# *Megaselia scalaris* (Diptera: Phoridae), a fly of forensic interest: advances in chronobiology and biology

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A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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# Abbreviations

**Insect Species**

| | |
|---|---|
| *A.fulica* | *Achatina fulica* (Férussac, 1821) |
| *A.versicolor* | *Acromyrmex versicolor* Pergande, 1894 |
| *A.pernyi* | *Antheraea pernyi* (Guérin-Méneville, 1855) |
| *A.museorum* | *Anthrenus museorum* (Linnaeus, 1761) |
| *A.pellio* | *Attagenus pellio* (Linnaeus, 1758) |
| *B. peregrina* | *Boettcherisca peregrine** Robineau-Desvoidy, 1830 |
| *B.similaris* | *Bradybaena similaris* (Férussac, 1821) |
| *B.fumigata* | *Byrsotria fumigate* (Guérin-Méneville , 1857) |
| *C.augar* | *Calliphora augur* Fabricius, 1775 |
| *C.chloropyga* | *Calliphora chloropyga* (Wiedemann, 1818) |
| *C.dubai* | *Calliphora dubia* (Macquart, 1855) |
| *C.hilli* | *Calliphora hilli* Patton, 1925 |
| *C.megacephala* | *Calliphora megacephala** (Fabricius, 1794) |
| *C.putoria* | *Calliphora putoria** (Wiedemann 1830) |
| *C.stygia* | *Calliphora stygia* (Fabricius 1782) |
| *C.vicina* | *Calliphora vicina* Robineau-Desvoidy, 1830 |
| *C.vomitoria* | *Calliphora vomitoria* (Linnaeus, 1758) |
| *Ch.albiceps* | *Chrysomya albiceps* (Wiedemann, 1819) |
| *Ch.chani* | *Chrysomya chani* Kurahashi , 1979 |
| *Ch.megacephala* | *Chrysomya megacephala* (Fabricius, 1794) |
| *Ch. nigripes* | *Chrysomya nigripes* Aubertin, 1932 |
| *Ch.pinguis* | *Chrysomya pinguis* (Walker, 1858 ) |
| *Ch.putoria* | *Chrysomya putoria* (Wiedemann 1830) |
| *Ch.rufifacies* | *Chrysomya rufifacies* (Macquart, 1842) |
| *Ch.villeneuvi* | *Chrysomya villeneuvi* Patton, 1922 |
| *C.septempuctata* | *Coccinella septempuctata* (Linnaeus, 1758) |
| *C.macellaria* | *Cochliomyia macellaria* (Fabricius, 1775) |
| *C.verena* | *Compsomyiops verena* Walker, 1849 |
| *C.tibialis* | *Conicera tibialis* Schmitz, 1925 |
| *D.carnivorus* | *Dermestes carnivorus* Fabricius, 1775 |
| *D.freshi* | *Dermestes frischi* Kugelann, 1792 |
| *D.lardarius* | *Dermestes lardarius* Linnaeus, 1758 |
| *D.maculatus* | *Dermestes maculatus* De Geer, 1774 |
| *D.melanogaster* | *Drosophila melanogaster* Meigen, 1830 |
| *D.pseudoobscura* | *Drosophila pseudoobscura* Frolova & Astaurov, 1929 |
| *E.persolla* | *Eumacronychia persolla* Reinhard, 1965 |
| *F.canicualris* | *Fannia canicularis* (Linnaeus, 1761) |
| *F.trimaculata* | *Fannia trimaculata* (Stein, 1898) |

| | |
|---|---|
| *H.ligurriens* | *Hemipyrellia ligurriens* (Weidmann, 1830) |
| *H.longipennis* | *Hippobosca longipennis* Fabricius, 1805 |
| *L.maderae* | *Leucophaea maderae* (Fabricius, 1781) |
| *L.ampullacea* | *Lucilia ampullacea* Villeneuve, 1922 |
| *L.caesar* | *Lucilia caesar* (Linnaeus, 1758) |
| *L.coeruleivirdis* | *Lucilia coeruleivirdis* Macquart, 1855 |
| *L.cuprina* | *Lucilia cuprina* (Wiedemann, 1830) |
| *L.illustris* | *Lucilia illustris* Meigen, 1826 |
| *L.sericata* | *Lucilia sericata* (Meigen, 1826) |
| *M.glabra* | *Madiza glabra* Fallen, 1820 |
| *M.abdita* | *Megaselia abdita* Schmitz, 1959 |
| *M.curtineura* | *Megaselia curtineura* (Brues 1909) |
| *M.giraudii* | *Megaselia giraudii* (Egger, 1862) |
| *M.rufipes* | *Megaselia rufipes* (Meigen, 1804) |
| *M.scalaris* | *Megaselia scalaris* (Loew, 1866) |
| *M.spiracularis* | *Megaselia spiracularis* Schmitz, 1938 |
| *M.rusticella* | *Monopis rusticella* (Denis & Schiffermüller, 1775) |
| *M.autumnalis* | *Musca autumnalis* De Geer, 1776 |
| *M.domestica* | *Musca domestica* Linnaeus, 1758 |
| *M.prolapsa* | *Muscina prolapse* (Harris, 1780) |
| *M.stabulans* | *Muscina stabulans* (Fallén, 1817) |
| *N.vitripennis* | *Nasonia vitripennis* Ashmead, 1904 |
| *N.bullata* | *Neobellieria bullata** Parker, 1916 |
| *O.aenesens* | *Ophyra aenesens* Wiedemann, 1830 |
| *O.spinigera* | *Ophyra spinigera** Stein, 1910 |
| *O.pumilum* | *Orthostigma pumilum* (Nees, 1834) |
| *P.vindemmiae* | *Pachycrepoideus vindemmiae* (Rondani 1875) |
| *P.ruficornis* | *Parasarcophaga ruficornis** (Fabricius, 1794) |
| *P.chrysostoma* | *Peckia chrysostoma* (Wiedemann 1830) |
| *P.americana* | *Periplaneta Americana* (Linnaeus, 1758) |
| *P.coeruleiviridis* | *Phaenecia coeruleiviridis** Macquart, 1855 |
| *P.regina* | *Phormia regina* Meigen, 1826 |
| *P.pictipennis* | *Phylloteles pictipennis* Loew, 1844 |
| *P.casei* | *Piophilia casei* (Linnaeus, 1758) |
| *P.terraenovae* | *Protophormia terraenovae* Robineau-Desvoidy, 1830 |
| *P.brunneus* | *Ptinus brunneus** Panzer, 1792 |
| *Puliciphora borinquenensis* | *Puliciphora borinquenensis* Wheeler, 1906 |
| *R.sanguineus* | *Rhipicephalus sanguineus* (Latreille, 1806) |
| *R.parallelocollis* | *Rhizophagus parallelocollis* Gyllenhal, 1827 |
| *R.prolixus* | *Rhodhinus prolixus* Stål, 1859 |
| *S.alienus* | *Saprinus alienus* LeConte, 1851 |

| | |
|---|---|
| *S.albiceps* | *Sarcophaga albiceps* Meigen, 1826 |
| *S. bullata* | *Sarcophaga bullata* (Parker, 1916) |
| *S.hirtipes* | *Sarcophaga hirtipes* Wiedemann, 1830 |
| *S.scrofa* | *Sus scrofa* Linnaeus, 1758 |
| *S.nudiseta* | *Synthesiomyia nudiseta* Van Der Wulp, 1883 |
| *T.fusca* | *Teichomyza fusca** (Macquart, 1835) |
| *T.obscurus* | *Tenebrio obscurus* Fabricius, 1792 |
| *T.sinuatus* | *Thanatophilus sinuatus* Fabricius, 1775 |
| *T.biselliella* | *Tineola biselliella* (Hummel, 1823) |
| *T.pellionella* | *Tineola pellionella** Linnaeus 1758 |
| *X.coerulescens* | *Xerosaprinus coerulescens* LeConte, 1851 |
| *X.vitiosus* | *Xerosaprinus vitiosus* LeConte, 1851 |

* **Species names are written as presented in the original publication**

**General Abbreviations**

| | |
|---|---|
| AFTER | Australian Facility for Taphonomic Experimental Research |
| CND | Control with no drug |
| CT | Computed Tomography |
| DAM | Drosophilia Activity Monitor |
| DD | Complete Darkness |
| DNA | Deoxyribonucleic acid |
| EM | Environmental monitor |
| ETSD | Elapsed time since death |
| EtOH | Ethanol |
| HWI | Hot water immersion |
| IR | Infrared |
| KI | Potassium Iodide |
| LAM | Locomotor Activity Monitor |
| LD | Light/Dark |
| LED | Light Emitting Diode |
| mPMI | Minimum Post Mortem Interval |
| maxPMI | Maximum Post Mortem Interval |
| mRNA | Messenger Ribonucleic acid |
| PCR | Polymerase Chain Reaction |
| R | Rhythmicity |
| RNA | Ribonucleic acid |
| SEM | Standard Error of the Mean |
| SEM | Scanning Electron Microscopy |
| TEM | Transmission Electron Microscopy |
| USA | United States of America |

# Abstract

*Megaselia scalaris* (Diptera, Phoridae) is a common species found amongst indoor and outdoor crime scenes and plays an important role in the decomposition of human remains and can be used following the forensic entomology approach for the estimation of the post mortem interval particularly in indoor cases. Several questions concerning the biology and the chronobiology of this species remain open.

Circadian clocks have evolved to synchronize physiology, metabolism and behaviour to the 24-h geophysical cycles of the Earth. The understanding of the circadian clock mechanism is a crucial element of forensic entomology because it is able to control routines such as feeding, mating, ovipositing or emergence times. To describe the behaviour and the potential role that the circadian clock may have on both the locomotor activity and emergence times of the *M.scalaris*, using Trikinetics technology, used previously in *Drosophilia* studies allows for factual data rather than observational data seen in many journals.

The activity rhythms of *M.scalaris* were monitored using light/dark photoperiods at 20 °C. Males and females both demonstrate that there are significant differences between dark and light conditions and further results establish that the flies are both diurnal and nocturnal in activity. The pupa emergence shows that there are different rhythms during full darkness conditions and light/dark conditions. In addition our experiments demonstrated that the activity of this species is clock regulated. Differences in locomotor activity between male and female flies were observed in the absence of food in continuous dark (DD), in contrast the activity of the two sexes don't differ in continuous light (LL) or in presence of food both in DD and in LL conditions. Cold White, Blue, Green, Red and Yellow lights were used to test the light attractiveness. Males and females show different behaviour. In contrast females seem to be attracted to red light and they don't present any directional behaviour under other light.

Colonisation of carrion by insects allow for the post mortem interval (PMI) to be determined. However it is thought by some, that flies are not active during the night time period and therefore are not able to oviposit during this time. To put that into a forensic context, if eggs were located on a cadaver, the conclusion would be that death occurred during the previous day or before. Determining nocturnal oviposition in forensically important flies is of fundamental importance so that the PMI can be determined correctly by the forensic entomologist. Our experiments have demonstrated that *M.scalaris* were able to oviposit in dark conditions during the night.

Insects colonise a cadaver in a predictable pattern otherwise known as the succession. Succession patterns may vary due to intrinsic and environmental factors, for example, has the cadaver been buried or is it located above ground. Colonisation in buried remains depend on the slower decomposition rate of buried bodies, reduced dispersion of the decomposition odours but as well the reduced accessibility to the body. Phoridae are commonly found amongst the entomofauna of exhumed bodies or coffins. The phorid *M.scalaris* has been reported as being able to dig up to 6 feet. Little information is available about the kind of soil this fly is able to dig through to reach a cadaver; two

different kinds of soil were investigated: sand and sandy loam garden soil. The results showed that *M.scalaris* was able to excavate garden soil but not sand.

Insect development rate is mainly temperature dependent, although other parameters like photoperiod, overcrowding and food availability can affect the developments. In addition several studies demonstrated that drugs and other chemicals can affect the growth of larvae, feeding on the dead body, leading to Inc.orrect mPMI estimations. Amitriptyline is a commonly used antidepressant in cases of major depressive disorder. It is a tricyclic molecule absorbed in the gastrointestinal tract and metabolized into the liver. This molecule shows a high toxicity results in cases of overdose. Studies on the effect of Amitriptyline on insect development and accumulation/excretion have been performed in the 1990's on *Parasarcophaga ruficornis* (Diptera: Sarcophagidae) and on *Calliphora vicina* (Diptera: Calliphoridae) whereas no data are available for other taxa. The results of these studies demonstrated the non-effect of the molecule on the growth rate. In the same years Amitriptyline and derivates have been isolated from empty puparia of *Megaselia scalaris* and from skin and faecal material of *Dermestes maculatus* (Coleoptera: Dermestidae) collected from a mummified body in New England. The aim of our study was to investigate the effect that Amitriptyline, often found on cadavers, may have on the development of *Megaselia scalaris*. Our experiment showed that Amitriptyline had no effect on the size but saw a decrease in the developmental time of *M.scalaris*, so the mPMI estimation can be affected if based on the larval size and not on the complete development.

The results reported and discussed in this thesis improve the knowledge about the biology of *M.scalaris* and its applicability in the forensic context.

# 1: Introduction

## 1.1 General Introduction to Forensic Entomology

An accurate estimation of the time since death is of fundamental importance in many forensic cases. Post mortem interval (PMI) or time since death on human remains can be estimated by a pathologist using different methods such as: *algor mortis* (temperature), *livor mortis*, *rigor mortis*, vitreous potassium concentrations, electrical excitability of skeletal muscles and gastric content (Henssge *et al*., 2002), however these methods can only be measured accurately in the two to three days following death (Amendt *et al*., 2004). An additional method that may help with the estimation of the time since death is the entomological approach. The collection and analysis of insect evidence from a forensic investigation may be used to assist in forensic, legal or medico-legal cases (Varatharajan and Sen, 2000).

The relationship between larvae on a cadaver and the oviposition of adult flies was not recognised during medieval times, however by the seventeenth century, insects were starting to be recognised and more understood which saw them becoming used to investigate crime scenes ( Smith, 1986; Schroeder et al., 2003; Amendt et al., 2004; Gennard, 2012) . As the centuries advanced so did Forensic Entomology which became its own branch of scientific study and entomology came into use in Europe in the 19$^{th}$ century (Schroeder *et al*., 2003). To date, forensic entomology is accepted as an important forensic tool in many countries (Catts, 1992; Greenberg, 1990a; Goff et al., 1991; Campobasso et al., 2001).

The Forensic Entomology discipline may also be applied to identifying pests that are found infesting food products (fruit, meat, etc.). In addition urban locations may see infestations in the home, work environment or businesses (Gennard, 2012).

Forensic Entomology may use the insect evidence to estimate the developmental time of insects whilst also observing the carrion insect succession to estimate time since death. As death may occur a variable amount of time prior to colonisation the entomologist will estimate the Minimum PMI ($_{m}$PMI) by determing the earliest period the body was colonised by insects to time taken for the collected specimens to reach a certain developmental stage (Villet *et al*., 2010). This time had also been defined by Byrd and Castner (2009) as the Time of Colonisation (TOC). The maximum PMI ($_{max}$PMI) can be

calculated from when the person was last seen alive to the discovery of the body (*Fig 1*) (Villet *et al*., 2010).

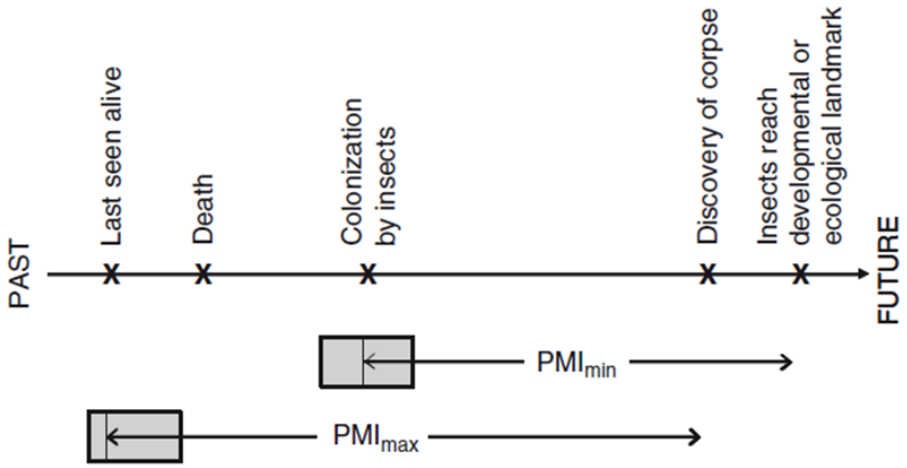



**Fig 1: Estimation of the post mortem interval time line of a general death investigation using $_{min}$PMI and $_{max}$PMI (Villet *et al*., 2010)**

Decomposition of human remains can begin quickly after death (approximately four minutes) but is dependent on many factors such as temperature, insect activity, soil type and how the body has been disposed of, for instance, whether the body has been buried, left in a cave or left on the surface open to the elements (Vass, 2001; Campobasso et al., 2001). There are four stages to the decomposition of human remains: autolysis, putrefaction, liquefaction and skeletisation (Dent *et al*., 2004). Whereas (Bornemissza, 1957) classified their decomposition stages by initial decay, putrefaction, black putrefaction, butyric fermentation and dry decay and commented on the affect that decomposition has on the underlying soils.

Many researchers use a wide variety of animal species as models for decomposition studies trying to draw conclusions about human decomposition from these studies. Due to the many differences (i.e. size, weight and hair) between some of the species used and humans, the validity of this research has been questioned in active court cases. Research showed that a suitable human model was found to be the domestic pig (*Sus scrofa* L). Side by side studies were completed in which pigs were found to have similarities such as, their bodies are moderately hairless, similar gut fauna and their skin

is similar to that of humans. A pig weighing 23 kg was found to be equivalent to that of an adult male torso (Anderson, 1996; Catts and Goff, 1992). Due to religious and ethical reasons, field studies of human decomposition were rare until the University of Tennessee Forensic Anthropology Centre came along with five other taphonomy centres in the USA were created and an Australian Facility for Taphonomic Experimental Research (AFTER) is currently being built. These facilities help to provide answers into decomposition under varying conditions and parameters.

## 1.2 The role of Diptera and other Arthropods in body decomposition and PMI estimation.

### 1.2.1 Introduction

Flies are the first colonisers on exposed cadavers but they play a fundamental role in decomposition as well on buried remains. Specific odours which are released during the different stages of decomposition will attract a wide variety of insect species, by identifying the insects present on the remains may help to estimate the stage of decomposition and the time since death (Smith, 1986). The most important insects used in the forensic entomology field are Diptera, Coleoptera, Lepidoptera and Hymenoptera (*Table 1*).

**Table 1: Summary of forensically important insects (Amendt *et al*., 2004).**

| Order/Family | Important genera |
|---|---|
| **Coleoptera/Beetles** | |
| Cleridae (Checkered beetles) | *Necrobia* |
| Dermestidae (Larder beetles) | *Attagenus, Dermestes* |
| Geotrupidae (Dung beetles) | *Geotrupes* |
| Histeridae (Clown beetles) | *Hister, Saprinus* |
| Silphidae (Carrion beetles) | *Necrodes, Nicrophorus, Silpha* |
| Staphylinidae (Rove beetles) | *Aleochara, Creophilus* |
| **Diptera/Flies** | |
| Calliphoridae (Blowflies) | *Calliphora, Chrysomya, Cochliomyia, Lucilia, Phormia* |
| Drosophilidae (Fruit flies) | *Drosophila* |
| Ephydridae (Shore flies) | *Discomyza* |
| Fanniidae (Latrine flies) | *Fannia* |
| Heleomyzidae (Sun flies) | *Heleomyza, Neoleria* |
| Muscidae (House flies) | *Hydrotaea, Musca, Muscina, Ophyra** |
| Phoridae (Scuttle flies) | *Conicera, Megaselia* |
| Piophilidae (Skipper flies) | *Piophila, Stearibia* |
| Sarcophagidae (Flesh flies) | *Liopygia, Sarcophaga* |
| Sepsidae (Black scavenger flies) | *Nemopoda, Themira* |
| Sphaeroceridae (Small dung flies) | *Leptocera* |
| Stratiomyidae (Soldier flies) | *Hermetia, Sargus* |
| Trichoceridae (Winter gnats) | *Trichocera* |
| **Lepidoptera/Buterflies** | |
| Tineidae (Clothes moths) | *Tineola* |
| **Hymenoptera/Wasps** | |
| Ichneumonidae (Ichneumon wasps) | *Alysia* |
| Pteromalidae (Fly wasps) | *Nasonia, Muscidifurax* |

* see abbreviations

Knowledge of succession patterns is important when trying to estimate the mPMI, as variations can occur in the visitation of insects at different intervals of the decomposition process. Research has been conducted to determine the insect succession that follows the decomposition process (Kasper *et al*., 2012).

Drugs, poisons or burnt cadavers are known to have an effect on both decomposition and insect succession which may affect the PMI estimation. In addition as well the body size and concealment of the body may impact the insect succession (Campobasso *et al*., 2001). Vanin and colleagues (2013) discuss a classic pattern of colonisation that is observed in cases of burnt carcasses, with insects usually attributed to different waves arriving at the same time.

The first studies on succession patterns have been produced in the XIX century and the first complete table was published by Megnin (1894) and updated by Smith (1973). Seasons can play a role in insect colonisation in temperature climate, but succession data in south China determined that 47 species of insect were identified on the carcasses. No obvious differences were seen in any of the four seasons and most of the necrophagous flies could be seen all year round with the exception of dermestid beetles which were absent during winter (Wang *et al*., 2008).

