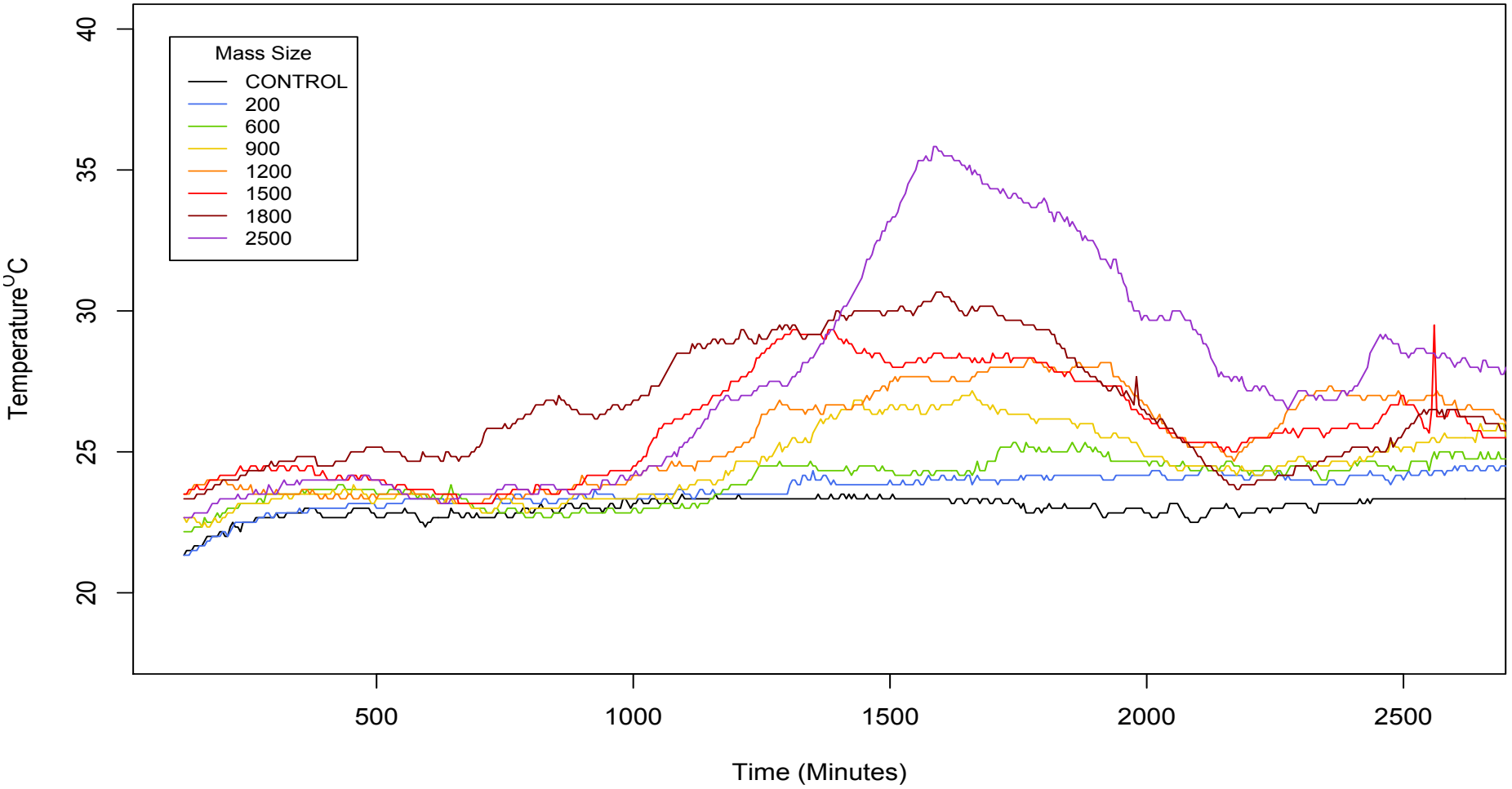


Figure 4.3. Mean mass temperatures (°C) vs. time (mins) for each of the mass sizes that displayed temperatures differing from ambient (control).

Even the smallest masses, which contained just 50 individuals, were recorded producing temperatures up to 2.5 °C warmer than ambient. Statistical analyses were carried out on the temperature data and showed that there were significant differences amongst masses of different size ($F_{11,24} = 12.70$, $p < 0.001$), with larger masses producing higher overall temperatures. Using the control group to represent ambient, the results showed that smaller masses (50-900 individuals) produced temperatures that were comparable to one another as well as to ambient ($p \geq 0.340$) (Table 4.1). Larger masses composed of 1200 larvae or more produced overall temperatures significantly higher than ambient as well as the majority of the smaller masses. However, despite larger masses producing more heat than ambient, they failed to be significantly warmer than masses of a comparable size. For example, the 2500 masses were not significantly warmer than the 1800, 1500 or 1200 masses whilst the 1200 masses was only warmer than the control and the masses containing 50, 100 and 150 larvae. This suggested that when comparing the thermal output in two aggregations, there needs to be a difference in mass size of approximately 1000 larvae or more for those temperatures to differ substantially.

Table 4.1. Results of the Tukeys test comparing temperatures in different sized aggregations. P-values shaded in red show where significant differences occurred between masses with Control (CTRL) representing ambient (22 °C ±1)

	CTRL	50	100	150	200	300	600	900	1200	1500	1800
50	1.00										
100	1.00	1.00									
150	1.00	1.00	1.00								
200	0.99	1.00	1.00	1.00							
300	0.98	1.00	1.00	0.99	1.00						
600	0.93	1.00	0.99	0.95	1.00	1.00					
900	0.34	0.90	0.58	0.40	0.98	0.99	1.00				
1200	**<0.01**	**0.04**	**<0.01**	**<0.01**	0.11	0.13	0.26	0.87			
1500	**<0.01**	**0.01**	**<0.01**	**<0.01**	**0.04**	**0.05**	0.10	0.65	1.00		
1800	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.03**	0.84	0.97	
2500	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.06	0.16	0.95

4.2.3 Peak temperatures

The maximum temperature was recorded for each of the 36 experimental masses and ranged from 23.5 °C in the 100 masses up to 36 °C in the 2500 masses. All temperature peaks occurred after 1200 minutes (20 hours) had elapsed. Figure 4.4 displays the data points and regression line for peak temperature against mass size with 95% confidence intervals. The plot gives an indication of the maximum temperatures that can be attained in different sized larval masses composed solely of *L. sericata* and reared at an ambient of 22 °C.

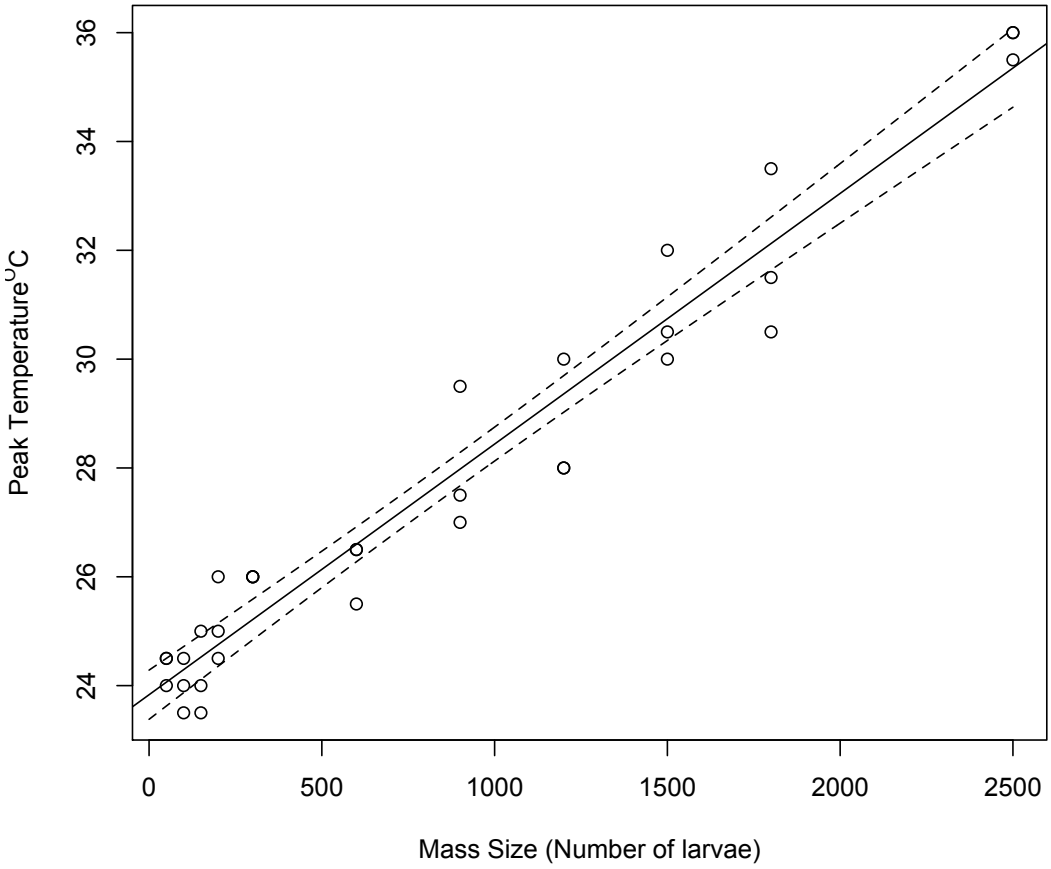


Figure 4.4. Peak recorded temperature (°C) for each of the experimental masses plotted with its regression line and 95% confidence intervals.

The regression line through the points produced a simple regression equation with "PEAK" representing the absolute maximum temperature recorded in the mass and "MASS" representing the number of larvae in the aggregation:

$$PEAK = 23.8 + 0.0046 \times MASS$$

This model fits the data well ($r^2 = 0.95$, $F_{1,34} = 685$, p-value < 0.001) and shows that there was a strong positive relationship between the recorded maximum temperature and the size of the maggot mass, with the peak temperature increasing by 0.46 °C for every additional 100 larvae.

4.2.4 Accumulated Degree Hours (ADH)

At 10 hours into development the first differences in ADH values were observed ($F_{11,24}$ = 2.44, p-value = 0.033), with the 1800 masses producing an ADH value greater than ambient (Figure 4.5, A). As time progressed and ADH values increased, pairwise comparisons showed that an increasing number of masses began to produce ADH values that differed from ambient as well as from each other (Figure 4.5, B). For example, at 30 hours, the 900 masses had an ADH value that was comparable to all the smaller masses, plus the 1200 and 1500 aggregations. However, it was significantly lower than the ADH calculated for the 1800 and 2500 masses ($p < 0.001$ in both cases). After 40 hours of feeding and development, maggot masses composed of 1200 larvae or more all had significantly higher ADH values than ambient (1200 masses: $F_{11,24}$ = 16.2, $p < 0.001$) (Figure 4.5, C), and masses up to 150 larvae. Still at 40 hours, the 1500, 1800 and 2500 masses produced significantly higher ADH values than masses containing up to 600, 900 and 1200 larvae, respectively. Twenty-six hours were required before the 1200 masses were capable of producing ADH values greater than ambient ($p = 0.048$). Larger masses composed of 1500, 1800 and 2500 larvae had ADH values exceeding ambient after 19, 10 and 22 hours, respectively. Despite the 2500 masses producing the maximum temperatures, the amount of time required for them to accumulate heat energy, as reflected in the ADH values, was longer in comparison to smaller masses. Aggregations composed of 1500 and 1800 individuals may not have reached as high a temperature as the 2500 masses, but they were capable of producing ADH values significantly greater than ambient several hours earlier.

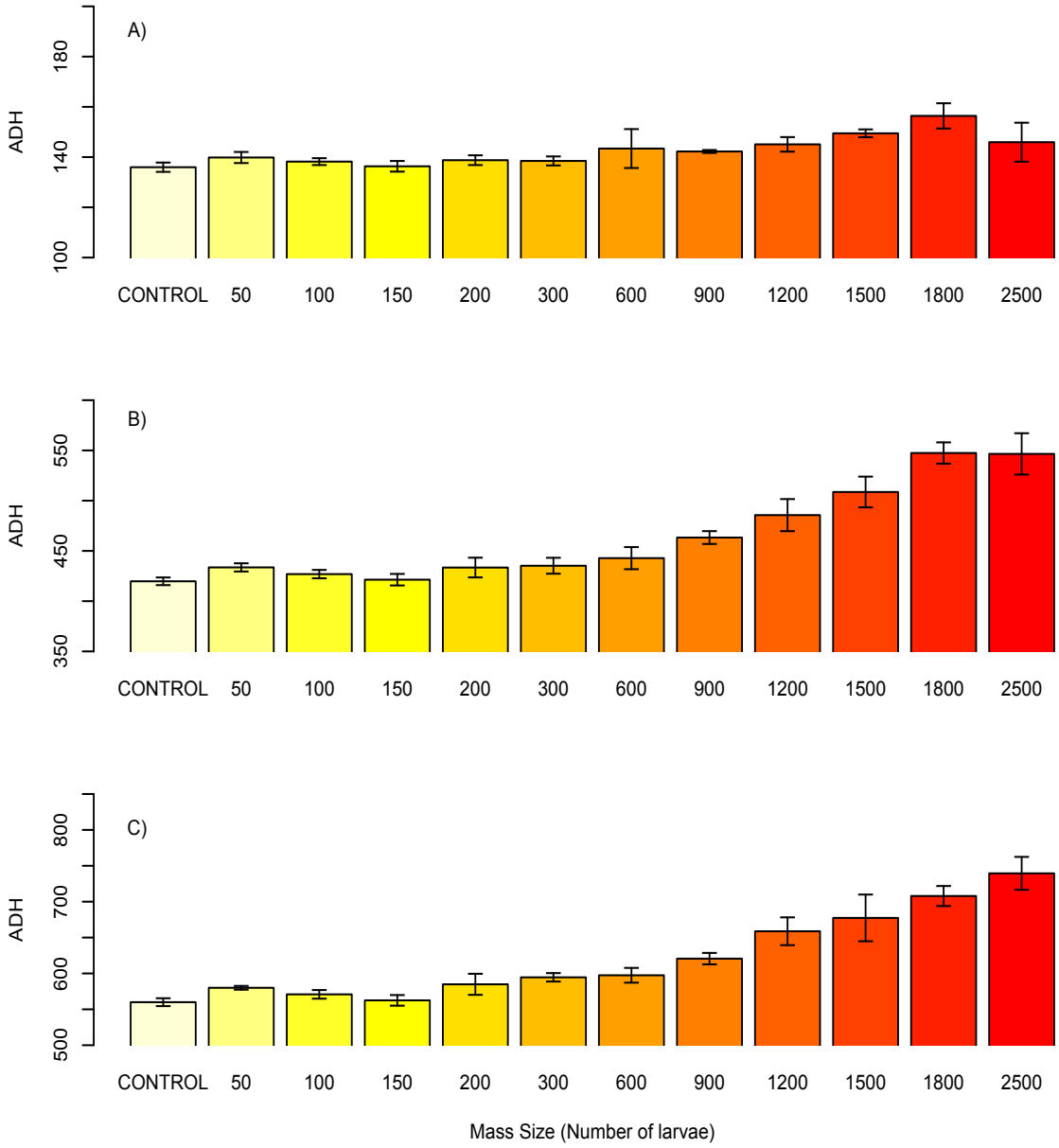


Figure 4.5. ADH values for each mass size at (A) 10 hours, (B) 30 hours and (C) 40 hours with associated standard error bars (calculated using a base temperature of 9 °C for the species *L. sericata*)

4.3 Discussion

The results of this experiment indicate that a strong positive association exists between mass size and heat generation, with temperatures rising as masses increase in size. This is most likely explained by escalating levels of metabolic heat emitted by increasing numbers of larvae feeding simultaneously in one area. This experiment showed that not only did mass temperatures increase with the size of

the aggregation, but the thermal output also increased as development progressed. This might have been a result of the larger body sizes observed in older larvae. It is possible that older, and hence larger larvae, had to raise their metabolism to process the greater amount of food ingested to sustain a larger body size. This idea is supported by studies that proposed heat emission was dependent on larval instar, with individuals in their 2nd larval instar emitting less heat than those in their 3rd larval instar (Charabidze *et al*., 2011; Joy *et al*., 2006; Rivers *et al*., 2010).

Although masses showed an initial increase in temperature, these highs were observed to drop several hours before migration. One explanation for this might be the splitting of larger masses into smaller aggregations. This was witnessed on several occasions, particularly in masses composed of 1200 larvae or more, and could be perceived as a form of thermoregulatory behaviour. By splitting into smaller masses larvae are removing their potential for producing critically high temperatures. A similar observation was reported from a study that monitored larval masses on rhinoceros carcasses in Africa (Richards *et al*., 2009). Here the authors reported that they observed spatial partitioning in mixed-species aggregations; species with a lower upper lethal temperature limit (*Chrysomya albiceps*) formed separate masses at the cooler periphery.

Thermoregulatory behaviour may be triggered in masses during early larval development when individuals are at their most vulnerable. This could explain why the 2500 masses took longer to accrue heat in comparison to the smaller masses. If the suggested upper lethal limit of approximately 35 °C for *Lucilia sericata* is in fact accurate (Charabidze *et al*., 2011; Wall *et al*., 2001), it would coincide with the maximum temperature recorded in the 2500 masses. The early

recognition of a potential problem, such as the presence of a large number of larvae that might result in thermal stress, could allow individuals to alter their behaviour accordingly, preventing temperatures becoming too high too soon. Research carried out by Byrd and Butler (1996, 1997, 1998) using temperature gradients suggested that larvae have a species-specific preferred temperature and will actively reposition themselves to regulate their temperature for accelerated development. They proposed that larvae would alternate between the hot feeding centre of the mass and the cooler periphery to prevent overheating, but perhaps at the expense of the amount of time spent feeding. This might explain the increased activity and movement observed in larger masses.

4.3.1 Peak temperatures in maggot masses

The graph of the linear regression is useful in that it gives investigators an indication of the types of temperatures that can be attained by even relatively small aggregations of *Lucilia sericata*. However, it is important to stress that this formula can only be assumed to be applicable over the mass sizes used in this experiment (50-2500 larvae) and at an ambient temperature of 22 °C. The plotted data points and their associated regression line propose that, as mass size increases, peak temperatures increase indefinitely with no signs of reaching a maximum level. In reality though, this is impossible, especially in large masses comparable to those found on cadavers. Evidence of a temperature plateau was presented by Slone and Gruner (2007) in plots that illustrated the relationship between mass volume (cm^3) and aggregation temperature (°C). Their graph indicated that as aggregations approached a volume of 1000 cm^3, heat emission began to level out at approximately 45-50 °C. These masses, comprised of several thousand larvae, were significantly larger than the aggregations used in the

current laboratory experiment, so perhaps it is not surprising that the temperature plot presented here shows no indication of this plateau. If the thermal output of a mass were to increase above the upper lethal temperature limit, then larvae would be effectively killing themselves with thermal stress. Although there was no evidence of larval mortality in this experiment, Slone and Gruner (2007) did report observing lifeless larvae on the periphery of large masses where temperatures were recorded as high as 50.7 °C.

4.3.2 Factors influencing heat generation

There are numerous other factors, aside from mass size/volume and age, which should be considered whilst attempting to model the thermodynamics of larval aggregations. By considering sources of variation, investigators can more accurately estimate developmental rates and, hence the mPMI for a case. For example, several studies have postulated that heat emission is strongly affected by mass density, with higher temperatures being recorded in denser, more tightly packed masses (Charabidze *et al*., 2011; Goodbrod & Goff, 1990). Maximum temperatures have been recorded for masses reared on 50-100% of their weight in meat but thereafter, the peak temperature decreased as the quantity of food increased (Charabidze *et al*., 2011). If this is true, then the masses observed in this study may not have reached their maximum potential for heat generation since they were specifically fed excess food to prevent overcrowding. Excess meat would provide a larger surface area, allowing all individuals in a mass the opportunity to access food simultaneously. This was observed in the current study, with several masses appearing "disc-like" in formation, composed of a single layer of larvae lined up in feeding rows with only their posteriors exposed. The same number of larvae feeding on a smaller surface area would likely form a

denser mass which could increase temperatures as a result of reduced evaporative cooling as well as displacement of competing larvae and an increase in muscular-activity related heat emission (Charabidze *et al*., 2011).

It should also be noted that different species are unlikely to share the same aggregating or thermal properties. A recent study, which investigated the development of two species of flies reared in different sized masses, reported that aggregations comprised of the flesh fly *Sarcophaga bullata* consistently produced higher temperatures than same sized masses of *Protophormia terraenovae*. This was attributed to the larger body size of *Sarocphaga bullata* (Rivers *et al*., 2010). Interspecific competition in mixed-species masses could also influence heat generation in aggregations. Although this was not a factor to be considered in the current experiment, researchers engaged in field studies involving natural colonization should be aware of its effects. These include spatial partitioning and species with a high upper lethal temperature raising the mass temperature to outcompete species with lower thresholds (Richards *et al*., 2009).

Internally generated heat in larval aggregations has the potential to impact significantly on mPMI estimates. This experiment has shown that even small larval masses of approximately 1200 individuals are capable of producing a level of heat significantly greater than ambient after as little as 26 hours. This microclimate may have influenced the development of larvae within the mass, which could have consequences for the field of forensic entomology. If the high temperatures produced by larval masses have disrupted feeding and development, then older larvae might be smaller and hence appear younger than they actually are. The problem then arises when these underdeveloped larvae are compared to

laboratory-reared larvae and aged without taking into account the mass size or thermal history. This could result in an underestimation when calculating the mPMI. Conversely, if the mass had produced a microclimate that favoured feeding and development, then sampled larvae might appear larger, and hence older, sooner than expected. This could then lead to an overestimation of the mPMI.

5 EXPERIMENT 2 – The Effect of Temperature on the Duration of Development

The results of the previous experiment indicate that a strong positive relationship exists between mass size and heat generation; aggregations composed of 1200 larvae or more produced temperatures that were significantly warmer than ambient. But does this mass-generated heat affect larval development rates and if so, at what point does it take effect? Since numerous papers have reported increased rates of development at higher temperatures (Anderson, 2000; Grassberger & Reiter, 2001; Nabity *et al*., 2006; Richards *et al*., 2008), it could be hypothesized that larvae feeding in larger masses, and hence exposed to warmer temperatures, are developing at faster rates in comparison to larvae reared under the same conditions but in smaller aggregations. Given that the thermal output of the mass appeared to increase as development progressed, it was assumed that the duration of the 2nd and 3rd larval instars would be reduced, whilst the 1st larval instar would proceed at the same rate regardless of the mass size. Therefore, the aim of the second experiment was to determine if larvae feeding in different sized masses developed at different rates and, if so, identify the stage or stages most affected.

Following this introduction, the methods section will include details on the experimental set up and statistical analysis. The subsequent results section will include quantitative analyses, including graphs that illustrate the thermal output of different sized aggregations during larval feeding and development. Grouped bar charts will be produced to show differences between mass and ambient ADH at specific developmental junctures whilst stacked bar charts will show the duration

of each developmental stage in different sized aggregations. A brief discussion will follow.

5.1 Materials and Methods

In order to reduce variation between experiments and facilitate comparisons, the experimental design and set up for this investigation closely matched that of the previous experiment, using the same equipment and laboratory conditions/settings. Eggs were harvested from the laboratory-reared colony of *Lucilia sericata* and placed in an incubator set at a constant temperature of 22 °C. These eggs were monitored at regular intervals for eclosion. The majority of egg batches were observed to hatch after 20 hours (+/- 1hr) or, given a constant temperature of 22 °C, 260 ADH. First instar larvae were then left to feed for approximately 14-16 hours, before being counted into different sized masses that contained either 300, 600, 1200, 1800 or 2500 individuals. Whilst masses composed of 50, 100, 150 and 200 larvae were studied in the previous experiment, they were omitted from this one since they were found to produce temperatures that were not statistically different from a 300 mass. These single species masses were placed directly onto lamb breasts held in 10 litre plastic containers before being returned to the incubator set at a constant 22 °C, 65% RH and 16:8 hour (light:dark) photoperiod. For each of the five mass sizes there were three replicates. Three controls were also monitored, which consisted of lamb breasts kept under the same conditions but with no larvae associated with them. In total there were 18 sets of recorded data.

5.1.1 Recording mass temperatures

Temperature readings for each of the masses were taken at regular five-minute intervals using a FLIR T425 thermal imaging camera on a time-lapse setting. The camera was attached to a tripod and positioned directly over the aggregation and food source (Figure 5.1). In the previous experiment and EL-USB-1 data logger (minus probes) was used and did show some success at recording mass temperatures. However, although the data logger allowed heat emission to be monitored at regular intervals over a set time, it was acknowledged that on occasion the temperature readings would not be representative of the maximum core temperature. There was also the risk that once a large aggregation had exhausted the food supply in a specific area, it would migrate to a new location on the meat and away from the data logger. This might have resulted in cooler, inaccurate temperatures being recorded immediately after a period of high heat emission. Unlike the data logger, the thermal imaging camera provided a clear image of the entire feeding area. This made it possible to locate the hottest point and monitor the distribution of heat throughout the aggregation at any one time.

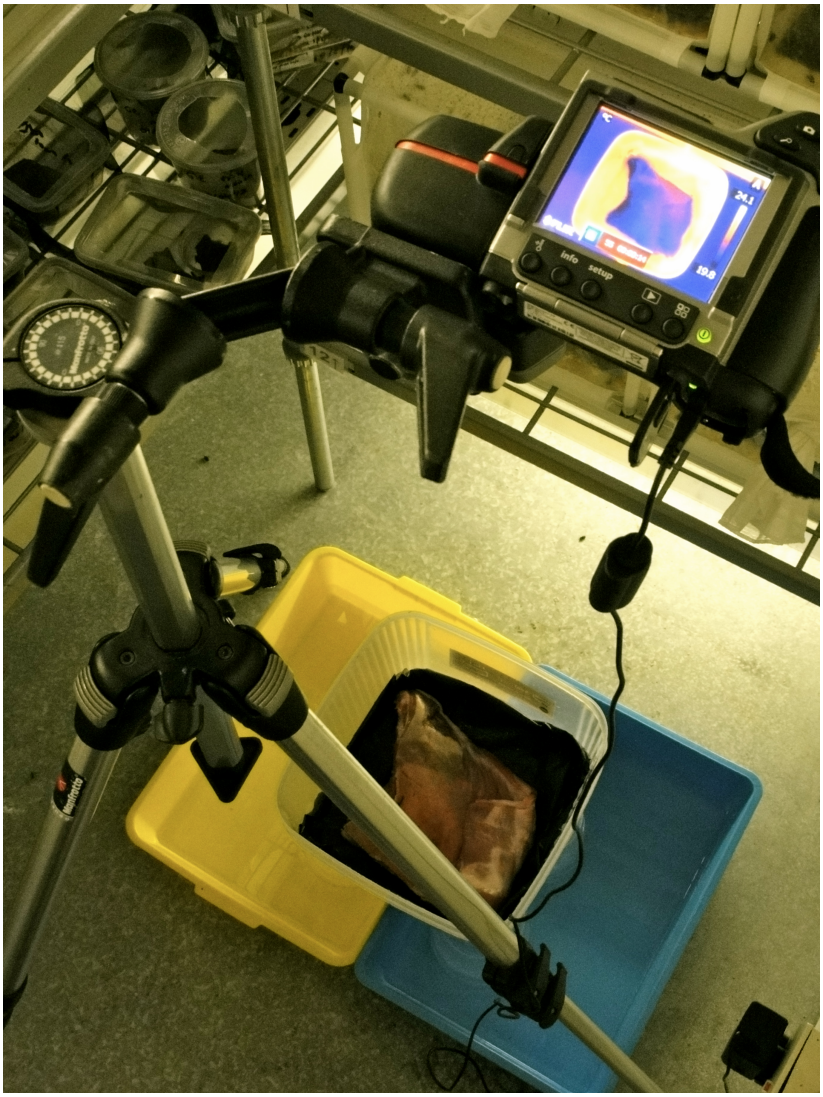


Figure 5.1. Experimental setup for data collection with the FLIR T425 thermal imaging camera positioned directly above the maggot mass and set to record temperatures every five minutes

Ten larvae were sampled from all parts of the mass (centre and periphery) at regular hourly intervals for the duration of the feeding stage of development. By examining their posterior spiracles under a Leica M80 stereomicroscope, individuals were identified as being in either 1st, 2nd or 3rd larval instar. A mass was classified as 1st, 2nd or 3rd instar based upon the stage at which more than 50% of individuals were for two consecutive samplings. All sampled larvae were immediately returned to the mass to prevent a reduction in overall mass size. The start of the post-feeding phase was identified as the point where 10% of the

original mass size (number of larvae) had migrated away from the aggregation and food source. At this point data collection ceased.

5.1.2 Statistical analysis

Data were analysed using the statistical package R (version 2.12.1). Diagnostic plots including a QQ-Norm plot and a Residual vs. Fitted plot showed the data to be normally distributed and of uniform variance in every case. A Mixed-Effects ANOVA model was used to compare overall mass temperatures in different sized aggregations. The results of this analysis were investigated further with a multiple comparison of means test, or Tukey's test. This identified which masses produced significantly different temperatures. Stacked bar charts were produced to show the duration of each developmental stage in different sized aggregations. These would help identify any changes in developmental rates as well as highlighting the stages that were most affected.

For each of the 15 experimental masses, values for ambient and mass ADH were determined using the calculations described in the methodology chapter. Mass ADH was estimated using the maximum temperatures read off the FLIR T425 thermal imaging camera. It was then possible to compare both mass and ambient ADH in different sized masses at specific developmental junctures. These comparisons were made using a Mixed-Effects model followed by a multiple comparison of means test where appropriate. This helped identify significant differences between ADH values across a range of mass sizes. Paired t-tests were used to determine if ambient and mass ADH values were significantly different from one another at each developmental juncture for all mass sizes.

5.2 Results

At the start of the experiment, two hours after initial setup, larvae had formed a single feeding mass on the meat. Aggregations were observed to remain at these initial feeding sites during the earlier stages of development until they had exhausted the immediate food supply. At that point they would move to another location where they continued to feed on the soft tissues whilst emitting increasing levels of heat (Figure 5.2a-d). This relocation of the mass usually occurred during late stage 2nd instar or early 3rd instar. At this point the entire mass could be observed moving along the periphery of the meat as a single unit in search of another feeding site.

In all nine of the larger masses (1200 larvae or more), single aggregations were observed to split into two smaller aggregations at some point during development (Figure 5.2c). On one occasion, a 2500 mass was observed dividing into three separate masses with each mass appearing equal in size (approximately 800-900 larvae). These split masses would then move away from one another and feed at opposite ends of the food source before they would reform into a single mass several hours later. As larvae developed and temperatures increased, individuals within the mass appeared more active, moving at a faster rate. Large masses seemed more humid, with condensation lining the container and liquefied tissue and foam accumulating around the aggregation, the latter caused by the churning motion of feeding larvae. On several occasions in the largest 2500 aggregations, early 3rd instar larvae were observed to leave the mass and wander around the edge of the container despite having not yet completed feeding. After several minutes these same individuals would return to the main aggregation. Larval dispersal was a slow and gradual process as opposed to a mass exodus, beginning

77-99 hours after oviposition. From the onset of the post-feeding phase, 2-10 hours were required for just 10% of the original mass size to disperse. As increasing numbers of larvae migrated away from the meat, the temperature of the masses began to drop (Figure 5.2f).

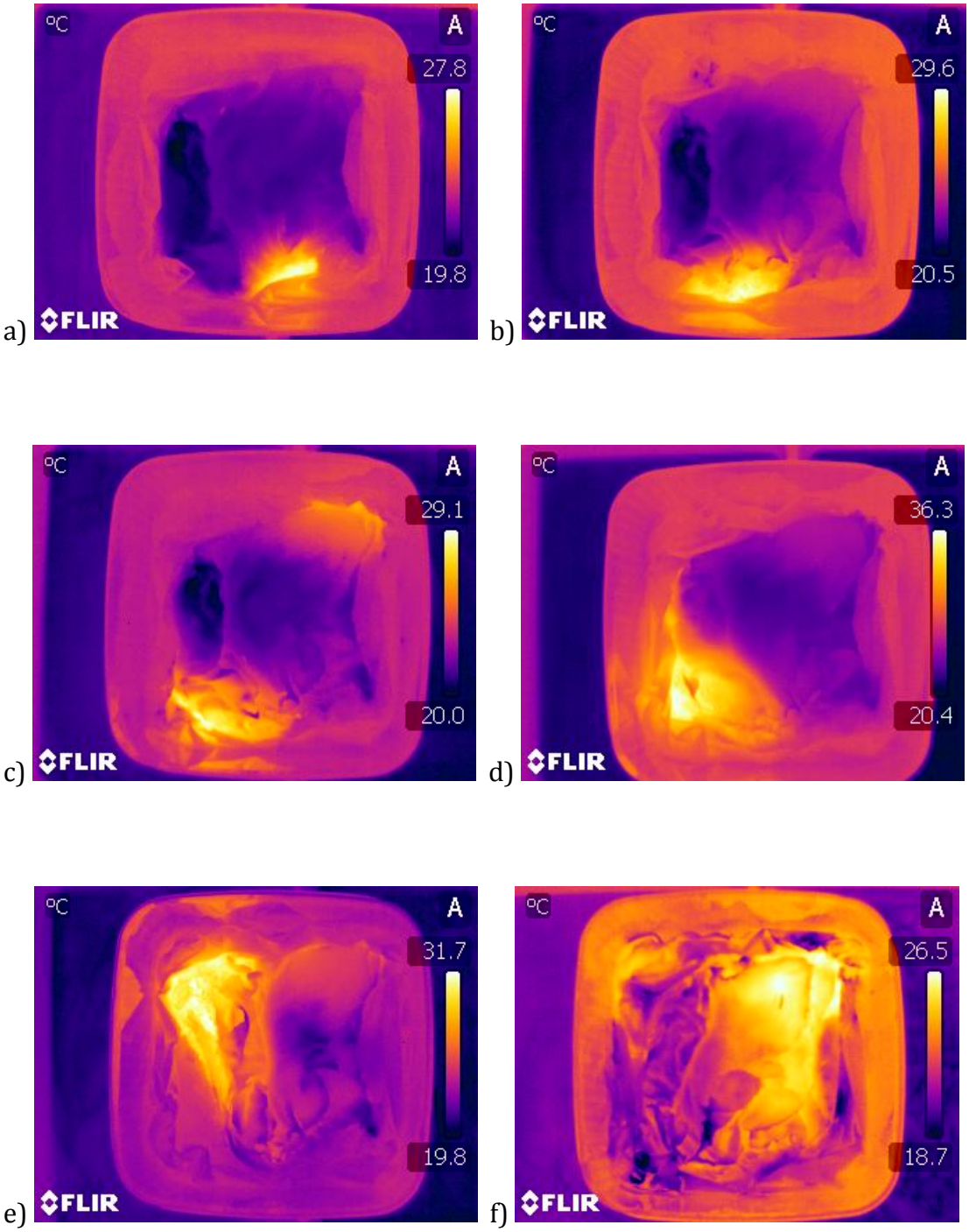


Figure 5.2. Thermal images of an 1800 mass taken at a) 20 hours (1st Instar); b) 29 hours (2nd Instar); c) 32 hours (3rd Instar); d) 37 hours (3rd Instar); e) 42 hours (3rd Instar) and f) 56 hours (3rd Instar – 4 hours prior to dispersal) after eclosion. The images show how the thermal output of the mass alters as a result of larval development or the aggregation splitting.

5.2.1 Overall mass temperatures

Recorded temperatures taken from the masses ranged from 19.4 °C (2.6 °C below ambient) to 36.7 °C (14.7 °C above ambient). Figure 5.3 shows the mean mass temperatures for each of the five different mass sizes and compares them to the control, which was used to represent ambient temperature. The graph is comparable to Figure 4.3 in Experiment 1, in that it shows the temperatures of the masses increasing in relation to size. However, in this experiment changes in heat emission appeared more gradual, with less exaggerated peaks or sudden drops in temperature. This could be attributed to the use of a thermal imaging camera as opposed to a data logger, the latter possibly recording drops in temperature as the aggregation moved away to feed elsewhere on the meat.