Blow flies (Calliphoridae), are in general the first colonisers of an exposed cadaver, attracted by the odour produced during decomposition. Females will oviposit within the first few hours after death however, differences in faunal colonisation have been observed depending on the environment in which the body was left (e.g. in water, in a vehicle, buried, indoors or outdoors, exposed to the elements, wrapped up in blankets or carpets) (Simpson and Knight, 1985). In forensic cases when working with flies of the first colonisation wave, PMI estimation can be calculated by determining the stage of development of the insect present on the body and correlating it with the temperature of the environment experienced by them prior to the body being discovered. An additional method involves looking at the appearance of specific arthropods and comparing the data to a succession model which may allow for both the minimum and maximum PMI to be calculated (Smith, 1986). Information about the cadaver may be provided when there is knowledge of the many insect species and the surroundings they inhabit (Varatharajan and Sen, 2000).

Succession patterns of carrion insects have a direct relationship to the decay rates of a human cadaver, Rodriguez and Bass (1983) observed that a cadaver with no insects was found to decompose and dry out slower, whilst Oliveira and Vasconcelos (2010) discuss the that time between death and skeletisation may be a lot shorter in tropical countries due to the constantly high temperatures, which could possibly reduce the time for successful data collection for the entomologist.

Oliveira and Vasconcelos (2010) describe the four main categories which house forensically important insects, these are:

- **Necrophagous insects** - feed only on decomposing tissue e.g. blowflies, hide/clown beetles,
- **Predators** (and parasites) of the necrophages – e.g. rove/ground beetles which ingest larvae and eggs of flies on the decomposing tissue,
- **Omnivores** – ingest live insects occupying the dead flesh e.g. ants and wasps,
- **Opportunist species** – appear due to the corpse being in their local environment e.g. Butterflies, spiders, mites and hoverflies.

Using exposed cadavers *(Table 2)*, a total of eight waves of arthropod succession was documented throughout the different stages of decomposition and has continually been updated throughout the years. Faunal succession using buried cadavers was also researched (*Table 3*) (Mégnin, 1894; Johnston and Villeneuve, 1897; Smith, 1986).

**Table 2: Faunal succession on exposed human cadavers. Based on Mégnin (1894) and tabulated by Johnston and Villeneuve (1897) and updated by Smith (1973).**

| Wave | Fauna | Condition of corpse | Approx. age of corpse |
|---|---|---|---|
| 1st | *Calliphora vicina* (Dipt., Calliphoridae)<br>*Calliphora.vomitoria* (Dipt., Calliphoridae)<br>*Lucilia* spp. (Dipt., Calliphoridae)<br>*Musca domestica* (Dipt.,Muscidae)<br>*Musca autumnalis* (Dipt.,Muscidae)<br>*Muscina stabulans* (Dipt.,Muscidae) | Fresh (changes with season) | First 3 months |
| 2nd | *Sarcophaga* spp. (Dipt., Sarcophagidae)<br>Cynomya spp. (Dipt., Calliphoridae) | Odour developed | |
| 3rd | *Dermestes* spp. (Col., Dermestidae)<br>*Aglossa* spp. (Lep., Pyralidae) | Fats rancid | |
| 4th | *Piophilia casei* (Dipt., Piophilidae)<br>*Madiza glabra* (Dipt., Piophilidae)<br>*Fannia* spp (Dipt.,Fanniidae)<br>Drosophilidae (Dipt.)<br>Sepsidae (Dipt.)<br>Sphaeroceridae (Dipt.)<br>*Eristalis* (Dipt., Syrphidae)<br>*Teichomyza fusca* (Dipt., Ephydridae)<br>*Corynetes,Necrobia* (Col., Cleridae) | After butyric fermentation<br>protein of caseic fermentation | 3 - 6 months |
| 5th | *Ophyra** spp. (Dipt., Muscidae)<br>Phoridae (Dipt.)<br>Thyreophoridae (Dipt.)<br>*Nicrophorus* spp (Col., Silphidae)<br>*Silpha spp.* (Col., Silphidae)<br>*Hister* spp. (Col., Histeridae)<br>*Saprinus* spp. (Col., Histeridae) | Ammoniacal fermentation<br><br>Evaporation of sanious fluids<br><br>Remaining body fluids now absorbed | 4 - 8 months |
| 6th | Acari | | 6 - 12 months |
| 7th | *Attagenus pellio* (Col., Dermestidae)<br>*Anthrenus museorum* (Col., Dermestidae)<br>*Dermestes maculatus* (Col., Dermestidae)<br>*Tineola biselliella* (Lep.,Tineidae)<br>*Tineola pellionella* (Lep.,Tineidae)<br>*Monopis rusticella* (Lep.,Tineidae) | Completely dry | 1- 3 years |
| 8th | *Ptinus brunneus* (Col., Ptinidae)<br>*Tenebrio obscurus* (Col., Tenebrionidae) | | 3 years plus |

*(Dipt; Diptera, Col: Coleoptera, Lep: Lepidoptera)*

**Table 3: Faunal succession on buried human cadavers. Based on Mégnin,(1894) and tabulated by Johnston and Villeneuve (1897) and updated by Smith (1973).**

| Wave | Fauna | Approx. age of corpse |
|---|---|---|
| 1st | *Calliphora and Muscina stabulans* | |
| 2nd | *Ophyra** spp. | |
| 3rd | Phoridae (*Conicera* may appear on surface) | 1 year |
| 4th | *Rhizophagus parallelocollis* (Col., Rhizophagidae)<br>*Philonthus* (Col., Staphylinidae) | 2 years |

#### 1.2.1.1 Colonisation of Buried Cadavers

Pastula and Merritt (2013) and Gunn and Bird (2011) comment that victims of fatal crimes are often found in shallow clandestine graves, this may be due to the assailants trying to dispose of the body rapidly, as digging a deeper grave would require more time and effort given the size and weight of an average adult human therefore the longer the body is in the assailants' possession the more likely they are to be caught. Gunn and Bird (2011) comment that on average, clandestine graves in the United Kingdom had an average depth of 0.4 m whilst those found in the USA were found to be a depth of 0.56 metres.

With the research done by Manhein, (1996) a preliminary experiment was set up by Pastula and Merritt (2013) in which two pigs were buried at 90 cm in different seasons. Results concluded that no insect activity was found at this depth in any of the experiments. Further experiments were set up using depths of 30 and 60 cm. The objective was to establish an insect succession database on buried carrion in the Michigan area by observing both arrival times and insect succession on buried pigs. The results showed that a cadaver buried at 30 cm would be colonised in less than two weeks as forensically important species were collected on day 5. Collection on day 7 from the burial at 60 cm found two Diptera species, one of which was *Megaselia scalaris.* It was thought that due to the smaller size of the collected flies that they would find it easier to manoeuvre through the spaces in the soil to reach the cadaver. At both 30 and 60 cm depths, larvae were regularly collected from the ventral torso, ear facing the soil, the fold of the legs and between the hooves rather than the usual sites such as the eyes, anus, mouth and nose. The authors suggested that the insects may have found protection at these points from rain that fell during the experiment.

Due to certain species, moving vertically away from the food source rather than horizontally, research by Balme *et al*. (2012) was undertaken using *Cochliomyia macellaria* (Diptera: Calliphoridae) and *Protophormia terraenovae* (Diptera: Calliphoridae) to determine if immature blowflies were able to complete their development process and emerge as flies when buried at different soil depths of 5, 25 and 50 cm. Their research revealed that the soil depth of 50 cm produced the least number of adult flies. Buried 3$^{rd}$ instar larvae were the most successful in survival whilst flies that were buried as pupae were the least successful. A further experiment was conducted in which 2$^{nd}$ instar larvae were buried with 30 g food, the soil was examined after a period

of time and the result showed that the insects were able to pupate near the soil surface therefore showing that they were able to move vertically in the soil which allowed for an increase in survival. Balme *et al*. (2012) then go on to discuss that should either *C.macellaria* or *P. terraenovae* fly puparia be found during an exhumation of a body that this may suggest the cadaver was colonised prior to burial.

Further work examining burrowing behaviour using *Chrysomya albiceps* (Diptera: Calliphoridae) and *Lucilia cuprina* (Diptera: Calliphoridae) was done by Gomes *et al*. (2009). Body weight and depth of burrowing were measured at different temperatures beginning at 15 $^{o}$C, increasing in 5 $^{o}$C increments up to 30 $^{o}$C. The results showed that temperature had an effect on both burial depth and body weight. At both lowest and highest temperatures, both species remained nearer the surface and the body weights were recorded to be lowest. Gomes and colleagues (2009) discuss that further studies are required using different species and also using temperature intervals that are less than 5 $^{o}$C should be researched.

It is important to understand insect burial behaviour to further comprehend the environmental parameters which may affect larval behaviour; this information may help to assist the entomologist in locating larvae which have buried down below the surface. Whilst some species e.g. *Megaselia scalaris* are able to burrow down on to buried cadavers numerous feet below the surface to colonise and complete their full developmental cycle, other species will reach their final development stage before moving away from the food source and burrowing to complete their final developmental stage (Gomes *et al*., 2009). Post feeding larval dispersal is the final development stage for larvae in which they are seen moving away from the food source, they do this to either look for another food source if their weight is not adequate or to locate a different area in which to begin the pupation process (Gomes *et al*., 2005). A review completed by Gomes *et al*. (2006) indicates all the work previously done on post feeding larval dispersal (*Table 4*). A protocol is also discussed to assist entomologists in larval or pupal location whilst assessing a crime scene.

**Table 4: Review of post feeding larval dispersion data (Gomes *et al*., 2006).**

| Species | Dispersal | Depth | Reference |
|---|---|---|---|
| Diptera Gen. spp. | X | | (Green, 1951b) |
| Diptera Gen. spp. | | X | (Lundt, 1964) |
| Diptera Gen. spp. | X | X | (Nuorteva, 1977)<br>(Gomes *et al*., 2005) |
| *Lucillia sericata,*<br>*Calliphora vicina*<br>*Phormia regina*<br>*Chrysomya rufifacies*<br>*Muscina stabulans*<br>*Cochlliomyia macellaria* | X | | (Greenberg, 1990a) |
| *Chrysomya rufifacies*<br>*Chrysomya chani*<br>*Chrysomya villeneuvi*<br>*Ophyra spinigera**<br>*Chyrsomya megacephala*<br>*Chyrsomya nigripes*<br>*Chyrsomya pinguis*<br>*Hemipyrelli ligurriens* | X | | (Omar *et al*., 1994) |
| *Phormia terraenovae* | X | | (Berrigan and Pepin, 1995) |
| *Chrysomya megacephala*<br>*Chyrsomya putoria* | X | | (Godoy *et al*., 1993) |
| Calliphoridae Gen. spp. | X | | (Bassanezi *et al*., 1997) |
| *Cochliomyia macellaria*<br>*Chrysomya putoria* | X | | (Tessmer and Meek, 1996) |
| *Protophormia terraenovae* | X | | (Benecke, 1998) |
| *Calliphora vomitoria*<br>*Lucilia caesar* | X | | (Kocarek, 2001) |
| *Chrysomya albiceps*<br>*Chrysomya megacephala*<br>*Lucilia cuprina* | X | X | (Gomes *et al*., 2002)<br>(Gomes *et al*., 2003) |

#### 1.2.1.2 Colonisation of Submerged Cadavers

Cadavers that have been emerged in water lose body heat twice as fast when compared to those in exposed conditions and therefore the decomposition process is delayed (Simpson and Knight, 1985).

Davis and Goff (2000) characterised the decomposition stages from a carcass being in a water environment as:

- **Fresh** – begins at the moment of death and continues until bloating is first observed.
- **Bloat/Floating/Buoyant** – the carcass begins to bloat from gases that are being produced from the activities of anaerobic bacteria.
- **Deterioration/disintegration** – skin begins to disintegrate and may flake off or break away.
- **Buoyant remains** – skin, tissue and parts of the bones remain partially suspended in the water with some parts being partially exposed.
- **Scattered Skeletal** – Fat and bones remain, no skin or soft tissues are present.

Anderson and Hobischak (2004) completed a similar study on the decomposition process of carrion in a marine environment. Freshly killed pigs were submerged at two different depths, 7.6 m and 15.2 m and secured so that they could either sink or float but were not able to drift away; time is recorded as the days elapsed since time of death (ESTD).

### 1.2.2 Application of Entomology to the Archaeological context: Archaeoentomology

Pettigrew (1834) discusses the unwrapping of mummies being the first scientific observations of archaeological flies. It was the entomologist F.W Hope who commented that the mummification process must be a lengthy process as he discovered two different species of fly puparia.

The most common insects seen within this specialisation are:

- **Flies**: fly remains e.g. puparia may give details about the presence and decay of organic material, season or exposure.
- **Ectoparasites**: fleas, lice and bedbugs which have a diet of human blood may help to interpret sanitary conditions.
- **Coleoptera**: beetles may indicate the environment and climate in which it was living.

In the early 1950's the Candelaria funerary cave located near San Pedro de las Colonias in the semi desert zone of the State of Coahuila, northern Mexico was excavated which produced many well preserved human remains within funerary bundles. The custom was used widely throughout America, from southwest USA to the Andes in which a corpse would be wrapped within a mat or cloth and bound with cords. The wrappings were thought to allow easier transport of the dead to the burial place. To some societies funerary bundles represented a chrysalis of a butterfly however there was no evidence to show that the Canderlia cave occupants shared this symbolism. Insects (*Table 5*) were recovered from different damaged parts of the funerary bundle and listed (Huchet *et al*., 2013a).

Current cases use Forensic entomology to estimate time since death and location of death, in addition Huchet (1996) and Dussault and Bain (2009) discuss a recently new field called Archaeoentomology, which is the collection and analysis of preserved insects from archaeological sites. Information gained through these findings can provide detailed information such as: environment, climate and living and burial conditions from the past. This discipline, that pulls together the principle protocols of Forensic Entomology and the aims of the archeoentomology has been created by Huchet (1996) and is called Funerary Archeoentomology. Reinhard and Araújo (2008) introduce the term Archaeoparasitology

to describe the study of all parasitological remains which have been excavated from both historic and recent archaeological contexts.

**Table 5: Summary of insect remains recovered from the funerary bundle sample set modified from (Huchet *et al*., 2013a).**

| Order | Family | Taxa | No. of Insects | *MNI |
|---|---|---|---|---|
| **Diptera** | Musicdae | *Synthesiomyia nudiseta* | >10 | 4 |
| **Diptera** | Fanniidae | *Fannia* spp. | 10 | 5 |
| **Diptera** | Sarcophagidae | Gen. spp. | >3 | 3 |
| **Coleoptera** | Dermestidae | *Dermestes* (*Dermestinus*) *carnivorus* | >9 | 5 |
| **Coleoptera** | Histeridae | *Saprinus alienus* | 1 | 1 |
| **Coleoptera** | Histeridae | *Xerosaprinus coerulescens* | 1 | 1 |
| **Coleoptera** | Histeridae | *Xerosaprinus vitiosus* | 1 | 1 |
| **Coleoptera** | Staphylinidae | Gen. spp. | 1 | 1 |
| **Coleoptera** | Anobiidae,Ptinidae | *Niptus* spp. | >10 | 8 |
| **Coleoptera** | Trogidae | *Omorgus* spp. | 2 | 1 |
| **Hymenoptera** | Formicidae | *Acromyrmex versicolor* | >10 | 3 |
| **Lepidoptera** | Tineidae ? | Gen. spp. | 1 | 1 |
| | | **Total** | >59 | 34 |

* *Minimal number of individuals*

The first case of canine ectoparasitosis was reported by Huchet *et al.* (2013b) in which well-preserved mummified dogs were discovered during archaeological investigations in El Dier, Egypt which were dated to Roman period (ca. 1$^{st}$ century A.D to 4$^{th}$ century A.D). One young dog, the time of death was estimated to be 4 to 5 months old was found to have clear signs of marked external parasitism. Under further examination still attached to the fur remnants was the presence of ticks, on inspection the right ear showed a high concentration of ticks. A sum of 61 tick specimens were collected from the dog and identified as 'brown dog tick' or *Rhipicephalus sanguineus* (Parasitiformis, Ixodidae). Twenty-three of these specimens were found still firmly attached to the inner

ear. *R. sanguineus* is also referred to as 'kennel tick' or 'pan tropical dog tick', the adults and nymphs are found attached to ears and shoulders of the host (*Table 6*).

**Table 6: Summary of ectoparasites and other insect remains recovered from the dog mummy (Huchet *et al.*, 2013b).**

| Order | Family | Taxa | Stage/Remains | Location |
|---|---|---|---|---|
| **Acarina** | Ixodidae | *Rhipicephalus sanguineus* | Adults/nymphs | Left ear/coat |
| **Diptera** | Hippoboscidae | *Hippobosca longipennis* | Adult | Coat |
| **-** | Calliphoridae | *Chrysomya albiceps* | Puparia (+few nymphs) | Coat/internal organs |
| **-** | Sarcophagidae | Gen. spp. | Puparia | Coat/internal organs |
| **Coleoptera** | Dermestidae | *Attagenus* sp. | Larval exuviae (cast skins) | Coat |
| **-** | - | *Anthrenus* sp. | Larval exuviae (cast skins) | Coat |
| **-** | - | *Dermestes* sp. | Larva (9$^{th}$ abdo. Segm.) | Internal organs |

### 1.2.3 Chronobiology and Forensic Entomology

Evolution of the circadian clock has allowed synchronization of behaviour, physiology and metabolism to the 24 hour geophysical cycles of the Earth. The understanding of the circadian clock mechanism is a crucial element of forensic entomology as it is able to control routines such as feeding, mating, ovipositing or emergence times (Vanin *et al*., 2012a).

Much research has been done with *Drosophila melanogaster* (Diptera: Drosophilidae) some of the work researched by Quinn *et al*. (1974); Salomon and Spatz (1983); Brembs and Heisenberg (2001) and Washington (2011) included the conditioning to colours and odours, research of colour vision and mating with different intensities of light.

Baldridge and colleagues (2006) investigated nocturnal ovipositing in necrophilous flies, and found that during 200 hours of bait being laid, nocturnal oviposition was recorded only once. Research of nocturnal ovipositing demonstrates a lack of consistency regards to the baits used (*Table 7*) as the foods used ranged from hedgehogs to pigs. One main observation found that certain species would not oviposit nocturnally on fresh bait but would wait for three to four days before laying their eggs. The authors that had successful results all observed a decline in the number of eggs laid at night when compared to daytime egg laying data.

**Table 7: Nocturnal oviposition data from different authors detailing bait, urban/rural area and light sources.**

| Bait | Light | R/U | Nocturnal species oviposition | Diurnal species oviposition | Author |
|---|---|---|---|---|---|
| **Hedgehogs** [T] | | U & R | None | *L.sericata*<br>*L. ampullacea*<br>*L.caesar*<br>*C.vicina* | (Amendt *et al.*, 2008) |
| **Beef liver** [F] | | U | None | *L. sericata*<br>*L.caesar*<br>*C.vicina*<br>*P. terraenovae* | |
| **Stock culture** [F] | LD 14:10 | N/A (Lab) | *Lucilia sericata* | - | |
| **White rat** [F] | Yes | U | None | None | (Baldridge *et al.*, 2006) |
| **White rat** [F] | No | U | None | None | |
| **Beef** [F] | N/A | R | *M.domestica*<br>*P.coeruleiviridis* | | |
| | Yes | R | *C.macellaria*<br>*N.bullata* | | |
| **Beef** [A] | Yes & No | R | None | None | |
| **Pig** [F] | - | - | | *P.coeruleiviridis*<br>*C.macellaria*<br>*M.domestica* | |
| **Ox liver** [F] | No | SR | None | *C.augar*<br>*C.dubai*<br>*C.hilli*<br>*C.vicina* | (George *et al.*, 2012) |
| **Rats** | Yes & No | U | *C.vicina*<br>*P.sericata* | N/A | (Greenberg, 1990b) |
| **Ground Beef** | Yes & No | U | *P.regina*<br>*P.sericata* | N/A | |
| **Beef liver** | No | R | *C. megacephala*<br>*Sarcophagidae sps.* | | (Pritam and Jayaprakash, 2009) |
| **Mutton** [T] | No | U | *C.megacephala*<br>*C.rufifcaies* | C.vicina | (Singh and Bharti, 2001) |
| **Mutton** [F] | No | U | *S.albiceps*<br>*S.hirtipes* | N/A | (Singh and Bharti, 2008) |
| **Rats** [T] | Yes & No | U&R | None | N/A | (Stamper and Debry, 2007) |
| **Rats** [T] | Yes & No | U & R | None | N/A | (Stamper *et al.*, 2009) |
| **Pork**[F] | No | N/A (Lab) | *Lucilia* spp.<br>*C.putoria*<br>*C.chloropyga* | *Lucilia* spp.<br>*C.putoria*<br>*C.chloropyga*<br>*C.megacephala* | (Williams, 2003) |
| **Rats**[T] | No | U | *Lucilia* spp. | | |
| **Pigs** [F] | No | R | None | *L.coeruleivirdis*<br>*P.regina*<br>*C. vomitoria*<br>*L.sericata*<br>*C.vicina* | (Zurawski *et al.*, 2009) |

*[T] = thawed, [F] = fresh, [A] = aged, R = rural, U= urban, SR= semi-rural*

### 1.2.4 Myiasis

Myiasis refers to the infestation of living tissue from either animals or humans by Diptera larvae. Human myiasis cases are more common in warmer climates and is prevalent in third world countries where many poor people live in unsanitary conditions, are malnourished and where the quality of health care is not first rate. Myiasis has also been stated to occur around the globe in different environments. In the veterinary field, myiasis is often referred to by other names e.g. fly-blown, fly strike (Rossi-Schneider *et al*., 2007). Myiasis is also reported to occur in both rural areas infection of livestock and domestic pets such as cats and dogs (Gabriel *et al*., 2008).