Different sized masses were capable of producing temperatures that varied significantly from one another ($F_{5, 12}$=78.25, $p < 0.001$). All of the experimental masses generated a thermal output that was greater than ambient. Pairwise comparisons between the five different mass sizes showed that the only masses producing temperatures that were not statistically different were the 300 and 600 aggregations ($p = 0.488$) (Figure 5.4). The average temperatures recorded in masses containing 300 and 600 larvae were 24.2 °C and 25.0 °C respectively, whilst in the largest 2500 masses mean temperatures were recorded at 30.3 °C.

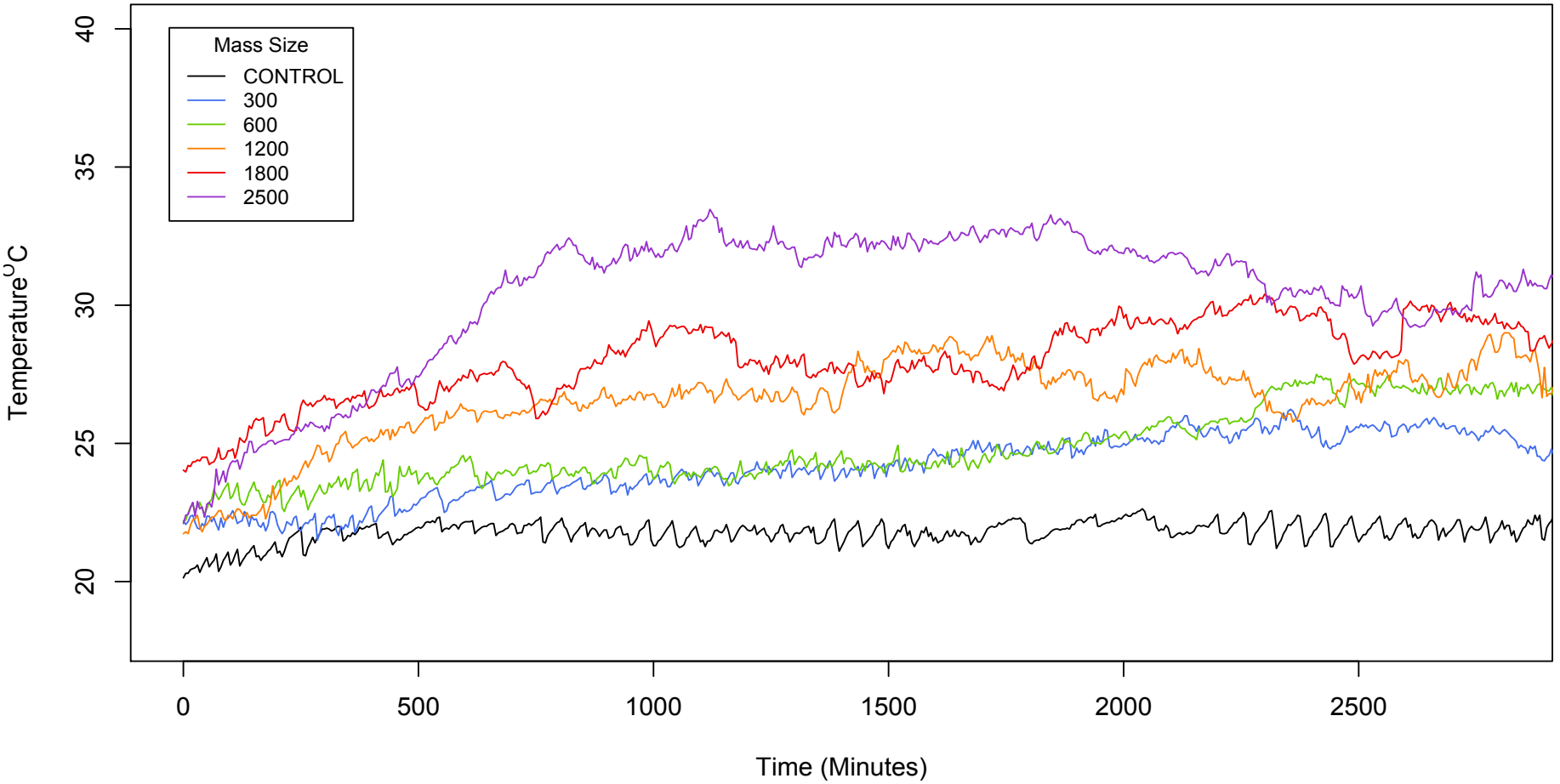


Figure 5.3. Mean mass temperatures (°C) vs. time (mins) for each of the mass sizes starting from mid 1st larval instar and reared at a constant temperature of 22°

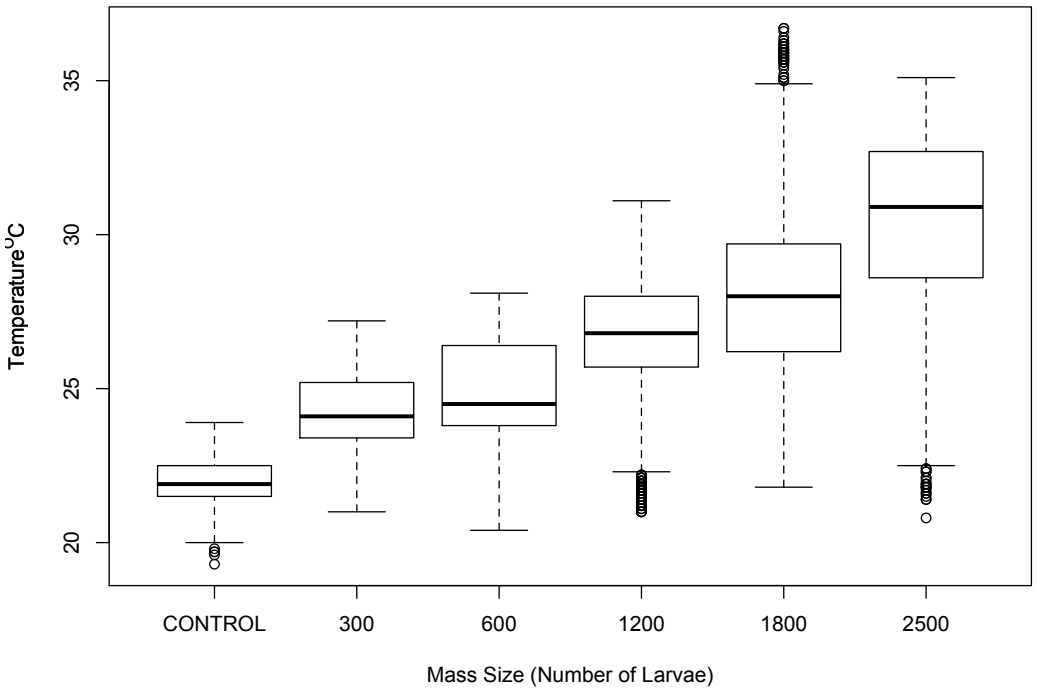


Figure 5.4. Mass temperatures (°C) for each of the experimental mass sizes plus control (ambient) whilst reared at a constant temperature of 22 °C (±1)

5.2.2 Accumulated Degree Hours

Mass and ambient ADH values were calculated for each of the 15 masses, which were then compared at specific stages of development. Since mass-generated heat is non-existent during the egg stage, ADH values were reported at the end of the 1st, 2nd and 3rd (feeding) larval instar.

5.2.2.1 End of 1st larval instar

Figure 5.5 shows both the mass and ambient ADH values for different sized masses at the point where 50% of the aggregation has molted from 1st to 2nd larval instar. At the end of 1st instar ambient ADH remained the same across all mass sizes ($F_{4,10}$=2.028, p=0.166), with values ranging between 555.1 and 585. ADH calculated using mass temperatures ranged from 554.1 to 601.4. These values were all the

same statistically, regardless of mass size ($F_{4,10}$=2.319, p=0.128). These results suggested that juvenile *L. sericata* required between 550 and 600 ADH to progress from oviposition to 2nd larval instar, irrespective of the size of the aggregation.

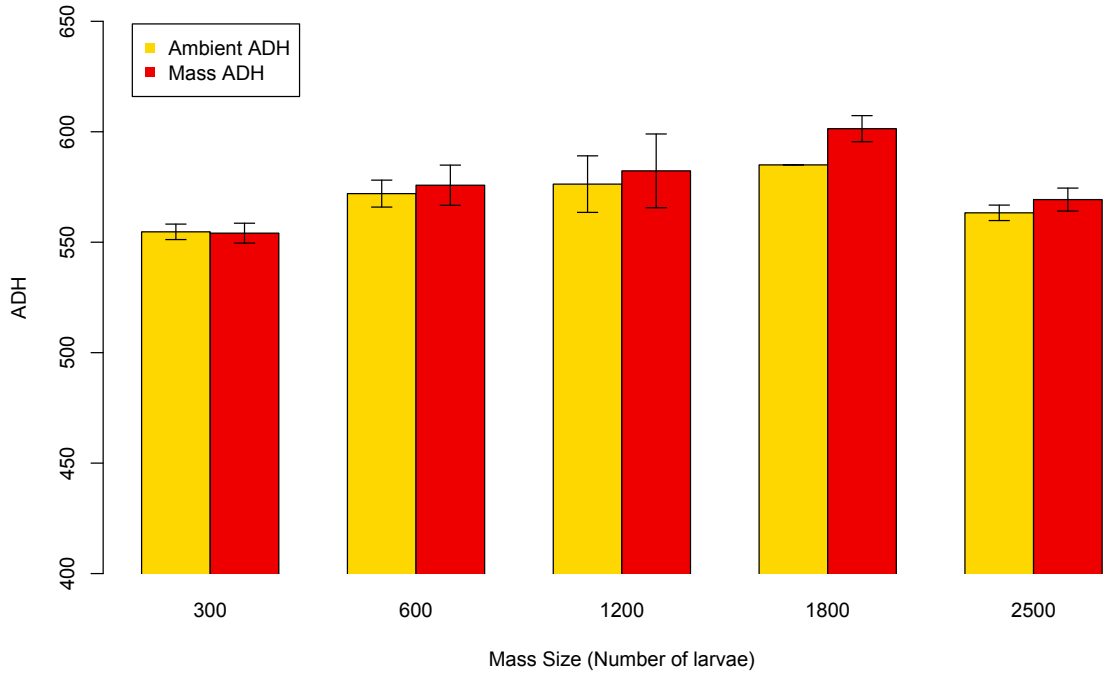


Figure 5.5. Mass and ambient ADH values with standard error bars for larvae reared in different sized masses at the end of 1st instar (constant ambient temperature = 22 °C ±1). Note there is no error bar for ambient ADH in the 1800 masses since all three masses reached the end of the 1st larval instar 45 hours after oviposition (at a constant ambient temperature of 22 °C this resulted in an ambient ADH of 585 in each case).

5.2.2.2 End of 2nd larval instar

At the end of the 2nd larval instar, mass ADH remained constant across all mass sizes ($F_{4,10}$=1.265, p=0.346) and ranged from 761 to 832.1 ADH (Figure 5.6). But the same consistency was not observed when comparing ambient ADH in different sized masses. Instead, as aggregations became progressively larger, ambient ADH began to decrease from 797.3 ADH in the 600 masses down to 693 in the 2500 aggregations, a difference of 104.3 ADH. These differences in ambient ADH are not significant ($F_{4,10}$=3.256, p=0.059). However, pairwise comparisons did confirm that the 600 and 2500 masses had significantly different ambient ADH values

(p=0.007). The observed decrease in ambient ADH with increasing mass size caused the ambient and mass ADH values to diverge away from one another. These differences were significant in aggregations composed of 600 larvae or more (p≤0.044). In the smaller 300 masses ambient and mass ADH differed by as little as 15.1 ADH, but in the large 2500 aggregations this difference had increased to 68.2 ADH.

Since the temperature of the mass represents the immediate microclimate in which larvae are developing, it was assumed that mass ADH gave the most accurate value for accumulated heat energy requirements in larvae. Therefore, based on mass ADH values calculated here, *L. sericata* larvae required a mean of 800 ADH to reach the 3rd larval instar.

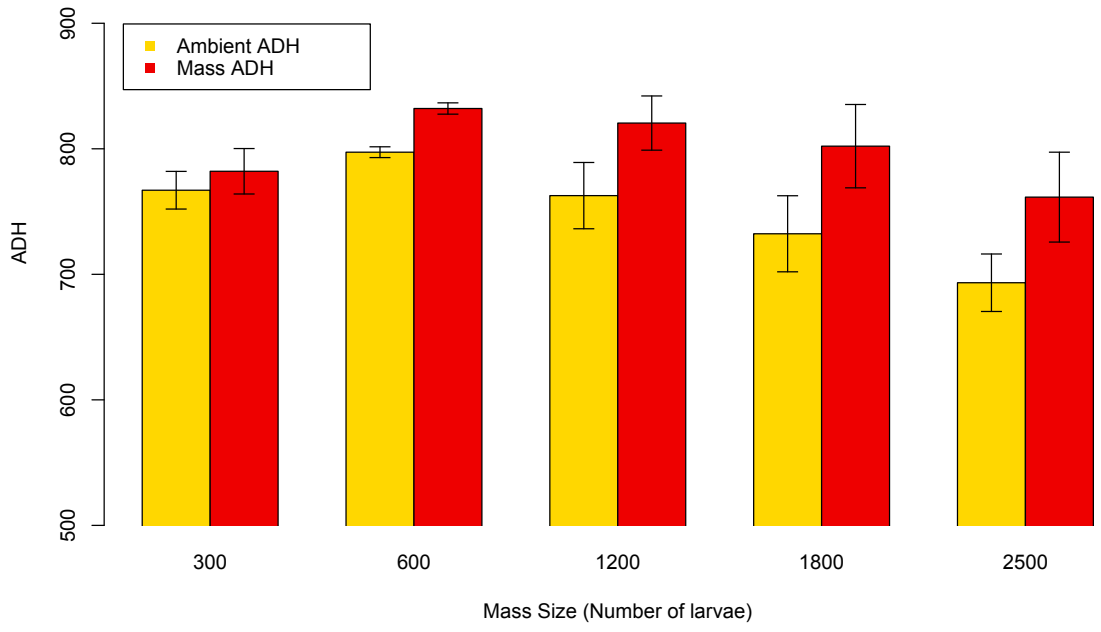


Figure 5.6. Mass and ambient ADH values with standard error bars for larvae reared in different sized masses at the end of 2nd instar (constant ambient temperature = 22 °C ±1)

5.2.2.3 End of 3rd (feeding) larval instar/start of dispersal

At the point where 10% of the larvae in the mass had dispersed, mass ADH still remained constant regardless of mass size ($F_{4,10}$=0.837, p=0.532). All larvae, whether developing in small or large aggregations, required 1309.5-1389.4 ADH (mean = 1365.4 ADH) to reach dispersal (Figure 5.7). But the same results were not recorded when the same comparisons were made with ambient ADH. At the start of the post-feeding period ambient ADH was observed to decrease significantly as mass size increased ($F_{4,10}$=4.434, p=0.026), with 2500 masses having a significantly lower ambient ADH value in comparison to the 300 and 600 masses (p≤0.027) (Figure 5.7). Larvae in these large 2500 aggregations required only 1048.7 ambient ADH to reach dispersal in comparison to the 1222 ADH recorded in the 600 masses. This gave a difference of 173.3 ambient ADH, which when converted to time, translated to 13.3 hours.

Figure 5.7 also shows how this decrease in ambient ADH resulted in the mass and ambient ADH values diverging away from one other as the size of the mass was increased. The differences between mass and ambient ADH at dispersal were significant in each of the experimental mass sizes (p≤0.013). By focusing on the 2500 aggregations, it is clear to see where problems might arise. Based on ambient temperature alone, larvae in these masses only needed 1048.7 ADH to reach dispersal. But in reality, and based on the mass temperatures, larvae had actually attained an ADH of 1388, a difference of 339.3 ADH.

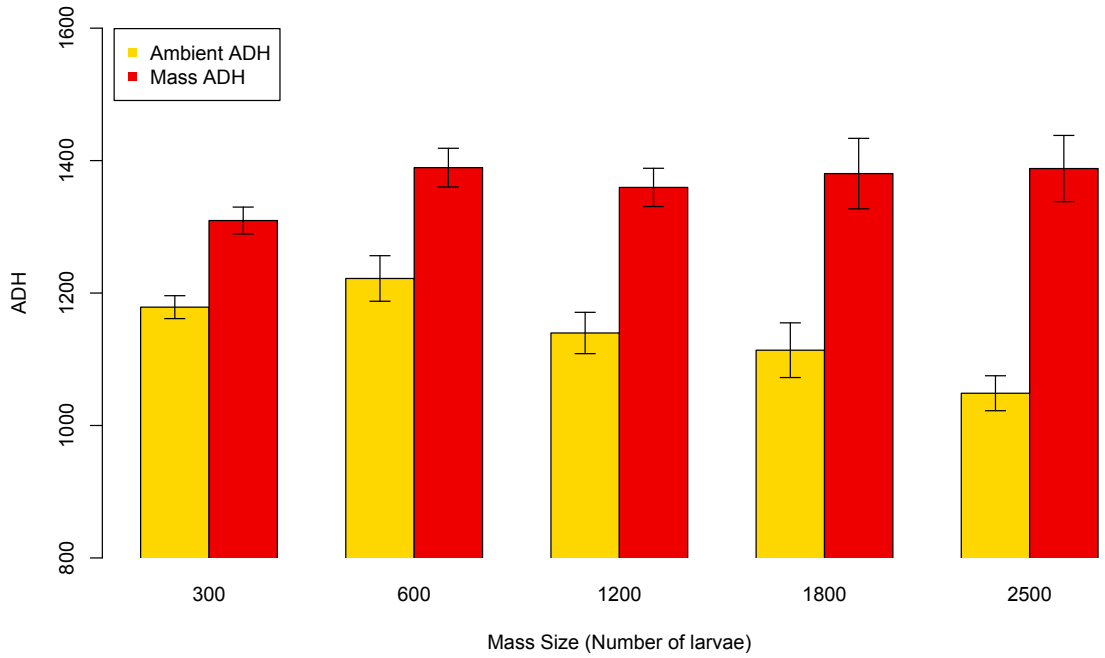


Figure 5.7. Mass and ambient ADH values with standard error bars for larvae reared in different sized masses at the start of dispersal (constant ambient temperature = 22 °C ±1)

5.2.3 Duration of developmental stages

By regularly monitoring larval development it was possible to calculate the duration of each developmental stage in different sized aggregations. Figure 5.8 shows the mean number of hours each developmental phase lasted, starting at oviposition and ending at dispersal, for larvae reared in different sized masses at a constant ambient temperature of 22°C.

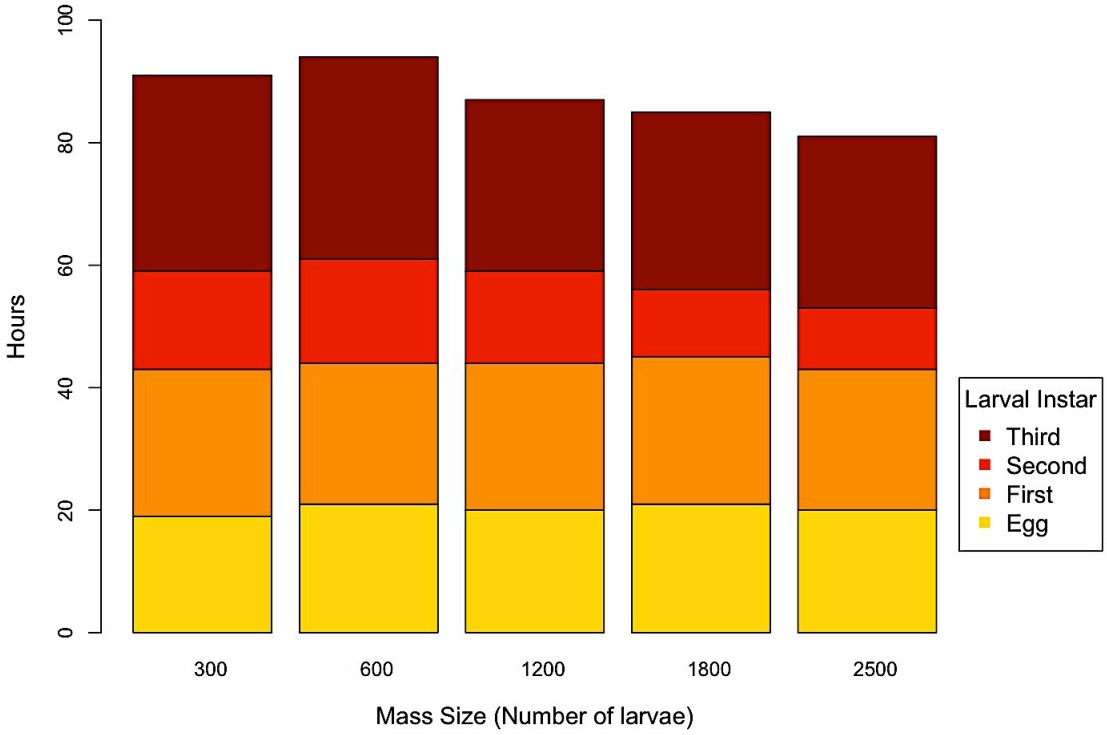


Figure 5.8. Duration of each stage of development (hours) for larvae reared in different sized masses at a constant ambient temperature of 22 °C (±1)

The duration of the egg phase remained constant at 19-21 hours, regardless of mass size, as did the 1st larval instar with a mean duration of 23-24 hours (± 0.58). However, differences in rates of development started to appear between masses when larvae reached the 2nd instar, approximately 43-45 hours after oviposition. At this stage developmental rates appeared to increase; with the duration of the 2nd larval instar decreasing with increasing mass size. For example, larvae in the 2500 masses required a mean of only 10 hours (± 3.00) to complete this stage, with one mass progressing through 2nd instar in as little as 7 hours. Meanwhile, larvae in the 300 and 600 aggregations required an average of 16-17 hours (± 1.53) to complete the same phase. Differences were observed during the 3rd larval instar, with individuals in the 2500 masses requiring 28 hours to complete this stage in comparison to the 32-33 hours recorded in smaller masses.

These accelerated rates of development during 2nd and 3rd larval instar resulted in the 2500 masses completing feeding an average of 13 hours (± 3.78) earlier than the smaller, cooler masses (Table 5.1). The slowest rate of development was observed in the 600 masses, where larvae required 90-99 hours (mean=94, SD=4.58) to progress from oviposition to dispersal. The fastest rate of development was recorded in the 2500 masses. In these aggregations larvae were observed to complete feeding in as little as 77-84 hours (mean=81, SD=3.51). The difference between the slowest developing mass and fastest was a considerable 22 hours.

Table 5.1. Mean number of hours and ADH (derived from mass temperatures) required for *Lucilia sericata* larvae to reach specific stages of development whilst feeding in different sized masses (constant ambient temperature = 22 °C (±1))

Mass		**Developmental Stage**			
		1st Instar	**2nd Instar**	**3rd Instar**	**Dispersal**
300	*Hours*	19	42.7	59	90.7
	ADH	247	554.1	782.1	1309.5
600	*Hours*	21	44	61.3	94
	ADH	273	575.8	832.1	1389.4
1200	*Hours*	20	44	58.7	87.7
	ADH	260	582.3	820.5	1359.5
1800	*Hours*	21	45	56.3	85.7
	ADH	273	601.4	802.1	1380.4
2500	*Hours*	20	43.3	53.3	80.7
	ADH	260	569.3	761.5	1388

5.3 Discussion

Warmer temperatures generated in larger masses influenced the developmental rates of larvae during their 2nd and 3rd instars. This coincided with when masses were at their warmest. An earlier study carried out by Rivers and colleagues (2010) also reported that faster rates of development were linked to increasing mass sizes. They claimed that the amount of time required to complete 1st, 2nd and 3rd instar was significantly shortened as mass size, and hence temperature, increased. Whilst the results of the current experiment agree with their observations regarding shortened 2nd and 3rd instars, there was no change recorded in the duration of the 1st larval instar, regardless of mass size. Although slight temperature increases were observed in recently formed 1st instar masses, thermal output was negligible and closely tracked ambient temperatures until larvae approached 2nd instar. At this point mass temperatures began to increase significantly above ambient, peaking during 3rd instar. This has been observed in other studies that have described how heat emission coincides with larval age, with 3rd instar masses generating more heat than 2nd instar masses (Charabidze *et al*., 2011; Cianci & Sheldon, 1990; Rivers *et al.,* 2010). It is these higher temperatures during 2nd and 3rd instar that influence larval development. The results of this experiment support this hypothesis, with the egg and 1st larval instar lasting for the same amount of time regardless of the mass size, whilst the 2nd and 3rd larval instars become progressively shorter as mass size increases. A faster rate of development might benefit larvae in two ways. Firstly, by reducing the duration of the feeding stage of development, larvae could reduce the risk of predation. Large masses feeding in one location (e.g., on carrion) are an easy

target for predators, so individuals would likely benefit by quickly completing feeding and migrating away from the mass as soon as possible. Secondly, carrion is a discrete and ephemeral source of food for necrophagous insects. If more eggs, and hence larvae, are oviposited on a cadaver than it can support, then food will be limited and larval growth may suffer. Therefore, individuals would benefit by completing the feeding phase of development as soon as possible, whilst nutrients are still available.

In 2001 Grassberger and Reiter published a paper that detailed the duration of each developmental stage for *L. sericata* larvae at a range of constant temperatures (Table 5.2). Although larvae were reared in small masses comprised of just 100 individuals, the results they produced might be useful when estimating the age of mass reared larvae. At a constant ambient temperature of 22 °C Grassberger and Reiter reported that *L. sericata* required 108 hours to reach dispersal (Table 5.2 – circled blue). This is an additional 27 hours compared to larvae reared in a 2500 mass at the same ambient temperature. But what if larvae sampled from a mass had their developmental duration estimated using the approximate temperature of the mass at each specific instar or stage? For example, a 2500 mass at an ambient of 22 °C would likely experience temperatures equal to ambient during the egg and 1st instar stages. As larvae develop mass temperatures might increase to 28 °C during the 2nd larval instar, and then peak at approximately 34 °C during the 3rd instar. Based on the data supplied by Grassberger and Reiter (2001) (Table 5.2 – circled red) the duration of development from oviposition to dispersal could be calculated as:

$$17 + 19 + 16 + 27 = 79 \text{ hours}$$

This is very close to the 81 hours reported in this experiment. Therefore, experiments that have monitored larval developmental at a range of constant temperatures could feasibly be used to estimate age in mass reared larvae. This would only be possible though if future research succeeded in producing more data that modelled the extent of heat production in different sized aggregations.

Table 5.2. Average minimum duration in hours of developmental stages for *Lucilia sericata* at a range of constant temperatures. When estimating the age of larvae sampled from a 2500 mass the data circled in blue gives age estimated using ambient temperature only (constant 22 °C) whilst the data circled in red gives age estimated based on the mean mass temperature for that developmental stage (Taken from: Grassberger & Reiter (2001))

	Duration (h)									
Stage	15°C	17°C	19°C	20°C	21°C	22°C	25°C	28°C	30°C	34°C
Eggs	31	28	24	22	19	17	14	11	10	8.5
1st Instar	56	39	27	24	23	19	16	11	10	9.5
2nd Instar	70	54	42	35	29	26	19	16	15	12
3rd Instar	115	79	60	53	47	46	36	30	27	27
Postfeeding	340	200	118	108	103	94	87	87	87	82
Pupa	(a)	442	293	209	158	137	125	120	119	120
Total	–	842	564	451	379	339	297	275	268	259

[a] No emergence of adults.

5.3.1 Factors affecting rates of development

Several studies have reported that larvae feeding in increasingly overcrowded conditions might experience a faster rate of development (Ireland & Turner, 2006; Shiao & Yeh, 2008). Crowded conditions in the large 2500 masses may have influenced rates of development in this experiment. However, efforts were made to limit the effects of overcrowding by using a large volume of meat. Lamb breasts used in this experiment weighed between 1000 and 1500 g. Even with 2500 individuals, the densest masses still only had a density of approximately 2.5 larvae/g of meat. Based on previous experiments this is classified as "un-crowded" (Charabidze *et al.*, 2013; Saunders *et al.*, 1999). Possible evidence for

overcrowding and intraspecific competition was observed during some of the sampling sessions, however. In several of the larger aggregations (1200+ larvae) some of the samples taken for larval aging were observed to include 1st, 2nd and 3rd instar larvae. Since all larvae originated from the same batch of eggs and were reared under the same conditions as well as provided with ample food (to avoid unnecessary overcrowding and competition) this was surprising. It suggests that despite the efforts taken to limit intraspecific competition, it was still prevalent in the larger masses. For some larvae to have reached 3rd instar whilst others remained in 1st, it is assumed they spent considerably more time at the centre of the mass feeding in comparison to their less developed counterparts, who might have been forced to spend an increased amount of time at the periphery.

Rates of development may also have been affected by the sampling techniques and frequency. Smaller masses that contained 300-600 larvae might have experienced increased disturbance during the hourly sampling session, which could have influenced their development. In smaller aggregations individuals would have had a greater probability of being sampled, possibly multiple times. Repeated handling of the same larva may have disrupted normal feeding behaviour and regular disturbance of the mass as a whole could have caused premature or temporary dispersal. This was not observed but should still be considered.

5.3.2 Mass vs. Ambient ADH

This experiment highlights the differences between ADH values derived from either mass or ambient temperatures, and the potential this has for creating errors when estimating larval age and hence mPMI. ADH values derived from mass temperatures showed consistency at each developmental stage across the entire

range of mass sizes. For example, at the start of dispersal ADH requirements for larvae in a 300 mass were the same as those in an 1800 or 2500 aggregation. It appeared that reaching the "pre-programmed" size for dispersal, as well as 2nd or 3rd instar, was dependent on the amount of heat energy accumulated as opposed to just the amount of time spent feeding. Since larvae develop in a microclimate that to some extent is independent of the ambient conditions, it is assumed mass generated heat drives their development as opposed to ambient. Therefore, the ADH values derived from mass temperatures should provide a more accurate representation of the accumulated heat energy requirements of larvae.

At the same developmental junctures, ADH values originating from ambient temperatures began to decrease as mass size increased, the differences becoming more exaggerated as development progressed, e.g., at the start of dispersal ambient ADH in a 300 mass > 1200 mass > 2500 mass. Since ADH is a fixed quantity of heat energy controlled by time and temperature, any differences in ADH observed under constant temperature conditions may be attributed to changes in time. In this experiment, since the ambient temperature remained a constant 22 °C for the duration, the observed decreases in ADH recorded in larger masses represented a reduction in time. Larvae had completed development in less time, as illustrated in Figure 5.5. This accelerated development has not been accounted for in ambient ADH estimates. Therefore, estimating larval age using ambient ADH instead of mass ADH will result in larger error ranges and a less accurate estimate of the mPMI.

The extent of these errors are made clear if larval ages estimated with mass ADH are compared to those estimated with ambient ADH. For example, an investigator

at a crime scene might collect *L. sericata* larvae that have recently dispersed from a nearby mass containing approximately 2500 larvae. From the results presented here the investigator knows that for *L. sericata* larvae to reach this stage they must have acquired a mean of approximately 1365 ADH. If they were to try and back calculate to the time of oviposition using a constant ambient temperature of 22 °C (22 °C – 9 °C = 13 °C) the ADH model would give a larval age of approximately 105 hours. However, if the investigator were to use the mean mass temperature for a 2500 aggregation instead (30.5 °C – 9 °C = 21.5 °C), then larval age would be estimated at 65 hours. The difference between these two estimated ages is a considerable 40 hours (nearly two days), which could have a significant impact on a forensic investigation. It is obvious that neither of these estimates match our recorded age of 80.7 hours. Yet, whilst ambient ADH overestimates age by 24.3 hours, mass ADH underestimates it by 15.7 hours, a much smaller margin of error.

5.3.3 Points to note

It should also be mentioned that larvae in the 600 masses experienced a slower rate of development than larvae in the smaller, cooler 300 masses. This could simply have been a result of the small sample size. With only three replicates for each experimental mass size, a 600 mass taking longer than expected to complete development, or even a 300 mass experiencing an accelerated rate, could distort the results slightly. Minor differences will have a larger influence on the overall results in small sample sizes.

6 EXPERIMENT 3 – The Effect of Mass Generated Heat on the Physical Measurements of Larvae

This experiment was carried out to investigate whether the size of a mass (i.e., the number of feeding larvae) had an effect on the two phenotypic traits commonly used to age larvae. The two traits most often used to age larvae (aside from the posterior spiracles) are the body length and the larval weight (fresh or dry). Individuals reared in small, medium or large aggregations had their lengths and weights measured at 70 hours development, as well as at the start of dispersal. These measurements were then compared to identify any differences in larva size that could be attributed to the size of the aggregation and its associated microclimate.

In the previous experiment, results indicated that warmer temperatures associated with larger masses influenced rates of development; they shortened the duration of the 2nd and 3rd feeding instars and culminated in an earlier than expected dispersal. Since larval body size increases as development progresses, an obvious assumption to make would be that individuals sampled from larger masses at set times during feeding (such as 70 hours) would be longer and heavier in comparison to larvae reared from smaller masses, as the former would have reached a more advanced stage of development. But would the size of the mass have an effect on larval length and weight at the start of dispersal? A number of papers have discussed how larvae cease feeding and start migration once they have reached what has been described as a "critical" size (Saunders & Bee, 1995). This implies that all larvae, regardless of the temperature or rearing conditions, will feed until they reach a species-specific length and/or weight, and only then

enter the post-feeding phase of development. However, other papers have described how larvae exposed to warmer temperatures develop at an accelerated rate but produce individuals with a reduced body size (Rivers *et al*., 2010; Tarone *et al*., 2011). Therefore, larvae sampled from large aggregations during late feeding or early post-feeding might appear stunted in size with shorter body lengths and lighter weights. Given that larval age, and hence mPMI, is estimated using these physical measurements, it is imperative that entomologists have a thorough understanding of the factors that might influence them.

The aim of this experiment was to determine if larvae reared in different sized masses varied in length and weight at two specific points in development. It was hypothesised that samples taken 70 hours after oviposition would confirm developmental differences, with larvae reared in large masses recorded to be larger than larvae from smaller aggregations. This experiment would provide evidence that either supported or refuted the suggestion that increased rates of development in hotter masses resulted in an overall reduction in body size at dispersal. If larvae from different sized masses were observed to differ in length and/or weight at the start of dispersal, then larvae might be inaccurately aged, which could ultimately lead to errors when estimating the mPMI.

The experimental setup and techniques used to collect the data are described in the subsequent methods section, along with details of the statistical tests used to analyse the data. The results section reports both quantitative and qualitative analyses. Boxplots were used to illustrate larval length and weight in different sized aggregations at both 70 hours development and dispersal, whilst scatter plots showed the relationship between length and weight. These findings were

then discussed in detail at the end of the chapter, along with any implications they might have on mPMI estimates.

6.1 Materials and Methods

First instar *L. sericata* larvae that had emerged approximately 12 hours earlier were counted out and randomly allocated to one of three different sized masses, which contained either 300, 1200 or 2500 larvae. These mass sizes were chosen since they represented the smallest and largest aggregations that were studied in both Experiments 1 and 2, whilst the 1200 masses had been used in the same experiments as an intermediate. This meant that for each of the three mass sizes data had already been collated regarding heat generation and developmental rates, which would be of use when analysing the results. Aggregations were placed onto lamb breasts held in 10 litre plastic containers and then stored in the incubator with controlled conditions of 65% RH, a constant temperature of 22 °C and a 16:8 hour (light:dark) photoperiod. No mass temperatures were recorded during this experiment but an EL-USB-1 data logger (minus probes) was used to take readings at five-minute intervals inside the incubator to ensure the ambient temperature remained constant.