In 1840, a paper titled 'On insects and their larvae occasionally found in the human body' written by Hope was amongst the earliest discussions about human myiasis (Hope, 1840).

Human myiasis is commonly caused by families of Calliphoridae, Sarcophagidae, Phoridae, Stratiomyidae and Oestridae however many other families of Diptera have been reported in myiasis cases. The majority of reported cases in human myiasis are known to be facultative (i.e. under varying conditions) wound myiasis mainly caused by Calliphorids (Sherman, 2000; Huntington et al., 2008). Dutto and Bertero (2011) comment that human myiasis may fall into one of two categories: specific myiasis (the flies require a live host for larvae to develop) and semi-specific myiasis (development that occurs in decomposing organic waste but occasionally may develop in/on living organisms) which does not cause tissue lesions, both these terms are also referred to as obligatory and facultative myiasis (Amendt, 2010).

Many cases of myiasis acquired in a hospital environment are often under reported or not reported at all, (*Table 8*) shows the reported cases between the years 1980 to 1998 (Joo and Kim, 2001).

**Table 8: Reported cases of nosocomial myiasis between 1980 and 1998 (Joo and Kim, 2001).**

| Diagnosis | Site of larvae | Age/Sex | Country | Author |
|---|---|---|---|---|
| Diabetic foot infection | Foot wound | 73 / NA | Germany | (Mielke and Schlote, 1980) |
| Diabetic foot infection | Foot wound | 66 / NS | Germany | |
| Heart surgery | Sternal wound | 73 / F | United States | (Jacobson *et al.*, 1980) |
| Heart surgery | Nose | 67 / M | United States | |
| Encephalopathy | Comatose | 8 / M | United States | (Magnarelli and Andreadis, 1981) |
| Multiple stab wounds | Sedated | 25 / M | India | (Gupta *et al.*, 1983) |
| Cerebrovascular accident | Comatose | 65 / F | Jamaica | (Rawlins and Barnett, 1983) |
| Terminal illness | NS | NS / M | United States | (Greenberg, 1984) |
| Renal failure | NS | NS / F | United States | |
| Diabetic hypersomolarity | Comatose | 64 / M | United States | (Smith and Clevenger, 1986) |
| Diabetic | Eyelid reconstruction | 63 / M | United States | (Bosniak and Schiller, 1990) |
| Diabetic | Leg ulcer | 57 / F | England | (Burgess and Davies, 1991) |
| Peripheral vascular disease | Leg ulcer | 79 / M | England | (Burgess and Spraggs, 1992) |
| Myocardial infarct | Tracheostomy wound | 82 / F | Canada | (Josephson and Krajden, 1993) |
| Vulvar condyloma | Vulvar wound | 19 / F | Honduras | (de Kaminsky, 1993) |
| Retropharyngeal abscess | Nose | 2 / M | Honduras | |
| Squamous cell carcinoma | Left temple wound | 73 / M | England | (Phillips and Marsden, 1993) |
| Right facial palsy | Oral cavity | 71 / M | Korea | (Chung *et al.*, 1996) |
| Premature | Vaginal orifice | Infant/ F | Israel | (Amitay *et al.*, 1998) |

In a first of its kind, a case reported by Benecke and Lessig, (2001) used an entomological approach to determine if neglect was evident prior to the death of a child. *Muscina stabulans* (Diptera: Muscidae), *Fannia canicularis* (Diptera: Fanniidae) and *Calliphora vomitoria* (Diptera: Calliphoridae) were found at the scene. *M. stabulans* and *F. canicularis* can both be found in an indoor environment. *M. stabulans* is highly attracted to human faeces but not as attracted to human cadavers, whilst *F. canicualris* is attracted to both urine and faeces, whilst *C. vomitoria* is amongst one of the first colonisers in cadavers. By collecting the insect evidence and determining the age of the developmental stages found, it was shown that the anal genital region of the child had not been cleaned for ~14 days whilst death occurred only 6-8 days prior to discovery.

In cases of neglect of the elderly not all cases involve violence and therefore the deceased may die from natural causes. Over the years there has become an increased awareness of malpractice cases towards the elderly in both the professional and personal environments. Determining if a care giver is guilty is difficult to judge however by bringing entomology in to the case helps to contribute further information surrounding the case that may have been originally missed.

Benecke *et al.* (2004) reported three cases in which not all cases were of neglect. The first case involved an elderly woman who was found in her apartment. The apartment was reported as being clean and only larvae and dead flies of *M.stabulans* were collected. By determining the developmental data it was decided that the minimal interval was around 21 days which would suggest misconduct by the carer. However in this case the elderly lady had dismissed her care giver and was not prosecuted for misconduct. In another case an elderly woman was found in her apartment which was described as untidy from non-organic items. Adult flies and larvae of *F.canicularis*, *M. stabulans* and adult *Dermestes lardarius* were found within the apartment. These species are often found inside human housing, however the finding of *F.canicularis* which is attracted by faeces and urine suggest some form of neglect as none of the species found had fed off the corpse.

### 1.2.5 Entomotoxicology

Introna *et al*. (2001a) comment that due to their feeding preferences of decomposing human tissue carrion feeding insects can be used for toxicological analysis for the purpose of identifying toxins and drugs in cases of poisoning or possible overdoses. Toxicological analyses include gas chromatography (GC), radioimmunoassay (RIA), gas chromatography/mass spectrometry (GCMS) and high performance liquid chromatography-mass spectrometry: the analysis used for human samples and biological fluids can be used for drug quantification from insect specimens (Campobasso *et al*., 2004b).

Entomologists began using insects to detect drugs from the 1980's. When the body is skeletonised and there is a complete absence of suitable tissue samples i.e. urine, blood or internal organs, then insects may be viewed as an alternative solution. Determining if drug abuse was a factor just before death is of major interest in entomotoxicology (Beyer *et al*., 1980).

Necrophagous species are often recommended for entomotoxicological analyses as they are often the first species to colonise the cadaver and their development is well studied as they are used to estimate post mortem intervals. Differences in drug concentrations are observed between the different instar feeding activities e.g. puparia drug concentrations are observed to be lower when compared with larvae. As development and oviposition may be affected by temperature and photo-periods, the toxicologist must have an understanding of the environmental factors that may affect insect development. Numerous drugs (*Table 9*) have been detected in insect tissues in many forensic cases however due to the differences in procedures e.g. oral/injection, drug stability, time before analysis and bacterial metabolism, requires research to understand how these parameters may affect the interpretation of the results (Gosselin *et al*., 2011b).

A study by Kintz *et al*. (1990c) found that drug concentrations were more stable in fly larvae than in post-mortem tissues with a greater sensitivity being obtained using fly larvae. Insects are often present for long periods of time and in large quantities when toxicological samples have diminished.

A review of publications featuring toxins and the name and development stage of the insect species along with the authors are listed in (*Table 9*).

**Table 9: Review of publications reporting toxic substances detected during different developmental stages of insects (Gosselin *et al.*, 2011b).**

| Toxic Substance | | Insect species | Developmental stage | Reference |
|---|---|---|---|---|
| **Alcohol** | Ethanol | Calliphoridae, Sarcophagidae | L | (Definis-Gojanović *et al.*, 2007) |
| | | *Phormia regina* | L | (Monthei, 2009) |
| **Drugs** | | | | |
| **Antidepressants** | Amitryptyline | Not specified | L | (Tracqui *et al.*, 2004) |
| | | *Lucilia sericata* | L | (Campobasso *et al.*, 2004b) |
| | | *Calliphora vicina* | C | (Wilson *et al.*, 1993) |
| | | | L, P | (Sadler *et al.*, 1997b, Sadler *et al.*, 1995) |
| | | *Dermestes maculatus* | E,F,Pu | (Miller *et al.*, 1994) |
| | | *Megaselia scalaris* | E, F, Pu | (Miller *et al.*, 1994) |
| | Clomipramine | Not specified | L | (Tracqui *et al.*, 2004, Kintz *et al.*, 1990a) |
| | | *Lucilia sericata* | | (Campobasso *et al.*, 2004b) |
| | Dothiepin | Not specified | L | (Tracqui *et al.*, 2004) |
| | Fluoxetin | Not specified | L | (Tracqui *et al.*, 2004) |
| | Nortriptyline | *Lucilia sericata* | L | (Campobasso *et al.*, 2004b) |
| | | *Dermestes maculatus* | | (Miller *et al.*, 1994) |
| | | *Megaselia scalaris* | | (Miller *et al.*, 1994) |
| | Trazodone | *Calliphora vicina* | L | (Sadler *et al.*, 1995) |
| | Trimipramine | *Calliphora vicina* | L | (Sadler *et al.*, 1995) |
| | Venlafaxine | Not specified | L | (Tracqui *et al.*, 2004) |
| **Barbiturates** | Amobarbital | Not specified | L | (Tracqui *et al.*, 2004) |
| | Barbiturates | Not specified | L | (Tracqui *et al.*, 2004) |
| | Phenobarbitol | *Cochliomyia macellaria* | L | (Beyer *et al.*, 1980) |
| | | *Lucilia sericata* | L | (Campobasso *et al.*, 2004b) |
| | | Not specified | L | (Tracqui *et al.*, 2004, Kintz *et al.*, 1990a) |

| | | | | |
|---|---|---|---|---|
| | Secobarbital | Not specified | L | (Levine *et al*., 2000) |
| | Sodium amylobarbitone | *Calliphora vicina* | L, P | (Sadler *et al*., 1997c) |
| | Sodium barbitone | *Calliphora vicina* | L, P | (Sadler *et al*., 1997c) |
| | Sodium brallobarbitone | *Calliphora vicina* | L, P | (Sadler *et al*., 1997c) |
| | Sodium phenobarbitone | *Calliphora vicina* | L, P | (Sadler *et al*., 1997c) |
| | Sodium thiopentone | *Calliphora vicina* | L, P | (Sadler *et al*., 1997c) |
| **Benzodiazepines** | Alprazolam | Not specified | L | (Tracqui *et al*., 2004) |
| | | *Calliphora vicina* | L, P | (Wood *et al*., 2003) |
| | Bromazepam | Not specified | L | (Tracqui *et al*., 2004) |
| | | *Piophilia casei* | L, P, A | (Kintz *et al*., 1990b) |
| | Clonazepam | *Calliphora vicina* | L, P, A | (Wood *et al*., 2003) |
| | Diazepam | *Calliphora vicina* | L, P, A | (Wood *et al*., 2003) |
| | | Chrysomya albiceps | | (Carvalho *et al*., 2001) |
| | | Chrysoma putoria | | (Carvalho *et al*., 2001) |
| | Flunitrazepam | *Calliphora vicina* | L, P, A | (Wood *et al*., 2003) |
| | Lorazepam | Not specified | L | (Tracqui *et al*., 2004) |
| | | *Calliphora vicina* | L, P, A | (Wood *et al*., 2003) |
| | Nordiazepam | Not specified | L | (Tracqui *et al*., 2004) |
| | | *Calliphora vicina* | L, P, A | (Wood *et al*., 2003) |
| | Oxazepam | Not specified | L | (Tracqui *et al*., 2004, Kintz *et al*., 1990a) |
| | | *Calliphora vicina* | L, P, A | (Wood *et al*., 2003) |
| | Prazepam | *Calliphora vicina* | L, P, A | (Carvalho *et al*., 2001) |
| | Temazepam | *Calliphora vicina* | L, P, A | (Sadler *et al*., 1995, Wood *et al*., 2003) |
| | Triazolam | Not specified | L | (Tracqui *et al*., 2004) |
| | | *Calliphora vicina* | L, P, A | (Kintz *et al*., 1990a) |
| | | Not specified | L | (Kintz *et al*., 1990c) |
| **Miscellaneous** | Amphetamine | Not specified | L | (Definis-Gojanović *et al*., 2007) |
| | | *Calliphora vicina* | L | (Sadler *et al*., 1997c) |

| | | | | |
|---|---|---|---|---|
| | Benzoylecgonine | Not specified | L | (Nolte *et al*., 1992) |
| | Cocaine | Not specified | L | (Nolte *et al*., 1992, Manhoff *et al*., 1991) |
| | | *Lucilia sericata* | L | (Campobasso *et al*., 2004b) |
| | Digoxin | Not specified | L | (Tracqui *et al*., 2004) |
| | Meprobamate | Not specified | L | (Tracqui *et al*., 2004) |
| | Nefopam | Not specified | L | (Tracqui *et al*., 2004) |
| | Sodium aminohippurate | *Calliphora vicina* | L | (Sadler *et al*., 1997c) |
| | Sodium salicylates | *Calliphora vicina* | L | (Sadler *et al*., 1997c) |
| | THC-COOH | Not specified | L | (Tracqui *et al*., 2004) |
| | 11-Hydroxy-THC | Not specified | L | (Tracqui *et al*., 2004) |
| **Opioids/opiates** | Codeine | *Lucilia sericata* | L, P, A | (Kharbouche *et al*., 2008) |
| | | Not specified | L | (Tracqui *et al*., 2004, Kintz *et al*., 1994) |
| | Methadone | *Lucilia sericata* | L | (Gosselin *et al*., 2010) |
| | Morphine | *Dermestes freshi* | L, P, A | (Bourel *et al*., 2001b, Bourel *et al*., 2001c) |
| | | Thanatophilus sinuatus | L, P, A | (Bourel *et al*., 2001b, Bourel *et al*., 2001c) |
| | | *Lucilia sericata* | L, P, Pu, A | (Bourel *et al*., 2001b, Hédouin *et al*., 1999) |
| | | Calliphora stygia | L | (Gunn *et al*., 2006) |
| | | | L, P, Pu, PP, A | (Parry *et al*., 2011) |
| | | *Calliphora vicina* | L, Pu | (Introna *et al*., 2001b) |
| | | *Calliphora vicina* | L, P | (Hédouin *et al*., 2001) |
| | | *Protophormia terraenovae* | L, P | (Hédouin *et al*., 2001) |
| | | Not specified | L | (Tracqui *et al*., 2004, Kintz *et al*., 1994) |
| | Opiates | *Lucilia sericata* | L | (Campobasso *et al*., 2004b, Introna Jr *et al*., 1990) |
| | Pholcodine | Not specified | L | (Tracqui *et al*., 2004) |

| | | | | |
|---|---|---|---|---|
| | Propoxyphene | Not specified | L | (Tracqui *et al*., 2004) |
| | | *Calliphora vicina* | C | (Wilson *et al*., 1993) |
| **Phenothiazine** | Alimezanine | Not specified | L | (Tracqui *et al*., 2004, Kintz *et al*., 1990a) |
| | Cholopromazine | Not specified | L | (Tracqui *et al*., 2004) |
| | Cyamezanine | Not specified | L | (Tracqui *et al*., 2004) |
| | Levomepromazine | Not specified | L | (Tracqui *et al*., 2004) |
| | | *Lucilia sericata* | L | (Campobasso *et al*., 2004b) |
| | | *Piophila casei* | L | (Kintz *et al*., 1990b) |
| | Thioridazine | *Lucilia sericata* | L | (Campobasso *et al*., 2004b) |

*L = larvae, P = pupa, PP = prepupa, A = adult, C= crop, F = frass, E = exuvia*

### 1.2.6 Imaging

The morphology of insects throughout their developmental stages has been conducted using both stereo and compound microscopes however advances in technology over the years have seen images using the scanning electron microscope, Transmission Electron Microscope, these techniques may have required considerable sectioning, dissection and more frequently the complete destruction of the specimen.

A newer imaging technique is that has been applied in insect morphology since the 1990's is the computer based 3D reconstruction however due to the early software programmes being used mainly for product design, many man working hours were required in the application of biological specimens. Today, software programmes have been much improved with minimal amount of time being needed to reconstruct the images (Friedrich and Beutel, 2008). Current X-ray micro-CT instruments are based on a similar principle to medical CT scanners. The specimen rotates inside the x-ray beam and the x-ray detector; this then acquires a large number of 2D angular projections which allows for the reconstruction of 3D imagery. For good imaging control with insects a low energy level is required as insects have a weak absorption due to their size (Gui *et al*., 2010).

Traditional approaches may make the analysis more subjective than objective which may result in a high level of error. It is the development of new technologies that allow for the increasing detail of anatomical and morphological changes. One such piece of technology is the Micro-computed tomography (micro-CT). Richards and colleagues (2012b) found that by using micro-CT with *Calliphora vicina* (Diptera: Calliphoridae) they were able to demonstrate the anatomical changes taking place within the pupae which could help to determine an accurate PMI. Stained specimens (aqueous Iodine 0.5 M solution) were mounted in a plastic drinks straw and scanned using energy at 80kV with current ranging from 50 to 100 µA. Reconstructions were done using CT pro 2.1 software (Nikon Metrology), renderings and slice stacks were created using VG Studio Max 2.1. Stained and unstained pupae at different development stages were dissected from the pupal cases and the specimens were photographed using light microscopy and external morphology was described. Overall it was determined that the specimens which had been stained for 7 days had the highest differentiation between tissue types. The disadvantages mentioned against using the micro CT are that the pupae would have to be

killed prior to imaging; this may cause problems where there are a limited number of specimens whilst the advantages include non-destructive and fast results.

Greco and colleagues (2011) discuss a new method for the non-invasive imaging of insects at microscopic level, it comes in the form of conventional micro focus and synchrotron based Micro CT, these are similar methods, currently used with medical CT scanners. This method allowed Greco and colleagues to be able to visualise an ancient social bee that had been preserved in amber however three different systems were used to perform these scans:

- A commercial bench top system (TOMOLAB)

  Energy = 40kV, Current = 200µA, Source to sample distance = 12cm, Isotropic voxel size = 8µm, Exposure time = 2.7s, Number of projections = 2400 over 360$^{o}$, Measurement time = 1h 48mins.

- SYRMEP beamline

  Energy = 15keV, Isotropic voxel size = 9µm, exposure time = 0.9s number of projections = 1800 over 180$^{o}$, measurement time = 1h 48mins

- Micro CT 40 system

  Energy = 45kV, Current = 177µA, Isotropic voxel size = 10µm, Number of projections = 1000 over 180$^{o}$, Measurement time = 10.5h.

Greco and colleagues concluded that they were able to accurately assess and visualise the anatomical characteristics of the ancient social bee using all three systems.

De Almeida and colleagues (2011) mention that to be able to comprehend an insect's development and function it is vital to be able to understand their anatomical structure. Traditionally anatomy and morphology is understood by using techniques such as dissection or by taking histological slices. Commonly used equipment that allows the viewing of 2D specimens Includes SEM (Scanning Electron Microscope) and either a stereo or compound microscope. By using x-ray computed tomography de Ameida and colleagues were able to research the microanatomy of *Rhodhinus prolixus* (Hemiptera, Reduviidae). Useful energy ranges are discussed and determined to be between 8 to

35keV. Methods include using 17keV at a distance of 10cm from the specimen position to allow the phase contrast technique to perform.

Post mortem CT scans have previously been used by Johnson and colleagues (2012) to determine the volume of maggot masses on deceased people. Maggot masses can generate a temperature increase which may cause increased growth rates and could be problematic for the entomologist also any physical evidence that may be on the cadaver could be destroyed. It was concluded that CT scanning was an accurate method for forensic cases in estimating and identifying the size of maggot masses and also helped with thermogenesis research.

A micro-CT study of overwintering seven spotted ladybird beetles *Coccinella septempuctata* (Coleoptera: Coccinellidae) was undertaken by Bell *et al*. (2012) in which different energy levels varied between 30kV and 50 kV. The lining of the malpighian tubules were visible on the scan and appeared to contain a dense radio-opaque material. Malpighian tubules are part of the excretory system; their openings are positioned at the mid and hind gut and they are variable in numbers as well as in form. Abdominal dissections were also done in 5 specimens to confirm their findings.

X-ray phase sensitive imaging and *PITRE* (Phase-sensitive X-ray Image processing and Tomography Reconstruction) software is discussed by Chen *et al*. (2012), this method uses phase shifts rather than absorption information which is currently used in x-ray absorption imaging/CT as the imaging signal. This allows the extension of possibilities of x-ray absorption imaging. Progress made in detector development allows the decrease of the pixel size whilst increasing the pixel number to allow for better reconstructions.

## 1.3 Introduction to Phoridae (Diptera)

Phoridae (Diptera) are made up of a large family of flies with ~ 3000 world species of which 300 occur in the British Isles (*Table 10*). They are often medium to minute in size and can be brown, black or yellowish in colour and have a distinctive wing venation (*Fig 3*). The hump backed fly feeds on a variety of different foods (polyphagous) as well as dead or decaying matter (sacrophagous) (Campobasso et al., 2004a; Disney, 2008). Some of these flies have been found to develop on peculiar media such as ripe banana (Karunaweera *et al*., 2002), boot polish (Lever, 1944) and emulsion paint (McCrae, 1967).

Phoridae are commonly referred to as humpbacked flies (due to their arched thorax when viewed from the side), coffin flies (due to their ability to burrow down into soil) and scuttle flies (due to their rapid surge of scuttling movement across surfaces rather than flying). Predominantly a warm climate species (Mediterranean distribution) but have been reported to have been found in central Europe as far north as Belgium, Netherlands and England and have also been found in Northern America (Disney, 2008).