Aggregations were then left to feed undisturbed for a further 38 hours until they reached 70 hours development (from oviposition). At this point the feeding masses were removed from the incubator and the larvae were immersed in 80 °C water for 30 seconds. This effectively killed them. Seventy hours was selected as the first sampling interval for several reasons. Based on the results obtained in Experiment 2, it was expected that at this time the majority, if not all, of the larvae

would have reached 3rd instar (Figure 6.1). This was important since it meant that sampled larvae had spent a considerable amount of time exposed to and developing in a microclimate specific to that particular mass size. If mass-generated heat did start to influence larval development during the 2nd instar as Experiment 2 suggested, then any influence it had on larval size should have been evident by 70 hours. The slightly larger size and thicker cuticle attributed to 3rd instar larvae also reduced the risk of any shrinkage during the killing process (Tantawi & Greenberg, 1993). Individuals were also more robust and easier to handle/measure during the 3rd instar in comparison to the smaller and more fragile 2nd instar larvae.

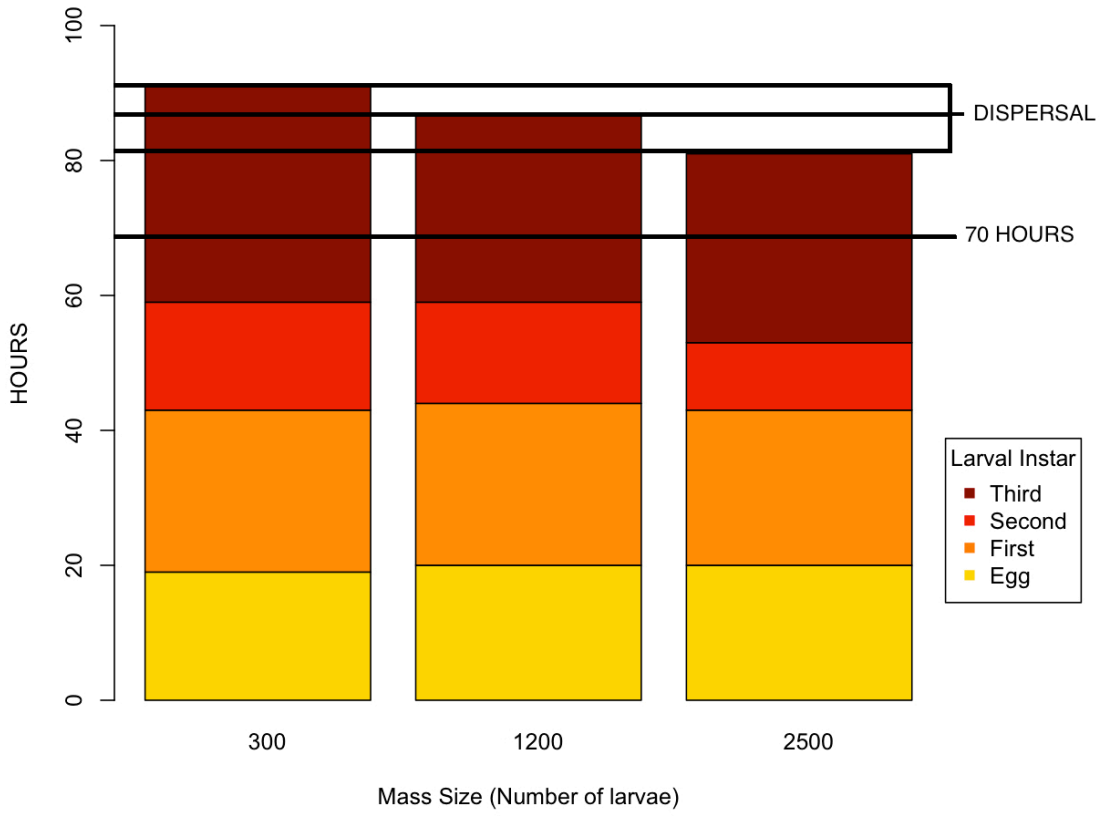


Figure 6.1. Stacked bar chart illustrating the sampling times for the three different mass sizes. Note that for "dispersal", masses composed of 300, 1200 and 2500 larvae were sampled at 91, 87 and 81 hours respectively (when 10% of the larvae had dispersed)

Immediately following death, 100 larvae were randomly sampled from each of the masses and their individual lengths and weights recorded. Larval length was measured to the nearest 0.01 mm using a Mitutoyo digimatic calliper whilst larval weight was recorded with a Denver Pinnacle PI-403 laboratory weighing balance to the nearest 1 mg. It should be mentioned that the absolute measurements taken for larvae were not as important as the relative measurements. This experiment was replicated a total of six times for each of the three mass sizes, which gave a total of 18 sets of data for larval size at 70 hours development.

The experiment was then repeated using another 18 masses composed of the same number of larvae and reared under the same conditions, but with samples taken at the start of dispersal as opposed to the 70 hour mark. Dispersal was identified as the point at which 10% of the larvae from each mass had migrated away from the meat and into the underlying trays. For aggregations containing 300, 1200 and 2500 larvae this was when 30, 120 and 250 individuals had completed feeding. Unlike the previous sample, which was collected at a set time across all masses, the timing of dispersal varied depending on the size of the aggregation. Experiment 2 showed this was after approximately 91 hours in the small (300) masses, 87 hours in the medium (1200) aggregations and 81 hours in the large (2500) masses (Figure 6.1). Using these times as indicators, masses were checked at regular hourly intervals on the run-up to dispersal. Larvae that had left the meat and container were collected and counted at each check. Once 10% of the original number of larvae had been accounted for, the mass was classified as "in dispersal" and the remaining larvae were killed and measured. Measurements were not taken from the larvae that had been collected from outside the container. Again,

six replicates were run for each of the three mass sizes sampled at dispersal, which gave a further 18 sets of recorded data.

6.1.1 Statistical analysis

Data were analysed using the statistical package R (version 2.12.1) (R Core Development Team, 2010). QQ-Norm plots and a Residual vs. Fitted plot were used to assess normality and the variances. These diagnostic plots showed the data to be normally distributed and of uniform variance in every case. A Mixed-Effects model was used to compare larval length and weights in different sized aggregations. This was to show whether larval size differed significantly between masses composed of 300, 1200 and 2500 individuals at 70 hours development and later at the start of dispersal. The results of these analyses were investigated further with multiple comparison of means tests, or Tukey's tests. These pairwise comparisons highlighted which of the three mass sizes were producing significantly different larvae. Scatter plots were constructed to illustrate the relationship between larval length and weight in relation to mass size at both sampling times. A Pearson's Correlation Coefficient test was run on this data to test whether these two measurement variables were related.

6.2 Results

6.2.1 70 hours development

Early observations were that larvae reared in different sized masses appeared to vary in length at 70 hours development, with length increasing in relation to increasing mass size (Figure 6.2).

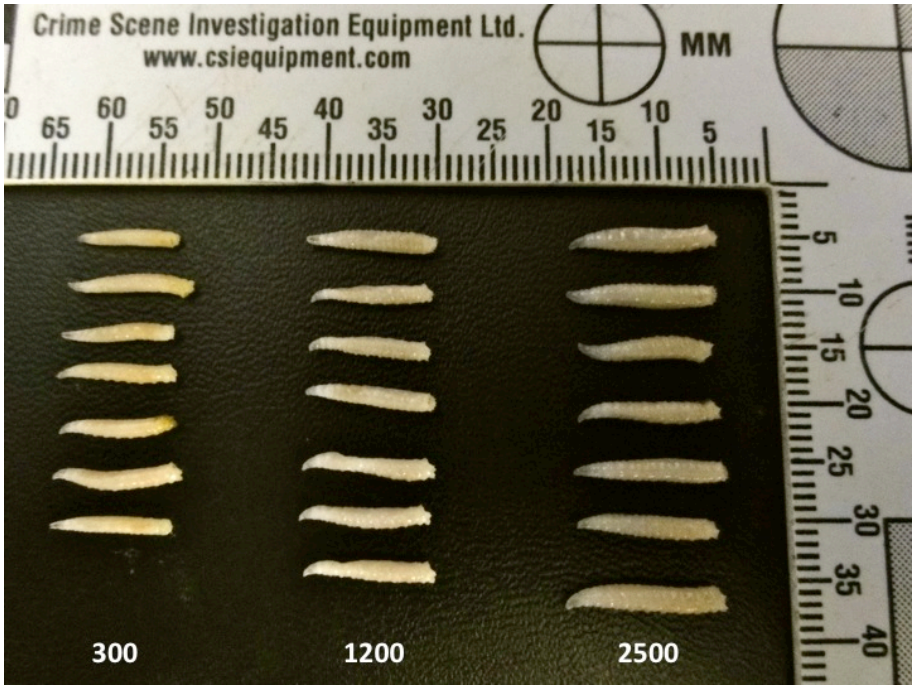


Figure 6.2. Visual comparison of larval lengths sampled from masses containing 300, 1200 and 2500 individuals at 70 hours development. Photographed larvae were randomly selected from the samples containing 100 individuals.

When tested statistically with a mixed effects model, results showed that there were significant differences when comparing larval lengths across a range of mass sizes ($F_{2,15} = 258.4$, $p<0.001$). As mass size increased, larval length also increased. Larvae from the 1200 masses (mean=13.05 mm, SD=0.49) were significantly longer ($p<0.001$) than individuals sampled from the 300 masses (mean=12.34 mm, SD=0.50) but shorter ($p<0.001$) than larvae from 2500 masses (mean=13.90 mm, SD=0.55) (Figure 6.3). Figure 6.3 also showed a large number of outliers in the 2500 masses. This implied that there was a greater range of larval lengths in larger aggregations, even when taking into account the number of constituent measurements.

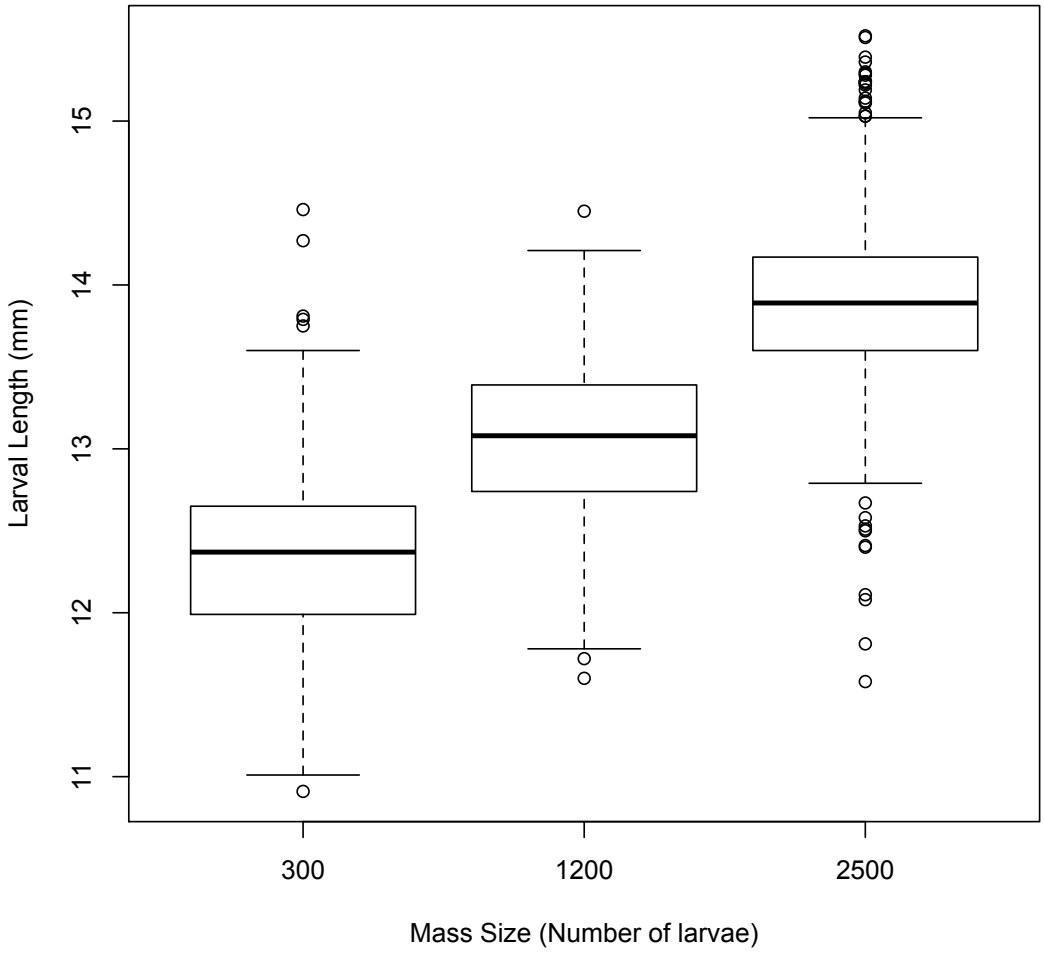


Figure 6.3. Larval lengths (mm) recorded in masses containing 300, 1200 and 2500 larvae at 70 hours development (constant ambient temperature = 22 °C ±1)

The same results were observed when comparing the weights of larvae sampled from different sized masses at 70 hours development, with weight increasing in relation to mass size ($F_{2,15} = 18.74$, $p<0.001$). Larvae reared in aggregations containing just 300 individuals were significantly lighter ($p=0.012$) (mean=25.99 mg, SD=4.38) than those reared in 1200 masses (mean=31.97 mg, SD=4.75), whilst larvae sampled from the large 2500 masses (mean=38.81 mg, SD=6.69) were significantly heavier than both the 300 ($p<0.001$) and 1200 ($p=0.003$) mass-reared larvae (Figure 6.4). A summary of this information for each of the three mass sizes is presented Table 6.1.

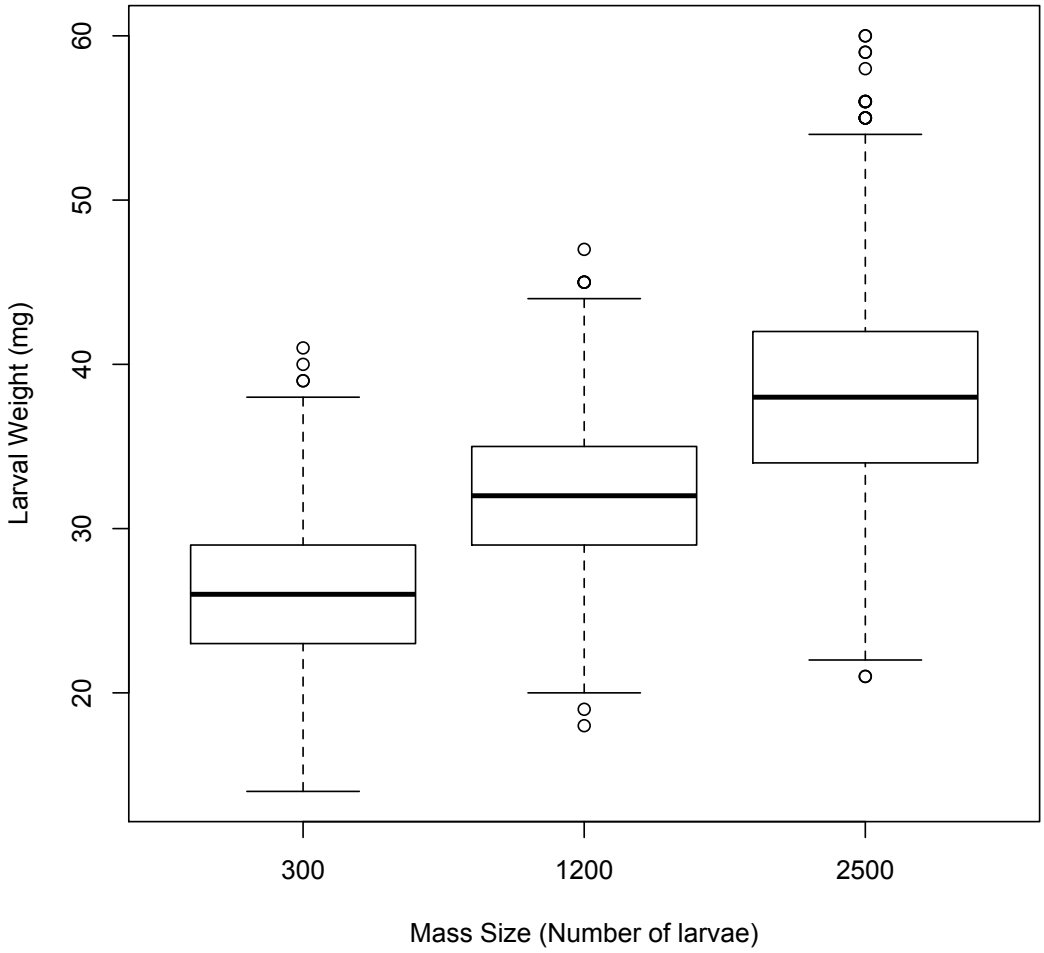


Figure 6.4. Larval weights (mg) recorded in masses containing 300, 1200 and 2500 larvae at 70 hours development (constant ambient temperature = 22 °C ±1)

Table 6.1. The mean lengths and weights (plus standard deviation) for larvae sampled from masses containing either 300, 1200 or 2500 larvae at 70 hours development

Mass Size		Mean	Standard Deviation
300	*Length (mm)*	12.34	0.50
	Weight (mg)	25.99	4.38
1200	*Length (mm)*	13.05	0.49
	Weight (mg)	31.97	4.75
2500	*Length (mm)*	13.89	0.55
	Weight (mg)	38.81	6.69

A strong positive correlation was shown to exist between larval length and weight at 70 hours development ($p<0.001$, $r=0.906$). As larval length increased, weight also increased. The graph also showed that individual plots clustered according to mass size (Figure 6.5). In the bottom left hand corner of the figure, where data points represent individuals with a shorter length and lighter body size, the 300 mass larvae were identified. In the top right hand corner the larger 2500 larvae were located. Meanwhile, the medium sized masses produced larvae with body dimensions that appeared to cluster in the centre, between the 300 and 2500 larvae. There was some overlap between the masses but each appeared to cover a specific size range.

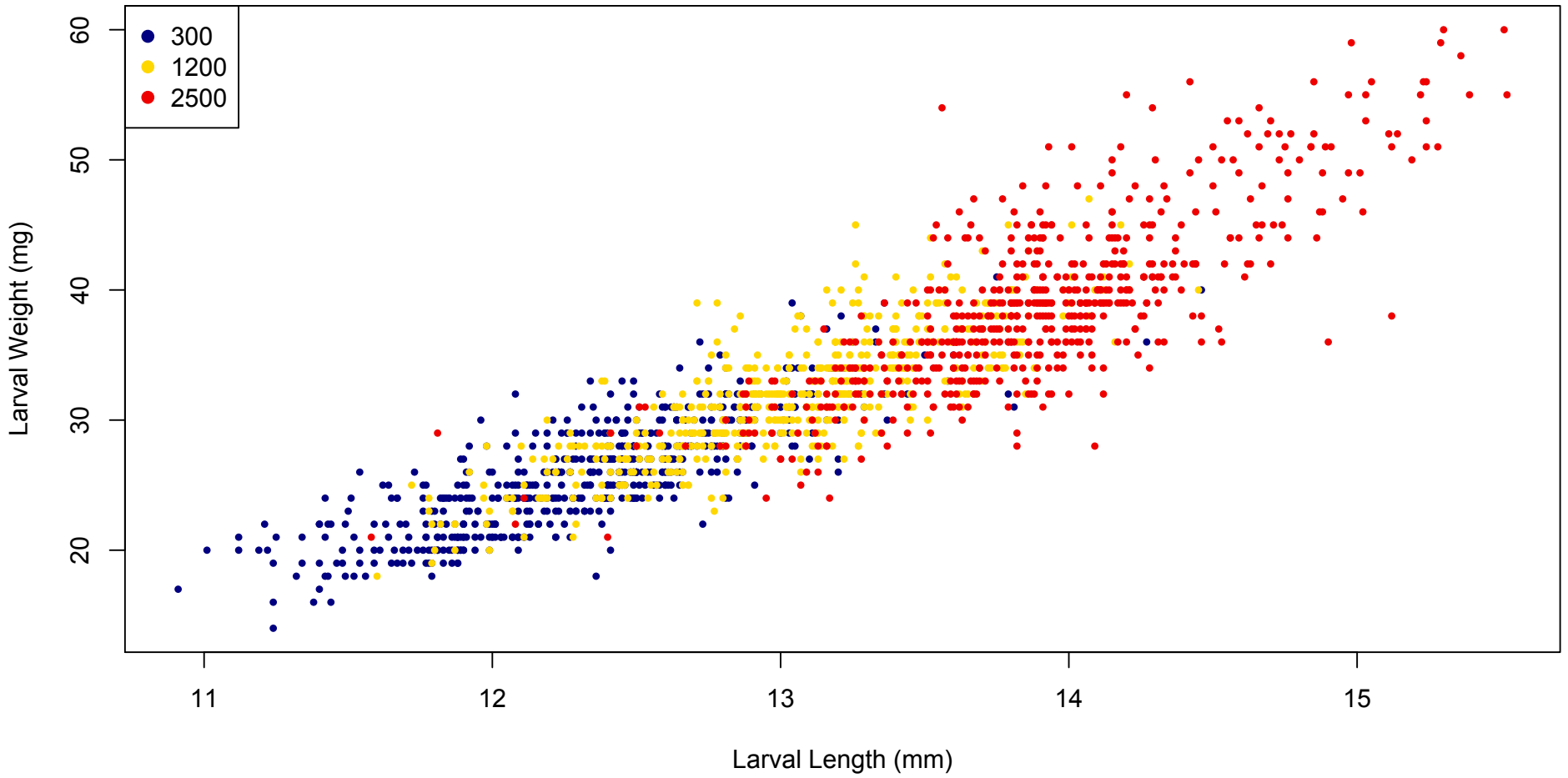


Figure 6.5. Scatter graph showing the distribution of individuals in relation to size (larval length and weight) and mass (number of larvae) at 70 hours development (constant ambient temperature = 22 °C ±1)

6.2.2 Start of dispersal

A visual inspection of larvae sampled at the start of dispersal indicated that all individuals, regardless of the mass they were sampled from, were of equal length and weight (Figure 6.6). The differences in body sizes that were observed between the three groups at 70 hours development no longer existed. Instead larvae sampled from masses containing 300 larvae appeared to be the same size as individuals sampled from the larger 1200 and 2500 masses.

Figure 6.6. Visual comparison of larval lengths sampled from masses containing 300, 1200 and 2500 individuals at the start of dispersal. Photographed larvae were randomly selected from the samples containing 100 individuals

When tested statistically with a mixed effects model, results showed that there were, in fact, no significant differences when comparing larval lengths in different sized masses at the start of dispersal ($F_{2,15} = 2.870$, $p=0.088$). Larvae from the 1200 masses (mean=15.53 mm, SD=0.79) were the same length as larvae sampled from the 300 (mean=15.17 mm, SD=0.80) ($p=0.087$) and 2500 masses

(mean=15.19 mm, SD=0.01) (p=0.105) (Figure 6.7). The mean lengths of larvae sampled from 300 masses and 2500 masses were equal (p=0.996).

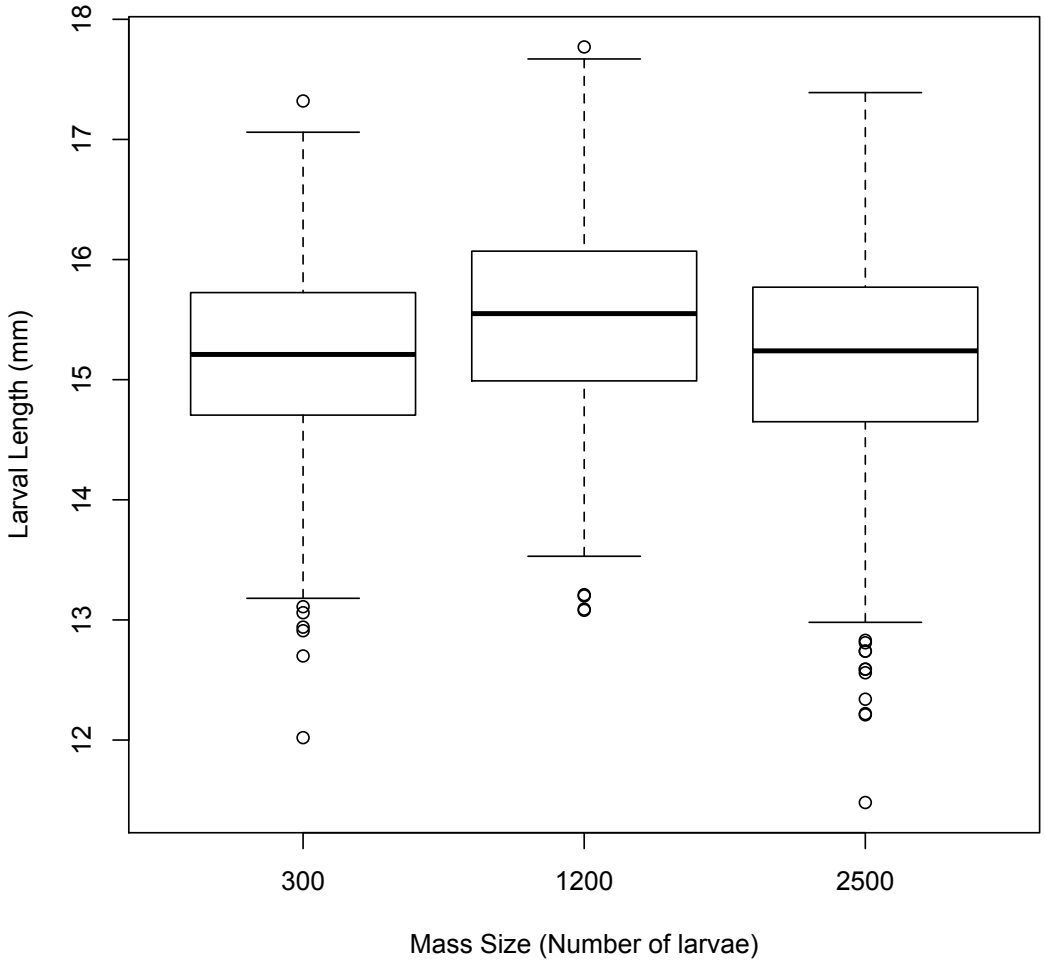


Figure 6.7. Larval lengths (mm) recorded in masses containing 300, 1200 and 2500 larvae at the start of dispersal (constant ambient temperature = 22 °C ±1)

When larval weights were compared at the start of dispersal, there were significant differences observed between the three mass sizes ($F_{2,15} = 4.157$, p=0.037). Pairwise comparisons showed that larvae reared in the 1200 aggregations (mean=60.07 mg, SD=7.22) were significantly heavier (p=0.018) than those sampled from the 2500 masses (mean=55.29 mg, SD=7.89). But larvae in the 300 masses (mean=56.20 mg, SD=7.33) were the same weight as larvae reared in both the 1200 aggregations (p=0.071) and the 2500 aggregations (p=0.864)

(Figure 6.8). A summary of larval lengths and weights in different sized masses at the start of dispersal is presented in Table 6.2.

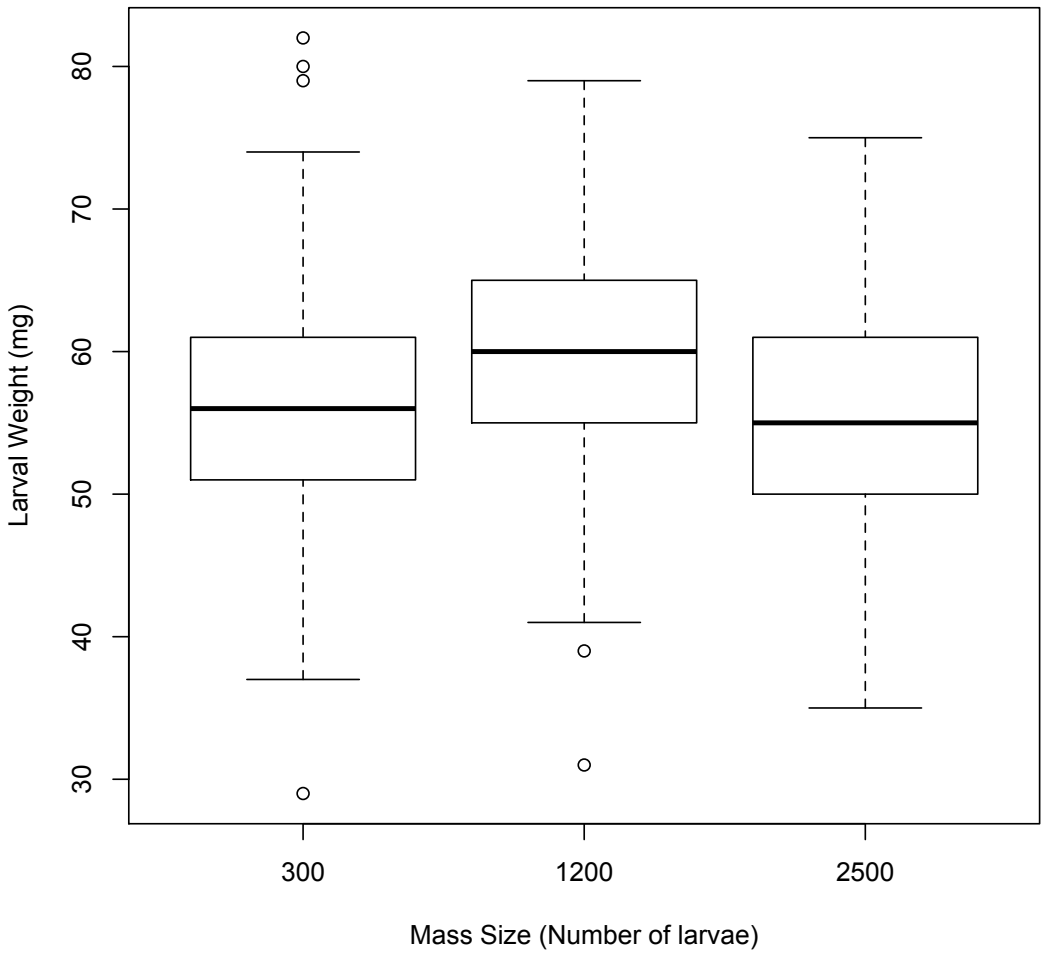


Figure 6.8. Larval weights (mg) recorded in masses containing 300, 1200 and 2500 larvae at the start of dispersal (constant ambient temperature = 22 °C ±1)

Table 6.2. The mean lengths and weights (plus standard deviation) for larvae sampled from masses containing either 300, 1200 or 2500 larvae at the start of dispersal

Mass Size		Mean	Standard Deviation
300	*Length (mm)*	15.17	0.80
	Weight (mg)	56.20	7.33
1200	*Length (mm)*	15.53	0.79
	Weight (mg)	60.07	7.22
2500	*Length (mm)*	15.19	0.01
	Weight (mg)	55.29	7.89

A strong positive correlation existed between larval lengths and weights at the start of dispersal ($p<0.001$, $r=0.822$). Not surprisingly, as larval length increased, weight also increased. When individual plots were identified by mass size, it was apparent that the clustering observed at 70 hours development no longer existed (Figure 6.9). Instead, all three of the different sized masses showed significant overlap in larval size. This implied that all larvae ceased feeding and started dispersal once they had attained a specific body size. Plots representing individuals from the 1200 masses appeared in higher numbers at the upper end of the scale as opposed to the lower end. This provided further evidence that larvae in these masses were slightly larger at dispersal than those in the 300 and 2500 aggregations.

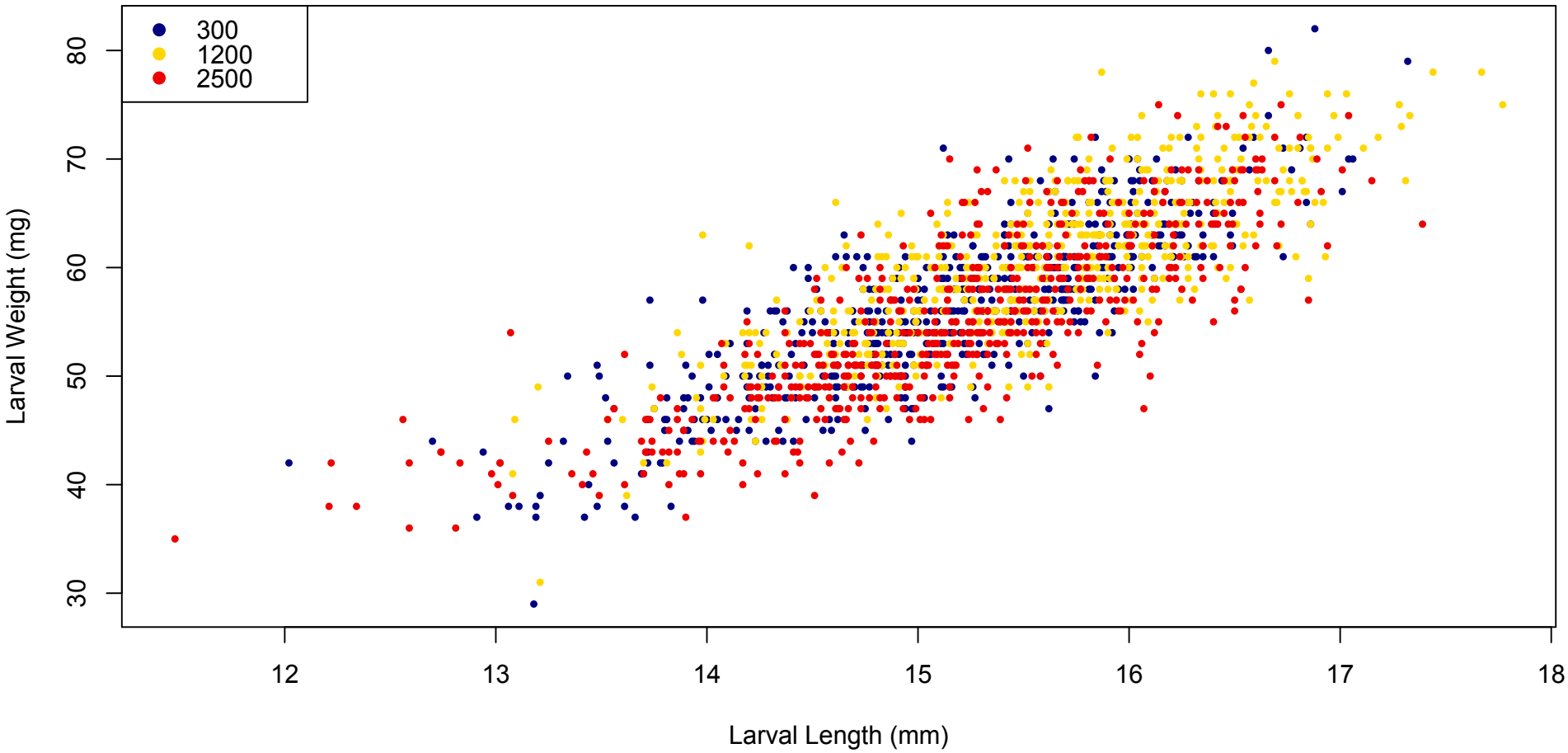


Figure 6.9. Scatter graph showing the distribution of individuals in relation to size (larval length and weight) and mass (number of larvae) at the start of dispersal (constant ambient temperature = 22 °C ±1)

6.3 Discussion

6.3.1 Length and weight at 70 hours development

At 70 hours development comparisons between larvae sampled from different sized masses showed that there were significant differences in lengths and weights. These results are not surprising though if one considers Figure 6.1, which shows the sampling points in relation to the progression of larval development in different sized masses. At 70 hours development larvae from the 300 masses had only molted into 3rd instar 11 hours previously and still required another 21 hours until they reached dispersal. They were approximately a third of their way through the feeding phase of 3rd instar. Meanwhile, larvae from the 1200 masses were approaching the halfway point of 3rd instar, which would have resulted in them being more developed and hence larger. At 70 hours development, the 2500 individuals were over halfway through 3rd instar, with only 11 hours remaining until they were due to start dispersal. Since individuals from these larger masses were closer to completing development, a process that is most likely accelerated by their warmer temperatures, they were expected to be larger in size compared to larvae from smaller masses that have experienced a slower rate of development.