### 1.3.1 Phoridae importance in Forensic Entomology

Phoridae are recorded to be amongst the fifth wave of insects *(Table 2)* that inhabit an exposed human cadaver. The cadaver at this stage is between four to eight months old depending on the climate, latitude, altitude, exposure, etc., the body would be fermenting ammonia and releasing a thin, fetid, greenish fluid consisting of serum and pus (Smith, 1986).

Environmental parameters may affect the colonisation of a cadaver, some of these parameters include: weather, geography, water, air exposure, sun exposure, bodies of water and moisture levels.

A delay in blowfly (Calliphoridae) arrival due to lack of entry (i.e. closed doors and windows) may be observed when a cadaver is located indoors, as a closed property could confine the odour of decomposition, therefore PMI using blowflies may be unreliable. The lack of entry for the larger flies allows much smaller flies such as scuttle flies (Phoridae) to be the primary ovipositor, suggesting in a case of similar circumstances

that the PMI for the Phoridae (*Table 10*) would be more reliable than that of the blowfly (Calliphoridae) (Reibe and Madea, 2010).

**Table 10: Summary of Phoridae genera (McAlpine, 1987).**

| | | | |
|---|---|---|---|
| *Abaristophora* | *Acontistoptera* | *Aenigmatias* | *Anevrina* |
| *Apocephalus* | *Auxanommatidia* | *Bactropalpus* | *Beckerina* |
| *Borophaga* | *Cataclinusa* | *Chaetopleurophora* | *Chonocephalus* |
| *Citrago* | *Commoptera* | *Conicera* | *Coniceromyia* |
| *Cremersia* | *Crinophelba* | *Diocophora* | *Diplonevra* |
| *Dohrniphora* | *Ecitomyia* | *Gymnophora* | *Hypocera* |
| *Lecanocerus* | *Megaselia* | *Metopina* | *Myriophora* |
| *Myrmosicarius* | *Pericyclocera* | *Phalacrotophora* | *Phora* |
| *Physoptera* | *Plastophora* | *Pseudacteon* | *Puliciphora* |
| *Rhyncophoromyia* | *Spiniphora* | *Stenophorina* | *Stichillus* |
| *Syneura* | *Triphleba* | *Trophithauma* | *Trophodeinus* |
| *Woodiphora* | *Xanionotum* | *Zyziphora* | |

Phoridae encountered in case studies between the period of June 2007 and June 2009 in Penang, Malaysia was reported by Thevan *et al*. (2010). All specimens were collected from decomposed cadavers during the autopsy in the Forensic Medicine Department, Penang Hospital. The first case comprises of a fully clothed 78 year old cadaver which was found lying face down on its right side and was in the mummified stage of decomposition. The insects present were collected and sampled. Two adult females and four puparia of *Megaselia scalaris* were identified. Phoridae pupae were located beneath the clothing, on the surface of the abdomen and on the right foot in second case involving a fully clothed cadaver which was in active decay. One pupae of *M.scalaris* was sampled, other Phoridae sampled compromised of *M.spiracularis*, *M.curtineura*.

Motter (1898) studied the fauna of the grave, 100 graves were exhumed over different time periods. In 37 of the burials, Phoridae puparia were identified and but not the species (*Table 11*). A further 50 burials (

*Table* 12) were exhumed, Phoridae puparia were found in 6 of the burials however the findings of the examinations were either unreliable or the specimens were not available.

To determine the entomofauna of buried bodies, skeleton studies were conducted. Unclaimed bodies from Municipal Cemetery of La Plata were donated to School of Medical Sciences, National University at La Planta for research and teaching purposes. The skeletons were received in numbered plastic bags which included sediments and external wrappings and death records (age, sex, nationality, date and cause of death, location at cemetery and date of exhumation) were also included. Insect remains were collected from an exhumed bundle of a 5 month male whose cause of death was non-traumatic cardiorespiratory arrest. The body had been buried into a 40cm deep grave underground in a soft wooden coffin which was made for rapid decomposition; the body had been clothed in woollens and a disposable nappy as well as wrapped in two woollen blankets. The exhumed bundle revealed insect species of forensic importance. *Megaselia scalaris* were amongst the first taxa to colonise the buried remains along with *Fannia canicularis* (Diptera: Fanniidae), *Muscina stabulans* (Diptera: Muscidae) and *Ophyra aenescens** (**see abbreviation)* (Diptera: Muscidae) (Mariani *et al*., 2014).

Bugelli *et al*. (2014) presented eight cases found between the months of June and November in central Italy in which bodies of elderly people and socially isolated people were discovered. Insects were collected during the body recovery and post mortem. In 75 % of the cases both Sarcophagidae and Calliphoridae species were present whilst 50 % of the cases had *Lucilia sericata* and *Chrysomya albiceps* and scuttle flies (Phoridae) were found in 37.5 % of the cases which confirmed the ability of indoor colonisation by this species.

**Table 11: Phoridae puparia found on 100 human cadavers buried for different periods of time (Motter, 1898).**

| Period of Interment | | Depth of grave (ft) | Character of soil | Degree of moisture | No. of Puparia | Period of Interment | | Depth of grave (ft) | Character of soil | Degree of moisture | No. of Puparia |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **3 years** | **1 month** | 5 | sc | 3 | 5 | 16 years | **5 months** | 9 | s | 2 | 218 |
| **3 years** | **2 months** | 6 | sc | 4 | 174 | **18 years** | **11 months** | 3 | s | 1 | 20 |
| **3 years** | **3 months** | 3 | s | 1 | 19 | **20 years** | **0 months** | 3 | s | 1 | 99 |
| **3 years** | **6 months** | 5 | sc | 2 | 280 | **20 years** | **- months** | 9 | s | 1 | 40 |
| **3 years** | **6 months** | 5 | sc | 2 | 291 | **20 years** | **3 months** | 5 | s | 1 | 48 |
| **4 year** | **1 month** | 5 | sc | 2 | 286 | **20 years** | **7 months** | 4 | s | 1 | 92 |
| **4 year** | **1 month** | 3 | sc | 1 | 306 | **20 years** | **9 months** | 6 | s | 1 | 45 |
| **4 year** | **1 month** | 5 | sc | 2 | 315 | **21 years** | **- months** | 3 | s | 1 | 38 |
| **4 year** | **2 month** | 5 | sc | 2 | 314 | **21 years** | **- months** | 3 | s | 1 | 31 |
| **5 years** | **5 month** | 5 | sc | 3 | 250 | **21 years** | **- months** | 6 | s | 1 | 51 |
| **6 years** | **0 months** | 5 | sc | 2 | 205 | **21 years** | **- months** | 3 | s | 1 | 54 |
| **7 years** | **0 months** | 4 | sc | 2 | 405 | **21 years** | **- months** | 4 | s | 1 | 62 |
| **7 years** | **7 months** | 5 | sc | 2 | 373 | **21 years** | **- months** | 6 | s | 1 | 64 |
| **9 years** | **9 months** | 5 | sc | 2 | 240 | **21 years** | **- months** | 4 | s | 1 | 68 |
| **10 years** | **- months** | 5 | sc | 3 | 228 | **21 years** | **- months** | 6 | s | 1 | 77 |
| **10 years** | **- months** | 5 | sc | 2 | 239 | **21 years** | **- months** | 6 | s | 1 | 82 |
| **12 years** | **11 months** | 6 | s | 1 | 89 | **27 years** | **- months** | 8 | s | 1 | 130 |
| **15 years** | **5 months** | 7 | s | 1 | 96 | **38 years** | **- months** | 6 | s | 1 | 126 |
| **15 years** | **10 months** | 4 | sc | 4 | 189 | | | | | | |

*Degree of moisture: 1= dry, 2= moist, 3= wet, 4= coffin submerged*

*Character of soil: s = sandy, c = clay, sc = sand/ clay mix*

**Table 12: Phoridae puparia found on 50 human cadavers buried for different periods of time (Motter, 1898).**

| Period of Interment | | Depth of grave (ft) | Character of soil | Degree of moisture | No. of Puparia | Other descriptions |
|---|---|---|---|---|---|---|
| **7 years** | **3 months** | 4 | sc | 2 | | No cadaver in coffin |
| **11years** | **0 months** | 5 | s | 1 | | |
| **11 years** | **7 months** | 5 | s | 3 | ? | |
| **14 years** | **5 months** | 6 | - | 1 | | Dry rotten rock |
| **14 years** | **8 months** | 5 | s | 1 | | |
| **24 years** | **5 months** | 5 | s | 1 | | |

*Degree of moisture: 1= dry, 2= moist, 3= wet, 4= coffin submerged*

*Character of soil: s = sandy, c = clay, sc = sand/ clay mix*

### 1.4 *Megaselia scalaris*

Phoridae have over 3000 world species, *Megaselia* one of the Phoridae genera, alone has over 1400 species worldwide and is the largest genus in the Phoridae family. *Megaselia scalaris* (Diptera: Phoridae) is a species that primarily favours a warmer climate however this species has been carried around the world by unsuspecting humans and observations and identification of this fly are becoming more common around the world. This species is able to survive in environments that avoid frost but when the temperatures begin to drop can move indoors to the warmer temperatures to breed (Turchetto and Vanin, 2004; Disney, 2008).

*M.scalaris* is a small (2 to 3 mm) blackish, brownish and yellowish minute fly with a small, rather flattened head. The thorax has a characteristic humpbacked appearance; the wings are commonly large with a distinctive venation (*Fig 3*). The legs are usually well developed with a stout, enlarged, laterally compressed hind femur. This species are also referred to as coffin fly, humpback and scuttle flies (McAlpine, 1987). *M.scalaris* has six stages to its life cycle, the adult (*Fig 2*), the egg (*Fig 16*), first instar larval stage (*Fig 15*), second instar larval stage (*Fig 16*), third instar larval stage (*Fig 17*) and pupa (*Fig 19*).

*M.scalaris* are frequently found in exhumed bodies and soils which supports the 'coffin fly' status (Campobasso et al., 2004a; Mariani et al., 2014). They are also reported to be amongst the fifth colonisation wave of insects (*Table 1*) that inhabit an exposed human cadaver. The cadaver at this stage is between four to eight months old, the body would be fermenting ammonia and releasing a thin, fetid, greenish fluid consisting of serum and pus. Observations show when a human cadaver is indoors with limited access to the larger flies, amongst the first wave of insects attracted is *M.scalaris* (Oliva, 2002; Reibe and Madea, 2010; Feng and Liu, 2013).

New reports of *M.scalaris* being present around the world in crime scenes or present on cadavers are becoming frequently reported. During the period 2005 to 2010, a review of forensically important entomological specimens that were collected in Malaysia from human cadavers to update the one previously carried out by (Reid, 1953; Lee et al., 1984; Lee, 1996; Hamid et al., 2003; Lee et al., 2004). Eighty cases in total were recorded

and 93 specimens were collected. 1.08% of specimens were identified as *M.scalaris* (Kavitha *et al*., 2013).

Trappings were conducted in Buenos Aires city, Argentine by Oliva (2001) using beef bait to determine the succession of insects (*Table 13*). The data shows that when a hiatus appeared in the month of February (middle of summer) and March-April (early fall) that *M.scalaris* had partially filled the traps.

Carvalho *et al.* (2004) explored succession using the carcasses of two domestic pigs (*Sus scrofa* L) weighing 17 kg. The carcasses were in an exposed open urban area in Campinas, Brazil and in direct sunlight. The experiment was run between the months of August and September. Further succession data was provided by Faria *et al*. (2013) in which *M.scalaris* was found only to be present in the forest during the dry decay stage during the humid season (October to April) and were not present at all during the dry season (May to September).

**Table 13: Monthly data of insect activity during 1996-1997 (Oliva, 2001).**

| Month | *C.vicina* | *P.sericata* | *Parasarcophaga* spp | *M.scalaris* |
|---|---|---|---|---|
| **January** | Not found | No oviposition | All stages | All stages |
| **February** | Not found | No oviposition | All stages | All stages |
| **March** | Not found | Not found | Not found | Larvae |
| **April** | Not found | Not found | Not found | Not found |
| **May** | Oviposition | Not found | Not found | Larvae, pupae |
| **June** | All stages | Not found | Not found | Pupae, adults |
| **July** | All stages | Not found | Not found | All stages |
| **August** | All stages | Not found | Adults | Not found |
| **September** | All stages | Not found | Larvae | Larvae, pupae |
| **October** | All stages | Oviposition | All stages | Pupae |
| **November** | All stages | All stages | All stages | All stages |
| **December** | All stages | All stages | All stages | Not found |


**Fig 2: Lateral view of an adult *Megaselia scalaris* fly, scale = 500µm, x40 magnification.**

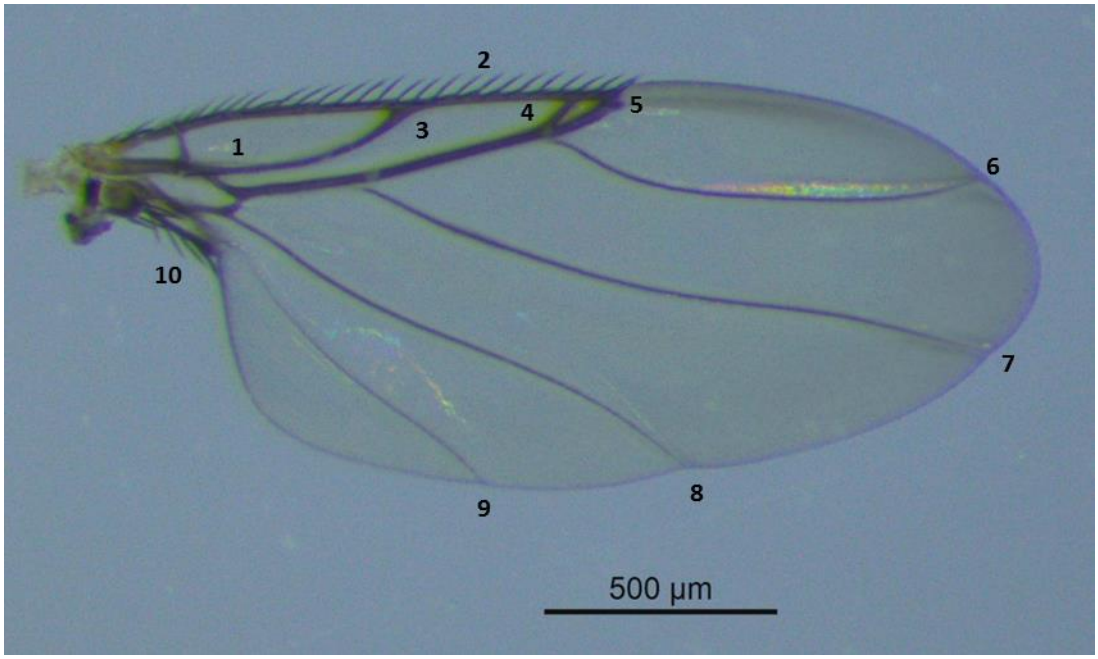



**Fig 3: Morphology of a left wing belonging to *Megaselia scalaris* (Diptera: Phoridae), scale = 500µm, x40 magnification.**
**1=Subcosta vein, 2 = costa cilia, 3= Radial 1, 4= Radial 2 + Radial 3, 5 = Radial 4 + Radial 5, 6= Medial 1, 7= Medial 2, 8= Anterior cubital 1, 9 = Anterior branch of cubitus 2 and anal vein 1, 10 = axillary bristles (McAlpine *et al*., 1981).**

Different fly species are used to calculate the PMI in warmer weather but it is understood that insect species are less active during the colder seasons. *M.scalaris* could be found over the whole year when researchers collected necrophagous insects throughout one calendar year in Germany (Schroeder *et al.*, 2003).

There has been much research done investigating development and growth rates of *M.scalaris*, as it is important to a forensic case to know at what temperature larvae were reared as the length of larvae may be used to give an estimation of age. Journal articles published on the duration of development of *M.scalaris* have been summarised below *(Table 14)*.

**Table 14: Duration of development of *Megaselia scalaris* by different authors (Disney, 2008).**

| °C | E | °C | E-FL | °C | E-PF | °C | E-P | Sex | Reference |
|---|---|---|---|---|---|---|---|---|---|
| **15** | 2 | - | - | 15 | 20.4-22.7 | 15 | (.45/-) | M+F | (Prawirodisastro and Benjamin, 1979) USA |
| **25** | 0.7 | - | - | 25 | 7.6-8.4 | 25 | 17.2-18.4 | M+F | " |
| **20** | 1.1 | - | - | 20 | 10.1-11.1 | 30.3 | 28.3 | M+F | " |
| **-** | - | - | - | - | - | 17 | ~60 | M+F | (Mainx, 1964) USA |
| **-** | - | - | - | - | - | 28 | 18- | M | " |
| **-** | - | - | - | - | - | 28 | 20- | F | " |
| **-** | - | - | - | - | - | 18.3-20.0 | 21-27 | M+F | (Patton, 1922) UK |
| **20.8** | 2 | - | - | 21.4 | 9.5 | 20.9 | 18.5 | M+F | (Leccese, 2004) Italy |
| **20.8** | 2 | - | - | 21.5 | 12 | 22.5 | 22 | M+F | " |
| **-** | - | - | - | 21 | 11.7-17.8 | 21 | 29.2-37.3 | M+F | (Trumble and Pienkowski, 1979) USA |
| **-** | - | - | - | 27 | 5.2-8.8 | 27 | 16.2-20.3 | M+F | " |
| **-** | - | - | - | 32 | 3.7-7.8 | 32 | 11.2-14.8 | M+F | " |
| **22** | 1.3 | 22 | 4.3 | 22 | 6.8 | 22 | 21.9 | M+F | (Greenberg and Kunich, 2002) UK |
| **29** | 0.75 | 29 | 2.87 | 29 | 4.27 | 29 | 11.2 | M+F | " |
| **-** | - | - | - | 23 | 5-6 | 23 | 16-18 | M+F | (Semenza, 1953) Italy |
| **27** | 0.7 | - | - | 27 | 7.7 | 27 | 19.0 | M+F | " |
| **20-25** | 2 | - | - | 20-25 | 8-11 | 20-25 | 22-25 | M+F | (Robinson, 1975b) USA |
| **-** | - | - | - | 21-33 | - | | 25 | M | (Benner and Ostermeyer, 1980) USA |
| **-** | - | - | - | 23-25 | - | | 25 | F | " |
| **23-27** | 2 | - | - | 23-27 | 7.3 | 23-27 | 18.7 | M+F | (Idris and Abdullah, 1997) Malaysia |
| **26-28** | 1 | - | - | 26-28 | 12-19 | 26-28 | 15-20 | M | (Tumrasvin *et al*., 1977) Thailand |
| **26-28** | 1 | - | - | 26-28 | 12-19 | 26-28 | 16-22 | F | " |
| **27** | 0.6 | - | - | 27 | 4.6-5.2 | 27 | 13.6-16.6 | M+F | (Amoudi *et al*., 1989) Saudi Arabia |
| **27** | 0.7 | - | - | 27 | 4-6 | 27 | 13-15 | M+F | (El-Miniawi and Moustafa, 1966) French |

*E =egg, E-FL = egg + feeding larvae, E–PF = egg + feeding stage larvae + post feeding stage larvae, E-P = egg + larvae + pupa, $^{o}C$ = temperature range, M=males, F=females.*

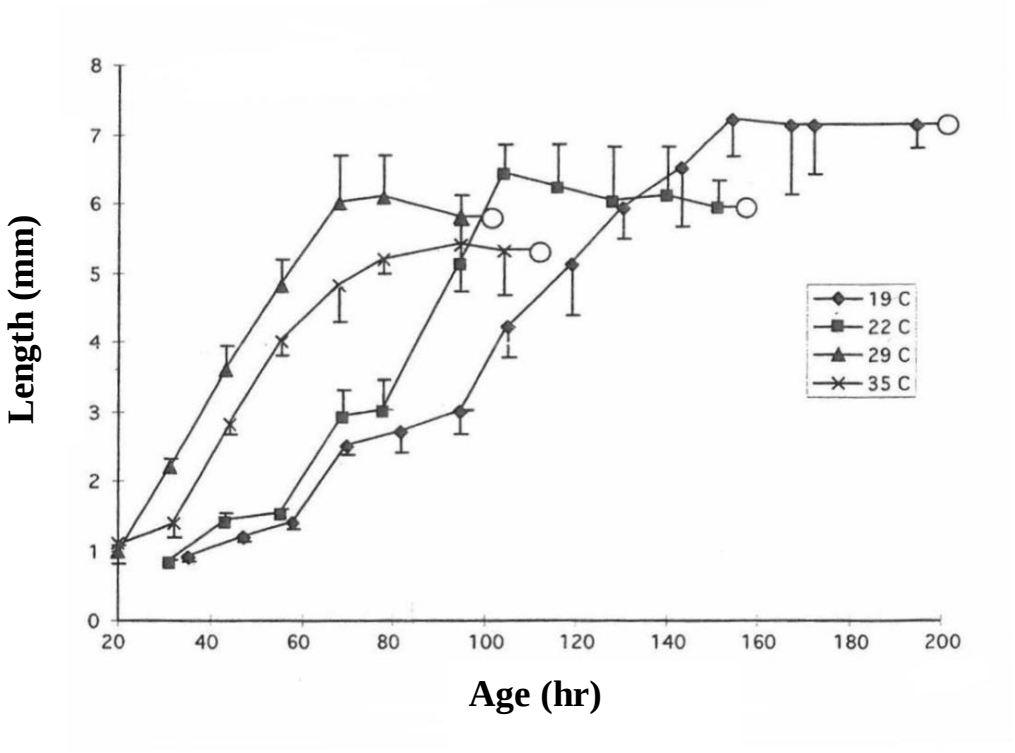



**Fig 4: Growth rate of *Megaselia scalaris* reared in four different temperatures, length (mm) vs. age (hr). Values are the mean and Standard deviation of the 5 - 8 largest larvae observed at each age (Greenberg and Wells, 1998).**

Further developmental studies include Greenberg and Wells (1998) who created a growth curve for *M.scalaris (Fig 4)* which compared length in millimetres (mm) to age (hours) for specimens reared at 19, 22, 29 and 35 °C. Harrison and Cooper (2003), compared the developmental rates between the *Drosophila melanogaster* (Diptera: Drosophilidae) and *M.scalaris*, a slower developmental rate was observed with *M.scalaris* which was more pronounced the lower the temperature. Recent work by Zuha and Omar (2014) compared developmental studies of *Megaselia scalaris* to previously published developmental data. They found that the developmental periods of their experiments were shorter than those previously published, 40.3% shorter than the findings of and 52.1% shorter than (Idris *et al*., 2001). The variances were explained by difference in lab equipment, food and sample volumes etc. The average daily egg production at 25 °C (*Fig 5*) was reported by (Prawirodisastro and Benjamin, 1979).

Additional observations of *Megaselia scalaris* include Benner and Ostermeyer (1980) who observed that female larvae at 25 °C pupate two days later than the males. Sex ratios were investigated by Semenza (1953); El-Miniawi and Moustafa (1966) Benner and Ostermeyer (1980); Macieira (1983) and Amoudi *et al*. (1989), they all

reported the male to female sex ratios can be affected by the temperature (23 °C = 1.28:1, 25 °C = 1.18:1, 25 °C to 30 °C = 1.0:1, 27 °C = 0.86:1). However with constant light and a relative humidity of 75% at 27 °C the ratio was reported at 0.43:1 but ranged from 0.13:1 to 0.60:1.

Water balance characteristics was studied by Rivers *et al*. (2013) in which *M.scalaris* was shown to have the lowest percentage body water content when compared to other forensically important fly species such as *Protophormia terraenovae* (Diptera: Calliphoridae), *Phormia regina* (Diptera: Calliphoridae), *Sarcophaga bullata* (Diptera: Sarcophagidae), *Lucilia sericata* (Diptera: Calliphoridae) and *Lucilia illustris* (Diptera: Calliphoridae). As the maggot mass increased there was no change in body water content, changes were observed only when the maggot mass contained over 1000 specimens and was a result of overcrowding. Having high dehydration tolerance and a low water content indicates that *M.scalaris* is able to colonise a dry carcass as discussed by Goff (2010) and Rivers and colleagues (2013).

Prawirodisastro and Benjamin (1979) researched the duration of the adult flies' life span calculating reproduction success. Specimens were kept in environmental chamber maintained at 15, 20 and 25 °C in a LD 18:6 photo-period. Overall 25 °C showed the most rapid development throughout the life stages. No adults emerged at 15 °C from the pupa (*Table 15 & Table 16*) and adults were noticeably less active at lower temperatures which was also commented on by (Mazyad and Soliman, 2006) whilst (Dian-Xing and Guang-Chun, 2014) found that a few adults were seen emerging at 15 °C. Overcrowding was seen to influence mean development times; larval development period was longer at 100 larvae per 10g food however at 200 larvae per 10g many underdeveloped larvae moved away from the food and died and the pupal development stage was shortened.

**Table 15: Average longevity and fecundity at 3 constant temperatures (Prawirodisastro and Benjamin, 1979).**

| Temperature (°C) | Sex | N | Longevity (Days) Mean ± SD | Fecundity Mean ± SD |
|---|---|---|---|---|
| 15 | ♀ | 10 | 51.0 ± 6.9 | 146.9 ± 51.3 |
| | ♂ | 19 | 43.0 ± 3.7 | |
| 20 | ♀ | 10 | 30.7 ± 12.5 | 591.7 ± 213.0 |
| | ♂ | 27 | 24.9 ± 1.5 | |
| 25 | ♀ | 10 | 29.9 ± 9.0 | 664.8 ± 254.1 |
| | ♂ | 30 | 24.8 ± 2.4 | |

**Table 16: Average pupal duration and survival of adults over three temperatures based on 250 male and female specimens (Prawirodisastro and Benjamin, 1979).**

| Temperature (°C) | Sex | Duration (Days) | Survival (%) |
|---|---|---|---|
| 15 | ♀ | - | - |
| | ♂ | - | - |
| 20 | ♀ | 17.5 ± 0.4 | 82.0 ± 9.3 |
| | ♂ | 17.7 ± 0.4 | 75.9 ± 10.3 |
| 25 | ♀ | 9.8 ± 0.2 | 95.8 ± 6.3 |
| | ♂ | 9.8 ± 0.2 | 91.6 ± 8.1 |

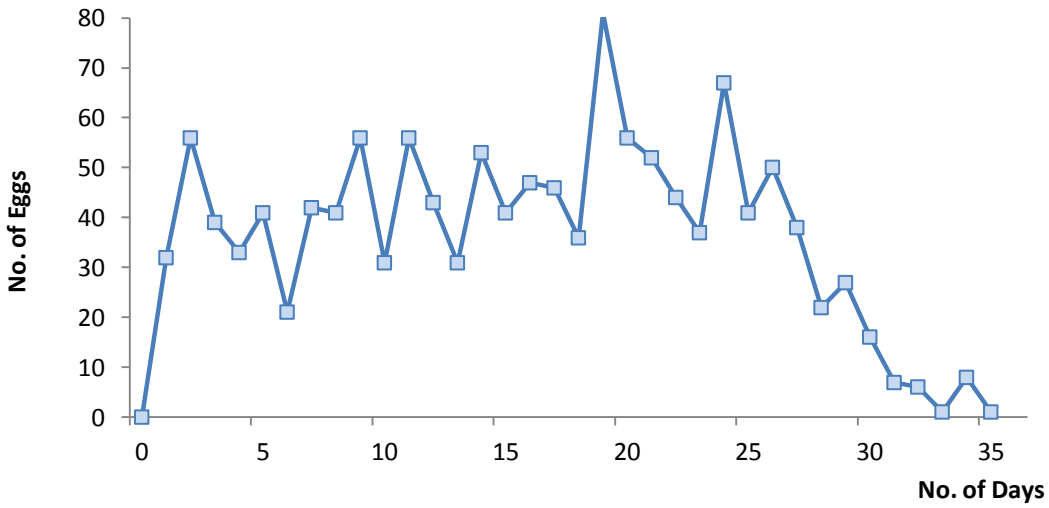



**Fig 5: Average daily egg production at 25 °C (Prawirodisastro and Benjamin, 1979).**

*Megaselia scalaris* adult flies are just one of the species that are able to burrow down into the soil and oviposit on to the corpse whilst other species lay their eggs on the soil surface and the newly hatched larvae reach the corpse by burrowing down through

the soil (Smith, 1986). Mariani *et al.* (2014) comments that *M.scalaris* larvae are able to burrow over 50cm to reach a buried cadaver. Buried corpses have four recognised waves (*Table 3*) which also shows Phoridae in the third colonisation wave when the age of the corpse is approximately 1 year old. Given the opportunity *M.scalaris* has been observed ovipositing at different stages throughout the decomposition process (Disney, 2008).

*M.scalaris* larvae have been defined as being predator, parasitoid or a parasite; this is supported by reports of:

- infestation of laboratory cultures by Robinson (1975a); Miller (1978); Gregorio and Leonide (1980); Garris (1983); Harrison and Gardner (1991); Andreotti *et al*. (2003); Zwart *et al*. (2005); Ruíz-Nájera *et al*. (2007); Costa *et al.* (2007); Miranda-Miranda *et al*. (2011); Batista-Da-Silva (2012) and Koch *et al*. (2013).
- infestation of food and seeds by Walter and Wene (1951); Disney (1994) and Karunaweera *et al*. (2002).
- the myiasis of reptiles and fish by Da Silva *et al*. (1999); De Morretti *et al*. (2006); Diclaro *et al.* (2011) and Vanin *et al.* (2012b).
- the myiasis in humans i.e. intestinal myiasis, urinary myiasis, wound myiasis etc. Trape *et al*. (1982); Singh *et al*. (1988); Sigh and Rana (1989); Carpenter and Chastain (1992); Hira *et al*. (2004); Mazayad and Rifaat (2005); Diaz (2006) and Wakid (2008).

*Megaselia scalaris* does however have its own predators and parasitoids, Marchiori and Barbaresco (2007) collected six specimens of *Pachycrepoideus vindemmiae* (Hymenoptera: Pteromalidae) from *M.scalaris* pupae. Disney and Munk (2004) also found that *Orthostigma pumilum* (Hymenoptera, Braconidae) were parasitoids of other *Megaselia* species and Disney (1983) comments that respiratory horns in *Megaselia* may be absent in the cases of parasitism by Hymenoptera, this is still to be confirmed.

### 1.4.1 Observations of *Megaselia scalaris* found in some Forensic Entomology cases

To determine characterisation of the different insects present on cadavers that arrived for autopsy at the Institute of Legal Medicine (ILM) in Pernambuco, Brazil, a survey was performed. Bait traps were placed around the ILM and were used for the collection of insects which took place three times a week for six months. Cadavers were examined immediately on entering the ILM prior to any washing. Overall during the study 4589 adult insects belonging to 24 species of dipteran families were collected. The frequency of *M.scalaris* was determined to be 23.1% and were collected from the ILM courtyard, storage room for putrefied cadavers, autopsy room and hospital garbage site (Oliveira and Vasconcelos, 2010).

In Recife, one of the largest cities in Brazil and one of the most violent cities in the country (homicide rates: 57.9 homicides/100,000 inhabitants) a study was carried out by Vasconcelos *et al*. (2013) to determine if larval competition during colonisation would favour a limited number of species in completing their developmental cycle on the carcass. A location frequently used for the clandestine disposal of cadavers was selected for the experiment. Numerous collections from 5 to 180 mins were taken immediately post death. To determine which species would continue to visit the carcass further collections were taken at 24, 48 and 72 hours. In the first few hours after death a total of 153 insects from 14 families were collected, this included species of Phoridae (24.2% of all adults), Sarcophagidae (18.3%), Piophilidae (10.5%), Calliphoridae (10.5%), Fannidae (8.5%), Chloropidae (6.5%), Muscidae (4.6%) and Dixidae (4.6%). *Megaselia scalaris* was observed to be the most abundant species at the period immediately after death and was seen within 30 mins post death but was also observed at different times up to 72 hours post death. Throughout the stages of decomposition *M.scalaris* larvae were not restricted in completing their larval development cycle.

A body of a male was found in his apartment in July in the municipality of Jaboatao dos Guararapes, Pernambuco state, Brazil. The deceased's flat was clean and tidy and the windows had been partially opened which allowed for limited access of insects. Death was estimated to have occurred five days prior to discovery of the body. On the head and trunk areas large larval masses were observed also under and between the clothes. Over a 180 minute sampling period, third instar larvae were collected from several parts of the body (10 insects per pot) and observed daily until emergence for identification. From the lab reared specimens, six species were recorded: *Chrysomya*

*albiceps* (Diptera: Calliphoridae) (65 %), *Chrysomya megacephala* (Diptera: Calliphoridae) (18.6 %), *Chrysomya putoria* (Diptera: Calliphoridae) (0.4%), *Megaselia scalaris* (Diptera: Phoridae) (15.2 %), *Fannia trimaculata* (Diptera: Fanniidae) (0.4%) and *Peckia chrysostoma* (Diptera: Sarcophagidae) (0.4%). Of the six species recorded three were the most abundant of which one was *M.scalaris* (Vasconcelos *et al*., 2014).

A fully clothed body of an adult male was exhumed in Bari, Southern Italy. He had been buried in a wooden coffin at a depth of 30 to 40 cm, one year after burial his body was exhumed. His cause of death was gunshot wound to the head and chest. On examination of the coffin revealed some very small holes which only small flies would be able to access. The corpse was covered with larvae, pupae and empty puparia. Examination of the insect remains revealed that only one species was present in the coffin and these were identified as individuals of *M.scalaris* (Campobasso *et al*., 2004a).

Other cases that involved *M.scalaris*, include a body found within a tightly sealed $7^{th}$ floor apartment in Japan (Greenberg and Wells, 1998).

Further forensic cases include myiasis; in which larvae were found on the deceased body of a female was discovered in her apartment. Young larvae of *M.scalaris* were found in the genital area of the body. The larvae were collected and reared in which the time interval for full development was considered too short for a complete life cycle and therefore myiasis was discussed (Dewaele *et al*., 2000).

Two cases of wound myiasis by *Megaselia* species in USA were reported, the first was (Sherman, 2000). Many cases of myiasis acquired in a hospital environment are often under reported or not reported at all, (*Table 8*) shows the reported cases between the years 1980 to 1998 (Joo and Kim, 2001).

### 1.4.2 Molecular identification of *Megaselia scalaris*

Insect sampling and identification plays an important role for the entomologist when trying to estimate the Post Mortem Interval. Many fly species such as Phoridae have similar morphology characteristics which pose a challenge when trying to determine identification as over 3,000 of these scuttle fly species have been identified so far. Morphological keys may be unavailable or hard to use for the different stages such as immature or adult. A DNA based method that allows for easier identification of fly species is beginning to be used more frequently. Molecular phylogenetic is approximating the evolutionary past based on the comparison of protein sequences or DNA (Baldauf, 2003). By amplifying suitable regions of the genome, amplicons are acquired. Commonly researched genes are: subunits I and II of the cytochrome oxidase (part of the respiratory chain within the mitochondrial membrane), ND5, ND1, 12S and 16S DNA (mitochondrial encoded) along with 28S, ITSI and II DNA (nuclear encoded) (Amendt, 2004). Cytochrome oxidase I (COI) gene was found to have a couple of advantages over other primers, as it appears to contain a larger range of phylogenetic signals when compared to other mitochondrial genes and the universal primers were found to be robust allowing for recovery of 5'. Using DNA based identification much research has been accomplished over the years from all around the world on many different species for the purpose of identifying forensically important flies (Wells et al., 2007; Nelson et al., 2007), (Wallman and Donnellan, 2001; Boehme et al., 2010). If a reference sequence matches a sequence from an unknown insect the conclusion would be that the unknown insect may belong to the same species of the reference sample or that the two taxa are identical. If differences are present in the sequence then interspecific and intraspecific variation information needs to be analysed to be able to evaluate the differences in the sequence. Using the cytochrome oxidase I gene (COI) and amplifying using polymerase chain reaction (PCR), the 658 base pair (bp) long region (without primer or 750 bp with primer) which in forensic entomology is a common molecular marker is then able to be sequenced and a neighbour joining phylogenetic tree can be generated (Boehme et al., 2010; Aly and Wen, 2013).

Rasmussen and Noor (2009) used pyrosequencing to sequence the genome of *M.scalaris* (*Fig 6*). Food associated insect pests were identified and sequenced (Cho *et al*., 2013) which included COI sequencing for *M.scalaris* (Genbank accession numbers: KC407773, KC407774, JN896297, JN896298, GU075400, JN896281 and JN896283).

Boehme *et al*. (2010) molecularly analysed *Megaselia scalaris*, *Megaselia giraudii*, *Megaselia abdita*, *Megaselia rufipes*, *Conicera tibialis*, and *Puliciphora borinquenensis*. The COI barcoding region was amplified, a total of 34 individuals were sequenced and aligned over 559 nucleotides of the COI barcoding fragment. The phorid sequences were deposited in GenBank under the following accession numbers: GU075399, GU075400, GU075401, GU075402, GU075403, GU075404, GU075405, GU075406, and GU075407. It was concluded that almost all specimens of one species showed identical nucleotide sequence except slight differences within the sequences of *M. rufipes*. Only two individuals shared the same haplotype while the remaining three specimens (*M.giraudii*, *M.abdita* and *M.scalaris*) differed to each other in one to two base pairs.

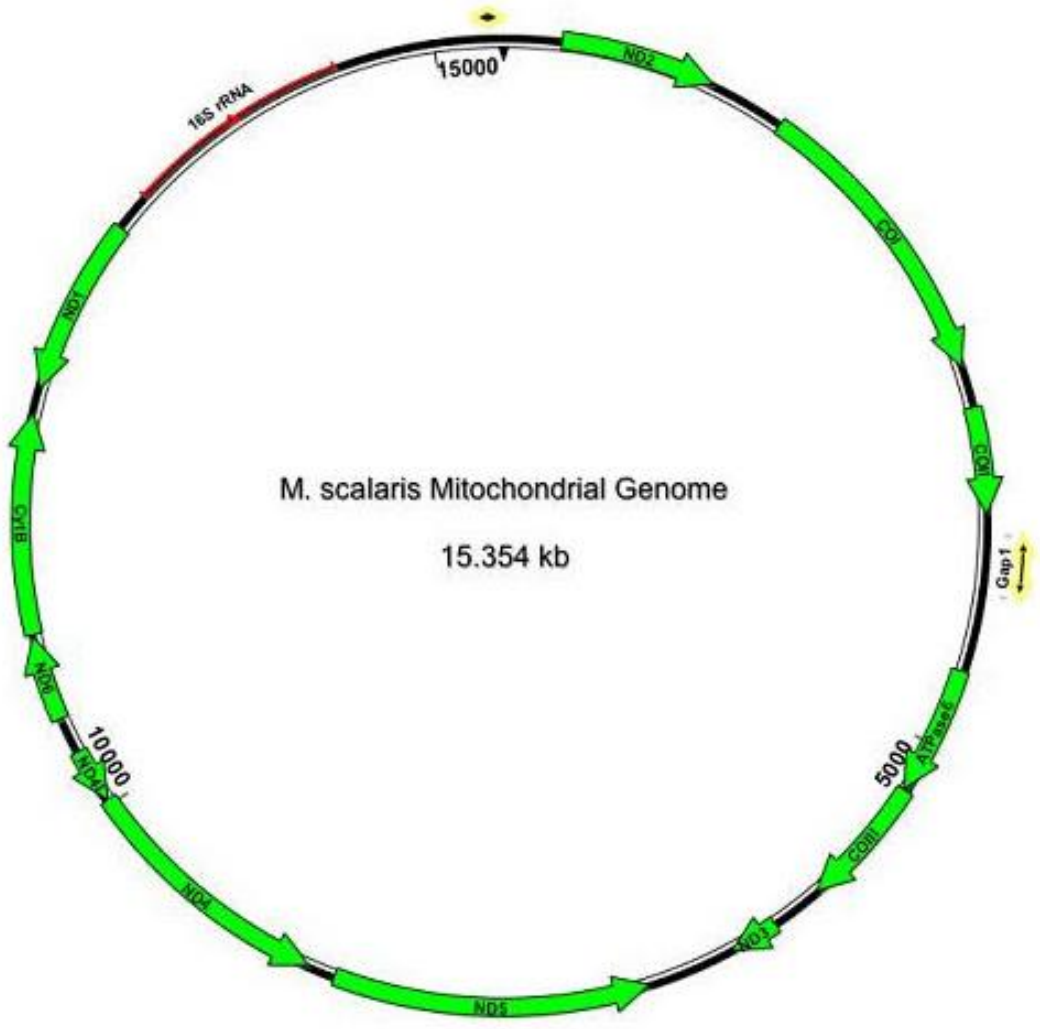



**Fig 6: Map of the *Megaselia scalaris* mitochondrial genome showing the positions of the protein-coding genes (green arrows), 16S ribosomal RNAs (red line) and the gaps in our sequence (external yellow arrows) (Rasmussen and Noor, 2009).**

### 1.5 Aims of Research

*Megaselia scalaris* is a common species found amongst indoor and outdoor crime scenes and plays an important role in the decomposition of human remains and can be used following the forensic entomology approach for the estimation of the post mortem interval particularly in indoor cases.

Several questions concerning the biology and the chronobiology of this species remain open. Only by researching and finding the answers to these questions will allow for the routine use of this species in forensic and legal investigations.

The most important questions include:

- period of activity and eggs laying time of this species,
- period of hatching and eclosion times from eggs and pupae,
- most important stimuli able to attract the flies to a body,
- effect of the food source in the species development,
- time of development related to the different photo-periods and temperatures
- estimation of the time of pupation
- estimation of the burial activity.

Studies have been conducted by numerous researchers on the developmental period of this species however different parameters were used by each of the groups, this has left the current data with inconsistencies and contradictions therefore the published data could not be used as comparison material. For the purpose of PMI estimation, developmental studies have been conducted using the same parameters such as constant temperature, time and size data. Studies were also conducted using different pabulum and drug induced food of different concentrations.

To further understand the behavioural patterns of this species the use of up to date technology may help us to be able to determine a more precise circadian clock rhythm. It has long been discussed by numerous researchers that flies are not active during the night time period, this also includes oviposition, understanding of the biological clock that controls this species may allow for a more precise estimation of the time since death.

Research has been completed to determine when this species is active, when they are able to oviposit and emerge as adults from their puparia.

Morphology imaging of *Megaselia scalaris* throughout the developmental stages has previously been conducted using SEM (Sukontason *et al.,* 2003; Sukontason *et al*., 2005), TEM (Wolf and Liu,1996) and both stereo and compound microscopes however new technologies are emerging which allow for a non-destructive approach, which may be required in either forensic or archaeological specimens.

This thesis was completed with the aim of giving robust answers to the above questions, based on experimental data collected meaning that other issues could be addressed which may allow us to be able to better understand the species *Megaselia scalaris* and the overall effect they may have in the entomological field. Permission has been granted for the reproduction of illustrations used throughout this thesis.