If larval age was estimated using length and/or weight for individuals still in the feeding phase, then the results could have a major impact on mPMI estimates. Third instar larvae collected from different sized aggregations could vary in size and misrepresent age. For example, if larvae were collected from a small 300 mass at approximately 70 hours development and aged using ambient temperature alone, then the estimated age would be reasonably accurate since smaller masses have been shown to produce a microclimate equal to ambient. This in turn would lead to a more accurate estimate of the mPMI. But, suppose larvae were sampled

from a large 2500 mass instead and aged using the same ambient temperature data. Based on the results from this experiment, individuals would be significantly larger than expected for that stage of development. Therefore, they would appear older in comparison (Figure 6.10). This size difference might result in larval age being overestimated by several hours, which could lead to an overestimate of the mPMI.

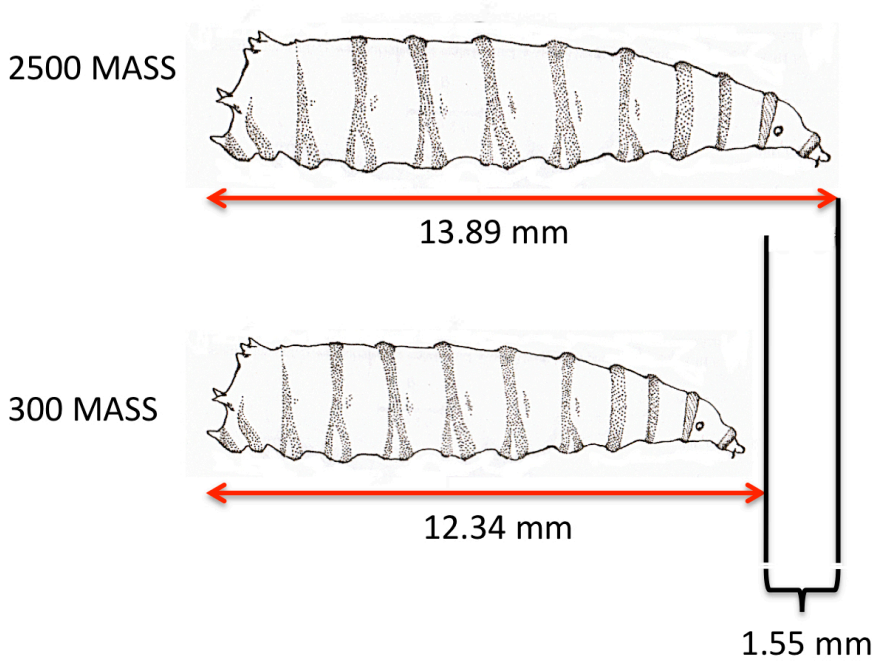


Figure 6.10. Differences in mean length at 70 hours development when comparing a larva reared in a 2500 mass with an individual from a 300 mass. The 1.55 mm difference in length could result in the larva sampled from the 2500 mass having its age overestimated by as much as 10 hours (after Greenberg & Kunich 2002; pp. 80)

6.3.2 Length and weight at the start of dispersal

At the start of dispersal all larvae were the same length regardless of mass size. This implies that larvae will continue feeding until they reach a maximum length, at which point they begin to migrate away from mass in search of a site for

pupariation. Based on the results from this experiment the mean length at the start of dispersal for *L. sericata* appears to fall between 15.17 and 15.53 mm. Once larvae attain this length they are considered large enough to successfully complete development. Therefore, the amount of time taken to reach this size determines the duration of the feeding period. In small masses larvae required a mean of 91 hours to reach this length, whilst in large masses 81 hours were required.

It is important to mention that even though the results of this experiment reported no significant differences in the lengths of larvae sampled from different sized masses at dispersal, the p-value itself ($p=0.088$) cannot be considered strong enough evidence. There is also the fact that significant differences were recorded in larval weights at dispersal, with the largest and the smallest aggregations producing the lightest larvae. For these reasons, factors that might explain the relationship between size (length/weight) and temperature effects (mass size) should be considered. Tarone and colleagues (2011) reported that *L. sericata* reared at two different temperatures (20 and 33.5 °C) produced pupae that differed significantly in length and weight. Pupae reared at 33.5 °C, which could be representative of larvae reared in the 2500 masses in this experiment, were significantly shorter and lighter than larvae kept 20 °C. The authors gave two explanations for this reduction in size. The first was that larval feeding rates were not able to keep pace with the faster metabolism associated with warmer temperatures (Tarone *et al*. 2011). The second was that an increased mortality at higher temperatures (like those associated with large masses) favoured smaller sizes in high temperature environments. If larvae were more likely to die in warmer conditions due to factors such as heat stress or anoxia, there might be a selection to complete development sooner but at the expense of body size (Tarone

et al. 2011). A comparable trade-off between body size and temperature was discussed by Rivers and colleagues (2010) after they monitored the levels of heat shock proteins (hsp) in the brains of mass feeding *Sarcophaga bullata* and *Protophormia terraenovae*. At high temperatures the heat shock response became active in synthesizing stress proteins. However, it has been demonstrated that this process occurs at the expense of normal protein production, which negatively affects larval growth (Rivers *et al*., 2010). The authors reported that this heat shock response occurred at temperatures in excess of 32 °C, which would have coincided with the temperatures reported for 2500 masses during the 3rd instar (Experiment 1 and 2). When Saunders and Bee (1995) studied the effect of density on the development of *Calliphora vicina* larvae, they claimed that in large, dense masses where the meat was overcrowded, larvae would lower their critical weight requirements for migration. The critical weight is defined as the minimum weight required for successful development (Saunders & Bee, 1995). Saunders and Bee (1995) reported that under optimal conditions the critical weight for *C. vicina* to complete feeding and pupate was 30 mg. However, in overcrowded conditions where food was scarce, larvae were observed to lower this weight and disperse at 15-20 mg. These larvae may have compromised on body size in order to escape a stressful overcrowded environment with high temperatures that could increase mortality rates.

These suggestions might help explain why larvae in the 2500 masses in Experiment 3 were lighter in weight than individuals in the 1200 masses at dispersal. However, the fact that they were the same size as larvae sampled from the 300 masses disputes this reasoning. Each of the suggested explanations for reduced larval lengths and weights relates back to temperature or overcrowding

and the fact that larvae in large masses were subjected to increasingly high temperatures, which caused thermal stress and disrupted normal larval development. Yet the small 300 masses, which have been shown to produce low levels of heat and a non-competitive feeding environment, produced larvae that were the same size as individuals reared in the 2500 masses. Perhaps rather than arguing that any size differences are a result of the stressful conditions in large masses, a more accurate explanation would be that the conditions of the 1200 masses are optimal and result in the most efficient feeding and development. It is possible that the "Goldilocks Principle" is in effect here, which states that something must fall within certain margins, as opposed to reaching extremes. Larvae developing in the medium sized 1200 masses may have the right balance, with enough individuals present to effectively break down soft tissue for feeding but without creating an overcrowded, stressful environment. Aggregations of this size have been shown to produce temperatures that are independent of ambient and consistently in the range of 25 °C – 30 °C, several degrees below the proposed upper temperature limit for *L. sericata* or the threshold for hsp production. The conditions in the intermediate 1200 masses might have sustained efficient feeding at optimum temperatures with minimal disruption to development, which resulted in larger body sizes.

Larvae might benefit from larger body sizes since the extra energy reserves could provide them with an opportunity to disperse further and for longer. The migratory phase of development is energetically expensive and can last for a number of days as individuals crawl several meters in search of a suitable site for pupariation. For *L. sericata* this may be significant as the species demonstrates a strong dispersal behaviour. Crawling large distances could leave them exposed

and vulnerable to predators and parasites. But it has been suggested that dispersing large distances away from carrion is actually an adaptation of some flies to avoid predation, bacterial attack and parasitism by insects such as the wasp *Nasonia vitripennis*, which attacks the puparial stages and arrives at carrion prior to dispersal (Greenberg, 1990; Rivers *et al*, 2011). A fast rate of development coupled with an optimum body size could facilitate an early migration before the first wasps arrive, providing larvae an opportunity to avoid parasitism. It has also been reported that crawling speed increases proportionally with body length and mass (Berrigan & Pepin, 1994; Charabidze *et al*., 2008). This faster rate of movement observed in larger larvae could also be advantageous in avoiding predators, further increasing survivorship.

6.3.3 Growth rates

For each of the three experimental mass sizes growth rates were estimated (Table 6.3). These were calculated by dividing the differences in mean length and weight between 70 hours and dispersal by the number of mean hours required to reach dispersal from the 70-hour point. For the 300, 1200 and 2500 masses this was 21, 17 and 11 hours respectively. It is important to stress though that these figures represented growth rates during 3rd feeding larval instar only and not overall growth rates from 1st instar to dispersal. The results showed that for both length and weight, growth rates were fastest in the 1200 masses, whilst they remained the same in the 300 and 2500 aggregations. Since growth rates are a function of time and length/weight, this implies that larvae in smaller masses have sacrificed time for size whilst the opposite has occurred in the large aggregations. In these masses larvae appeared to sacrifice physical size in order to complete development earlier. Saunders and Bee (1995) reported that optimal body

weights were not achieved in large masses. A possible explanation for this could be the reduced nutrients available per larva and increased volume of waste products that might negatively affect growth.

Table 6.3. Mean growth rates in terms of length and weight for each of the three mass sizes during 3rd larval instar

Growth Rates	**Mass Size**		
	300	**1200**	**2500**
Length (mm/hr)	0.13	0.15	0.12
Weight (mg/hr)	1.43	1.65	1.45

7 EXPERIMENT 4 – Feeding and Development in Solitary Larvae

The previous three experiments have all focused on the development of larvae reared in aggregations. Regardless of whether they contained 50 individuals, or 2500, these masses were observed to form without fail in the laboratory, usually during late-1st instar. Various reasons have been proposed as to why larvae have evolved to form feeding masses. In nature, social behaviours are adaptive, which means that being social ultimately increases fitness in some way. It could therefore be assumed that there are benefits for larvae feeding in a mass. In order to gain an insight into the advantages of this gregarious behaviour, the current experiment was designed to make comparisons with larvae that had been reared in the absence of a mass.

Numerous research papers have reported that the formation of a mass is beneficial for larvae since it maximises their feeding efficiency by producing sufficient proteolytic enzymes for effective tissue breakdown. This allows them to extract more nutrients (Cianci & Sheldon, 1990; Goodbrod & Goff, 1990; Green *et al*., 2003; Hobson, 1932; Ireland & Turner, 2006; Richards *et al*., 2009; Schoofs *et al*., 2009). In comparison, it has been suggested that solitary larvae struggle to penetrate their food or produce enough enzymes to effectively liquefy soft tissues for ingestion (Charabidze *et al*., 2013; Green *et al*., 2003; Greenberg & Kunich, 2002). Green and colleagues (2003) studied single-reared *Phormia regina* larvae and compared their development with larvae reared in small groups. They reported that the former demonstrated stunted growth, lighter pupal weights and experienced a slower rate of development. But the groups used in their study

were composed of only ten individuals (Green *et al*., 2003). This infers that mass-generated heat was unlikely to be a factor of any significance, and the differences between mass and single-reared larvae could potentially be greater. Therefore, the first aim of this experiment was to monitor the development of solitary larvae and determine whether individuals feeding in isolation exhibited poor growth, as reflected in slower rates of development and stunted body sizes at dispersal. This was achieved by monitoring their developmental duration, recording their lengths and weights at the end of feeding, and then comparing these measurements with those taken from mass-reared larvae in Experiments 2 and 3.

The second aim of the experiment was to determine how much meat (g) individual *L. sericata* larvae consumed before they completed the feeding phase of development and left the food. Various papers have reported that 1 g of meat per larva was sufficient for successful development (Ireland & Turner, 2006; Saunders & Bee, 1995). However, this weight did not represent what was actually consumed by a larva, but what was "sufficient" for development. This implies that in reality individuals consume less than 1 g of meat. Data concerning food consumption in individual larvae is scarce. To date, the author has not found any published research that reports the volume of food required for the successful development of a single *L. sericata* larva. For the sake of future experiments, there is a need to understand the nutritional requirements of larvae.

Following this introduction, the methods section outlines the experimental setup, the equipment used and describes how data were collected. The subsequent results section includes both quantitative and qualitative analyses. Bar charts are used to illustrate any differences in developmental duration. Boxplots illustrate

the lengths and weights of solitary larvae at the start of dispersal with larvae reared in different sized aggregations. A brief discussion follows, which explains the findings in relation to what has already been published on the topic of single reared larvae.

7.1 Materials and Methods

The experimental design used to study solitary larvae was somewhat different to the previous experiments that had focused on larval aggregations. *Lucilia sericata* eggs were harvested from laboratory reared adults and kept in an incubator programmed to maintain a constant temperature of 22 °C. Eggs were checked every one to two hours during the day and were observed to hatch after approximately 20 hours (+/- 1 h), which was an equal duration to the egg stages monitored in earlier experiments at the same temperature. Two to three hours after the first larvae had hatched, individuals were carefully transferred to glass test tubes set up as depicted in Figure 7.1. Larvae were transferred so soon after emergence to ensure that individuals only experienced ambient temperatures for the duration of development and had no exposure to any mass-generated heat, even the low levels that may occur during 1st instar. Damp cotton wool or paper towel was inserted into the bottom of the test tube below the meat to help maintain a humid environment and reduce the risk of desiccation. However, care was taken to ensure the two did not touch since there were concerns the paper towel could draw liquid out of the meat, which might have increased water loss and influenced the final volume of the soft tissue.

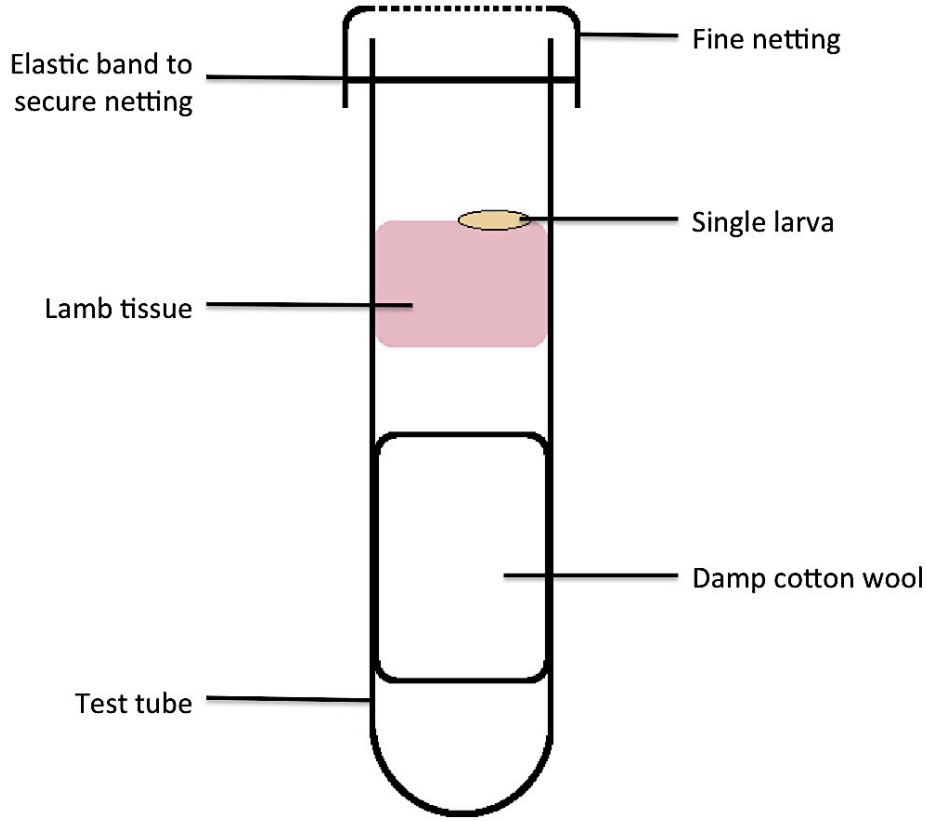


Figure 7.1. Experimental setup for monitoring the development of solitary larvae

The meat was sourced from a lamb joint, which maintained consistency with the previous experiments that had also used lamb as a food source for developing larvae. Cubes of soft tissue were cut to measure approximately 2 cm^3 and weighed to the nearest 0.001 g using a Denver Pinnacle PI-403 weighing balance. All the pieces of meat used in this experiment weighed between 3-5 g, which was in excess of the recommended 1 g per larva. Efforts were made to ensure each piece of meat was uniform in its toughness. All of the food samples contained just soft tissue with no cartilage or fat attached and were sourced from fresh joints of meat as opposed to the lamb that been frozen and thawed. The soft tissue was then inserted into the test tube so it was positioned approximately 2-3 cm above the paper towel and 4-5 cm below the test tube opening. A single larva was placed on top of the meat and fine netting was secured around the opening of the test tube

with an elastic band, which allowed air to circulate but prevented larval escape. The test tube was then returned to the incubator which was set at a constant temperature of 22 °C, with a 65% RH and a 16:8 hour (light:dark) photoperiod.

The initial plan was that larvae in this experiment would be sampled at regular hourly intervals, their instar determined by examination of their posterior spiracles under a Leica M80 stereomicroscope. This would make it possible to identify the times at which larvae molted from 1st to 2nd larval instar and then 2nd to 3rd larval instar, as well as the start of dispersal. However, during trials, larvae responded negatively to this repeated handling, with many individuals demonstrating a high mortality rate or an abnormally long developmental period. Because of this, the decision was made that larvae would be left to feed undisturbed without handling until they started to display dispersal behaviour; migrating away from the meat and attempting to exit the test tube. At this point the larva was identified as having completed feeding and the time was noted. The post-feeding larva was then killed by immersion in 80 °C water for 30 seconds before having its length measured to the nearest 0.01 mm with a Mitutoyo Absolute Digimatic Calliper and its weight recorded to the nearest 1 mg with a Denver Pinnacle PI-403 weighing balance. The remaining meat was then carefully removed from the test tube and re-weighed. By subtracting this final weight from the starting weight it was possible to estimate what volume of meat had been consumed.

Twenty test tubes were prepared in this manner, each containing a single larva originating from the same egg batch, and monitored simultaneously in the incubator. This was replicated on another two occasions over subsequent weeks,

which produced data for a total of 60 larvae. These replicates accounted for any variation in the development of larvae from different egg batches. Controls in the experiment consisted of the same lamb meat left to decompose in test tubes under the same conditions and for the same length of time but without the presence of a larva. By weighing the control meat before and after the experiment it was possible to estimate how much weight loss was attributed to decomposition and water loss in the absence of feeding larvae. This could then be incorporated into the calculations used to estimate food consumption by a single larva. Six controls were set up and monitored for each of the three replicates, which gave a total of 18 controls.

7.1.1 Statistical analysis

Data were analysed using the statistical package R (version 2.12.1) (R Core Development Team, 2010). QQ-Norm plots were used to test for normality whilst a Residual vs. Fitted plot was used to assess the variances. These diagnostic plots showed the data to be normally distributed and of uniform variance in every case. Therefore a Mixed-Effects ANOVA model was used to compare larval lengths and weights at the start of dispersal. This was used to test whether solitary larvae differed significantly in size compared to larvae reared in masses composed of 300, 1200 or 2500 individuals. Length and weight data for the mass-reared larvae at dispersal originated from Experiment 3. Since rearing conditions in the laboratory remained the same during both experiments, this was considered acceptable and expedient. The results of these analyses were investigated further with multiple comparisons of means tests, or Tukey's tests. The use of pairwise comparisons pinpointed where any differences existed.

A correlation coefficient was used to test if the length and weight measurements recorded for solitary larvae at the start of dispersal were related to the amount of food individuals had consumed. Despite both length and weight data for larvae being normally distributed, the weight data for food consumption was found to be not normally distributed (Shapiro Test: w= 0.923, p-value= 0.003). Therefore a Spearman's correlation was used to investigate the relationship between food consumption and larval body size. Scatter plots were produced to illustrate the findings.

7.2 Results

Solitary larvae appeared to be much less active in comparison to the mass-reared larvae that had been studied in earlier experiments. In fact, on several occasions, some individuals were so sedentary it was difficult to determine if they were still alive. All of the experimental larvae were observed to spend the majority of their time on the meat, only occasionally leaving it to climb the sides of the test tube. Twenty-six of the larvae were observed to burrow into the soft tissue to feed whilst the remainder preferred to feed at the surface. Larvae that fed in an isolated environment also exhibited a high mortality rate. Of the 60 individuals monitored, only 51 survived to reach the start of dispersal (mortality rate = 15%).

7.2.1 Duration of the feeding stage

In the absence of a mass, larvae experienced a considerably slower rate of development. Whilst the mass-reared larvae monitored in Experiment 2 completed feeding approximately 81-97 hours after oviposition (dependent upon mass size), a single larva required between 120-240 hours (5-10 days). However,

only one larva required 240 hours to reach dispersal; all others completed feeding within 192 hours. The mean number of hours required for solitary larvae to reach dispersal was 151.8 (6.3 days) (±22.65 hours), which was approximately 1.5 times slower than the slowest feeding mass (Figure 7.2).

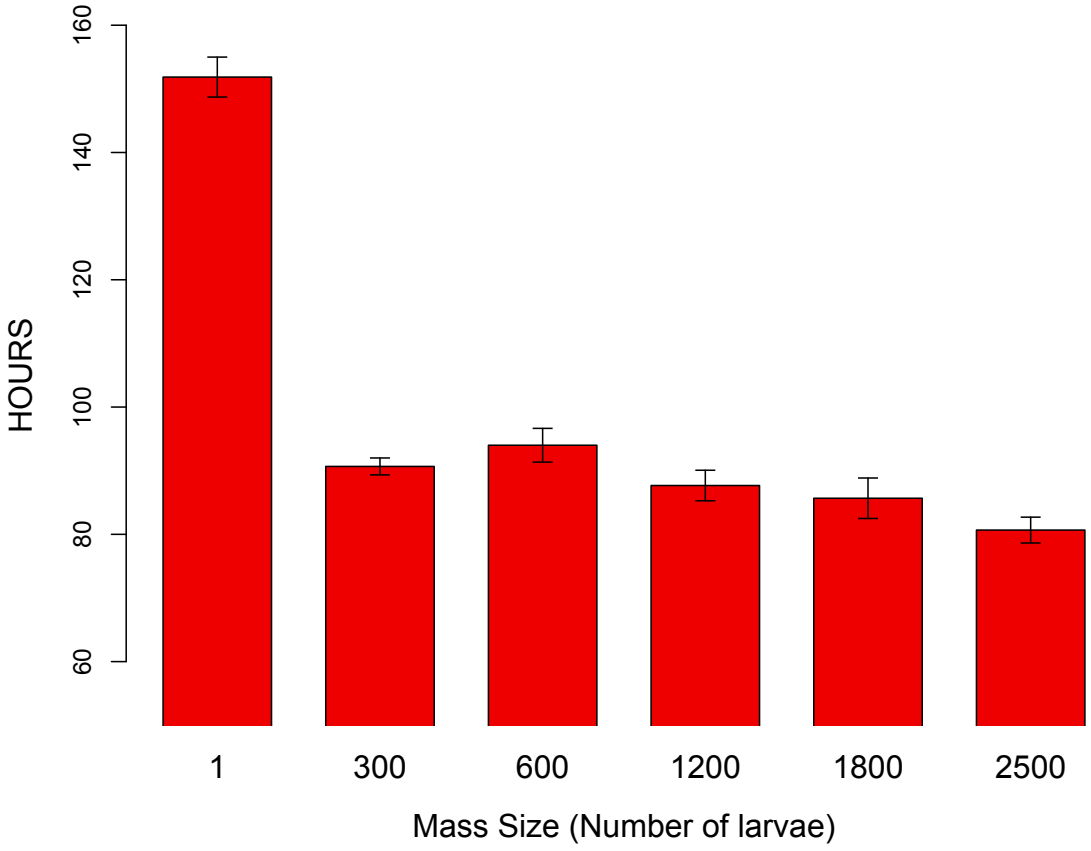


Figure 7.2. The mean number of hours (with standard error bars) required for single and mass reared *L. sericata* larvae to reach the post-feeding stage of development (constant ambient temperature = 22°C ±1). (NB. Data for masses 300-2500 taken from Experiment 2)

7.2.2 Size at dispersal

At the start of dispersal, solitary larvae were significantly shorter than larvae that had been in reared in masses ($F_{3,17}$=14.09, p<0.001). Whilst larvae sampled from masses were all the same length at dispersal (mean length = 15.17-15.53 mm), individuals that had developed in an isolated environment were observed to

disperse with a mean length of only 13.98 mm (±1.24), approximately 1.5 mm shorter than their mass-reared counterparts (Figure 7.3).

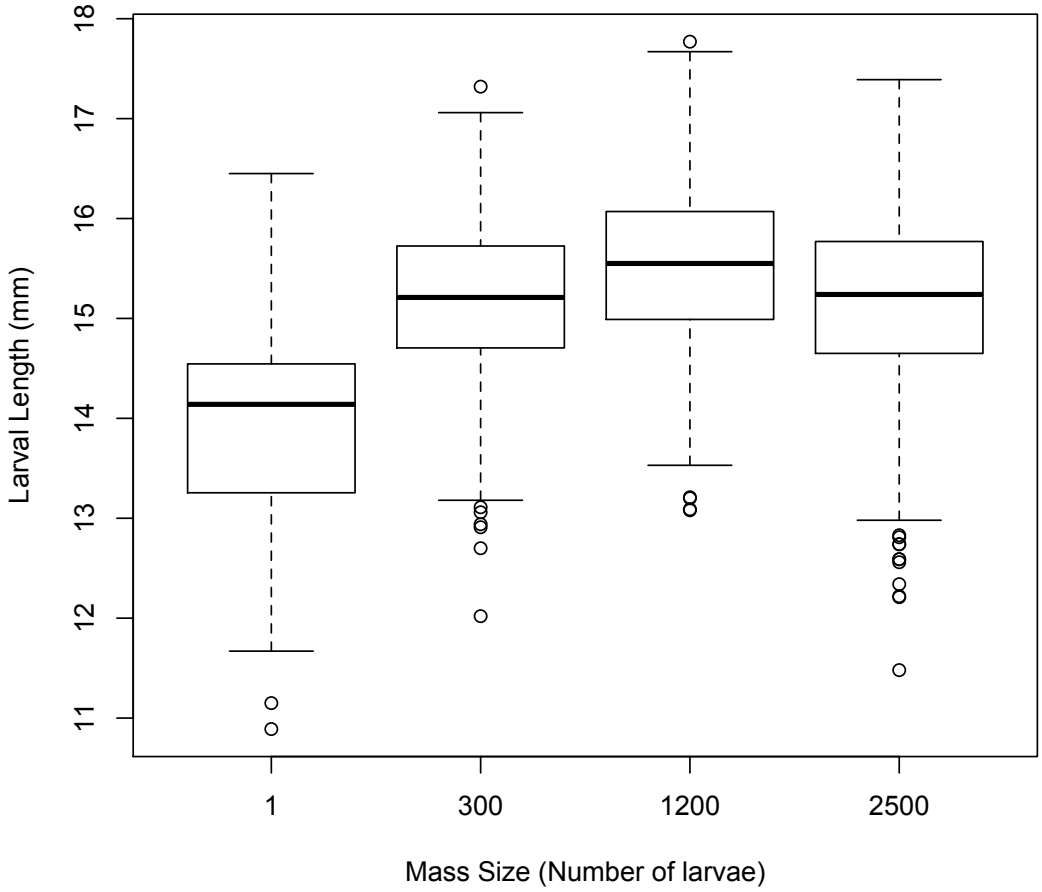


Figure 7.3. Larval lengths (mm) recorded in solitary and mass reared larvae (300, 1200 and 2500 individuals) at the start of dispersal (constant ambient temperature = 22 °C ±1). (NB. Data for the masses 300-2500 taken from Experiment 3)

Similar results were reported when larval weights at the start of dispersal were compared. When tested with a mixed effects model, the results showed that there were significant differences between solitary and mass larvae ($F_{3,17}$=7.55, p=0.002). At the end of feeding, solitary larvae had a mean weight of 49.33 mg (±8.95), which was significantly lighter than the mean weights of larvae sampled from masses composed of 300 (mean=56.20 mg, SD=7.33) and 1200 individuals

(mean=60.07 mg, SD=7.22) (Figure 7.4). However, despite having a p-value approaching significance (p=0.051), pairwise comparisons between individuals reared in isolation and those in large aggregations containing 2500 larvae (mean=55.29 mg, SD=7.89), showed that they were statistically not different in weight, with a difference of only 5.96 mg. The mean lengths and weights of larvae from different sized aggregations, along with minimum and maximum measurements, were reported in Table 7.1.

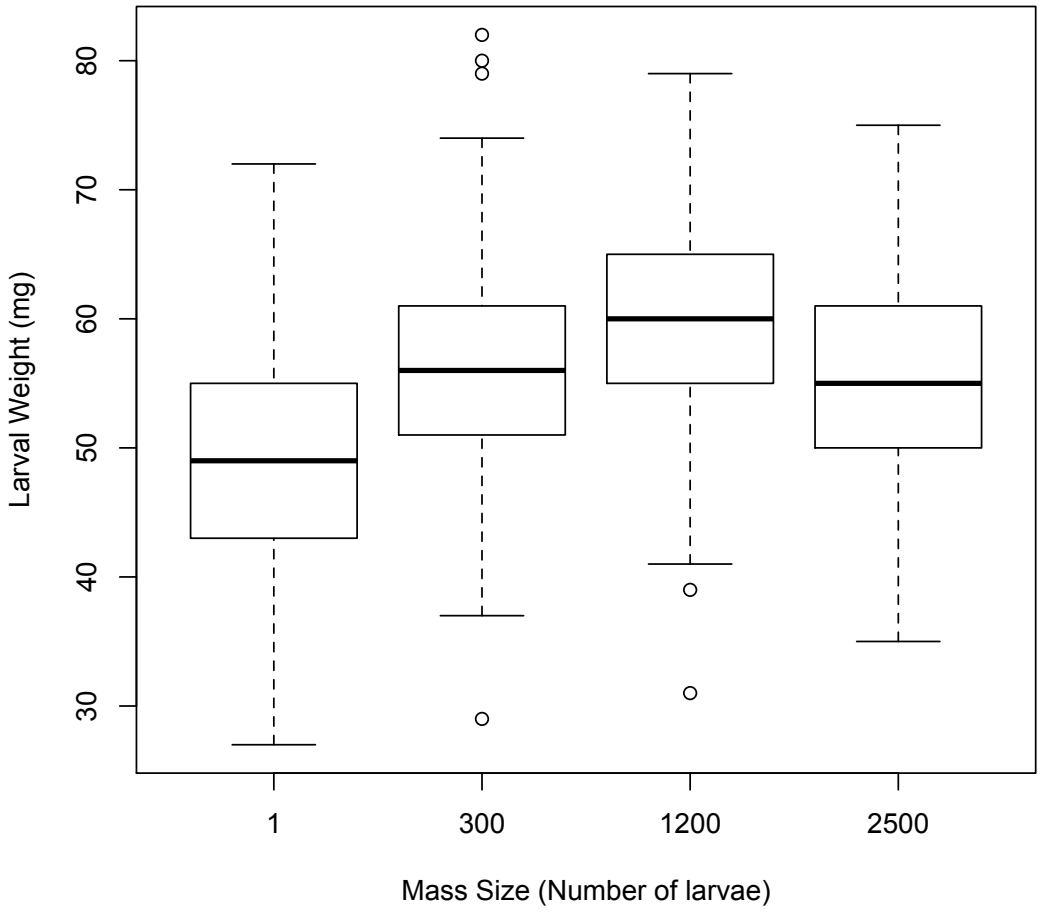


Figure 7.4. Larval weights (mg) recorded in solitary and mass reared larvae (300, 1200 and 2500 individuals) at the start of dispersal (constant ambient temperature = 22 °C ±1). (NB. Data for the masses 300-2500 taken from Experiment 3)

Table 7.1. The mean lengths and weights (plus standard deviation) for larvae reared in different sized aggregations at the start of dispersal (constant ambient temperature = 22 °C ±1)

Mass Size		Mean	Standard Deviation
1	*Length (mm)*	13.98	1.24
	Weight (mg)	49.33	8.95
300	*Length (mm)*	15.17	0.80
	Weight (mg)	56.20	7.33
1200	*Length (mm)*	15.53	0.79
	Weight (mg)	60.07	7.22
2500	*Length (mm)*	15.19	0.01
	Weight (mg)	55.29	7.89

7.2.3 Food consumption

By recording the before and after weights of the 18 samples of control meat, it was possible to calculate weight loss as a consequence of desiccation. Over a period of 150 hours (mean developmental duration in solitary larvae) the average reduction in the weight of meat was 0.638 g (or 14.63% of the original weight). Therefore, in the absence of any feeding larvae and over a period of 150 hours, the tissue samples used in this experiment were expected to lose approximately 0.638 g of their original weight through water loss. It is important to note though that some larvae spent up to 192 hours feeding and the estimated weight reduction of 0.638 g will differ between meat samples depending on their volume and surface area. For each of the 51 larvae that had successfully reached dispersal, 0.638 g was subtracted from the overall weight difference recorded in the soft tissues they had fed on. This gave an estimate for the amount of food consumed by a solitary larva. For *Lucilia sericata*, individuals were found to consume an average of 0.603 g

(±0.31) of meat during the feeding period. However, food intake varied considerably between individuals and ranged from 0.099 g to 1.434 g.

A Spearman's correlation coefficient was calculated for the data. This was to identify whether there was any correlation between the amount of food consumed by a larva and its length at dispersal. The results showed that there was no correlation between larval length and food consumption (n=51, r_s= -0.219, S=26935.66, p=0.123) (Figure 7.5). The same results were observed when investigating the relationship between food consumption and larval weight, with again no correlation between the two being identified (n= 51, r_s= -0.203, S=26579.21, p=0.154) (Figure 7.6). Therefore, the amount of soft tissue consumed by individual larvae did not appear to be related to their length and/or weight at the start of dispersal, i.e., the largest individuals were not necessarily the ones that had consumed the greatest volume of food.