# 2: Morphology

## 2.1 Morphology

### 2.1.1 Introduction

Changes to the morphology of *M.scalaris* throughout the developmental stages has been conducted using Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and both stereo and compound microscopes as previously discussed which may have required considerable sectioning, dissection and more frequently the complete destruction of the specimen.

*Megaselia scalaris* larvae go through numerous changes in size and weight during their development cycle; these measurements are used by forensic entomologists to estimate the age of the larvae along with other morphological characters. Estimating pupal age is increasingly harder to determine due to the lack of morphological changes that occur outside the pupa. To be able to describe anatomical and morphological changes that occur during metamorphism without destroying the sample relies on newer advanced technology such as X-ray computed micro-tomography (micro-CT) (Feng and Liu, 2013).

Richards and colleagues (2012b) explored the methods currently used with pupae which may help to determine PMI. Measurements of weight and larval length are the main techniques in determining the developmental age of the larvae. However the same method cannot be used for determining pupal age, techniques presently used include:

- Killing of the pupae, removing the case and observing the specimen under a light microscope.
- Identifying morphological markers on the pupal case observed during development.
- Histology which looks at changes that occur within the internal morphological structures such as structural changes in salivary glands in larvae during pupation (Levy and Bautz, 1985).
- Recently scanning electron microscopy (SEM) has been used to try and determine the developmental rates of blowfly pupae however it was found that further work is required if this method is to be made reliable when calculating PMI.

Different staining methods which would allow for a high contrast in 3D imaging was researched by Metscher (2009). The smaller specimens were imaged using an Xradia Micro XCT system whilst specimens above 5mm were imaged using a Skyscan 1171 scanner. The stains of interest to the research consisted of a PTA stock solution (PTA), Iodine aqueous solution (IKI) and an Iodine alcohol solution (I2E, I2M). It was determined that the better stains were either the PTA solution or the aqueous iodine solution. Aqueous Iodine solution was found to penetrate into the tissues more rapidly and was able to stain the specimen within a few hours and was found to be a robust contrast stain. As PTA is a larger molecule the solution was found to require overnight incubation to diffuse into the tissues but the pupae must be pierced for this to happen.

Different imaging techniques used to investigate the external morphology of *M.scalaris* have included SEM and TEM.

- Developmental differences between the first and second instar larvae were researched by Boonchu and colleagues (2004). Observed were changes in both the posterior and anterior spiracles and little change in the structure of the mouthparts. They concluded that morphology between first and second instars were very similar however even fewer changes were observed between second and third instars.
- Mouthparts of the *M.scalaris* was researched by Sukontason and colleagues (2003) to determine sexual dimorphism. It was concluded that differences were seen between the two sexes, with the most distinctive feature of *M.scalaris* sexual dimorphism is the surface structure of the labellum, females have an entirely smooth labellum whereas the males labellum is entirely covered with microtrichia.
- The ultrastructure of the ommatrichia in *M.scalaris* was studied by Sukontason and colleagues (2005) to gain a further understanding of the compound eye structure. They concluded that the *M.scalaris* have numerous ommatrichia however there was no differences noted between the sexes though further work is required to determine the exact function of the ommatrichia in the *M.scalaris*.
- Sukontason and colleagues (2006) also examined *M.scalaris* puparia to determine if any morphological features were present. It was concluded that whilst the pupa is of a uniform shape, under a high magnification many different structural features are present i.e. intersegmental spines along the dorsal and lateral

segments. Two straight slits are present on the anterior spiracles with one end closed whilst the other is open. The papillae on the respiratory horn may be species specific in arrangement, shape and number. This may help to differentiate the *M.scalaris* from other fly species which are of great importance in forensic cases.

- Further work includes the identification of forensically important fly eggs using researched by Sukontason and colleagues (2004). Characteristics were based on length of the egg, width of plastron and morphology of plastron area surrounding the micropyle and chorionic sculpturing. It was determined that the chorionic sculpturing allowed for identifying species which was more apparent on some species when compared to others.
- Using TEM, Wolf and Liu (1996) investigated the fine shell structure of both the *M.scalaris* and *M.spiracualaris*. Concluded was that when comparing the *Megaselia* species alongside *Drosophila* that the *Megaselia* species had thicker chorions. This may help prevent environmental hazards such as bacterial attack and dehydration. An electron dense lamina was also discovered this is thought to represent the wax layer found in *Drosophila* which serves as resisting layers in an aqueous environment.

### 2.2.2 Experimental Design

#### 2.2.2.1 Adult, Egg and Larval Imaging

Images were taken of the throughout the different stages of development using both a Leica M60 microscope at different magnifications imaging was completed using Leica software and a Keyence VHX-1000 (*Fig 7*) with digital optical system, a 54 megapixel camera, 3D measurement software and magnification from 20 x to 2500 x with auto magnification recognition software.

The life cycle of *M.scalaris* consists of six parts, the adult (*Fig 9*), the egg (*Fig 12, Fig 13* and *Fig 14*), no staining was required with the eggs as sufficient detail was captured, first instar larval stage (*Fig 15*), second instar larval stage (*Fig 16*), third instar larval stage (*Fig 17*) and pupa (*Fig 19*). The hot water immersion (HWI) killing method was used on both larvae and pupa, whilst recently deceased flies were used for imaging.


**Fig 7: Keyence VHX-1000 digital microscope.**

#### 2.2.2.2 Pupal Imaging

Flies used were from lab colonies maintained and reared on pet food and kept under constant conditions at 20 ± 0.5 $^{O}$C using LD 12:12 photo-period. White prepupae were collected and observed every hour for pupation, once pupated, each puparia was sexed and separated into petri dishes. Each dish contained three males and females and damp tissue to prevent dehydration.

Specimens were removed from the Incubator at set time intervals: 0, 14, 36, 50, 71, 86, 100 % throughout the developmental period. Time 0% was the initial pupation formation plus 30 min max to prevent disruption of the pupation process.

Initially prior to the removal of the pharate, microscopy images were taken of the pupae exoskeleton (*Table: 3 & 4*) to display the changes over time under a Leica M60 microscope at x 25 magnification and Keyence VHX-1000 with digital optical system at x 50 magnification, both microscopes used white illumination. Once the external images were taken the pupa were then fixed using the HWI method. The developing fly was then dissected from the pupa case and imaged using the method above (*Table: 5 & 6*).

At the end of the experiment it was noted that the unused specimens emerged from pupae within 2 hours of the last sample therefore time 100 % was within the very final stages of adult emergence.

#### 2.2.2.3 Nikon XT H 225 Series Used for Internal Imaging

Nikon XT H 225 series X-ray and CT technology (*Fig 8*) was initially used to investigate the anatomical and morphological changes taking place within the puparia of *Megaselia scalaris*. This research was completed in collaboration with Dr Peter Laity and Dr Paul Bills.


**Fig 8: Nikon Metrology XTH 225**

Nikon datasheet (Nikon, 2012) shows the system operates from 30 to 225 kV and 0 to 1000 µA. Reconstruction software used was CT pro software and VG studio max 2.1 was used for the 3D renderings and slice stacks. The operational method used during the experiment was:

- Energy = 50kV,
- Current = 200µA,
- Measurement time = 1h 30mins.

The pupae used in the initial experiments were of undetermined age as the initial experiments were preliminary attempts at imaging to determine the best parameters for both x-raying and positioning of the pupae.

Three different techniques were initially used:

- Dry
- Stored in 70% Ethanol (EtOH),

- Stained in Potassium Iodide aqueous solution (IKI) for 1, 3, 5 and 7 days.

The specimens in EtOH were removed and imaged immediately or left to air dry for one hour to determine if this had any effect on the internal specimen (i.e. dehydration).

Two different mounting mediums were used:

- Polyethylene cylindrical mounting media.
  (A body portion that has the same diameter throughout from start to finish)

- Polyethylene tapered mounting media.
  (A body portion that has a cylindrical shape with the outer periphery having a larger diameter than that of the distal end portion)

Different supports were used alongside the mounting mediums to investigate which method would offer the most support to prevent movement during imaging, these were:

- Spongy Phenolic Foam
- Petroleum Jelly

All of the above were used together in different arrangements to determine if they had any effect on the resulting image. To prevent the dehydration of the specimens' Petroleum Jelly was used.

Images of both unstained and stained *M.scalaris* adult flies and larvae were also captured to try and understand the positioning of the internal structures that we may see.

#### 2.2.2.4 Elettra Synchrotron Used For Internal Imaging

Beam time was accepted at Elettra Synchrotron in Trieste, Italy. Using a lower energy higher resolution images were taken. This research was completed in collaboration with Dr Lucia Mancini (Beamline Scientist - SYRMEP Group).

The operational method used during the experiment was:

- Energy = 10 to 14 keV,
- Current = 200µA,
- Filter = Aluminium
- Exposure = 800 m sec
- No of projections = 1800 over 180 $^{O}$
- Measurement time = 46 mins.

Image pro plus, VHR grablink and IDL Image software was used for the image rendering and reconstructions.

Pupa of both sexes was collected at 0, 10, 30, 60, 80, and 100 % of their development. The samples were by fixed by HWI and stained with a 0.5 Iodine solution for 3 hours and stored in 70 % EtOH solution until imaging. Time 0 % was the initial pupation formation plus 30 mins to prevent disruption of the pupation process. The unused specimens emerged from pupae within 2 hours of the last sample therefore 100 % was within the very final stages of adult emergence. Aging of pupa will be determined by combining both internal and external morphological markers.

### 2.2.3 Results

#### 2.2.3.1 Adult, Egg and Larval Imaging

The adult fly (*Fig 9*) is small and is brown/yellowish in colour with dark eyes. Females are larger in size than the males and also have rotund white abdomens that are occupied with eggs.


**Fig 9: Lateral image of *Megaselia scalaris* adult fly. Scale 500µm, x40 magnification**

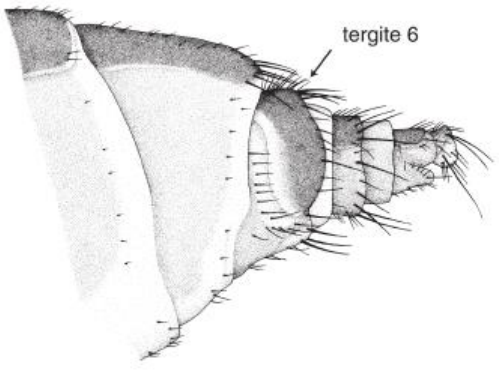



**Fig 10: Abdominal section of female *Megaselia scalaris* (Brown and Oliver, 2007)**

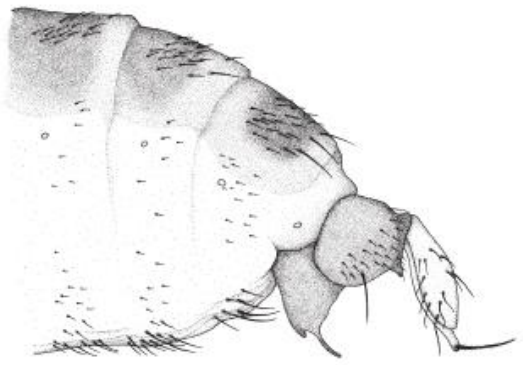


**Fig 11: Abdominal section of male *Megaselia.scalaris* (Brown and Oliver, 2007)**

The female sclerite (*Fig 10*) of segment/tergite 6 extends laterally. The male's final segments (*Fig 11*) are easily recognisable (Brown and Oliver, 2007).

*Megaselia scalaris* eggs (*Fig 12, Fig 13* and *Fig 14*) are white in colour, boat like in shape as half the egg is curved whilst the other half is flat. Polygonal patterns cover the curved half of the egg whilst plastron occupies the other half.

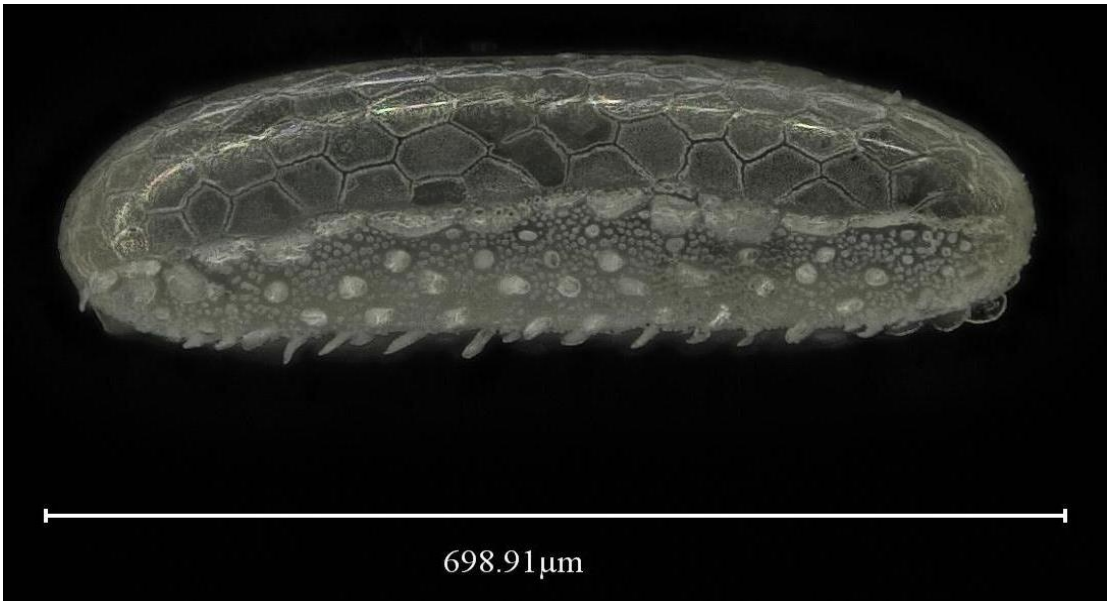



**Fig 12: Lateral image of *Megaselia scalaris* egg using Keyence digital microscope at x300 magnification.**

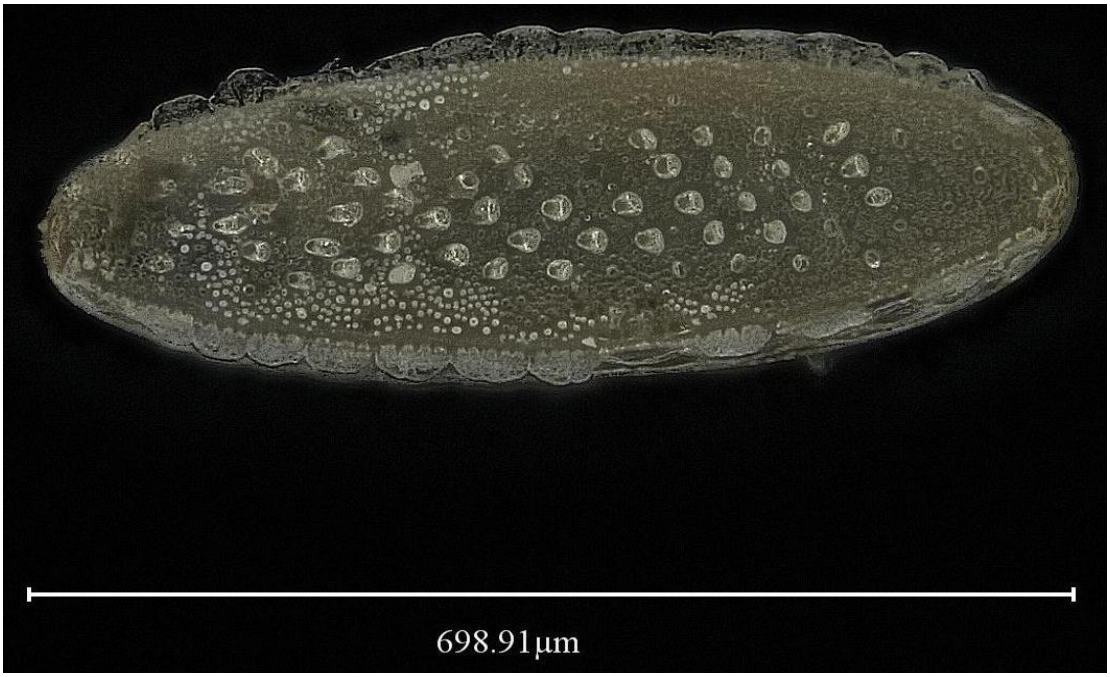



**Fig 13: Ventral image of *Megaselia scalaris* egg using Keyence digital microscope at x300 magnification.**

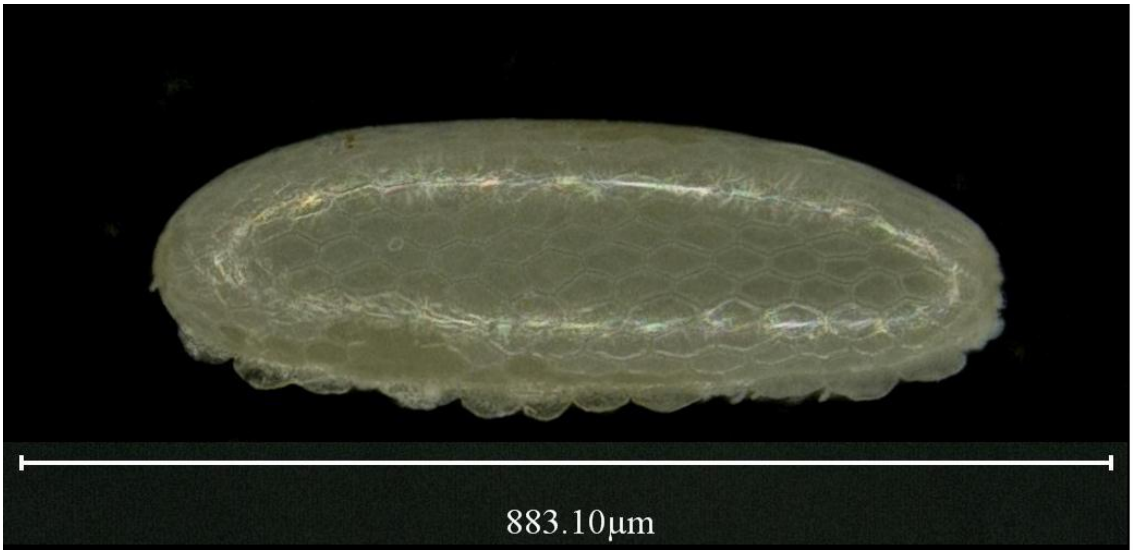



**Fig 14: Dorsal image of *Megaselia scalaris* egg using Keyence digital microscope at x250 magnification**

All *Megaselia scalaris* larvae are cylindrical in shape with narrowing towards the head. Larvae have 12 abdominal segments in which bilateral spines are present from the third thoracic to the last abdominal segment. Posterior spiracles are present on the 12$^{th}$ abdominal segment and appear cone like in shape which protrude dorsally.

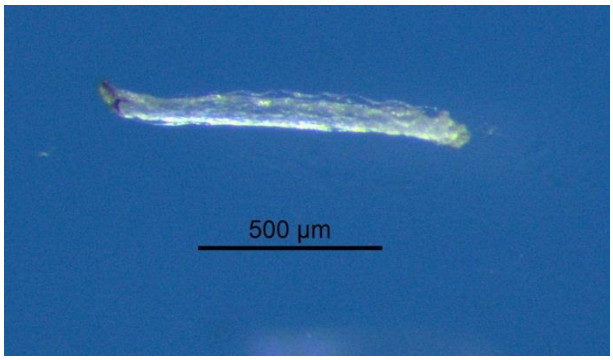



**Fig 15: Lateral image of a first instar *Megaselia scalaris* larvae taken using Leica M60 microscope at x40 magnification, scale = 500µm.**

First instars (*Fig 15*) are white with some transparency after eclosion from the egg.

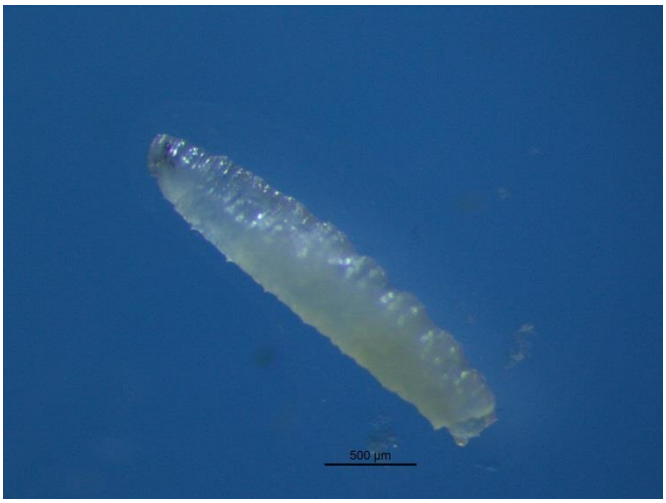



**Fig 16: Lateral image of second instar larvae of *Megaselia scalaris* taken using Leica M60 microscope at x40 magnification, scale = 500µm.**

Second instars (*Fig 16*) lose their transparency during this stage and take on a more cylindrical shape.