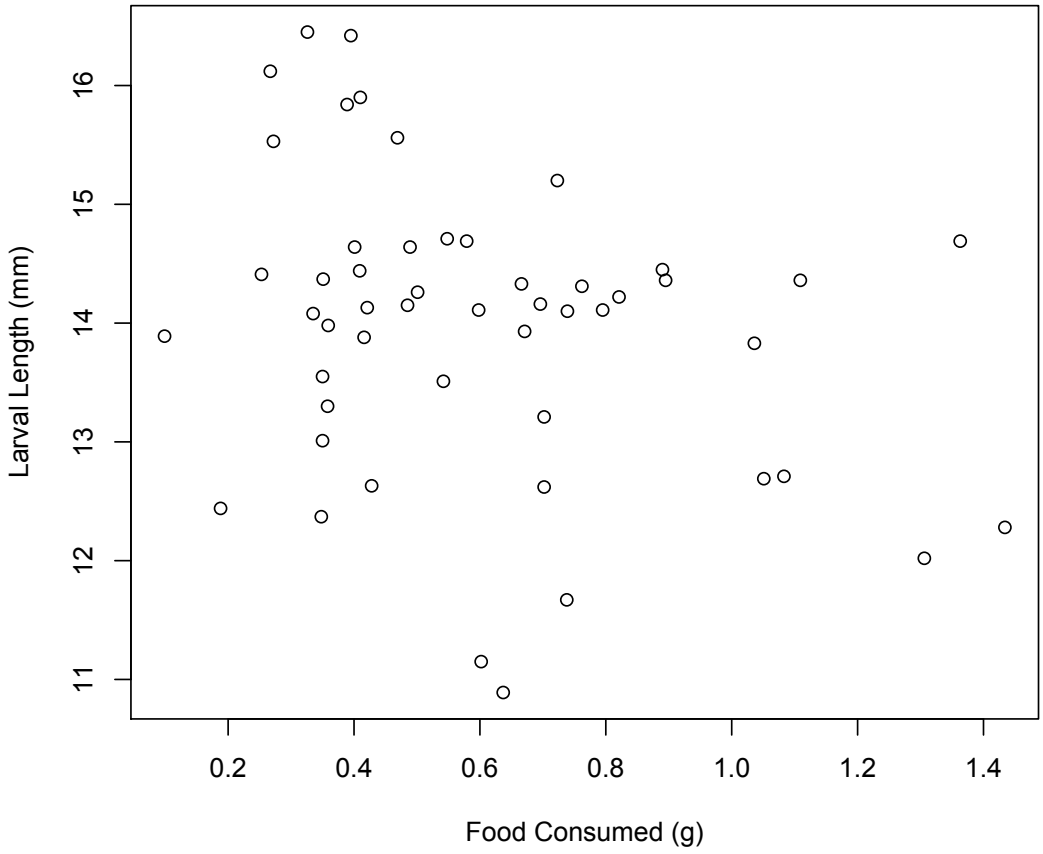


Figure 7.5. Amount of food consumed (g) and the length (mm) of solitary larvae show no discernible relationship

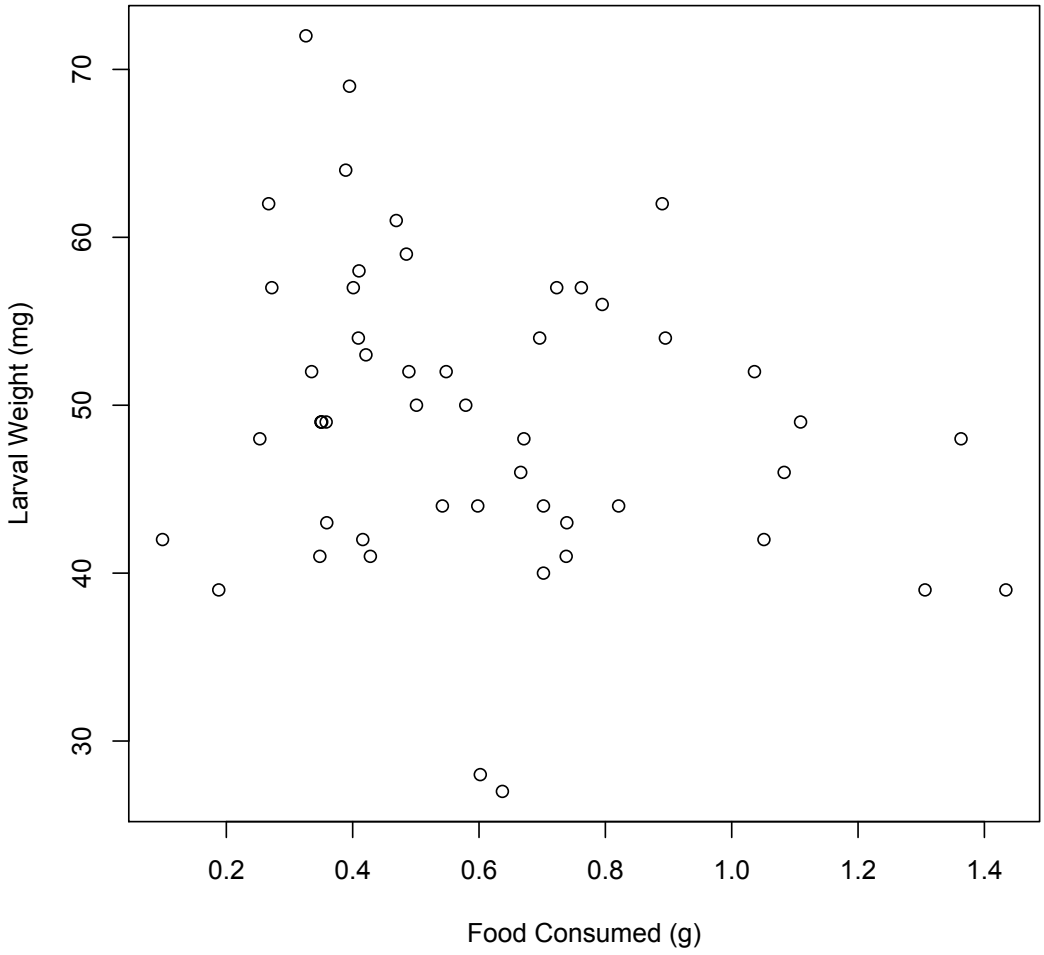


Figure 7.6. Amount of food consumed (g) and the weight (mg) of solitary larvae show no discernible relationship

7.3 Discussion

Larvae forced to feed in isolation demonstrated a slower rate of development and a reduced body size at the start of dispersal when compared to larvae reared in a mass. This supported the idea that larvae benefited from feeding and developing within an aggregation.

7.3.1 Feeding duration

The slower rates of development associated with solitary larvae have been reported for other species in numerous papers. For example, Ireland and Turner (2006) monitored the effect of larval crowding on the development of *Calliphora vomitoria* larvae in a controlled laboratory environment. They established a series of cultures with different degrees of larval crowding by transferring 1, 5, 10, 20, 50, 75 and 100 newly hatched larvae to a 10 g piece of meat maintained at an ambient of 20 °C. The authors reported that as larval density increased the rate of development was accelerated, with larvae reared in the largest (and densest) masses showing adult eclosion three days earlier than single larvae (Ireland & Turner, 2006). Similar results were published by Saunders and Bee (1995) as well as Green and colleagues (2003), who investigated the effects of larval crowding on *Calliphora vicina* and *Phormia regina* respectively.

7.3.2 Larval size

Larvae reared in groups were found to be up to 10.74 mg heavier and 1.55 mm longer at dispersal than individuals that developed in an isolated environment. This supported work published by Green and colleagues (2003). They investigated whether single feeding *Phormia regina* larvae developed differently compared to individuals reared in groups of ten. They observed that larvae feeding in groups, albeit small groups, were more than 14 mg heavier at pupation compared to those reared in isolation (Green *et al*., 2003).

The accelerated rates of development and larger body sizes observed in larvae sampled from aggregations could be a result of the warmer temperatures and the greater volume of tryptic and alkaline secretions attributed to larger, denser

masses (Green *et al*., 2003; Ireland & Turner, 2006; Mackerras & Freney, 1933). It is possible that larvae forced to feed in isolation are unable to secrete sufficient enzymes to digest an adequate amount of food for efficient development. Green and colleagues (2003) claimed that solitary larvae were poorly adapted to feed in the absence of other individuals, and rather than burrow into the food to feed on soft tissue, they fed on the liquid exuding from it. However, in the current study this behaviour was observed in only half of the larvae, whilst the other half disappeared from view completely as they burrowed into the meat.

7.3.3 Food consumption

In this experiment, a single *Lucilia sericata* larva was estimated to consume a mean of 0.603 g (±0.31) of soft tissue during the feeding stage of development. This is of the same order that has been reported for *Calliphora vomitoria* larvae. For this species, Ireland and Turner (2006) reported that approximately 0.5 g of pig muscle was sufficient to ensure full development of a single larva. These weights are half of what has been recommended in the past for various blowfly species. It should be noted though that food consumption in *Lucilia sericata* larvae appeared to differ considerably between individuals, ranging from 0.099 g to 1.434 g. Charabidze and colleagues (2013) reported comparable results after they investigated the foraging behaviour of *L. sericata* larvae. The researchers dissected and measured larval crops from 3rd instar larvae and reported that there was a great heterogeneity in their surface areas. They attributed this finding to variation in food intake between individuals as opposed to the genetic background of the population, since all larvae had originated from the same strain. This could also be the case with the results presented here, since all larvae originated from the same stock and were maintained under the same conditions in the laboratory.

For the duration of the experiment, solitary larvae were rarely seen to leave the meat. But despite spending the majority of their time in contact with a viable source of food, it was hard to determine if individuals were feeding the entire time due to their small size and the handling restrictions set for the experiment. It has been suggested in previous studies that larvae will feed continuously until the post-feeding phase of development, ingesting food faster than they can digest it (Greenberg & Kunich, 2002). This constant feeding hypothesis was also described by Huckesfeld and colleagues (2010), who used it to explain how the motor patterns of feeding *Calliphora vicina* larvae did not change due to sensory input. Green *et al.* (1983) reported that 84% of the lifetime of *Drosophila* larvae is spent feeding. However, this behaviour cannot be generalized to necrophagous larvae because they consume a different type of food.

When larvae ingest liquefied material, dark patches of the gut are typically visible through the semi-translucent cuticle. It has been proposed in the past that the amount of food inside the crop can be used as an indicator of the food intake of necrophagous larvae (Charabidze *et al*., 2013). In this experiment, the crop of single-feeding larvae was never visible through the cuticle. Since food entering the alimentary tract goes through the crop before moving on to the oesophagous and gut, the fact that the crop was never visible in the solitary larvae implied that it was never full. This suggested that food was being utilised as soon as it was ingested, rather than being stored and purged from the crop at a later time. Perhaps it was the case that for solitary larvae, food intake was unable to keep pace with the metabolism.

7.3.4 Mortality rates

In the previous experiments, which focused on larvae feeding and developing in aggregations, no deceased individuals were observed, regardless of the mass size. However, when solitary larvae were studied, a 15% mortality rate was recorded. The deceased larvae were not handled or disturbed during the experiment, were provided ample food, maintained at a constant ambient temperature of 22°C and originated from a number of different egg batches. Therefore, it was unclear what contributed to this higher than expected level of mortality. In another study, Ireland and Turner (2006) reported that single feeding *Calliphora vomitoria* larvae, whether reared on liver, brain or muscle, showed a 100% survival rate. However, they found that this survivorship decreased as mass density was increased. In fact, for the densest masses (100 larvae/10 g meat) reared on brain, percentage survival for adults was as low as 3% (Ireland & Turner, 2006).

It is possible that the high mortality rates of *L. sericata* observed in the current experiment were a result of inefficient feeding and nutrient restriction. If solitary larvae were unable to secrete sufficient enzymes for tissue breakdown, then they might have starved to death as a result of not being able to access the nutrients in the soft tissue. It is also possible that the increased mortality rates were influenced by the methods used to set up the experiment. Unlike the previous experiments, solitary larvae were transferred to test tubes approximately one to two hours after hatching. These minute and fragile 1st instar larvae could have been damaged during handling, which might have reduced their fitness and ultimately led to their death. The high mortality rates observed in solitary larvae, combined with the extended feeding period and reduced body sizes at dispersal,

provide evidence supporting the theory that larvae benefit by aggregating during the larval stage.

8 EXPERIMENT 5 – The Movement of Larvae in a Mass

In the previous experiments (Experiments 1-2) large masses were recorded reaching highs of 36 °C. The fact that no deceased larvae were observed in any of these large aggregations could mean one of three things. Firstly, it could provide further evidence that the upper lethal temperature threshold for *Lucilia sericata* is higher than 36 °C. Secondly, larvae in these laboratory-reared masses might have experienced increased mortality rates that manifested themselves during the later stages of development, falling outside the scope of this project (e.g. failed pupariation or adult eclosion). Or thirdly, the absence of any deceased larvae at these temperatures could indicate that there is some form of thermoregulatory behaviour occurring within the mass.

For *Lucilia sericata* there is still uncertainty as to where exactly the upper lethal temperature threshold lies, with reports ranging from 35 °C (Wall *et al*., 2001) up to 47.5 °C (Richards *et al*., 2009). Several researchers have documented large maggot masses associated with decomposing animal carcasses producing temperatures as high as 50 °C (Deonier, 1940; Marchenko, 2001; Richards & Goff, 1997; Slone & Gruner, 2007), which were several degrees warmer than the highest proposed upper lethal limit of 47.5 °C (Richards *et al*., 2009). Whilst deceased larvae were reported on the periphery of some of these large aggregations (Kelly *et al.,* 2009; Slone & Gruner, 2007), the majority of the larvae were able to continue feeding despite being exposed to extreme temperatures. These observations imply that larvae within a mass are able to regulate their temperature and avoid overheating.

Naturally occurring temperature gradients exist in large aggregations. A number of entomologists have suggested that larvae use these gradients to regulate their own temperature, circulating between the hot centre where they feed and the cooler periphery (Ames & Turner, 2003; Catts, 1992; Charabidze *et al*., 2011; Hückesfeld *et al*., 2011; Rivers *et al*., 2011; Slone & Gruner, 2007). Further evidence supporting this idea of larval rotation was presented by Byrd and Butler (1996, 1997, 1998). Using temperature gradients in the laboratory they demonstrated that larvae actively repositioned themselves in order to regulate their temperature for accelerated development. This behaviour could result in the average temperature experienced by a larva to be lower than the hottest temperature in the mass, which might explain how individuals avoid the potentially lethal temperatures at the core.

Whilst many entomologists (Ames & Turner, 2003; Byrd & Butler, 1996; Catts, 1992; Charabidze *et al*., 2011; Hückesfeld *et al*., 2011; Rivers *et al*., 2011; Slone & Gruner, 2007) have described the circulating movement of larvae, very little research has been carried out on the subject that has succeeded in producing quantitative data to support the hypothesis. To date, the author is yet to find a research paper that has successfully tracked or recorded the movements of a larva within a feeding mass. This paucity of research in the field of entomology may be due to a lack of suitable tagging or marking techniques available for blowfly larvae, which have made it near impossible to discern an individual from a cohort. But a recent publication by Moffatt (2013) has highlighted a new technique for dye tagging larvae that could now facilitate studies on their movement. The current experiment used this new dye tag, known as VIE (see section 3.5), and the methods described by Moffatt (2013) to mark 3rd instar *Lucilia sericata* in laboratory-reared

masses. Four different coloured fluorescing tags were injected into four larvae, which were then returned to a feeding aggregation. The positions of these larvae were then recorded at regular intervals to determine how much time individuals spent at the periphery and/or the centre. The aim of this experiment was to produce quantitative data that could be used to either support or refute the theory of larval rotation in a mass.

Following this introduction, the materials and methods section describes the experimental set up as well as the sampling methods used. The subsequent results section includes both quantitative and qualitative analyses. Boxplots and histograms were produced to illustrate the proportion of time tagged larvae spent at either the periphery or the centre of the mass. The final discussion attempts to explain what may have influenced the movement of larvae within the aggregation.

8.1 Materials and Methods

At the start of the experiment early 3rd instar larvae were already feeding in masses composed of 500 individuals. These larvae had hatched from eggs approximately 48 hours earlier and had been counted out into masses whilst still in the 1st larval instar. Each of these masses was reared on a piece of soft tissue (lamb) weighing approximately 150 g, which produced a mass density of 3.5 larvae/g. An aggregation of this size and density was judged suitable for the experiment since it was dense enough to promote the formation of a mass without being so large that tagged larvae would be easily lost within the aggregation. During the 2nd instar, larvae were checked at regular 2-3 hour intervals in order to identify the point at which the majority of the mass had molted into 3rd instar.

Once larvae had reached this stage of development, four individuals were randomly selected and removed from the mass so they could be injected with the VIE tag.

Third instar larvae were deemed the youngest that could be tagged since Moffatt (2013) reported that the gauge of the needle was limited by the viscosity of the elastomer and that a needle this size was too large to inject 1st and 2nd instar larvae. However, it could be used on early 3rd instar *Calliphora vicina* larvae with a minimum length of 8 mm (Moffatt, 2013). According to Northwest Marine Technology Inc., the smallest fish to be tagged are 8 mm damselfish. They also warn that as the size of the target animal decreased, the risk of causing damage whilst tagging increased (Northwest Marine Technology, 2008). Given that massing occurs more in the 3rd instar, it was decided that this was the best stage for studying larval movement anyway, regardless of the restrictions placed on tagging smaller larvae. Each of the sampled larvae were injected with one of four different fluorescing colours (orange, pink, green or blue) using the methods described in section 3.5. Using different coloured tags made it possible to monitor the movements of four individuals in a mass at any one time (Figure 8.1).

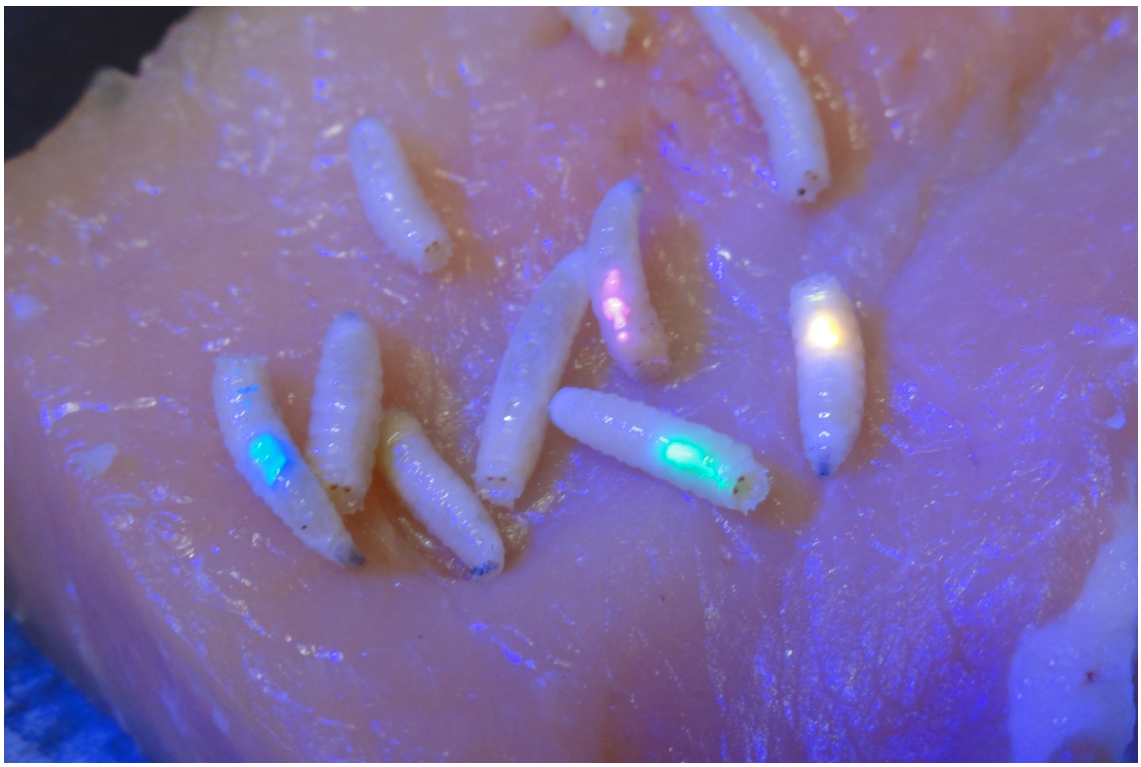


Figure 8.1. Four 3rd instar *L. sericata* larvae tagged with blue, green, pink and orange VIE as viewed under a UV light source. Note how the orange elastomer appears yellow in colour as it fluoresces. The pink tag has also separated into a series of "scatter tags" whist the blue tag has migrated forwards from the initial injection site

Before they were returned to the mass, tagged larvae were placed on a small piece of meat for 30 minutes. This was to ensure that larvae continued to move and feed as normal and that no subsequent death occurred as a result of the tagging procedure. Once these individuals were observed to commence normal behaviour, they were returned to the aggregation. Shortly thereafter data collection began. The mass was checked at ten-minute intervals over a four-hour period and each of the tagged larvae had their position within the mass recorded. If larvae were observed they were recorded as being at the periphery. If not they were assumed to be inside the aggregation feeding. A total of 15 masses were prepared using these methods, each containing four tagged larvae. All of these aggregations were housed in an incubator set at a constant temperature of 22 °C with a 65% RH and

16:8 hour (light:dark) photoperiod for the duration of the experiment. At the conclusion of the experiment data was available for a total of 60 individuals.

Mass temperatures were not recorded for any of the 15 aggregations monitored in this experiment due to the thermal imaging camera not being available. However, in earlier tagging trials, which used masses of the same size and density, temperature readings were taken intermittently using the FLIR T425 whilst larvae fed during 3rd instar. Temperature readings for masses containing 500 larvae and reared at a density of 3.5 larvae/g were consistently in the range of 25-27 °C during 3rd instar.

8.1.1 Statistical analysis

Data were analysed using the statistical package R (version 2.12.1) (R Core Development Team, 2010). Given that the data were found to be normally distributed, a Grubbs' test was carried out to detect any outliers. A mixed-effects generalized linear model was carried out on the data to determine whether there are any significant differences between i) masses and ii) maggots within a mass in terms of the proportion of time larvae spent at the periphery. A histogram was produced which illustrated the proportion of time larvae spent feeding, i.e. the proportion of time they were reportedly inside the mass and not observed at the periphery. Marked on the histogram were the 1.96 x standard deviations, which indicated where 95% of the data fell. To establish whether or not larvae differed in the number of times they changed location in the mass (centre/periphery), a new variable was made for each of the tagged larvae in R. This variable recorded whether an individual's position had changed from the previous observation and

was analysed with a mixed effects generalized model. This could then be used to represent how quickly larvae moved through the mass.

8.2 Results

8.2.1 Casual observations

Individuals tagged with VIE are easily identified within a mass of 500 larvae. The use of a UV light source enhanced the identification process and allowed tagged larvae to be located within seconds without disturbing the aggregation or triggering negative phototaxic behaviour. However, whilst the orange and pink tags were easy to distinguish, there were occasional problems when trying to differentiate between the green and blue tags. Altering the angle of the light rectified this issue.

8.2.2 Proportion of time spent at the centre/periphery

One larva, recorded to have spent 88% of the observation period at the periphery, was identified as an outlier and removed from the data set. This individual was observed barely moving for long periods of time at the surface of the meat away from the aggregation. It is possible that this larva was injured in some way during the tagging process. For the remaining 59 larvae, the amount of time they spent at the periphery appeared to differ between masses as well as individuals (Figure 8.2). In one aggregation larvae spent a mean of 28% of their time at the periphery whilst in another the mean proportion of time individuals spent at the periphery was nearly double at 54%. When tested with a mixed-effects generalized linear model, these differences between masses were found to be significant (X^2= 27.8, df= 14, p= 0.015). The same results were observed when comparisons were made

between larvae within a mass; individuals from the same aggregation differed significantly in the proportion of time they spent at the periphery (X^2= 78.9, df= 58, p= 0.035). These results suggested that the amount of time a larva spends at a particular location is very variable.

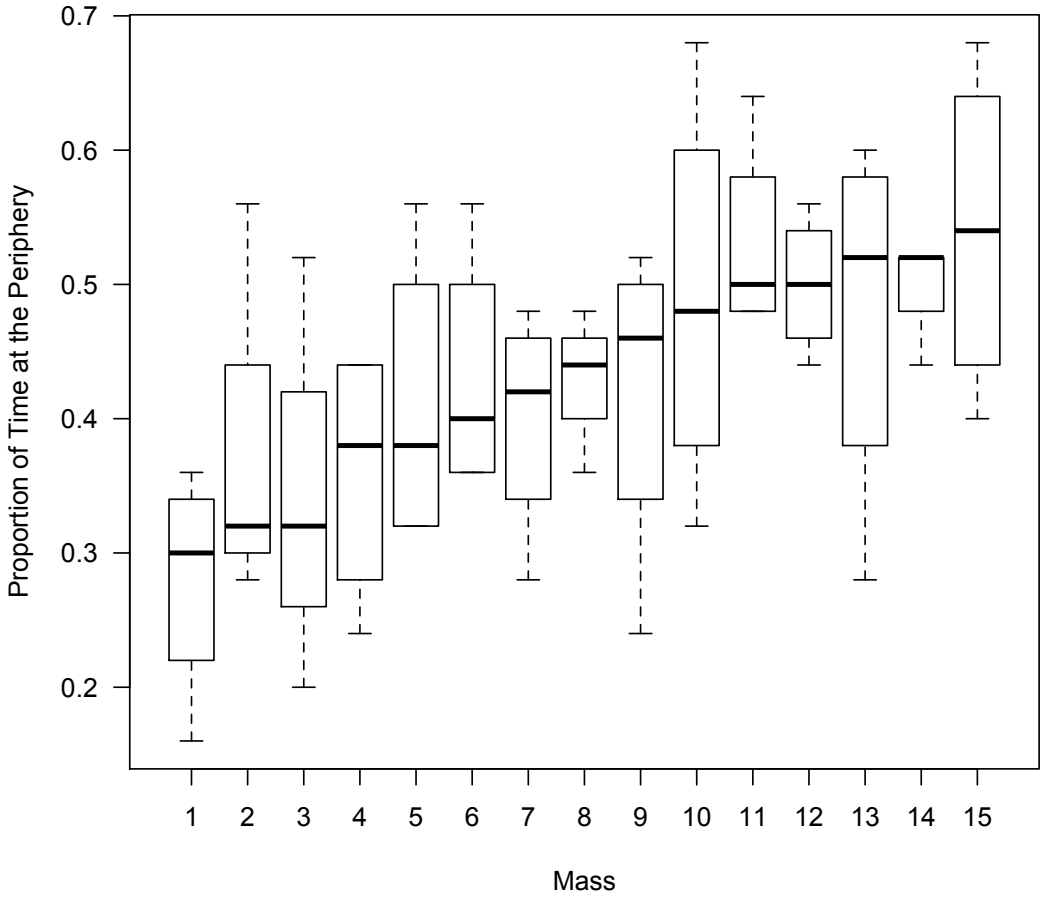


Figure 8.2. Boxplot showing the proportion of time tagged larvae spent at the periphery in each of the 15 experimental masses. Each of the masses had 4 larvae tagged, aside from Mass 2, which had 3 since one was identified as an outlier and removed. (*NB. Masses are arranged sequentially along the x-axis with increasing medians. This was for ease of interpretation)

None of the tagged larvae were observed to spend more than 84% of their time inside the mass. At some point they all ceased feeding to make an appearance at the periphery. The majority of larvae appeared to divide their time fairly between

the two locations, with individuals spending a mean of 55.9% of their time inside the mass (44.1% of their time at the periphery). However, the proportion of time spent feeding at the centre appeared to vary among individuals and ranged from 32-84% (Figure 8.3).

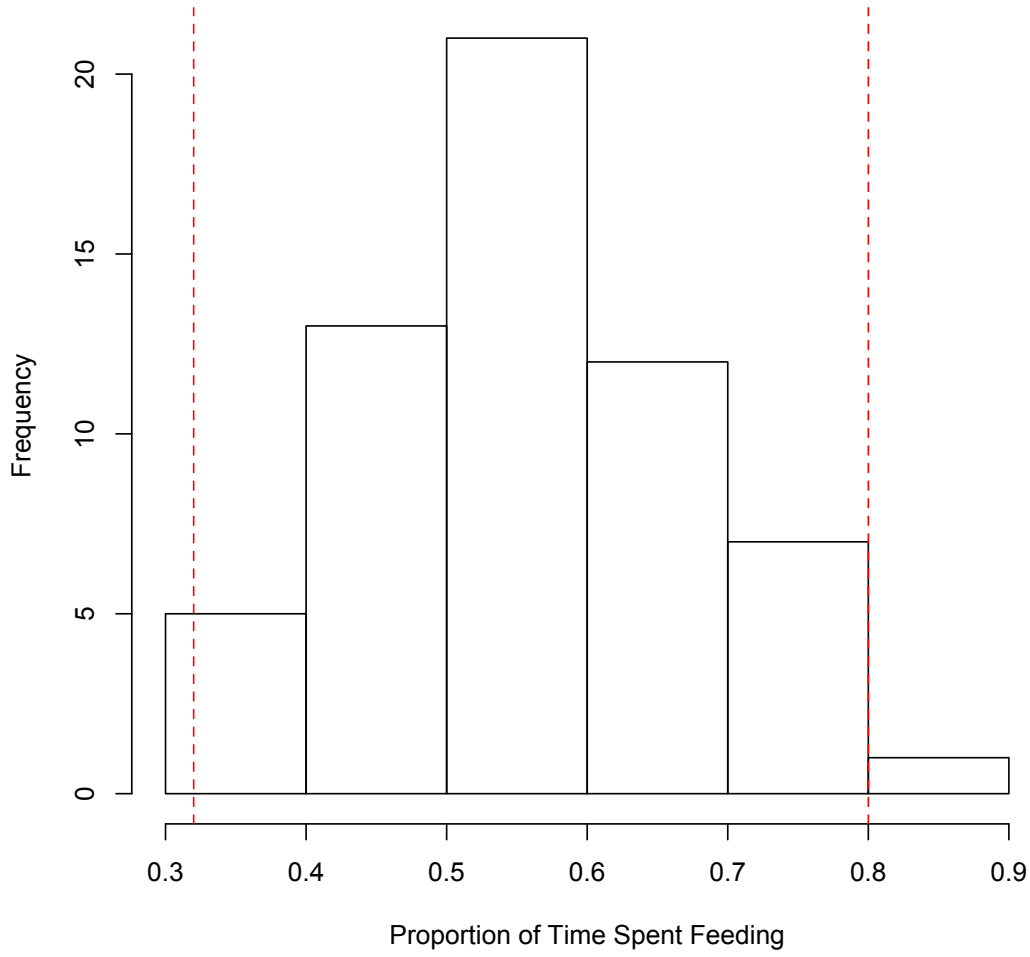


Figure 8.3. Histogram illustrating the proportion of time 3rd instar larvae were observed to spend inside the mass with 1.96 standard deviations (red dashed lines). All masses were composed of 500 larvae and reared at an ambient temperature of 22 °C (±1) and a density of 3.5 larvae/g

9.2.3 Rate of movement within the mass

Larvae appeared to be in a constant state of movement. Differences were observed in the rate at which some individuals circulated through the mass. For example, one larva was always recorded to spend at least two consecutive observations at the periphery (20 minutes total) before disappearing into the mass where it would remain for the following two to three observations (20-30 minutes). This suggested a slow rate of movement through the mass. But another larva sampled from the same mass appeared to circulate between the centre and the periphery at a faster rate. For each successive observation (every five minutes) this individual was recorded at a different location. Yet despite these two larvae appearing to move differently through the mass, by the end of the observational period both had accumulated the same amount of time at the periphery. However, when tested statistically there were no significant differences between (X^2= 18.5, df= 14, p= 0.186) or within (X^2= 64.3, df= 58, p= 0.267) masses when comparing the number of times larvae were observed to change their position. All tagged larvae alternated between the periphery and centre at the same rate.

8.3 Discussion

8.3.1 Movement of larvae in a mass

Larvae reared in masses are in a constant state of motion, alternating between the centre of the aggregation, where they presumably feed, and the periphery. There are a number of reasons that could explain why larvae reposition themselves in this way, including thermoregulation, foraging behaviour, competition and hypoxic conditions. The idea that larvae feeding in aggregations are capable of regulating their temperatures to avoid overheating has been described on numerous occasions (Amendt *et al*., 2011; Ames & Turner, 2003; Catts, 1992; Charabidze *et*

al., 2011; Hückesfeld *et al*., 2011; Kelly *et al*., 2009; Sharanowski *et al*., 2008; Slone & Gruner, 2007). Previous research has demonstrated that larval masses are self-organized and extremely sensitive to numerous parameters of their surroundings, appearing to continually re-orientate themselves to adapt to their environment (Charabidze *et al*., 2008). When confronted with a sudden change in temperature, *Calliphora* larvae have been shown to demonstrate a reflex-like evasive behaviour, retracting their anterior segments and crawling away from unfavourable temperatures (Hückesfeld *et al*., 2011). This thermotaxic behaviour could influence larval movement inside a mass, directing individuals away from the hot feeding centre and out towards the cooler periphery where they might experience evaporative cooling and avoid lethal temperatures (Ames & Turner, 2003; Catts, 1992; Charabidze *et al*., 2011; Hückesfeld *et al*., 2011; Rivers *et al*., 2011; Slone & Gruner, 2007). If larvae in a mass are able to perceive temperatures, then it would also support Byrd and Butler's research on temperature gradients (1996, 1997, 1998), which discussed how larvae regulated their body temperature by positional effect within the mass. They argued that this behaviour allowed larvae to maintain a preferred temperature for accelerated development.

However, since the masses observed in this experiment contained only 500 larvae, it is unlikely that the rotation observed was a result of individuals regulating their temperature to avoid overheating. Based on the results of Experiment 1 and 2, aggregations of this size are not expected to generate mass temperatures exceeding 27-28 °C (peak temperatures recorded in the slightly larger 600 masses), several degrees cooler than the proposed stress inducing temperatures recorded in large masses. Studying larval movement in heat stressed aggregations would have required working with exceptionally large masses capable of

producing temperatures of 40 °C or more. In the laboratory this would be unfeasible. Therefore, it is assumed that the movement observed in this experiment was influenced by other factors aside from mass size and heat generation. One possible cause of rotation might be mass density. The aggregations studied here had a density of 3.5 larvae/g, which whilst not considered "crowded", could still have resulted in competition for food and hence larval displacement. Charabidze and colleagues (2013) also linked the movement of larvae in a mass to foraging behaviour. They claimed that larvae do not feed continually and regulate their foraging behaviour. It is this foraging behaviour, where individuals move out to the periphery to search for new feeding sites, which creates a permanent movement, or turnover, within the mass. Larvae feeding in a mass might also experience periods of little (hypoxia) or even no (anoxia) oxygen, especially if the mass is dense or partially submerged in decompositional fluids. Hoback and Stanley (2000) described carrion as a hypoxic microhabitat, where larvae and bacteria with high oxygen demands removed it from the surrounding air. Therefore it is possible that larval hypoxia contributes to mass rotation, with individuals moving away from the centre to more oxygenated areas at the periphery (Hoback & Stanley, 2000). It seems likely that the circulating behaviour observed in masses is a result of all of these causes.

8.3.2 Consequences of larval movement

How larvae move through a mass may have consequences for individuals in terms of development. Many of the tagged larvae appeared to divide their time evenly between the two locations, spending approximately half of their time at the periphery and half at the centre. Repeatedly changing their position in the mass like this this implies that larvae do not feed continuously. This could result in an

overall reduction in body size for individuals feeding in aggregations where this behaviour is prevalent. It could be assumed that if larvae spend more time at the periphery or moving through the mass, then it would result in less time being at the centre feeding. It should also be noted that despite larvae spending a mean of 55.9% of their time inside the aggregation, this proportion of time varied significantly between individuals and masses. For example, whilst five of the 59 larvae were observed to spend 30-40% of the observation period at the centre, seven individuals spent 70-80% of their time in the same location. It's possible that intraspecific competition was occurring within the masses, with some larvae dominating at the centre whilst others were forced out to the periphery. This might influence food consumption between individuals. Larvae spending a higher proportion of their time at the centre would be expected to have an increased access to nutrients, which might consequently result in larger body sizes. In comparison, larvae that have been forced out to the periphery by more dominant individuals would have reduced access to food and might therefore experience slower development and smaller body sizes. It could therefore be assumed that larval rotation might negatively affect some individuals, reducing their fitness and probability of survival. As well as influencing body size, larvae spending a greater proportion of their time at the periphery might also suffer from an increased risk of predation in comparison to individuals who are more protected in the centre of the mass.

It is important to mention that even with thermoregulatory behaviour there are still instances where larvae have reportedly suffered high mortality rates as a result of thermal stress. Slone and Gruner (2007) described how they observed lifeless larvae at the periphery of large aggregations whose temperatures peaked

at 50.7 °C, whilst Kelly and colleagues (2009) reported similar findings after they monitored the decomposition of wrapped and unwrapped pig carcasses in South Africa (Kelly *et al.,* 2009). The high mortality rates described here suggest that thermoregulation in masses may only be effective up to a point. It is possible that there is a critical mass size, and hence temperature, where larvae are unable to regulate or escape such extreme heat. There are also other factors to consider that might contribute to larval mortality at high temperatures. For example, in the aforementioned Kelly *et al.* (2009) study, the low survivorship could be attributed to a build up of noxious gases (carcasses were wrapped in plastic sheets) as well as the high mass temperatures.

8.3.3 Potential sources of error

The results of this study implied that all the tagged larvae moved between the periphery and the centre at the same rate. However, there are a number of factors related to the tagging and sampling techniques used that could have affected these findings and should to be taken into consideration. It is plausible that some larvae may have been injured during the injection process. This could have reduced their ability to crawl and restricted their movements within the mass. The timing of observations might also have influenced the results. If larval rotation is indeed cyclic, then there was the risk that the observations made at regular ten-minute intervals could have inadvertently fallen in sync with the rotational pattern of some larvae. The timed observations and larval rhythms may have been running concurrently.

8.3.4 Visible Implant Elastomer as a future tagging tool

Visible Implant Elastomer clearly has potential as a tag for blow fly larvae. VIE was easy to use and made it possible to discern individuals from the rest of the cohort with ease, either in natural light conditions or with the use of a UV torch. This experiment was not designed to investigate mortality rates in tagged larvae but Moffatt's (2013) paper reported that individuals injected at the D11 site had an 80% survival rate. Of the 60 larvae tagged in this experiment, only one appeared to have been injured or negatively affected by the process. However, it is possible that some of the larvae tagged might have failed to emerge as adults had the experiment been extended to include the later stages of development. Even though tagging with VIE has been shown to affect mortality in small target animals such as larvae, it is likely that this could be remedied with practice (Butt *et al*., 2009; Moffatt, 2013; Northwest Marine Technology, 2008).

8.3.5 Future research projects

Whilst this experiment provided an insight into the movement of larvae through a mass, there are numerous factors that could potentially influence this behaviour and would benefit from further investigation. One such area of research might be the movement of tagged larvae in different sized aggregations, investigating whether behaviour was influenced by heat generation. A possible hypothesis might be that as masses became larger, and hence hotter, larvae might rotate through them at a faster rate, reducing the amount of time they spend at the centre. This would likely cause the histogram in Figure 8.2 to shift or skew to the left as larvae increase time spent at the periphery in an attempt to avoid thermal stress. The effect of the photoperiod on larval movement could also be explored

with VIE. This could be of interest since fluorescing VIE tags would easily facilitate observations in a dark environment. There is also the option of comparing movement in different species of blowfly larvae. One way to achieve this would be to rear masses in the laboratory composed of two different species. By tagging individuals of each, researchers could investigate whether different species move or behave differently whilst coexisting in the same mass.

In summary, larvae feeding in an aggregation are in a constant state of motion, alternating between the hot centre and the cooler periphery. Whilst the average larva was recorded to spend 55.9% of their time at the centre feeding, the amount of time spent at this specific location varied considerably between tagged individuals and ranged from 32% up to 84%. These differences in the amount of time spent at the centre of the mass may be influencing larval feeding behaviour and development. Therefore, the consequences of larval rotation were further investigated in the following experiment.

9 EXPERIMENT 6 – The Consequences of Larval Rotation

The previous experiment illustrated that larvae rotate through a mass, alternating between the centre and the periphery. This pattern of movement is thought to be a consequence of thermoregulation, foraging behaviour, competition and the need for oxygen (Charabidze *et al.,* 2013; Hoback & Stanley, 2000; Richards *et al*., 2009; Rivers *et al.,* 2011; Slone & Gruner, 2007). Depending on the size or the condition of the mass, each of these factors may carry a different weight. For example, small masses may not be able to generate levels of heat high enough to trigger thermoregulation, but they could be dense enough to result in larval displacement. But whilst the behaviour is likely to convey advantages to individuals in crowded conditions (avoiding overheating, intermittent access to food and oxygen) there may also be disadvantages associated with it. If larvae are rotating through a mass, then the time spent at the periphery or moving through the aggregation comes at the expense of the time spent feeding at the centre. Less time spent feeding might reduce nutrient intake, which would be expected to negatively affect larval growth, producing smaller individuals with reduced fitness.

Experiment 5 also provided evidence that this circulating behaviour might have a competitive edge. Whilst individuals were observed to spend a mean of 55.9% of their time feeding at the centre, the proportion of time spent at this particular location varied significantly among individuals, ranging from 32-84%. This implied that some larvae dominated at the centre whilst others were forced out to the periphery. One could hypothesise that larvae spending a greater proportion of their time at the centre would have increased access to food and nutrients, which

would allow them to attain larger body sizes and increase their fitness. In contrast, larvae spending a greater proportion of their time at the periphery would have limited access to food and experience cooler temperatures, which could negatively affect their growth and development. This might result in samples taken from masses showing a large amount of variation in individual lengths and weights.

The aim of this final experiment was to determine what the consequences might be for individuals participating in rotational behaviour. This was achieved by altering mass density, which in turn promoted larval displacement, and investigating the effect this movement had on food consumption. Physical measurements taken from larvae at 90 hours development were used to give an indication of the relative amount of time individuals had spent feeding, which could then be linked back to their movement. For example, in low-density aggregations larvae would be expected to experience minimal rotation. A larger surface area for feeding should result in low levels of mass-generated heat and reduced competition for food, which are two of the conditions that could be responsible for larval displacement. Under these circumstances larvae should be able to spend a maximum amount of time feeding efficiently at the centre, which could result in all sampled larvae demonstrating maximum length and weight. In contrast, larvae feeding in high-density aggregations with a reduced surface area might be expected to experience increased displacement as individuals are repeatedly forced out to the periphery to make room for others at the centre. It could be hypothesised that larvae sampled from these aggregations would yield shorter lengths and lighter weights since individuals sacrificed time spent feeding at the centre for time spent at the periphery or moving through the mass.

Charabidze and colleagues (2013) used a similar method to study foraging behaviour in *Lucilia sericata*. However, rather than altering mass density and using body size as a measure of time spent feeding, they used variation in the surface area of larval crops to describe foraging behaviour. They dissected and measured the crops of 117 larvae that were all the same age and found that their surface areas ranged from 0-16.6 mm^2. It was suggested that this heterogeneity in crop surfaces resulted from their food intake behaviour and not from their morphology. Rather than feed continuously, the authors claimed that larvae regulated their foraging behaviour, with some individuals consuming food whilst others spent time at the periphery. This created a permanent movement inside the mass. They described how even in small (200 larvae), low-density aggregations (2-2.5 larvae/g) where heat generation was negligible and competition for food was not an issue, larvae would still move towards the periphery in search of new foraging areas. This created a mass "turnover" or rotation comparable to that observed in Experiment 5.

Following this introduction, the experimental setup and methods used are described in full. This includes information on why specific densities were chosen for this experiment and what statistical analyses were carried out on the data. In the results section, box plots and variances were used to illustrate differences in larval lengths and weights at various densities. These findings are then discussed in detail.

9.1 Materials and Methods

9.1.1 Selecting appropriate larval densities

Extensive background reading and numerous trials in the laboratory were carried out before the start of this experiment to help determine a suitable range of mass densities. In this experiment, all of the masses were composed of the same number of larvae but reared on various quantities of food. A reduction in the surface area of meat available for feeding meant that larvae were encouraged to aggregate in an increasingly small area and form denser, more tightly packed masses, which would encourage larval rotation.

The selection of a low-density mass was relatively straightforward since the only requirements were that there was an excess amount of food. This was important since easy access to meat and low-stress temperatures would allow all the larvae to feed simultaneously and reduce the probability of any larval displacement resulting from competition or temperature avoidance behaviour. Byrd and Butler (1996) claimed that a density of 1 larva/2 g meat (or 0.5 larva/g) prevented any build up of metabolic heat whilst Wallman and colleagues (2009) suggested that 170 larvae on 120 g meat (or 1.5 larvae/g) caused mass temperatures to increase slightly above ambient. For these reasons, and the fact that *L. sericata* larvae have been shown to consume less than 1 g of food during development, 0.5 larvae/g was selected as the lowest mass density.

Once the minimum density had been established, the number of larvae used to make up experimental masses could be determined. With a density of 0.5 larvae/g, a large mass composed of 1000 individuals would have required 2 kg of meat. For practical reasons, when taking into consideration the number of replicates needed

and the potential cost, this mass size was rejected. Instead masses composed of 500 larvae were chosen, which meant that the maximum volume of meat required at any one time would be 1 kg. When it was time to purchase meat for the experiments, local suppliers sold pre-cut lamb joints, which weighed approximately 900 g. This resulted in an actual mass density of 0.55 larvae/g.

Once mass size was determined, the next task was to select an appropriate "high density" aggregation for the experiment. It was vital that these high-density masses were dense enough to promote larval movement without creating an overly stressful environment that might severely affect larval development or force individuals to disperse prematurely. Saunders and colleagues studied larval development at two densities: 1 larva/g and 7 larvae/g (Saunders *et al*., 1999). They described the higher density masses as "severely overcrowded" and reported that larvae in these aggregations experienced an estimated 60% mortality as well as a reduction in body size. Meanwhile Goodbrod and Goff (1990) claimed that maximum lengths and weights in *Chrysomya megacephala* larvae occurred at densities of 2 larvae/g liver, whilst minimum lengths and weights were observed at 40 larvae/g. But at densities this high there was a rapid depletion of food, which resulted in maggot masses only being maintained for a short period of time before larvae dispersed prematurely in search of alternative feeding sites. For these reasons, as well as convenience, the maximum density chosen for the experiment was 5 larvae/g meat.

9.1.2 Experimental setup

The materials and methods used to carry out this experiment closely matched those described in Experiment 3. *Lucilia sericata* eggs were harvested from

laboratory-reared adults and kept in an incubator maintained at 22 °C. Larvae were observed to eclose after approximately 20 hours. Recently emerged larvae were then left in the incubator for a further 12 hours before they were counted and randomly allocated to masses, each composed of 500 individuals. These masses were then placed onto soft tissue (lamb to maintain consistency with previous experiments) that had been cut and weighed to the nearest 0.1 g using a Denver Pinnacle PI-403 weighing balance. Larvae were reared on 100, 150, 350, 500 or 900 g of meat, which produced mass densities of 5, 3.5, 1.5, 1 and 0.5 larvae/g respectively. These feeding masses were then stored in 10 litre plastic containers and maintained in the incubator at 22 °C with 65% RH and a 16:8 hour (light:dark) photoperiod for the duration of the experiment.

Masses were left to feed at these different densities until they reached 90 hours development (from initial oviposition). Ninety hours was chosen as the sampling point as opposed to 70 hours since it meant that larvae had spent the maximum amount of time possible exposed to and developing in the conditions imposed on them without running over into the post-feeding phase. Therefore any effect density, and hence larval rotation, might have on body size would have manifested itself to a level that was measurable. None of the masses sampled at 90 hours showed any signs of dispersal. Since no comparisons were being made with larvae sampled at 70 hours development, this later sampling time was not deemed to be problem. When taking samples, masses were removed from the incubator and larvae killed by immersion in 80 °C water for 30 seconds. These larvae were then left to dry for 10-15 minutes on paper towels before 100 individuals from each mass were randomly selected and their lengths and weights recorded. Also

weighed was the remainder of the meat, again to the nearest 0.1 g. There were six replicates for each of the five different densities, giving a total of 30 sets of data.

9.1.3 Statistical analysis

Comparing larval size in different density masses

Data were analysed using the statistical package R (version 2.12.1) (R Core Development Team, 2010). Larval lengths and weights were compared to determine whether individuals reared in different density masses were significantly different in size at 90 hours development. Diagnostic plots showed the length and weight data to be normally distributed in every case. A Residual vs. Fitted plot showed the data to be of uniform variance. Therefore, a Mixed-Effects model followed by a multiple comparison of means test was carried out on the data. This highlighted if and where size differences existed between different density masses. Boxplots were used to illustrate the results.

Comparing variances in different density masses

To help quantify larval movement variances were calculated for each of the five different densities using the length and weight data. The variance of a random variable, such as larval length or weight, should give an indication of how widely spread the values are. If variances were observed to increase with increasing density, then this would imply that the data points were becoming more spread out from the mean and each other. But a decrease in the variance would suggest that the values were lying closer to the mean and the sampled larvae were equal in size. To compare variances, F and p-values were calculated in R. The p-value was multiplied by two to make it a two-tailed test and then by ten since there were ten F tests in total. The Bonferroni correction was used on the data to reduce the

chances of obtaining any false-positive results since multiple pairwise tests were performed on the data set. Given that the Bonferroni correction is considered the simplest and most conservative method to control for error, any significant differences after its use should be meaningful.

Estimating food consumption

For each of the experimental masses, the final weight of the meat was subtracted from the start weight, the difference then divided by 500 (the number of larvae in each aggregation). This provided an estimate for the mean amount of food (g) consumed by each larva in the mass. These values were then compared with one another to determine if food consumption varied in different density aggregations. They were also compared with the estimated food intake for larvae reared singly (Experiment 4). However, it should be noted that sample sizes for food consumption differed between solitary and mass reared larvae; 51 values for solitary larvae compared to 6 values for each of the five different densities. This was because the recorded weights for food consumption in solitary larvae were actual values, as opposed to the mean values that were estimated for mass reared larvae. To account for differences in the sample size, randomization was carried out on the single larvae, which reduced the sample so it equalled that of the mass-reared larvae. This was achieved by producing eight random groups in R. For each of these eight groups of single feeding larvae, means were calculated for food consumption, which were then compared with the means for larvae feeding at different densities. Diagnostic plots for between group comparisons showed the data to be normally distributed and a Residual vs. Fitted plot demonstrated uniform variance in every case. Therefore, any comparisons were made with a

Mixed-Effects ANOVA model followed by a multiple comparison of means Tukey's Test.

9.2 Results

9.2.1 Comparing lengths and weights in different density masses

Significant differences were observed when comparing the lengths of larvae reared in different density masses at 90 hours development ($F_{4,25} = 18.65$, $p<0.001$). As the density of the mass increased, the length of the larvae sampled decreased (Figure 9.1). Larvae which fed in the densest masses (5 larvae/g) had a mean length of 14.00 mm (±0.83), which was 1.23 mm shorter ($p<0.001$) than larvae sampled from the 0.5 larvae/g masses (mean length= 15.23 mm, ±0.58). The results of the Tukey test showed significant differences ($p\leq0.031$) in length in all bar two of the pairwise comparisons. Larvae reared in the 5 and 3.5 larvae/g masses didn't show any statistical difference in lengths ($p= 0.929$) as did larvae sampled from the 1 and 1.5 larvae/g aggregations ($p= 0.995$). It is also interesting to note that the error bars in Figure 9.1 appeared to be comparable in length for all densities between 0.5-3.5 larvae/g, but were bigger for the 5 larvae/g masses. This implied that there was more variation in larval length in the densest aggregation.

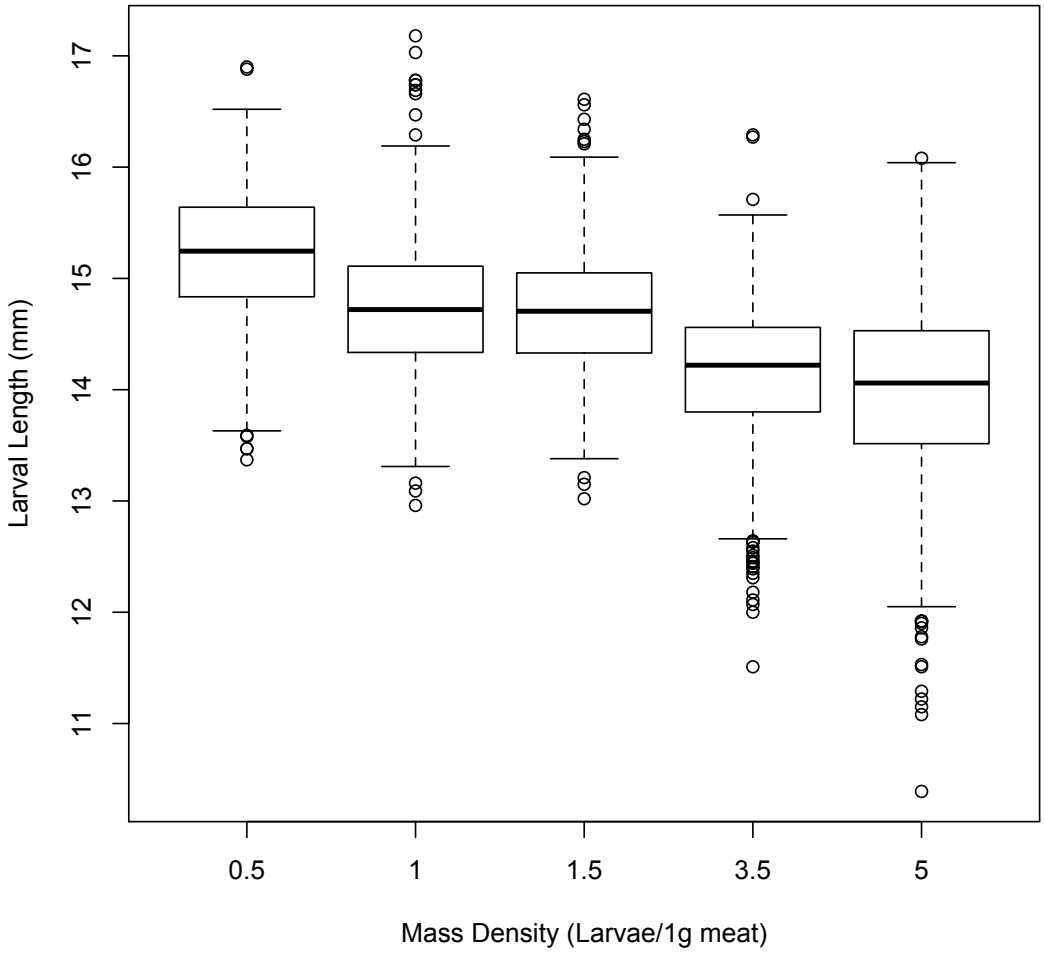


Figure 9.1. Larval lengths (mm) at 90 hours development for individuals reared in different density masses, each containing 500 larvae (constant ambient temperature = 22 °C ±1)

Significant differences were also observed when comparing the weights of larvae reared at different densities ($F_{4,25} = 11.47$, $p<0.001$). Larvae reared in low-density masses were significantly heavier in comparison to individuals from high-density aggregations (Figure 9.2). For example, at densities of 0.5 larvae/g, larvae weighed an average of 54.65 mg (±5.60) after 90 hours. But as density was increased to 5 larvae/g, mean larval weight dropped to 42.97 mg (±7.58). Figure 9.2 also gave error bars for the 5 larvae/g masses that appeared considerably larger in comparison to lower density aggregations. However, the results of the

pairwise comparisons indicated that larval weights differed less between densities. In fact, all except one of the pairwise comparisons that showed significant differences ($p \leq 0.022$) involved the 0.5 larvae/g masses. These low-density aggregations consistently produced larvae that were significantly heavier than anything sampled from the higher density masses. All other masses produced larvae that were the same weight, regardless of their density (aside from the 1 and 3.5 larvae/g masses ($p = 0.036$)).

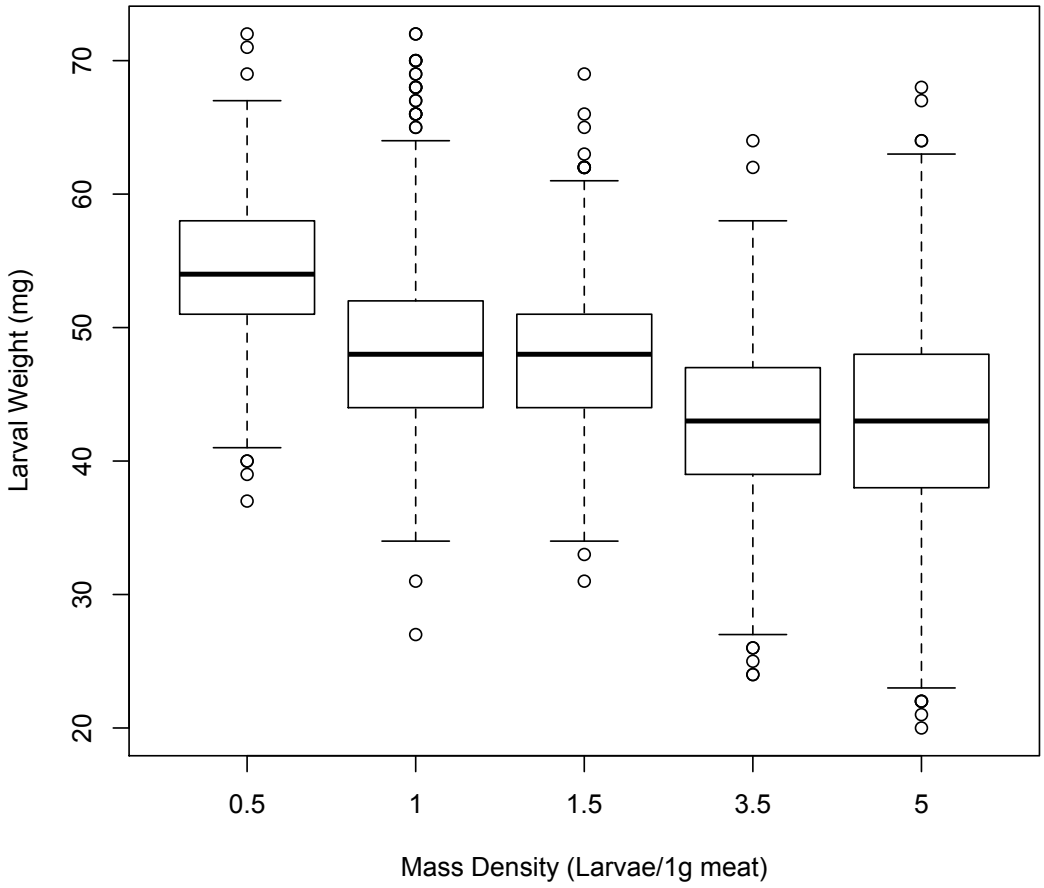


Figure 9.2. Larval weights (mg) at 90 hours development for individuals reared in different density masses, each containing 500 larvae (constant ambient temperature = 22 °C ±1)

9.2.2 Comparing variances in different density masses

Length Variances

Variances were calculated for each of the five mass densities using the length data (Table 9.1). The largest variance was recorded in the densest masses. In these 5 larvae/g masses the variance was calculated to be 0.682, which was significantly greater than all the lower density variances ($p<0.001$) and twice the variance recorded in the 0.5 larvae/g masses (var = 0.341). This implied that in denser, more tightly packed aggregations, the spread of larval lengths was significantly greater than that observed in less dense masses (Figure 9.3). The smallest variance was recorded at a density of 1.5 larvae/g. This variance of 0.297 was significantly smaller than all other variances apart from that reported for the 0.5 larvae/g masses ($p=0.854$) and inferred that the larvae reared in the medium density masses were the most uniform in length.

Table 9.1. Variances in length recorded for larvae reared in different density aggregations at 90 hours development (ambient = 22 °C ±1)

Density (Larvae/g)	Variance
0.5	0.341
1	0.386
1.5	0.297
3.5	0.445
5	0.682

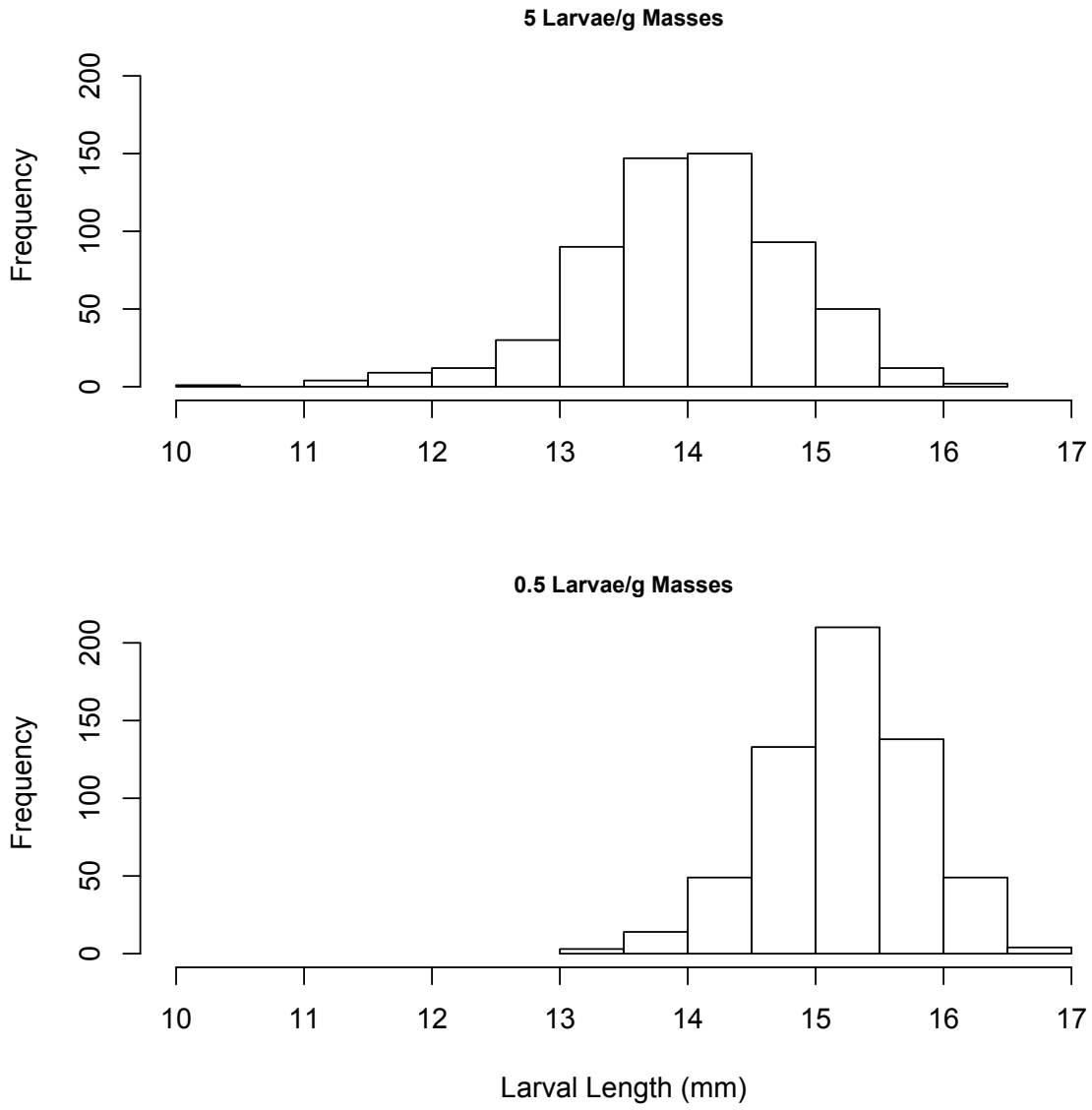


Figure 9.3. The distribution of larval lengths (mm) in the 5 larvae/g and 0.5 larvae/g masses at 90 hours development

Weight Variances

Using the weight data, variances were calculated for each of the five different densities (Table 9.2). The largest variance calculated was 57.50 in the dense 5 larvae/g masses. This was significantly greater than the variances recorded for all the lower density aggregations ($p \leq 0.011$), apart from the 1 larva/g masses, which had a variance of 47.68 ($p = 0.221$). Again, the 1.5 larvae/g masses produced the smallest variance (var= 28.28). The large variance observed in the 1 larva/g masses was unexpected and seemed unusually large (var= 47.68) considering it

had originated from what is often classified in the literature as a "low density mass". This large variance at 1 larva/g was not mirrored in the length data. It was also noted that masses alike in density (e.g. 5 and 3.5 larva/g; 3.5 and 1.5 larvae/g etc.) produced significantly different variances ($p \leq 0.018$).

Table 9.2. Variances in weight (mg) recorded for larvae reared in different density aggregations at 90 hours development (ambient = 22 °C ±1)

Density (Larvae/g)	Variance
0.5	31.39
1	47.68
1.5	28.28
3.5	38.09
5	57.50

9.2.3 Food consumption by larvae in different density masses

There were significant differences in the amount of food consumed by larvae reared in different density masses ($F_{5, 32} = 125.42$, $p<0.001$)(Figure 9.4). For larvae feeding in a mass, the amount of food consumed by an individual varied considerably and ranged from 0.062 g up to 0.308 g. Larvae reared at a density of 1 larva/g were shown to have the highest food intake, with individuals estimated to have consumed a mean of 0.217 g (±0.06) of meat during the first 90 hours of development. This was significantly greater than the 0.090 g (±0.02) consumed by larvae in the dense 5 larvae/g masses ($p< 0.001$). However, it is interesting to note that regardless of density, all mass-reared larvae consumed significantly less food than the larvae reared singly in Experiment 4 ($p< 0.001$). *Lucilia sericata* larvae feeding in the absence of a mass were observed to consume 3-7x the amount of

food reportedly consumed by mass-reared larvae (Figure 9.4). Pairwise comparisons showed that larvae reared in masses of a similar density consumed the same amount of food (Table 9.3). However, there are various factors that need to be taken into account concerning these estimates for food consumption, which will be detailed in the discussion.

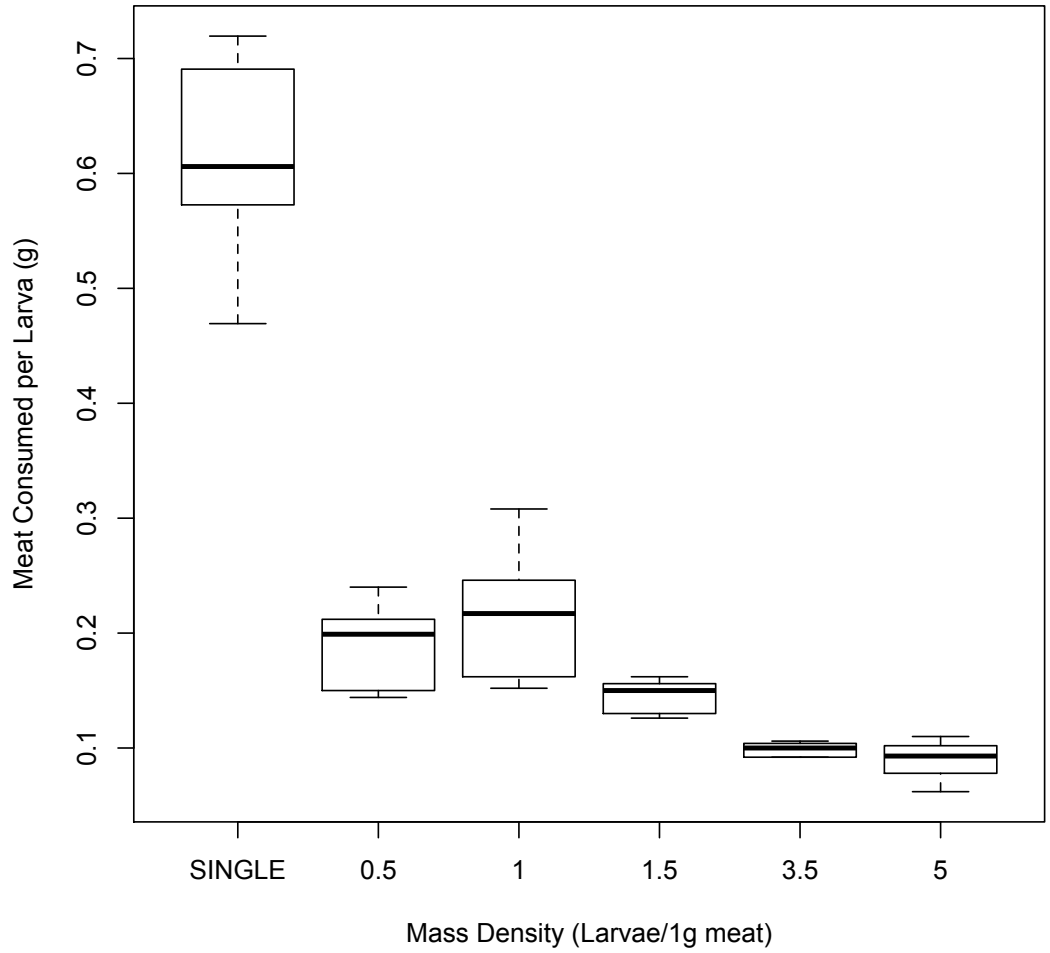


Figure 9.4. Food consumption (g) for solitary (SINGLE) and mass feeding larvae reared at different densities (each mass contains 500 larvae)

Table 9.3. Pairwise comparisons of food consumption estimated for solitary larvae (SINGLE) and individuals reared in different density masses

	Density (Larvae/g)					
	SINGLE	**0.5**	**1.0**	**1.5**	**3.5**	**5**
SINGLE		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
0.5			0.934	0.586	**0.013**	**0.004**
1.0				0.106	**<0.001**	**<0.001**
1.5					0.546	0.334
3.5						1.000
5						

9.3 Discussion

This experiment was designed to investigate the effect of larval movement on food consumption, and hence body size, by altering the density of the mass and comparing the physical measurements of larvae. This would give an indication of the relative amount of time individuals had spent feeding, which could then be linked back to their movement. The results showed that larvae sampled from different density aggregations varied significantly in length and weight, with body size decreasing with increasing density. These differences between masses, as well as the larger variances recorded in dense aggregations, implied that not only does larval movement influence development, it also appears to have a competitive edge, which allows some individuals in an aggregation to experience accelerated growth in comparison to other members of the cohort.

9.3.1 Low-density aggregations – Minimal rotation

Larvae feeding in low-density aggregations composed of 500 larvae were expected to experience low levels of mass-generated heat and have increased access to food for feeding. It was assumed that under these conditions there would be little need for larval rotation and the behaviour would be reduced to a minimum level. In these low-density masses (0.5-1 larva/g), the larger body sizes and small variances recorded at 90 hours supported the hypothesis that in an uncrowded and non-competitive environment larvae are able to feed efficiently. It was assumed that these larger larvae had spent a greater proportion of their time at the centre of the mass as opposed to the periphery. Here individuals would have had increased access to nutrients, facilitating rapid growth and larger body sizes. Evidence supporting this can be found in Figure 9.4, which illustrates how individuals reared in 0.5 larvae/g masses consumed a mean of 0.19 g of food whilst larvae feeding at densities of 5 larvae/g only ingested 0.09 g. The observed uniformity in size infers that all the larvae sampled from these low-density masses were feeding for an equal amount of time. This was not surprising though since an excess amount of food would have permitted all individuals the opportunity to feed simultaneously and removed the need for displacement behaviour.

9.3.2 High-density aggregations – Maximum rotation

By reducing the volume of meat available for feeding, larvae were encouraged to form denser masses, which resulted in increased larval displacement. Individuals sampled from these high-density masses (5 larvae/g) produced significantly smaller larvae with large variances. This overall reduction in body size at 90 hours implied that individuals in these aggregations had experienced a slower rate of development; presumably this was because time spent feeding was being

sacrificed for time spent at the periphery or moving through the mass. The estimated food consumption for larvae reared in these dense aggregations supported this idea, with individuals reared at 5 larvae/g ingesting a mean of 0.09 g of food, which was significantly less than that consumed by larvae in 0.5 and 1 larva/g masses. These results contradict the published findings reported for other experiments where larval density was altered, which claimed that increasing density resulted in faster growth rates during the feeding stages of development (Goodbrod & Goff, 1990; Rivers *et al*., 2010). However, these studies investigated other species and not *L. sericata*. Perhaps more importantly, they altered larval numbers as opposed to the volume of food available, which could have had a different effect on heat generation and thus developmental rates.