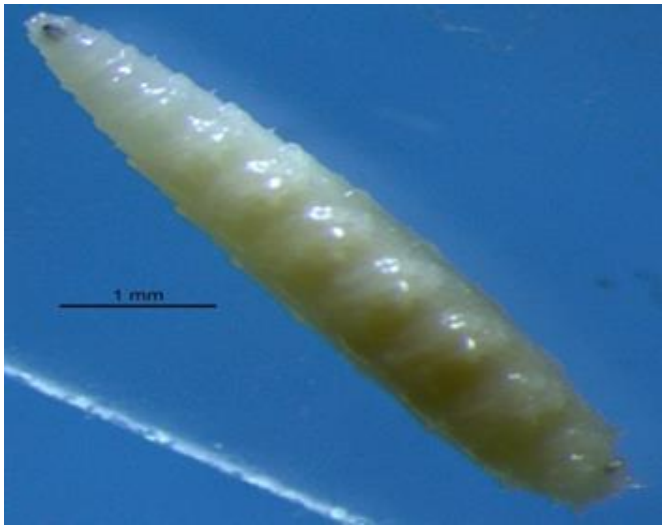



**Fig 17: Lateral image of *Megaselia scalaris* third instar larvae taken using Leica M60 microscope at x32 magnification, scale = 1mm.**

Third instar (*Fig 17*) is the last stage prior to then becoming post feeding in which they empty their gut and then move away from their food source and begin the metamorphic process into a pupa, the last developmental immature stage.

Larvae are able to swallow air (*Fig 18*) which allows them some buoyancy to prevent them from drowning.

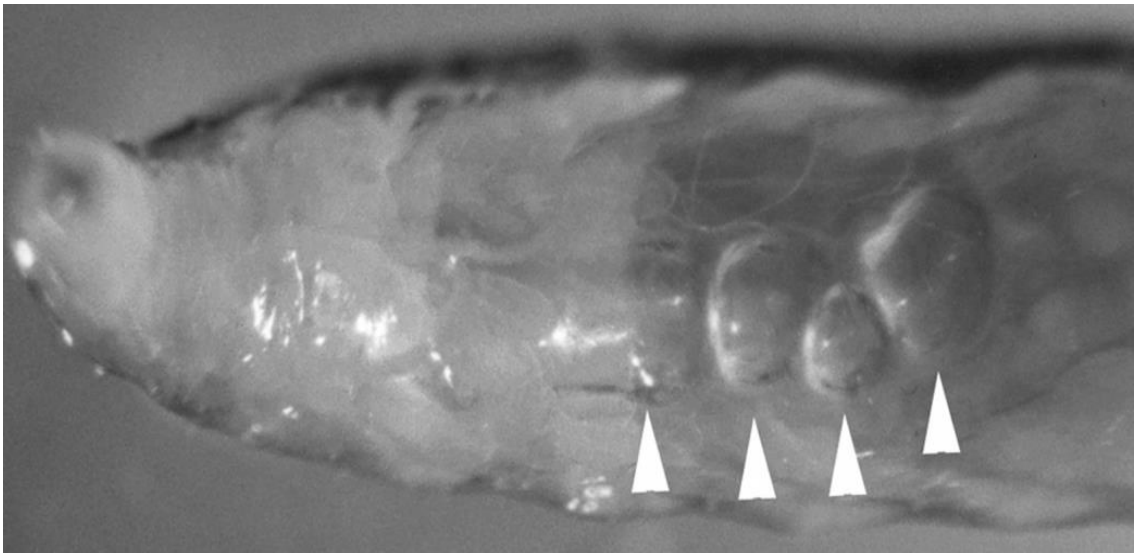


**Fig 18: Air swallowed by *Megaselia scalaris* larvae creating bubbles to prevent drowning when in aqueous solution**
**(Harrison and Cooper, 2003)**

#### 2.2.3.2 Pupal Imaging

At the beginning of the metamorphic process the puparia is soft and white (*Table 17*) the pupa will harden and turn brown over a short period of time.

The puparia contains both anterior and posterior spiracles along with respiratory horns which extrude through the posterolateral wall (*Fig 19*). The horns which curve at the apex are slender and long. On the surface of the horns are papillae which form a spiral pattern, whilst we were able to see these on a higher magnification images, we did not research these features further. Each puparia is dome shaped and oval in appearance. The anterior spiracle contains two straight slits in which one end is closed whilst the other remains open (Sukontason, 2006).

Due to females being larger than males (Carareto and Mourão, 1988; Disney, 2008 and Dian-Xing, Guang-Chun, 2014), logically their puparia are larger allowing for easier sex determination. At the end of the metamorphosis process the adult fly will emerge from the hardened puparia.

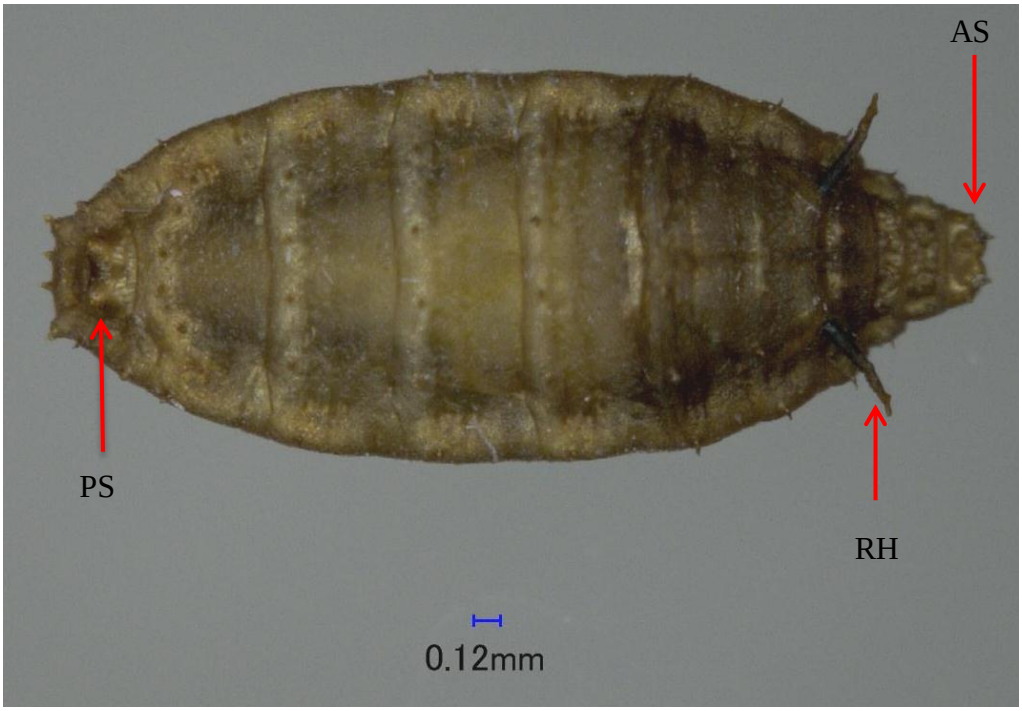
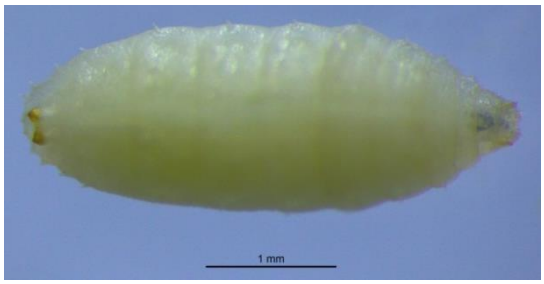
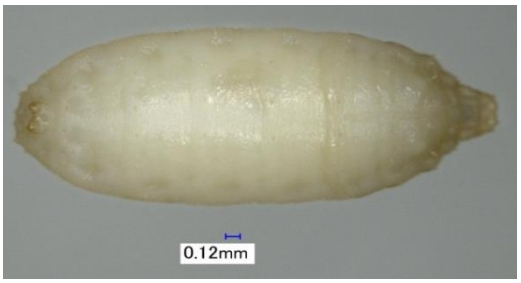


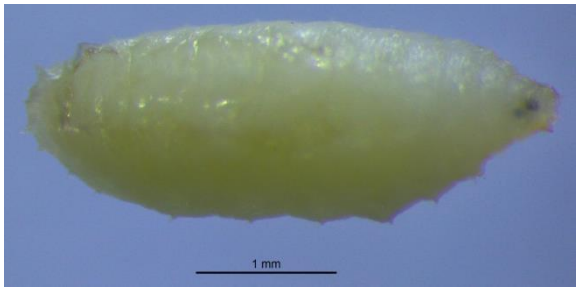

**Fig 19: Dorsal image of a ten day old *Megaselia scalaris* puparia, x26 magnification (PS =posterior spiracle, AS = anterior spiracle, RH = respiratory horn).**

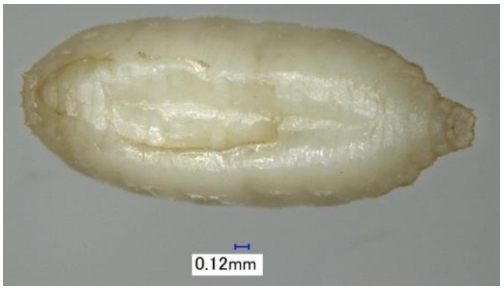
In order to correlate the changes that occurred over time through the development stage of pupa to fly, analysis and was performed (*Table 17*) at 0, 14, 36, 50, 71, 86, 100 % throughout the developmental period. Time 0% was the initial pupation formation plus 30 minutes max to prevent disruption of the pupation process. The pictures on the left hand side of the table were taken with the Leica microscope whilst the right hand side images were taken with the Keyence digital microscope. Ventral and dorsal images of the puparia are displayed.

**Table 17: Dorsal and ventral images from the pupal development phases of a male *Megaselia scalaris*. Numbers under the images represent the developmental time since pupation. Images on the left side of the table were acquired using the Leica microscope, images on the right side were acquired using the Keyence microscope.**

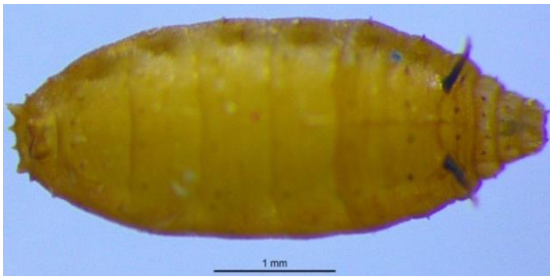


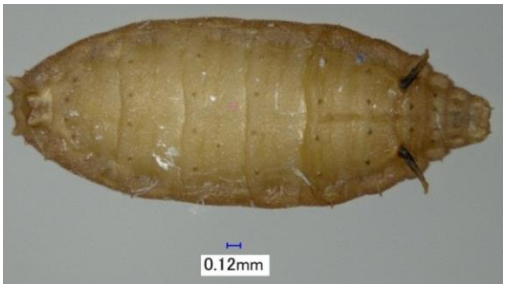



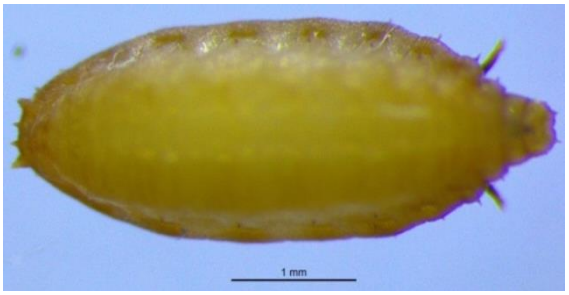



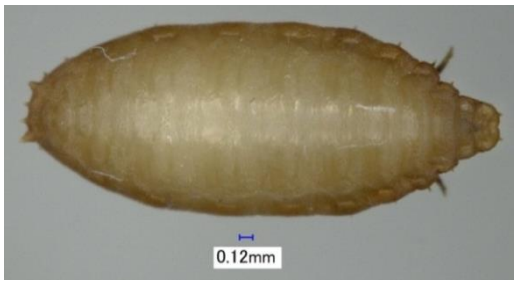




**0%** **0%**

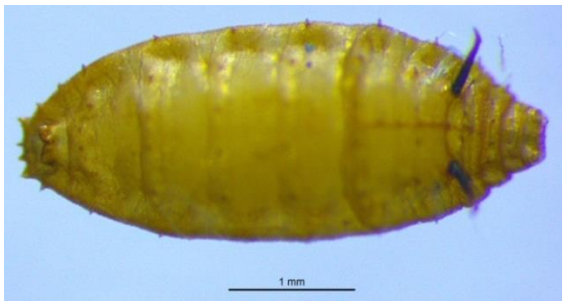


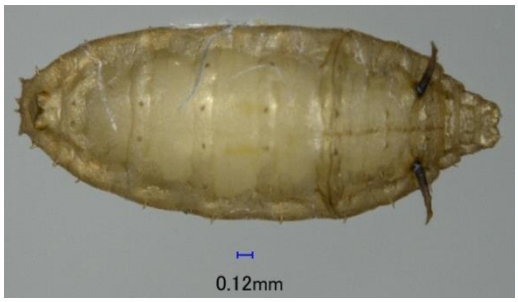



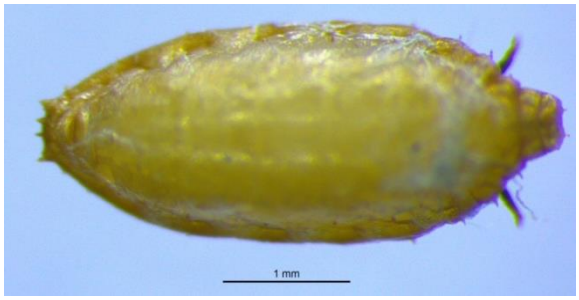



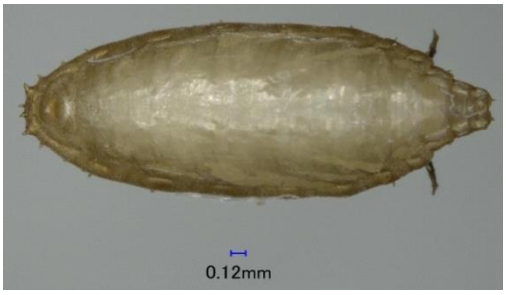

**14%** **14%**



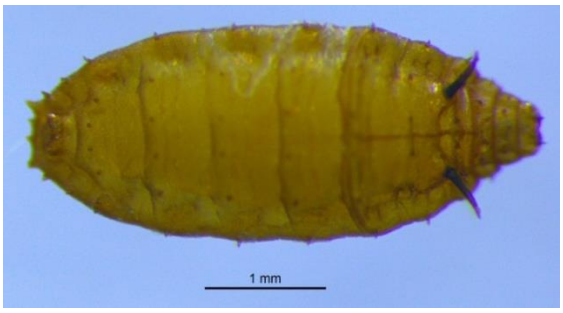



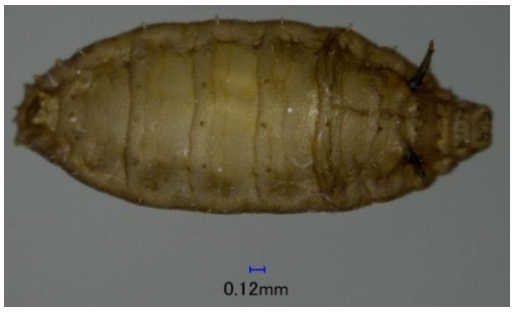



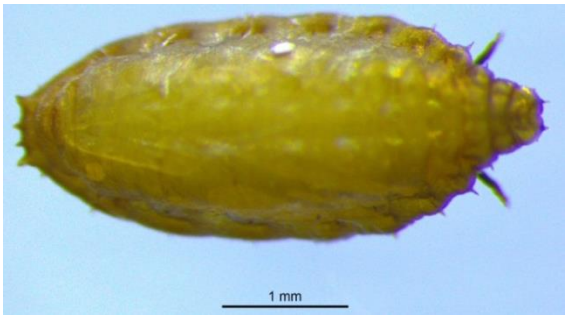

**36%**



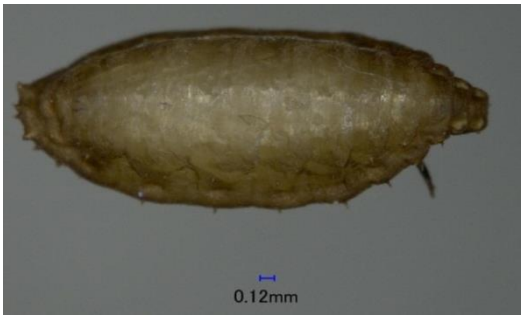

**36%**



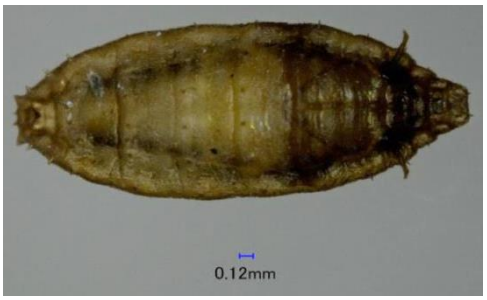




**50%**




**50%**



**71%**


**71%**




**86%**


**86%**




**100%**


**100%**

Other than size, males and females both have very similar characteristics throughout their development. The observations noted (*Table 18*) are those that can be observed through the transparent pupal case.

**Table 18: Sequence of visible events during pupal metamorphism of both *Megaselia scalaris* sexes.**

| Time (%) | Observations |
|---|---|
| 0 | White prepupae which has stopped moving completely. |
| 14 | Respiratory horns have everted.<br>Exoskeleton changes colour from white to yellow then to light brown. |
| 36 | No further colour change, white adult can be seen taking shape within the transparent puparia. |
| 50 | Further darkening of exoskeleton, clear legs seen through transparent puparia. |
| 71 | Head, thorax and abdomen can be defined. Edges around the adult are beginning to darken, thorax has darkened and scutellum present, hair present on thorax, legs have darkened in colour. Slight darkening around head down to thorax. |
| 86 | Head and thorax very dark, wings appear black and black eyes can be seen. |
| 100 | No further change. |

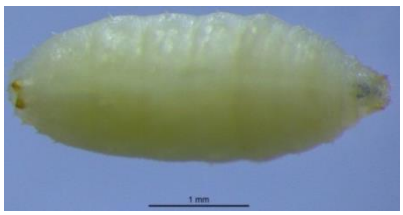
#### 2.2.3.3 Pharate Imaging

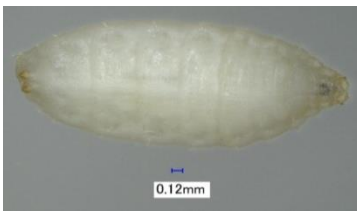
In order to correlate the changes that occurred over time through the development stage of pupa to adult fly, the pupal cases were fixed using the hot water immersion method and the pharate was removed by gently dissecting the pupal cage away from the pharate.

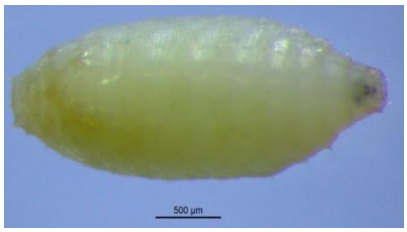
The pharate was removed at 0, 14, 36, 50, 71, 86, 100 % throughout the developmental period. Time 0% was the initial pupation formation plus 30 minutes max to prevent disruption of the pupation process. Prior to the respiratory horns emerging the pharate was not possible to remove therefore time 0 % is the pupal case.

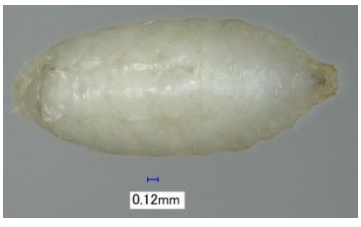
Dorsal, ventral and lateral images were taken (*Table 19*) of the pharate. The top rows of images were taken using the Leica microscope whilst the bottom rows of images were taken using the Keyence digital microscope. The observations are noted in (*Table 20*).