Previous studies have reported smaller sized individuals during the larval, pupal and adult phases resulting from an increase in density. Researchers claimed this was due to larval overcrowding, food shortages and a build of larval excrement, which caused the meat to become tainted (Ireland & Turner, 2006; Saunders & Bee, 1995). Whilst not mentioned specifically in these papers (Ireland & Turner, 2006; Saunders & Bee, 1995), it seems likely that "overcrowding" includes the effect of rotation, since this condition would result in larval displacement. However, densities where these factors had a significant impact on larval size ranged from 10-40 larvae/g, which were considerably denser than the maximum 5 larvae/g investigated here (Goodbrod & Goff, 1990; Ireland & Turner, 2006; Rivers *et al*., 2010). It should also be noted that on no occasion did food become depleted in any of the aggregations. Rivers and colleagues (2010) reported decreasing puparial weights for larvae reared at increasing densities and claimed it was a consequence of heat shock proteins (hsps) being synthesised at the expense of

normal protein synthesis. However, for many insect species, hsps are only expressed as part of the heat shock response if the temperatures they are exposed to are warmer than 32 °C. Masses composed of 500 larvae and reared at an ambient temperature of 22 °C were not expected to exceed temperatures of 28 °C, which was the maximum temperature recorded in the slightly larger 600 masses during Experiments 1 and 2. And whilst Charabidze and colleagues (2011) reported that increasing density resulted in an increase in heat emission, this was only observed in masses exceeding a density of 7.5 larvae/g (approximately 3000 larvae on 400 g of meat), considerably more crowded than the conditions experienced by larvae in the current experiment. Therefore, larvae in this experiment were only ever exposed to temperatures less than or equal to 28 °C, several degrees cooler than the minimum 32 °C required for hsp production. However, it is important to bear in mind that the production of heat shock proteins can also be triggered by exposure to other kinds of environmental stress conditions, including starvation and oxygen deprivation. Both of these factors could be prevalent in dense aggregations. And whilst none of the masses used in this experiment generated temperatures high enough to significantly impact larval development, there may have been slight variations in temperature between different density masses that could have influenced growth rates. Rivers and colleagues (2013) also reported that of six necrophagous fly species studied, *Lucilia sericata* was the most resistant to the effect of overcrowding. They claimed that larvae of this species demonstrated no decrease in pupal size as mass density increased, and were observed to function normally in large, overcrowded aggregations (Rivers *et al*., 2013). Given this information, it seems reasonable to assume that the reduced body sizes observed in the current experiment were at

least partly influenced by larval movement, since many other suspected causes can be ruled out.

The large variances in length and weight recorded in high-density aggregations indicated that larvae did not rotate evenly through the mass. Samples taken from these masses contained a number of very large and very small individuals. This suggested that larvae feeding in the same aggregations and exposed to the same conditions were experiencing different growth rates. Some larvae appeared to spend a considerable amount of time at the centre feeding and attaining larger body sizes. But for these individuals to dominate at the centre, it is likely that others were forced out to the periphery. These larvae would have experienced cooler temperatures and a restricted access to food, which could have resulted in poor development and smaller body sizes. These findings supported the results from Experiment 5, which showed that significant differences existed between individuals in the amount of time they were observed at a particular location. Therefore, whilst larval rotation allows all individuals an opportunity to feed (as demonstrated by the fact that all sampled larvae were over 10 mm in length and 20 mg in weight), the amount of time they spend ingesting food is reduced, with evidence of intraspecific competition existing within the mass.

9.3.3 Consequences of rotation and smaller body sizes

For behaviour such as larval rotation to exist, there must be advantages associated with it. Researchers have suggested that larvae circulate between the centre and the periphery in order to regulate their temperature, forage for new feeding areas and obtain oxygen. However, despite these benefits, this experiment has highlighted a potential disadvantage to the behaviour. The reduced body sizes

resulting from the increased amount of time spent at the periphery or moving through the mass could be representative of a slower rate of development. Less time spent feeding at the centre might increase the duration of larval development since it takes a longer period of time to consume enough nutrients to reach the required critical weight for a specific stage of development (Rivers *et al*., 2011; Saunders & Bee, 1995). It has been suggested though that in overcrowded mass, this is likely countered by the warmer temperatures associated with larger aggregations, which would explain why some larvae appear to experience accelerated development at higher densities (Rivers *et al.,* 2011). However, masses in the current experiment were composed of just 500 larvae, making it unlikely that mass-generated heat would have had the same impact on any growth rates in this study.

Since data collection ceased at 90 hours development in every case, it is not possible to say whether the different density masses completed feeding at different times, or if the significant differences in body sizes still existed at the start of the post-feeding period. Therefore, we can only speculate if rotation affected overall developmental rates or had a lasting effect on the physical measurements of blowflies later in the lifecycle. If high-density masses did continue to produce undersized larvae at the start of dispersal, then it could lead to a reduction in puparial size, as well as lighter adult weights at emergence (Goodbrod & Goff, 1990; Levot *et al.,* 1979; Saunders & Bee, 1995). If larvae were to enter the post-feeding stage of development with a reduced body size, then it could have a negative impact on their fitness and survival. Smaller larvae with limited energy reserves may be restricted in the distances they are able to migrate, whilst their shorter lengths would produce slower crawling speeds (Charabidze *et al*., 2008). If

individuals are forced to pupate close to the body or in a suboptimal location they might be at risk from predation. Smaller weights at pupariation could result in larvae failing to complete development or undersized adults emerging. Stunted adults are still capable of laying full sized, viable eggs but in smaller numbers (Ireland & Turner, 2006; Saunders & Bee, 1995).

9.3.4 Points for consideration

Despite the results for estimated food consumption in different density masses supporting the findings, there are a number of issues regarding the collection of this data that need to be considered. Firstly, no controls were used to estimate weight reduction in meat as a result of water loss. Therefore, larvae feeding in masses might actually have consumed less than calculated if some of the recorded weight loss was a result of desiccation. However, on the other hand, the presence of a mass might have prevented water loss due to moisture from larval excretions, secretions and even respiration. Also, comparisons between mass-reared larvae and solitary larvae need to be made with caution since the masses were sampled at 90 hours whilst single larvae were sampled at dispersal, which in some cases was after a period of 190 hours. Despite this 90 hours sampling point yielding larvae what were considered late 3rd instar (feeding) and on the verge of dispersal, no larvae from the masses were observed having already completed feeding. Therefore, food consumption in mass-reared larvae may have been underestimated.

10 General Discussion

The objective of the research was to model the thermodynamics of maggot masses on decomposing carrion. This was essentially a three-part project. The first phase of the research was to monitor heat generation in different sized aggregations. Once a relationship between mass size and temperature had been established, the study shifted focus to concentrate on the effect this mass-generated heat had on larval development. Finally, the research project investigated how larval aggregations influenced the behaviour of the individuals feeding in them. This was achieved by conducting a series of controlled experiments in the laboratory, which produced quantitative data for statistical analysis and interpretation.

10.1 Summary of the Findings

Before a discussion of some of the major themes is presented, it might be expedient to first concisely state again the main findings.

10.1.1 Mass temperatures

The first and most integral part of the research was to determine whether different sized aggregations produced temperatures that differed from one other as well as from ambient. This was investigated by rearing masses containing different numbers of larvae at a constant ambient temperature of 22 °C and recording their temperatures for the duration of feeding. The results showed that a strong positive association existed between mass size and heat generation, with temperatures rising as masses increased in size. Even aggregations containing as few as 50 individuals were able to produce temperatures approximately 2.5 °C warmer than ambient, whilst masses composed of 2500 larvae peaked at 36 °C, 14

°C warmer than ambient. Once aggregations exceeded a minimum size of 1200 larvae, they were capable of producing temperatures that were considerably warmer than ambient.

10.1.2 Larval growth and development

The higher temperatures recorded in larger aggregations were observed to increase the rate of development during the 2nd and 3rd larval instars. This coincided with when masses were at their warmest. These accelerated rates of development during the later stages of feeding resulted in the largest aggregations (2500 larvae) dispersing an average of 13 hours earlier than the smaller, cooler masses. As a consequence of this faster development, larvae reared in different sized aggregations varied significantly in body length and weight despite being the same chronological age. But whilst larvae feeding in larger masses experienced a shorter developmental duration, optimal growth rates were recorded in the intermediate sized aggregations. This indicated that medium sized masses provided more ideal conditions for feeding and development.

10.1.3 Larval behaviour

Compared to solitary larvae, individuals reared in a mass appeared to benefit from a faster rate of development, reduced mortality and larger body sizes at dispersal. Larvae were observed to rotate through the mass, alternating between the periphery and the centre where they were presumed to feed. Individuals spent a mean of 55.9% of their time at the centre. However, this proportion of time varied significantly between larvae, with tagged individuals observed to spend anywhere between 32% and 84% of their time inside the aggregation. Larval rotation appeared to influence development, as reflected in the lengths and weights of individuals sampled from denser aggregations. Time spent at the periphery or

moving through the mass resulted in larvae spending less time at the centre feeding. This reduction in food consumption meant larvae exhibited shorter lengths and lighter weights. Larval movement appears to be influenced by a number of variables including mass temperature, density, foraging behaviour and oxygen availability, all of which require further investigation.

10.2 Evolutionary Advantages of Maggot Masses

For larval aggregations to form and persist there must be some evolutionary advantages that are selected for. Evidence supporting this was illustrated in the experiment that compared solitary feeding larvae with mass-reared larvae, the former demonstrating slower rates of development, smaller body sizes at dispersal and an increased mortality. The gregarious behaviour observed in blowfly larvae is consistent with the Allee effect theory, which asserts that the presence of many congeners in a single place confers benefits to all individuals (Boulay *et al*., 2013; Rohlfs & Hoffmeister, 2003; Stephens & Sutherland, 1999). For larvae in an aggregation these benefits might include increased feeding efficiency and faster developmental rates, or a reduction in the risk of predation and environmental stresses.

10.2.1 Reduced risk of predation

The gregarious behaviour of blowflies during the larval stage of development may be an efficient mechanism to increase survival. Greenberg (1991) suggested that female blowflies oviposited large numbers of eggs in mounds to reduce the risk of predation, with eggs at the periphery being sacrificed whilst those at the centre have an increased chance of survival. The same idea can be applied to larvae in a

mass. Crowded conditions in large aggregations could mean individuals, particularly those feeding at the centre, have a reduced risk of attack by predators. If predators were to visit heavily infested carrion they might be overwhelmed by the large numbers of larvae, so even after feeding a proportion of the mass would still remain to complete development. This is comparable to the Selfish Herd theory observed in a variety of animals, where individuals within a population reduce their predation risk by putting other conspecifics between themselves and predators.

Warmer temperatures in larger masses could also reduce the risk of predation by accelerating development and shortening the larval phase, which is when individuals are most vulnerable (Boulay *et al*., 2013; Cinaci & Sheldon, 1990; Richards *et al*., 2009). If larvae can complete feeding and disperse away from the mass and carrion quickly, then predators would have less time to find and consume them. An individual larva is harder to locate than a mass and pupating under soil or leaf litter might afford some protection from predators. Meanwhile, Rivers and colleagues (2011) proposed that the smaller puparia resulting from accelerated development in warmer masses (Rivers *et al*, 2010) could reduce the risk of parasitism. They claimed that the nutritious value of these smaller pupae was greatly diminished for parasitoids, resulting in some parasitic wasps like *Nasonia vitripennis* (Hymenoptera: Pteromalidae) rejecting them as hosts if they were deemed too small.

10.2.2 Feeding efficiency

Carrion is a nutrient rich, yet discrete and ephemeral source of food, which can be completely depleted in a single generation (Rivers *et al*, 2011). Cianci and Sheldon (1990) described how maximum energy is present at the moment of carrion deposition, with no additional energy entering the system. Therefore, necrophagous larvae must have adapted highly specialized strategies to extract the maximum amount of energy in the shortest possible time. This should allow them to exploit the resource and complete development whilst food is still available. As suggested in Experiment 4, an individual larva may be incapable of penetrating tissues fast enough to meet nutritional needs, which could lead to starvation. Elsewhere, a study by Rivers and colleagues (2010) implied that for some species (*Sarcophaga bullata* and *Protophormia terraenovae)* normal growth and development was only possible if aggregations exceeded a minimum number of individuals. Therefore, another benefit of larval masses might be the collective effort of a large number of individuals piercing soft tissues with mouth hooks, combined with the mass release of digestive enzymes (Goodbrod & Goff, 1990; Hobson, 1932; Rivers *et al*, 2011). One might also speculate that an increase in larval secretions, coupled with the liquefied soft tissues resulting from mass feeding behaviour, could help reduce the risk of desiccation as well as facilitating evaporative cooling.

Heat generation in larval aggregations could also assist in feeding efficiency. Not only would a large mass increase the amount of enzymes produced, spreading them over a larger surface area, but the warmer temperatures associated with them could promote increased enzyme activity (Rivers *et al*, 2011). This might result in a more rapid breakdown and digestion of tissues. Warmer temperatures

have also been shown to increase the rate at which food travels through the larval gut, allowing individuals to consume food at a faster rate and complete development earlier (Greenberg & Kunich, 2002). On heavily infested cadavers where the number of larvae exceeds the carrying capacity of the carrion, individuals would benefit from a more rapid growth rate, which would allow them to complete feeding before food becomes exhausted.

10.2.3 Protection against cool temperatures

The formation of an endothermic mass could provide larvae protection from any sudden or temporary drops in ambient temperature (Campobasso *et al*., 2001; Rivers *et al*, 2011). This benefit has been reported by several researchers, including Huntington and colleagues (2007), who observed masses still feeding in morgue coolers, and Deonier (1940), who reported a larval mass remaining active at ambient temperatures as low as -4 °C. It has been suggested that larvae direct much of their metabolic energy to the production of new biomass during the feeding stage of development, which leaves them poorly buffered against suboptimal temperature conditions (Cianci & Sheldon, 1990). Therefore, it could be assumed that the warmer microclimate associated with larger masses may reduce stress and allow individuals to function at their maximum efficiency by maintaining temperatures higher than the lower developmental threshold.

10.2.4. Interspecific competition

Since female blowflies oviposit in such large numbers, possibly to ensure at least a proportion of their offspring survive, inter- and intraspecific competition in aggregations could be severe (Rivers *et al*., 2011). However, for necrophagous

species with a high upper temperature threshold, the extreme temperatures generated in some large aggregations could provide them with a competitive edge. Williams and Richardson (1984) studied the growth energetics of four Calliphoridae species, all with different optimal and lethal temperatures. They found that at temperatures exceeding 35 °C the only species that produced viable larvae was *Lucilia cuprina*, which had an optimal temperature of 40 °C and a lethal temperature of 40-45 °C. Richards and colleagues (2009) reported similar results after investigating the thermal ecophysiology of seven species of carrion feeding blowflies. They observed that species with a high upper lethal limit dominated in large, hot maggot masses and suggested that blowflies with a lower upper threshold might have evolved breeding strategies to avoid development in large aggregations (e.g., females oviposit on smaller carcasses, larvae mass at the cooler periphery) (Richards *et al*., 2009). Therefore, larvae with a high tolerance to heat might benefit from feeding in a mass since it would provide them a competitive advantage over potential rivals.

10.3 The Impact of these Findings

It has been hypothesised in numerous publications that aggregations of feeding larvae produce temperatures warmer than ambient, which influences their rate of development (Amendt *et al*., 2011; Campobasso *et al*., 2001; Catts, 1992; Charabidze *et al*., 2011; Goodbrod & Goff, 1990; Huntington *et al.,* 2007; Ireland & Turner, 2006; Marchenko, 2001; Slone & Gruner, 2007). But despite entomologists appearing to be in agreement about this, very little research has been carried out in a controlled environment that has produced data from which mass thermodynamics can be modelled. To meet the Daubert standard set by the courts

it is imperative that evidence pertaining to larval development and succession studies is supported with a statistical background. Therefore, what separates this thesis from previously published work on larval masses is that this research produced quantitative data that was analysed statistically to back up its findings. This thesis has the potential to impact on both forensic casework and how research is conducted in the laboratory. Details of how the findings influence these two areas are outlined below:

10.3.1. Laboratory research

The methods used to carry out research vary considerably between researchers, institutions and experiments. Many of the papers that have reported developmental rates for larvae under various conditions have done so using individuals reared in different sized aggregations. For example, Grassberger and Reiter (2001) reported rates of development for *Lucilia sericata* at a range of constant ambient temperatures. But these larvae were reared in groups of approximately 100 individuals, which have now been shown to be capable of producing temperatures about 2.5 °C warmer than ambient. Elsewhere, studies exploring the effects of nutrition on development have used larvae reared in groups of 10 (Green *et al*., 2003) or 20 individuals (Clark *et al*., 2006). Although these masses remain small, there is still a risk they could influence the findings, whilst a lack of consistency between experiments in regards to rearing conditions means that the results reported cannot be accurately compared. Consequently, if the methods used to calculate larval age are derived from studies where individuals were reared in a mass then, depending on the mass size and its potential for heat generation, what we consider to be a "standard" rate of development could actually be false. To reduce error in mPMI estimates based on

larval development it may be necessary to produce a set of standards for the laboratory that factor in mass size. This would ensure that future studies are controlling for the temperature variable, which would ultimately result in more consistent developmental data being generated. Data could then be compared and collated, which would help create an overall image of mass thermodynamics that could be applied to individual cases.

10.3.2 Forensic casework

The results of this research imply that internally generated heat in larval aggregations has the potential to impact significantly on mPMI estimates. Larvae sampled from large masses, particularly during the 3rd instar, might appear older than they actually are due the accelerated rates of development experienced under warmer conditions. Therefore, if mass-generated heat is not taken into consideration it could result in an overestimation of the time of death. Alternatively, if masses were to exceed a critical size, the extreme temperatures sometimes associated with them might disrupt feeding and development. This would result in stunted larvae being sampled that could be mistaken for younger individuals.

Very rarely is mass size or volume recorded or even mentioned in reports that investigate the use of larval growth and development for mPMI estimates (Arnaldos *et al*., 2005; Benecke, 1998; Introna *et al*., 1998). If the presence of a maggot mass is mentioned, then it is uncommon for details to be included in regards to its size, temperature or thermal history. This is most likely due to the lack of research that, until now, has been able to model heat generation in different sized aggregations. But even with the findings presented in this thesis, there are

still difficulties in incorporating these temperatures into mPMI estimates. Several researchers have emphasized the need to record mass temperatures at a crime scene (Amendt *et al*., 2007; Campobasso & Introna, 2001; Greenberg & Kunich 2005; Haskell *et al*., 1997). But it is unlikely that these temperature readings will accurately reflect the thermal history of the mass. There are numerous factors that can interact to influence the temperature of an aggregation, including species composition and exchanges with the surrounding environment. Investigators arriving at a scene may be aware of each of these factors, but they would still struggle to say with any certainty how exactly they interacted to shape the thermal history of each individual mass in an uncontrolled environment.

During the course of this research, a possible method for estimating the age of mass-reared larvae and reducing error in subsequent mPMI estimates has come to light. For *Lucilia sericata* sampled from an aggregation containing 2500 larvae or fewer and exposed to an ambient temperature of approximately 22 °C, larval ages could be taken directly from this thesis. However, it is very unlikely that masses at a crime scene would meet these criteria exactly. In Experiment 2 it was proposed that studies reporting developmental durations for different species at a range of constant temperatures could feasibly be used to estimate age in mass-reared larvae. This was demonstrated with the aid of Grassberger and Reiter's (2001) developmental data for *L. sericata*. Given that mass temperatures have been shown to have little or no effect during the earlier stages of development (egg – 1st instar), ambient temperatures alone could be used to estimate the duration of each of these periods. But if the larvae sampled are in their 2nd and 3rd instar, then it is likely that mass temperatures have started to have an effect on development. At this point investigators should attempt to incorporate the mean mass temperature

in age estimates for larvae. However, mean temperature values for different sized masses at specific times during feeding will only be available if more research like this is carried out in the future. It is also important to note though that species ovipositing on carrion several days after its initial exposure could experience an accelerated development during the egg and 1st instar stages if a large mass is already well established on the body. Whilst initial colonisers will experience a gradual increase in temperature that might eventually influence the later stages of development, late arrivals will immediately be deposited into a warmer microclimate. Therefore, knowledge of insect succession should also be incorporated into mPMI estimates to identify species that may have experienced a faster rate of development from the outset.

An alternative to estimating larval age with absolute mass temperatures would be to substitute them with a temperature that has been shown to result in the fastest rate of development. This idea was first proposed by Byrd and Butler (1996, 1997, 19980) and is based on the principle that mass temperatures are not uniform, with the centre of the mass being several degrees warmer than the periphery. They suggested that larvae actively repositioned themselves within an aggregation to experience an accelerated rate of development. The current research project has demonstrated that larvae move through the mass, alternating between the centre and the periphery. Whether this movement is a consequence of feeding, respiration, thermoregulation, or a preference for accelerated development, the author cannot say. It is possible that all four are responsible in regulating larval movement. Whatever the cause, it seems reasonable to assume that the maximum temperature recorded at the centre of a mass will not be the temperature experienced by an individual larva for the duration of development. If larvae are

using these naturally occurring temperature gradients to control for accelerated development, then using a species' preferred temperature could provide the minimum age and PMI estimate. It should also be stressed that regardless of the methods used to estimate larval age, error ranges should always be included.

The thesis also highlights the need to reassess the ADD/ADH models used for larval development. Comparisons between mass and ambient ADH values indicated that as masses began to produce temperatures significantly warmer than ambient, their associated ADH values also began to differ. By the start of dispersal, mass ADH was significantly greater than ambient ADH for a range of mass sizes. Therefore, if larval age were estimated with ambient ADH instead of mass ADH it might result in larger error ranges and a less accurate estimate of the mPMI. To avoid this, ADD/ADH models need to be reworked to account for the effect of the microclimate (Rivers *et al*., 2010). It is unclear how this might be achieved though, given the number of factors that can influence heat generation in individual masses, especially at an outdoor scene. It has also been suggested that using ADD/ADH summation models is more accurate during the earlier stages of development (egg, 1st and 2nd instar), since these are the shortest developmental stages (Tarone & Foran, 2008). The results of the current research supported this suggestion. As larval development progressed, differences in ADH became more exaggerated as values diverged further away from one another. This would cause error rates to increase and confidence intervals to become wider, particularly by the start of the post-feeding phase. Again, this could be accounted for by giving large age ranges to older larvae.

10.4 Future Studies

There is still a great deal to understand concerning the thermodynamics of maggot masses and their effect on larval development and behaviour. All future research, regardless of whether it focuses on larval aggregations or not, would benefit from the inclusion of replicated growth rate data obtained under a variety of variables and temperatures. Conducting future experiments under standardised conditions would also help facilitate direct comparisons amongst species and populations. Whilst designing the methodology for this research, efforts were made to utilise equipment and techniques that could be easily replicated, the aim being that this thesis would provide a framework for future studies. If researchers can collect and analyse data using the same methods then it would help create an overall image of heat generation in larval aggregations that could be applied to forensic casework.

During the course of this project a number of potential areas for future research were highlighted. The development of novel research tools like VIE and the increased availability of new technology such as hand-held thermal imaging cameras could open up a number of research opportunities. The potential use of VIE to monitor the movements of individual larvae has already been discussed in Experiment 5, but other areas that would benefit from further research are considered below.

1. Extending this research to other species

Reproducing this research with other species of forensic importance, such as *Calliphora vicina, Calliphora vomitoria* and *Lucilia caesar*, could help determine

whether species differ in terms of their heat generating properties. An earlier study has suggested that variation in body sizes and species-specific upper lethal temperature limits will influence heat generation in aggregations as well as larval behaviour (Rivers *et al*., 2010). Should different species be studied using these same methods and found not to differ from one another, it may be possible to generalize mass temperatures. For the mixed-species masses occasionally encountered at a crime scene, being able to rule out a species-specific effect on heat generation would make it easier to incorporate mass temperatures into mPMI estimates. It is feasible that entomological data obtained using the same methods and standards could then be collated in a database, which could account for species, population, ambient temperature, photoperiod, humidity and food type, as well as mass size.

2. Preferred growth rates

In Experiment 3 it was mentioned that growth rates for 3rd instar *L. sericata* larvae appeared to be fastest in intermediate sized aggregations. It was proposed that medium sized masses provided optimal conditions for feeding and development (i.e., sufficient larvae to breakdown soft tissue and maintain mass temperatures at an optimum level without producing competitive or heat stressed conditions). More work could be done on this topic, looking at a greater range of mass sizes to try and produce a quantitative relationship between mass size and growth rate. This might allow researchers to identify the preferred mass size for larval development. It would also be interesting to determine whether the preferred mass size for larval development varied between species, based on either the upper or lower developmental temperature thresholds.

3. Fieldwork

Although there is a lot to be learnt from controlled laboratory experiments, they are limited in their applicability to forensic casework. Earlier in the thesis it was acknowledged that the equipment available and indoor setting placed restrictions on the size of the masses that could be studied in the laboratory. Masses composed of 2500 larvae have been referred to as "large" aggregations throughout this research despite being considerably smaller than many of the aggregations that have been observed colonizing corpses (Richards *et al.*, 2009; Slone & Gruner, 2007). If masses with a mean average temperature of 30 °C (peaking at 36 °C) are shortening the feeding phase of development by 13 hours in the laboratory, then it could be assumed that larvae feeding on heavily infested carrion exposed to temperatures of 40-50 °C are subjected to even faster rates of development. This provides rationale for fieldwork on the subject, which would supplement the data obtained from laboratory studies and make it more applicable to forensic casework. But despite field studies providing an opportunity to monitor mass thermodynamics in a more realistic setting, working in an uncontrolled environment introduces variables that could complicate analyses (e.g., species composition, sun exposure, temperature fluctuations). With new, affordable technology such as thermal imaging cameras available on the market, it should be easier to monitor temperatures in large masses. These cameras might be the simplest and least invasive tools available for recording temperatures. However, it should be noted that thermal imaging cameras report surface temperatures. For smaller masses in the laboratory that are only ever 1-2 layers deep this was not considered to be a problem. But out in the field extensive masses on larger carrion can be several centimeters deep in places (Slone & Gruner, 2007), meaning that the

temperatures inside the mass could be significantly different to those recorded at the surface. Further research would have to confirm this though.

4. Incorporating mass heat into forensic casework

Another important area for future research would be to investigate ways to incorporate mass heat into mPMI estimates. If laboratory research is complimented with observations out in the field, then an overall image of mass thermodynamics can be produced. The next phase of the research would then be to apply these findings to casework. One way to achieve this would be to establish a standard procedure for investigators at the scene that would allow them to estimate mass size, and hence its temperature and/or thermal history. This could involve the use of a grid system to estimate surface area coverage or be as simple as measuring or weighing the mass at the scene. If an investigator was able estimate the size of the mass (based on surface area, weight or volume) they could then refer back to papers that report the findings of laboratory and field studies. This would help them to model the thermal history of the crime scene mass, which in turn would lead to better assessment of larval age and more accurate PMI estimates.

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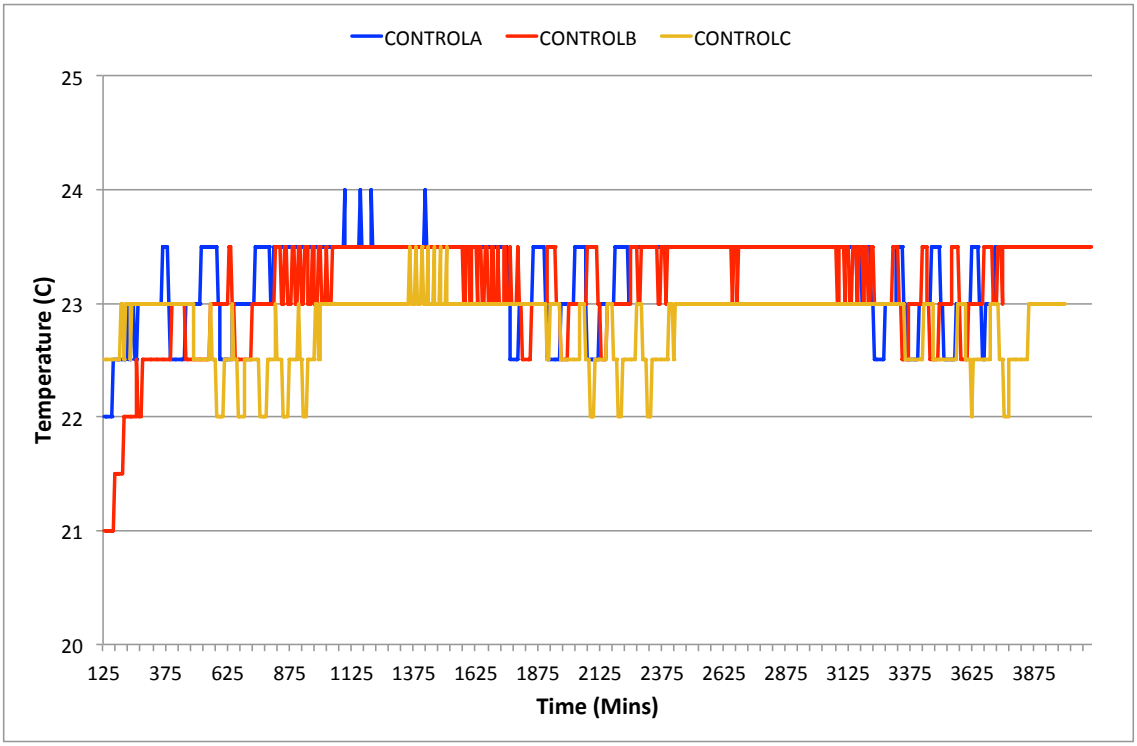
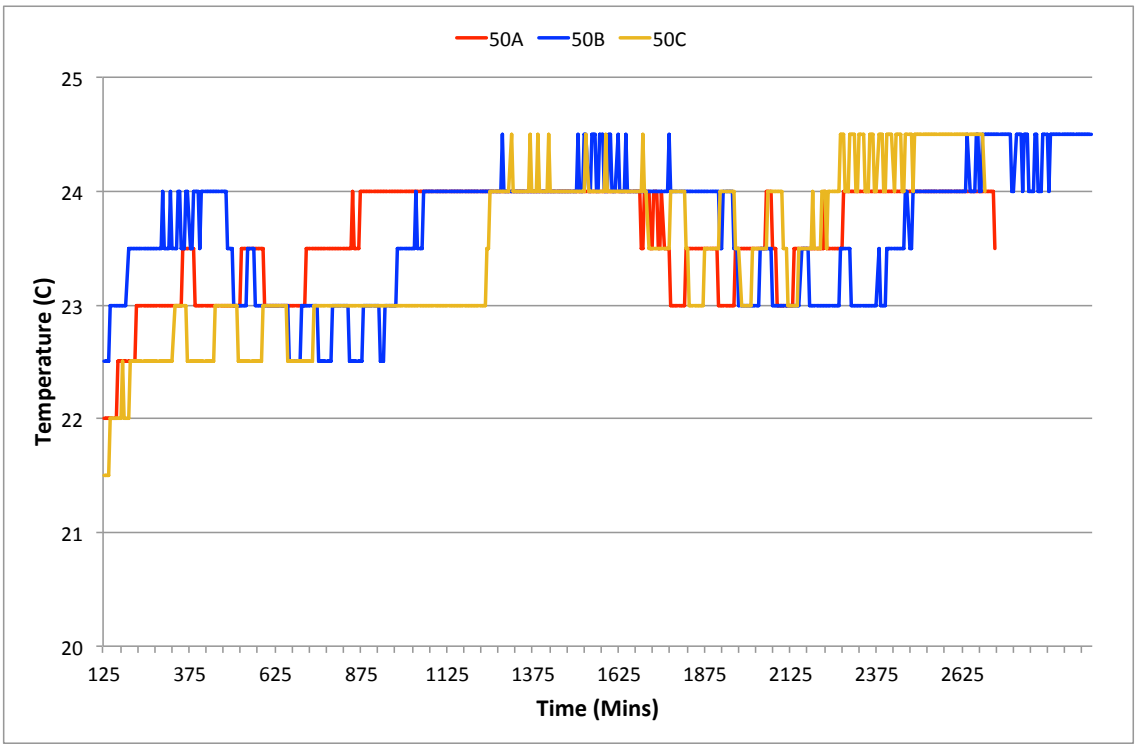
12 Appendices

APPENDIX 1



Temperatures recorded in the three Controls at an ambient of 22 °C with an EL-USB-1 data logger (minus probes)



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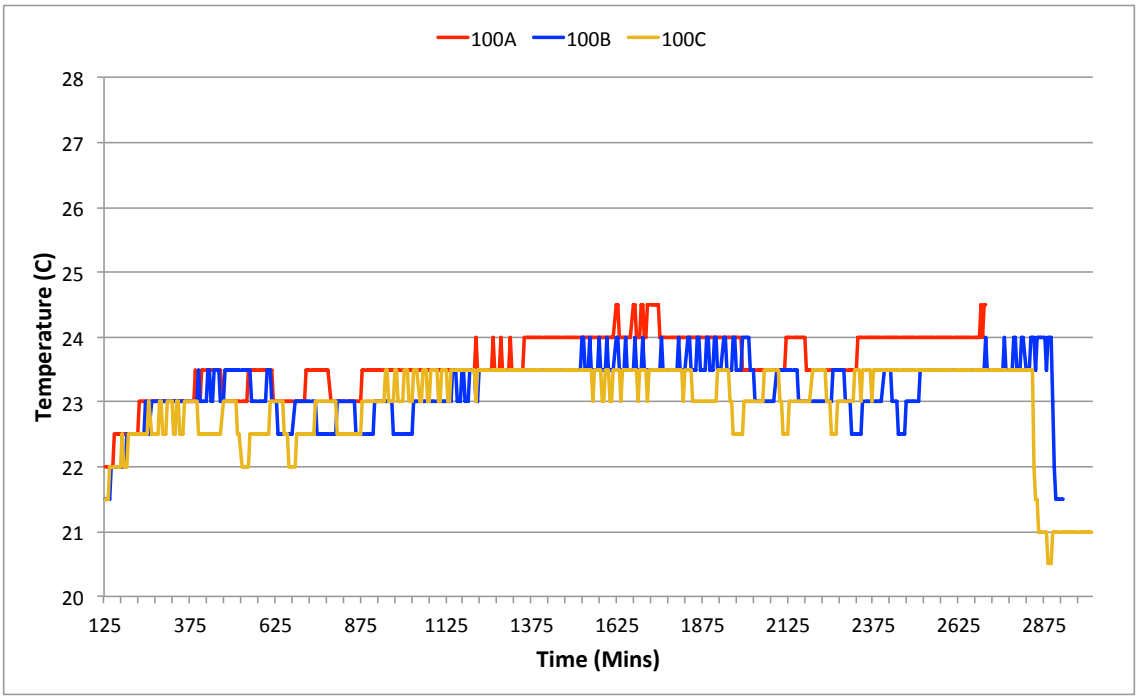

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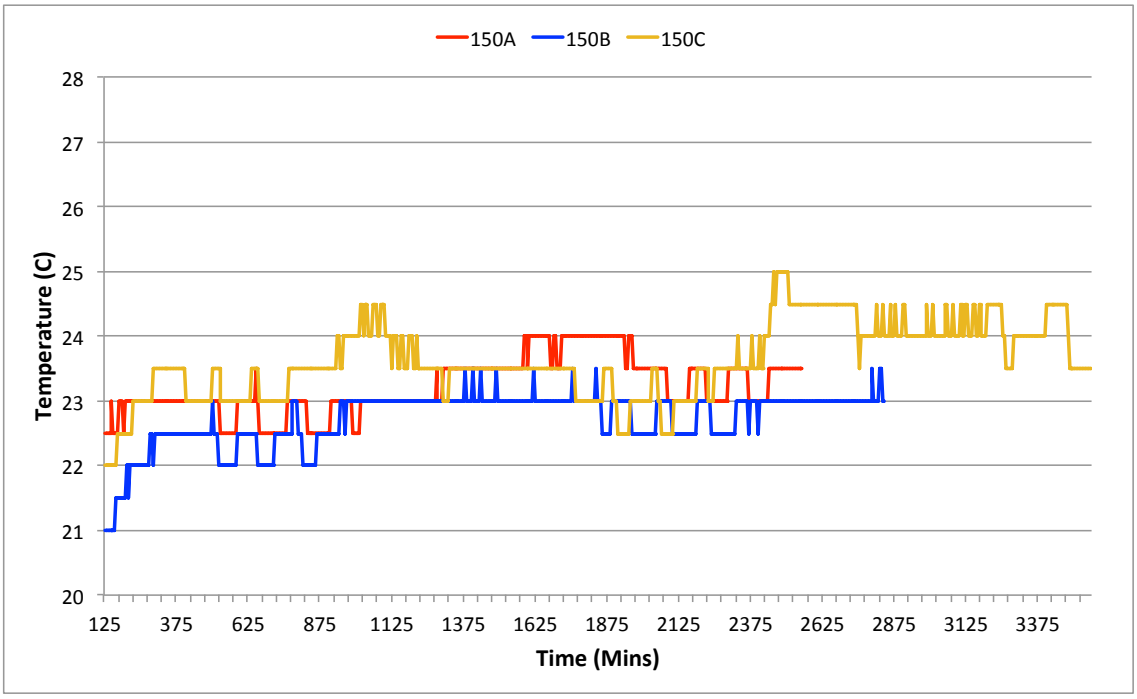

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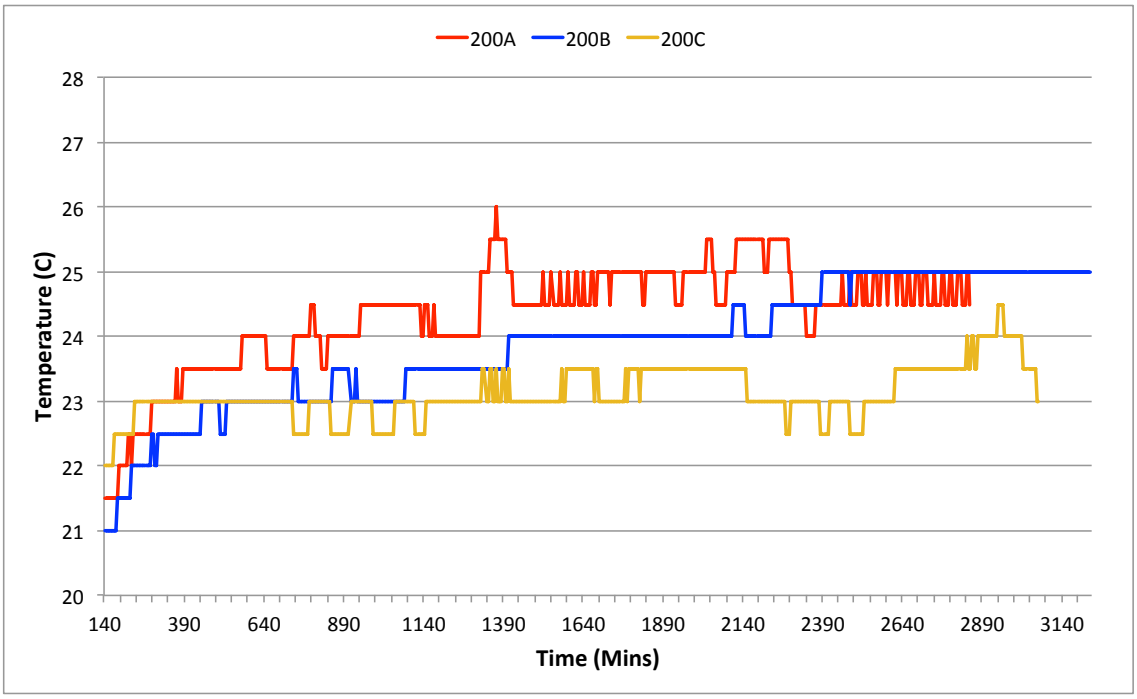

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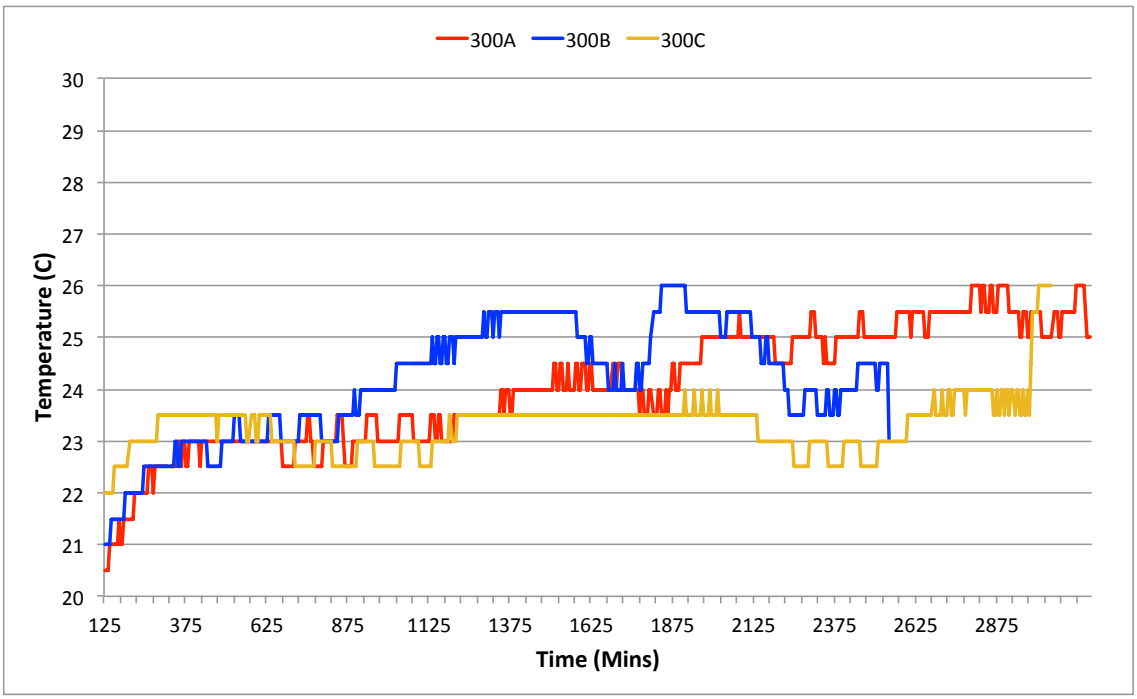

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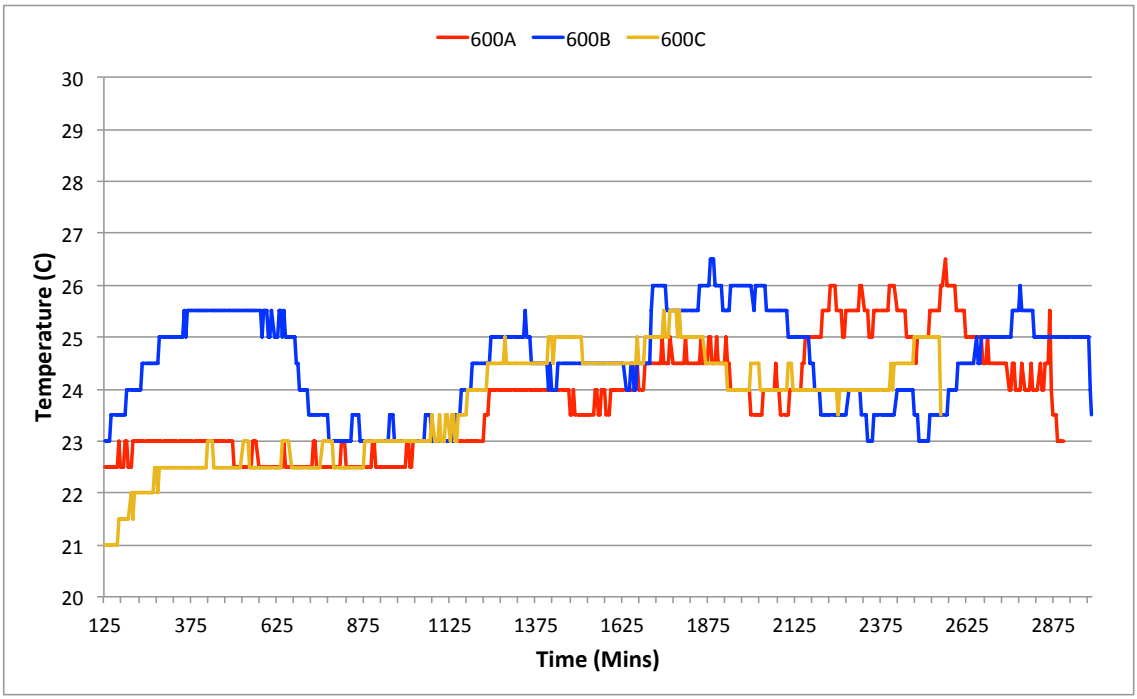

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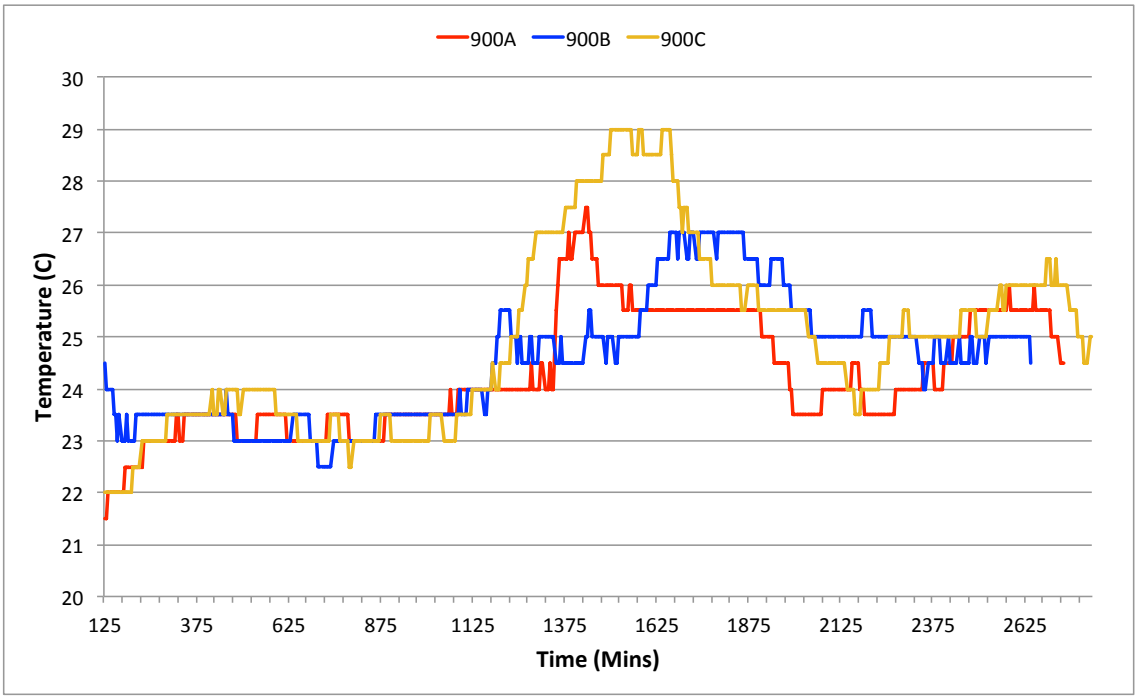

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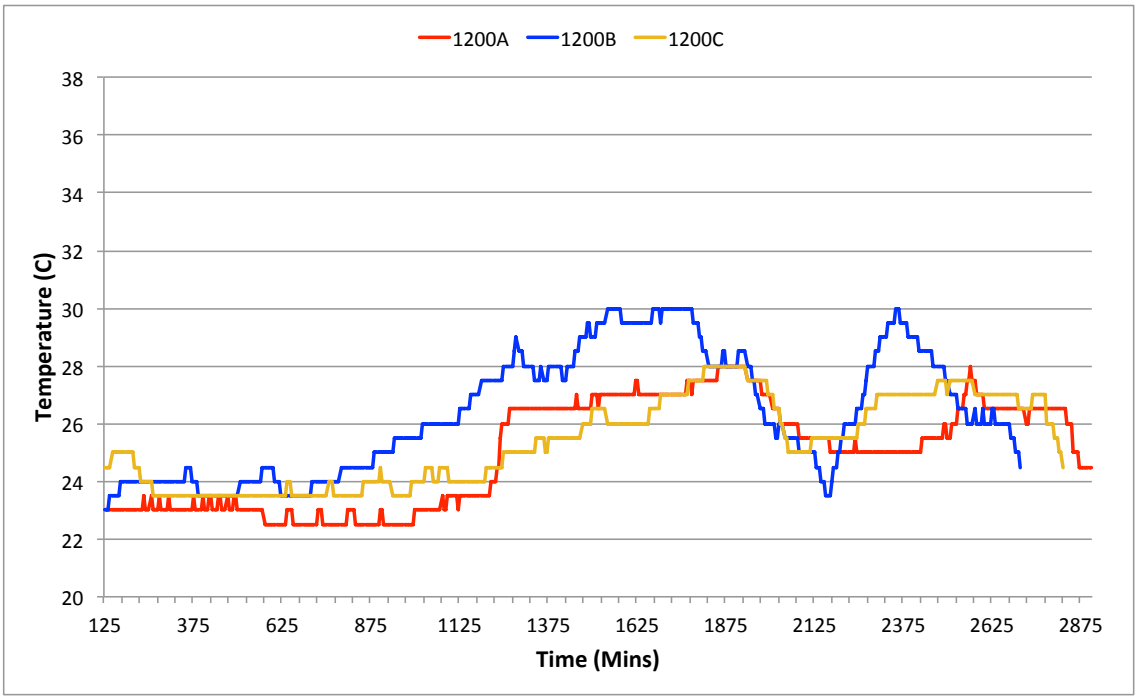

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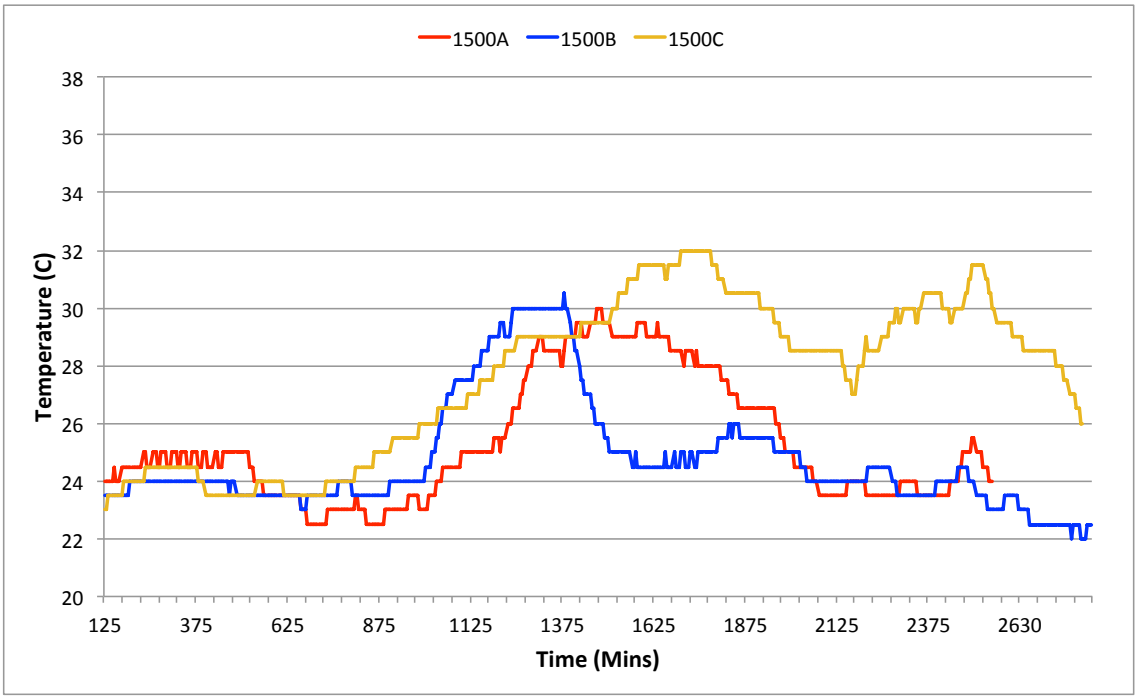

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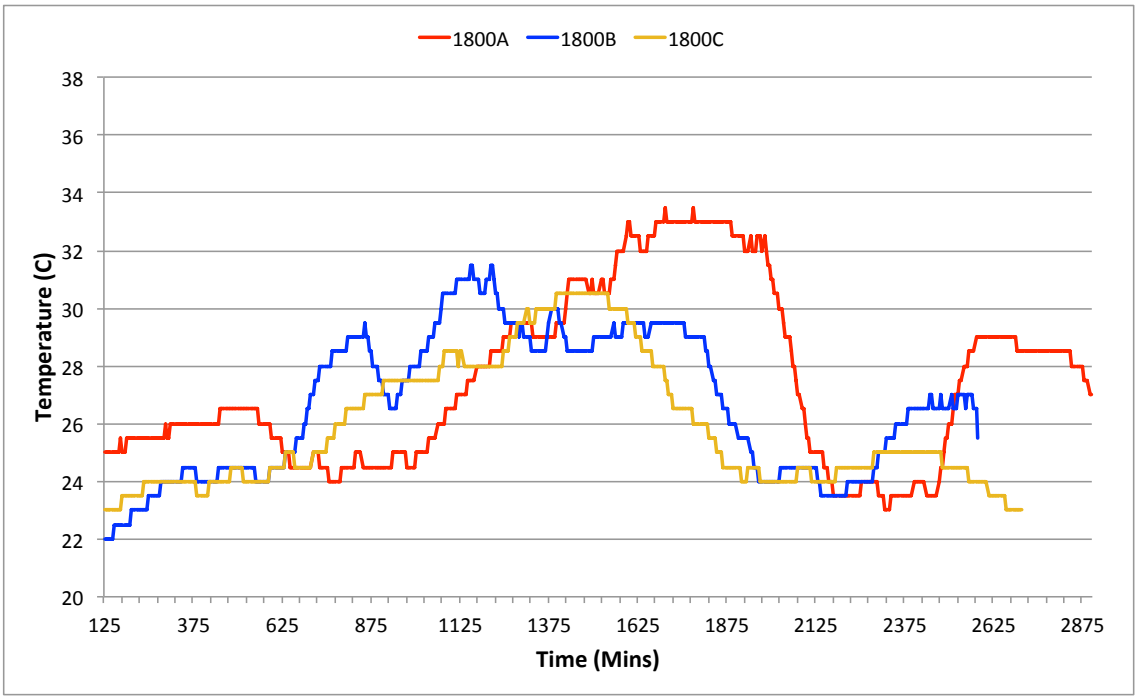
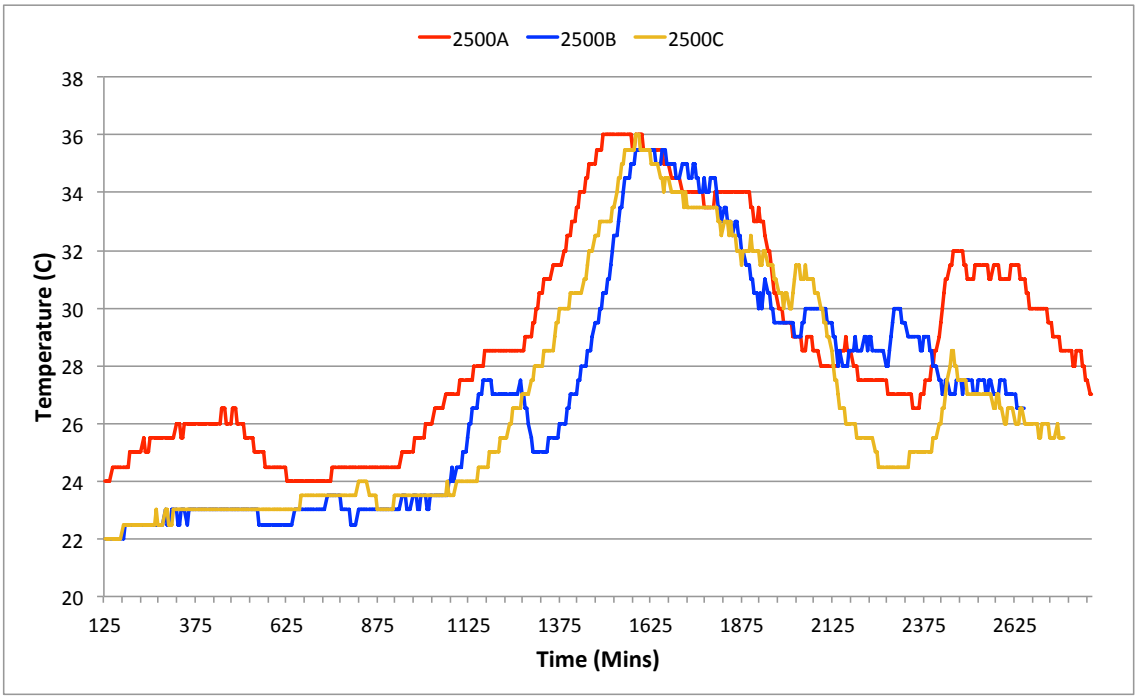
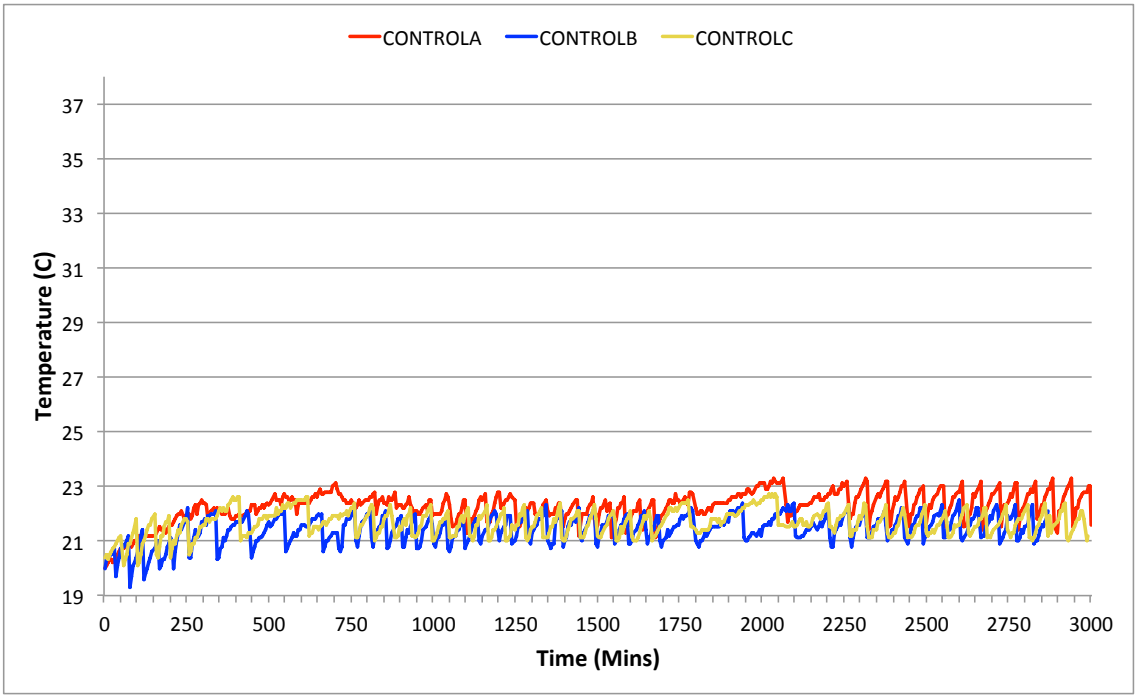


Temperatures recorded in the three masses containing 1800 larvae at an ambient of 22 °C with an EL-USB-1 data logger (minus probes)

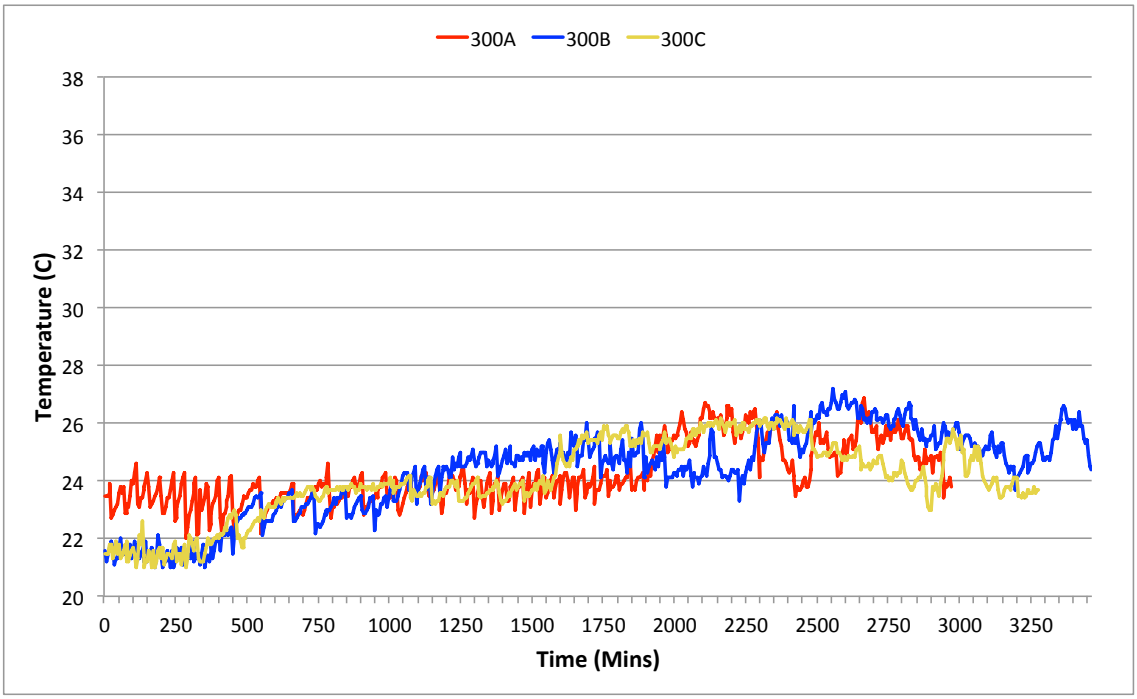


Temperatures recorded in the three masses containing 2500 larvae at an ambient of 22 °C with an EL-USB-1 data logger (minus probes)

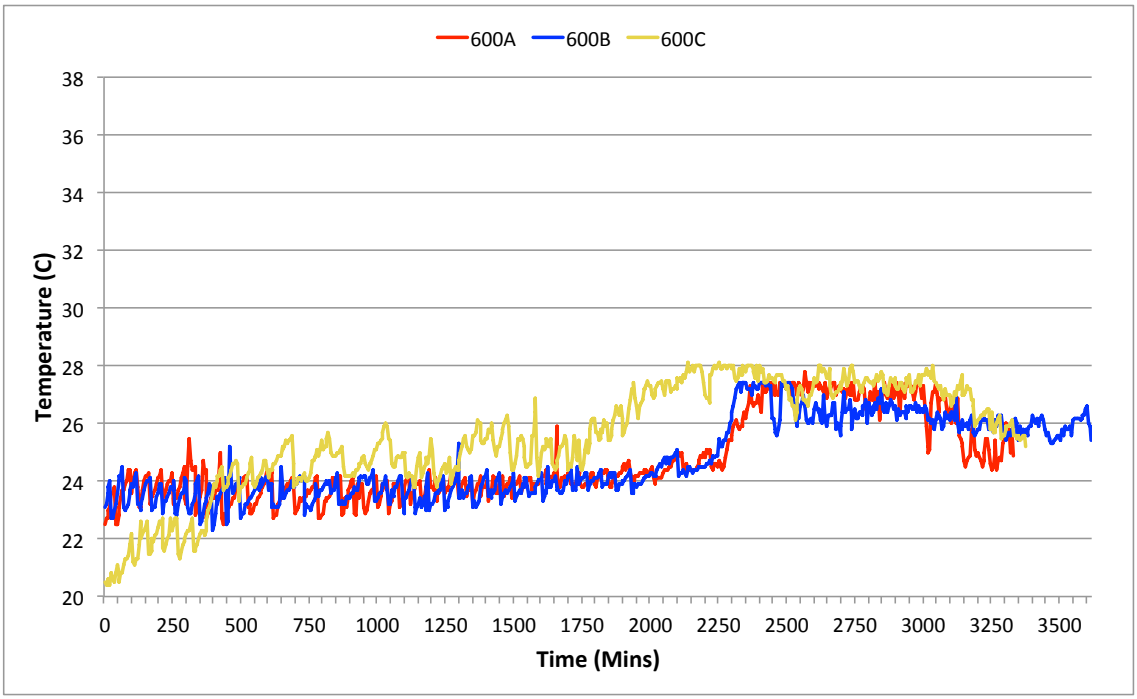
APPENDIX 2



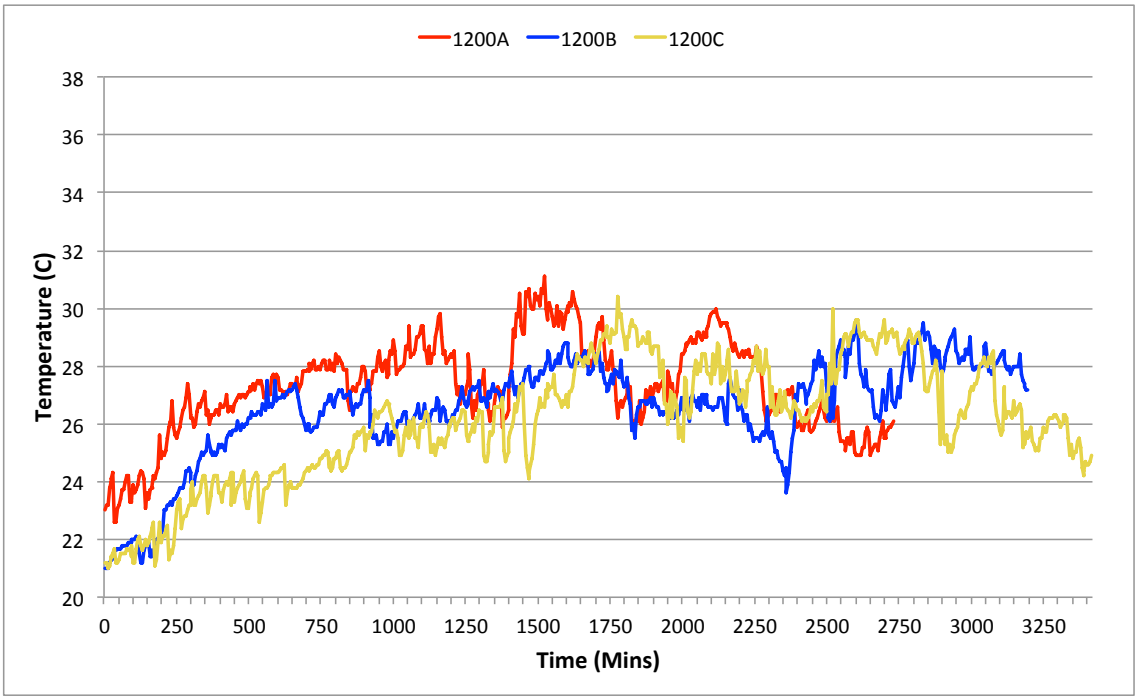
Temperatures recorded in the three controls at an ambient of 22 °C with a FLIR T425 Thermal Imaging Camera



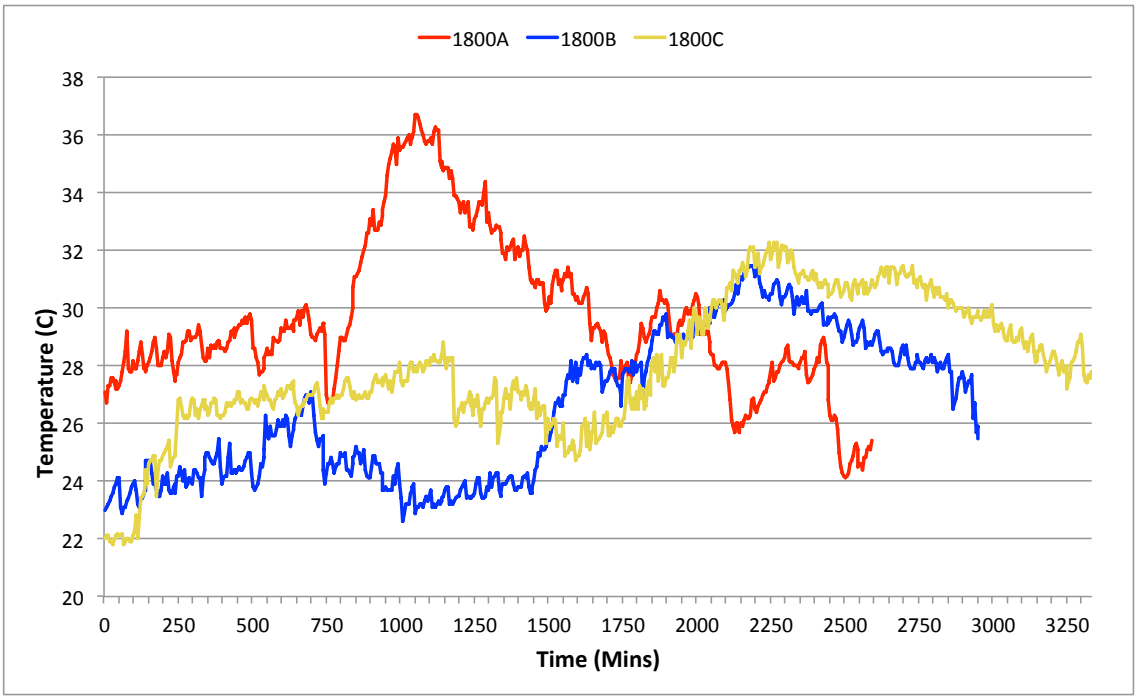
Temperatures recorded in the three masses containing 300 larvae at an ambient of 22 °C with a FLIR T425 Thermal Imaging Camera



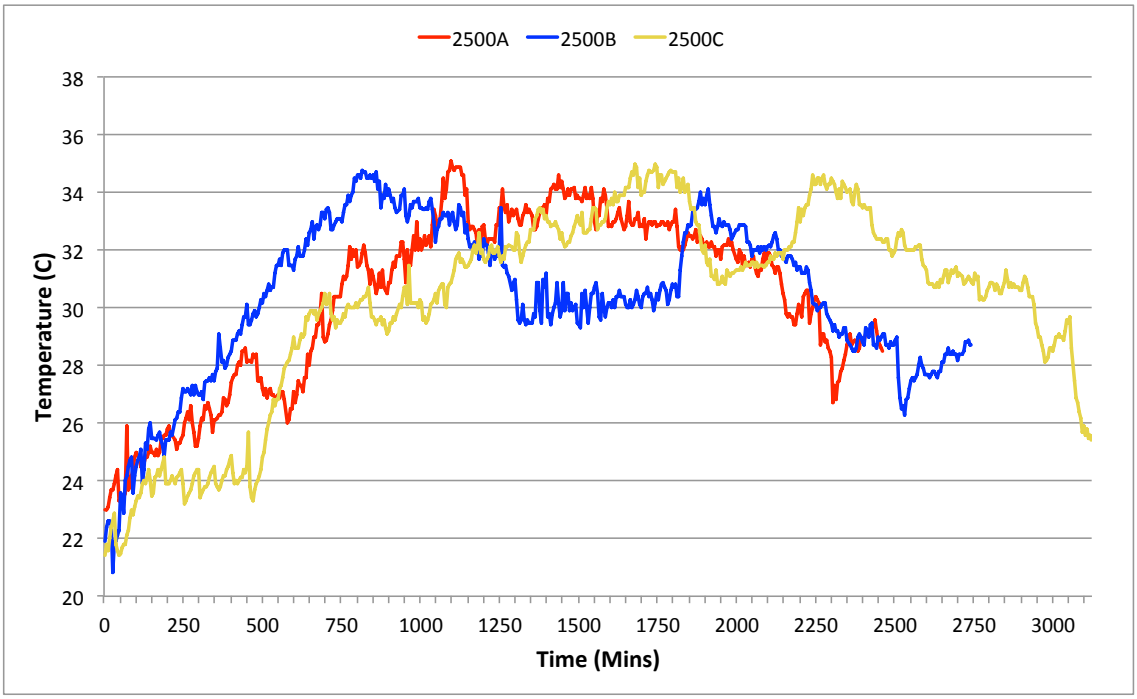
Temperatures recorded in the three masses containing 600 larvae at an ambient of 22 °C with a FLIR T425 Thermal Imaging Camera



Temperatures recorded in the three masses containing 1200 larvae at an ambient of 22 °C with a FLIR T425 Thermal Imaging Camera



Temperatures recorded in the three masses containing 1800 larvae at an ambient of 22 °C with a FLIR T425 Thermal Imaging Camera



Temperatures recorded in the three masses containing 2500 larvae at an ambient of 22 °C with a FLIR T425 Thermal Imaging Camera