**Table 19: Dorsal, lateral and ventral images showing the morphology of pharate *Megaselia scalaris* male. Numbers under the images represent the developmental time since pupation. Images on the top of the table were acquired using the Leica microscope; images on the bottom were acquired using the Keyence microscope with the exception of 0% in which right side images were taken using Leica microscope and the left side images were taken using a Keyence microscope.**

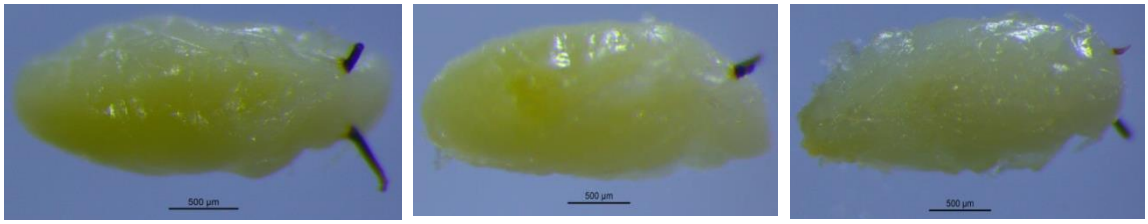


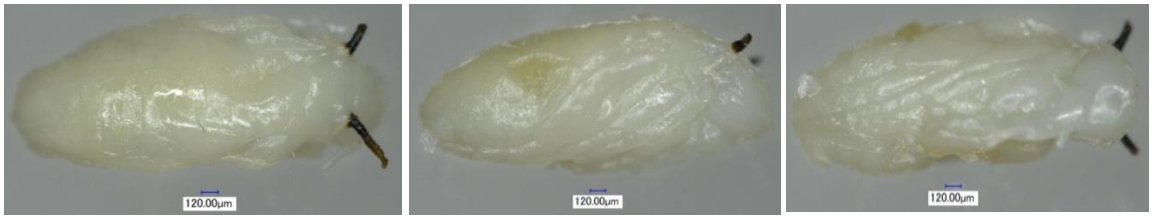



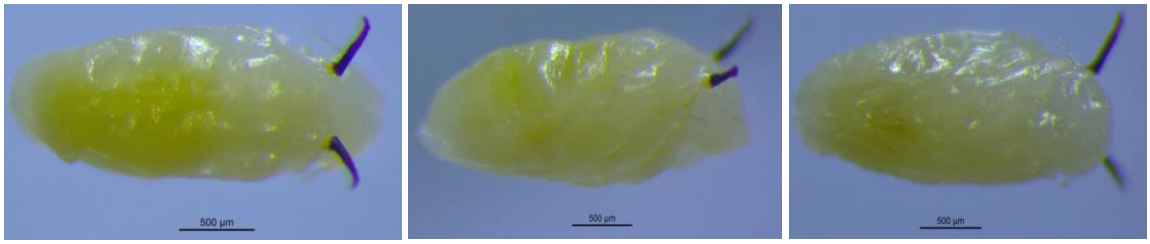



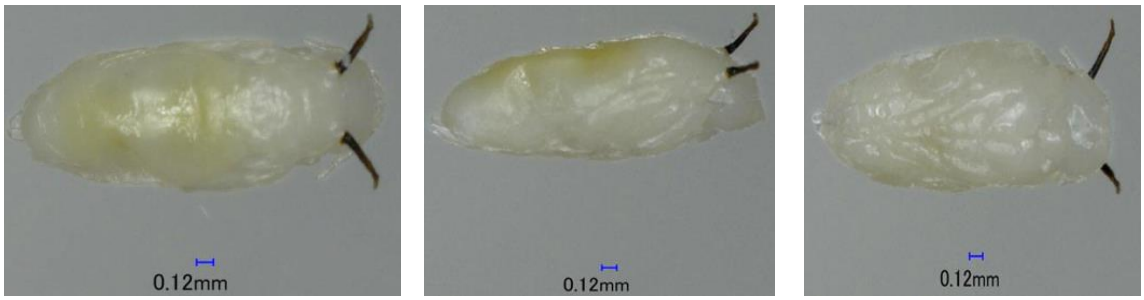

**0%** **0%**


500 µm
500 µm
500 µm

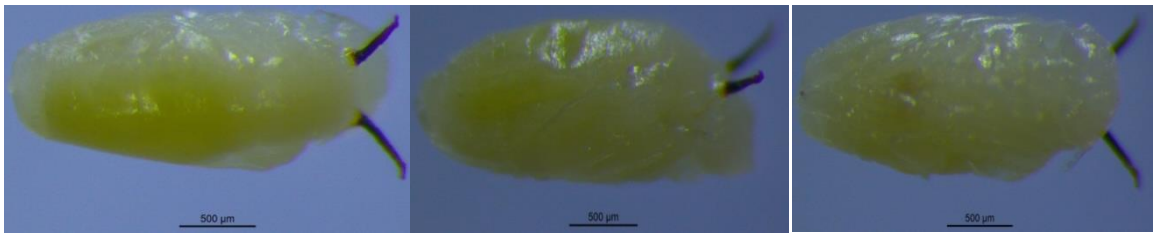

120.00µm
120.00µm
120.00µm

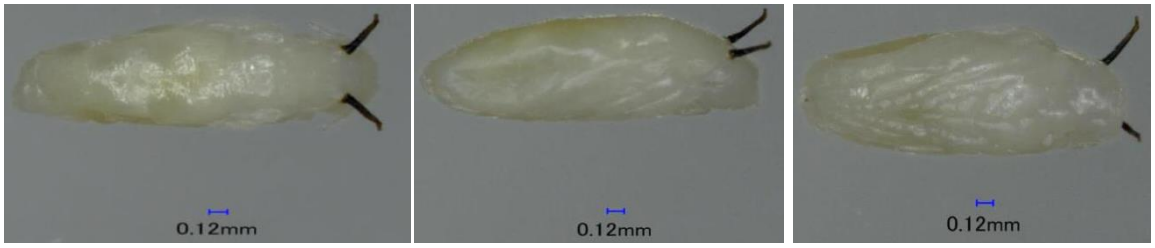
**14%**


500 µm
500 µm
500 µm

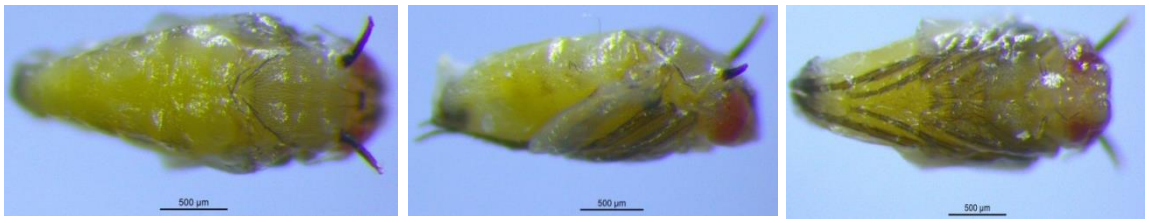

0.12mm
0.12mm
0.12mm

**36%**


500 µm
500 µm
500 µm

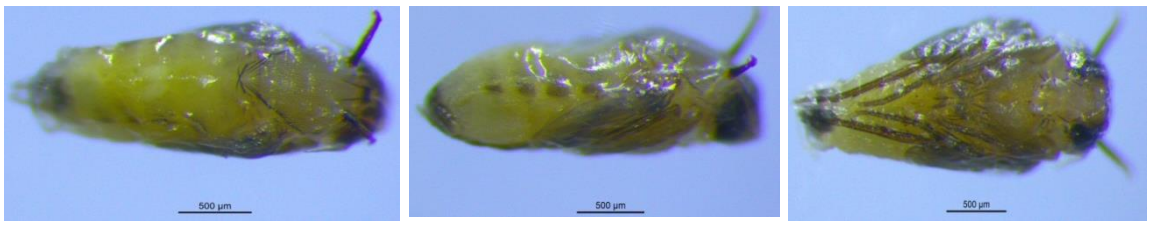

0.12mm
0.12mm
0.12mm

**50%**


500 µm
500 µm
500 µm


120.00µm
120.00µm
120.00µm

**71%**


500 µm
500 µm
500 µm


0.12mm
120.00µm
120.00µm

**86%**



**100%**


**Newly Emerged**

**Table 20: Description of morphological developmental changes of pharate *Megaselia scalaris* male.**

| Time (%) | Observations |
|---|---|
| 0 | White prepupae which has stopped moving completely. |
| 14 | Respiratory horns have everted. Outline of eyes, legs and wings can be observed. Mouth parts can be seen along with antennas which are currently white. |
| 36 | Small colour changes in the abdomen. White adult can be seen taking shape. |
| 50 | Body starting to take further shape. Legs and wings more prominent. |
| 71 | Head, thorax and abdomen can be defined. Edges around the abdominal tergites are becoming darker in colour, thorax has darkened and scutellum present, hair present on thorax, legs have darkened in colour. Slight darkening around head down to thorax. White wings and dark legs can be observed. A protective membrane surrounding the fly can be clearly seen. |
| 86 | Head and thorax very dark, wings appear darker in colour and black eyes can be observed. Stripes more prominent on the abdomen. |
| 100 | Protective membrane coming away from the adult fly ready for emergence. |

#### 2.2.3.4 Internal Imaging Collected Using Nikon XT H 225 series

In order to associate the changes that occur over time throughout the development stage within the puparia, X-ray tomography was performed using a Nikon XT H 225 series X-ray and CT scanner. The puparia images (*Fig 20* to *Fig 38*) were taken at different periods throughout the developmental stage and images taken. Stains had previously been used by Metscher (2009) and Richards and colleagues (2012b), this was an area that was also looked at. Images were taken using dry specimens, specimens that had been stored in 70% EtOH and specimens stained with an aqueous potassium iodide stain. The images were taken using 50kv energy and between 150 and 200µA current.

**Dry Images**

**Fig 20: Dorsal surface rendering of dry female *Megaselia scalaris* pupa.**

**Fig 21: Ventral surface rendering of dry female *Megaselia scalaris* pupa.**

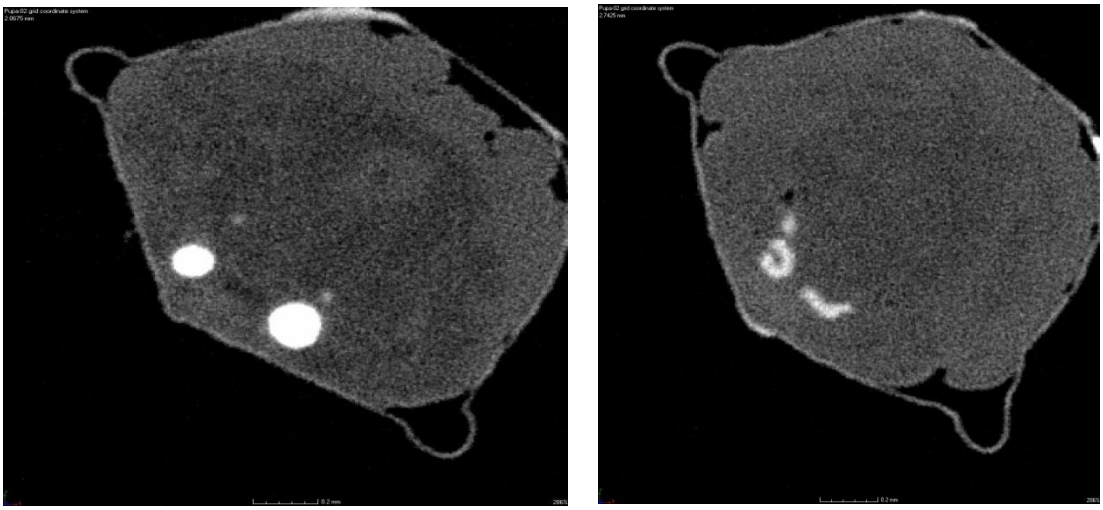


**Fig 22: Coronal images of female *Megaselia scalaris* dry pupa.**

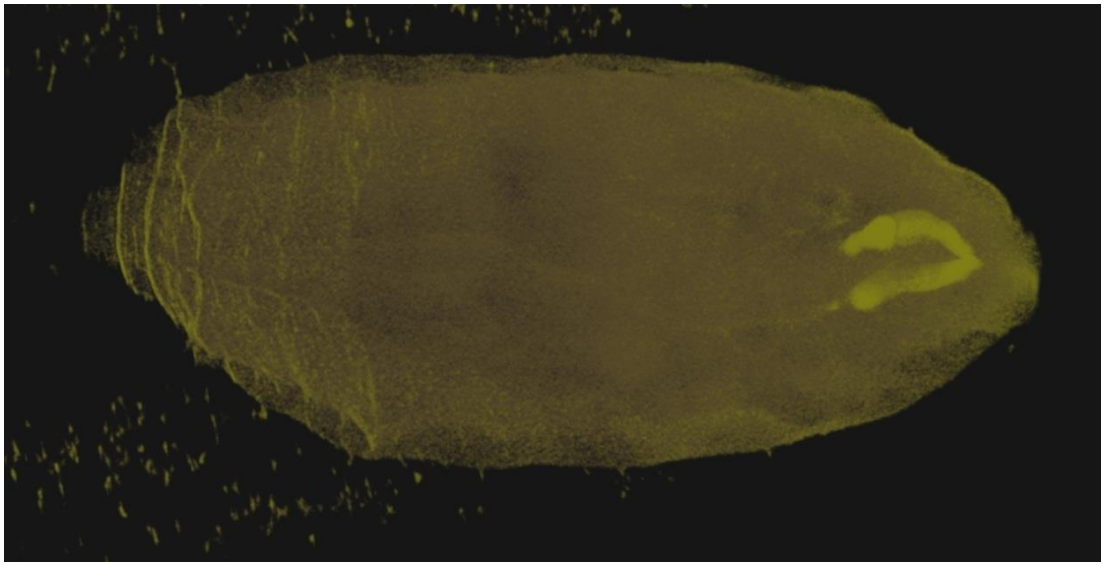


**Fig 23: Surface rendering of dry male *Megaselia scalaris* pupa.**

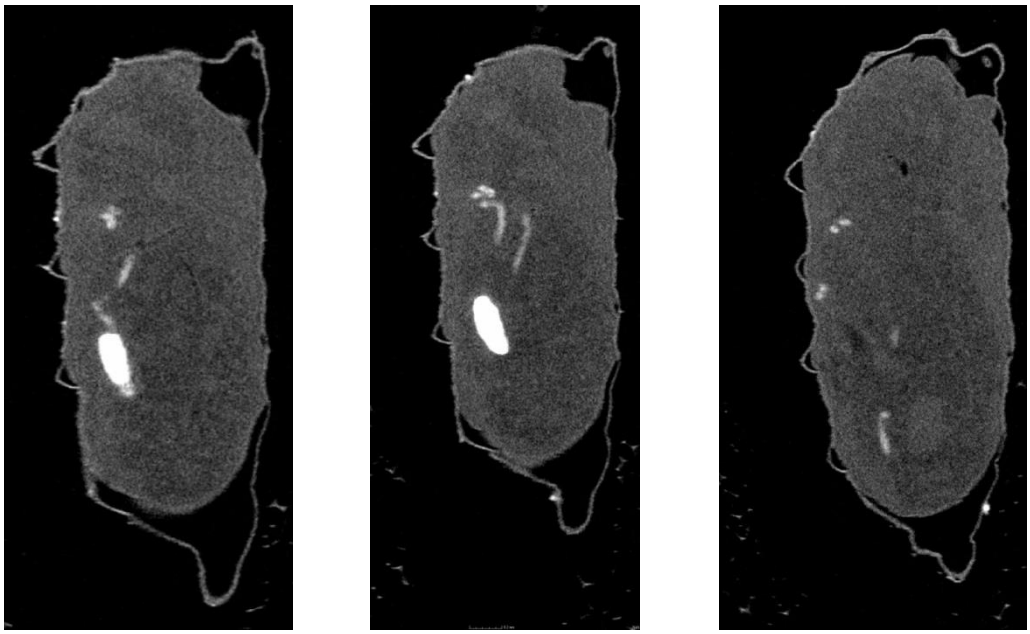


**Fig 24: Sagittal images of female *Megaselia scalaris* dry pupa.**

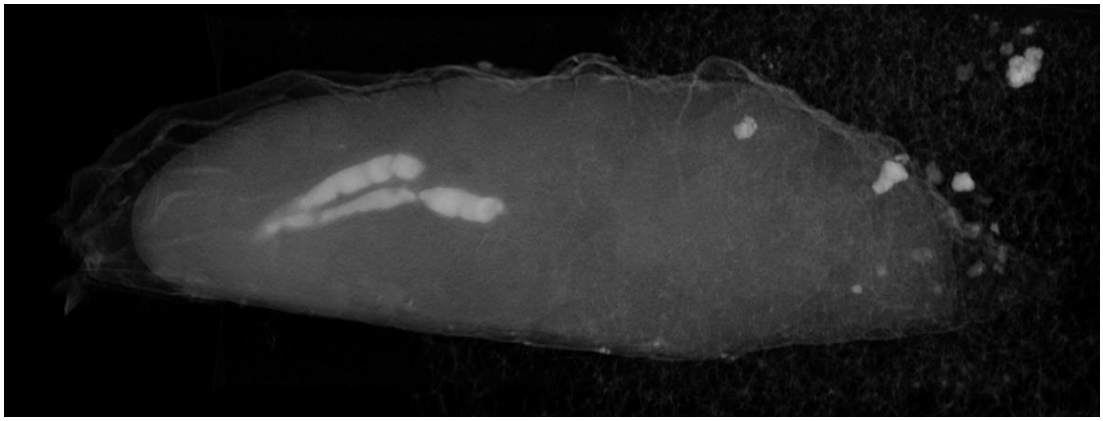


**Fig 25: Surface rendering of Megaselia scalaris pupa supported with oasis foam.**

## Stored in 70% Ethanol

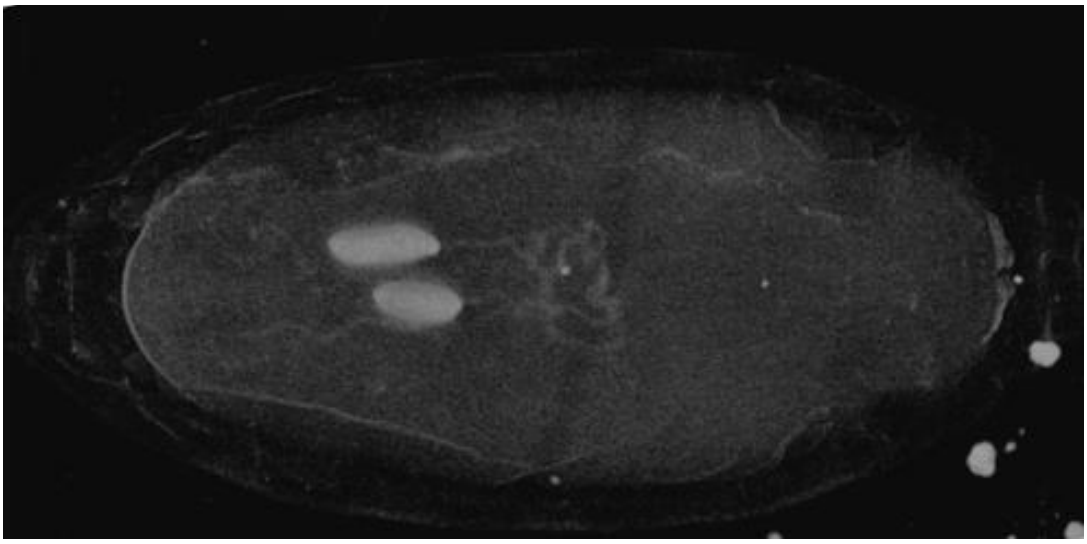


**Fig 26: Dorsal surface rendering of *Megaselia scalaris* pupa,**
**Stored in 70% EtOH and imaged in petroleum jelly.**

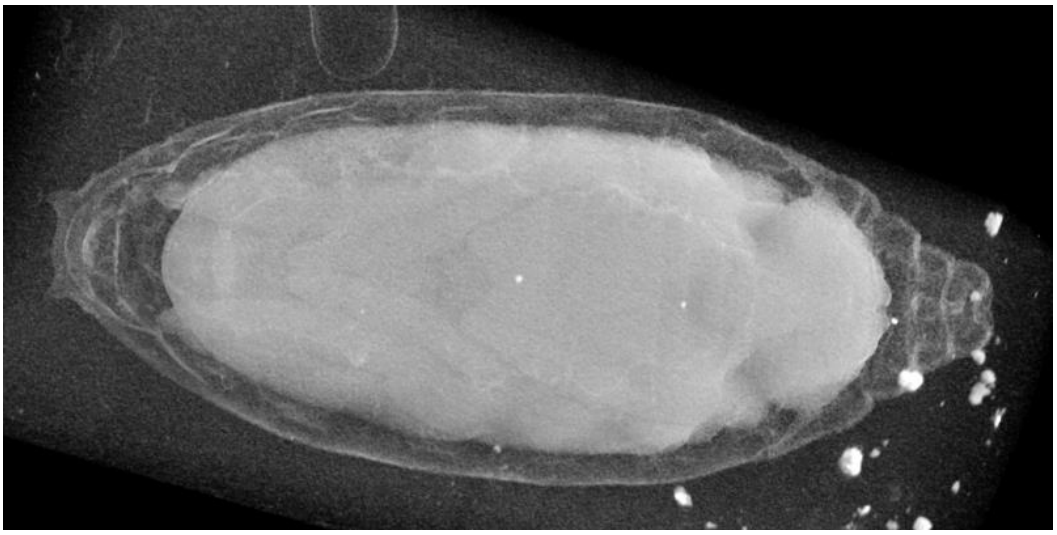


**Fig 27: Ventral surface rendering of *Megaselia scalaris* pupa**
**Stored in 70% EtOH and imaged in petroleum jelly.**

## Potassium Iodide Staining

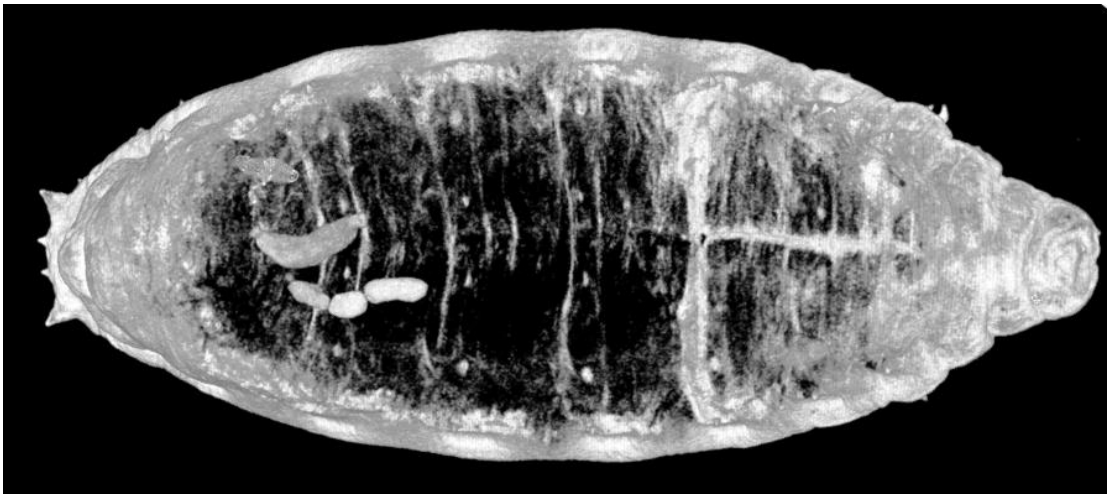


**Fig 28: Dorsal surface rendering of *Megaselia scalaris* pupa stained with KI for 24 hours Imaged in petroleum jelly.**

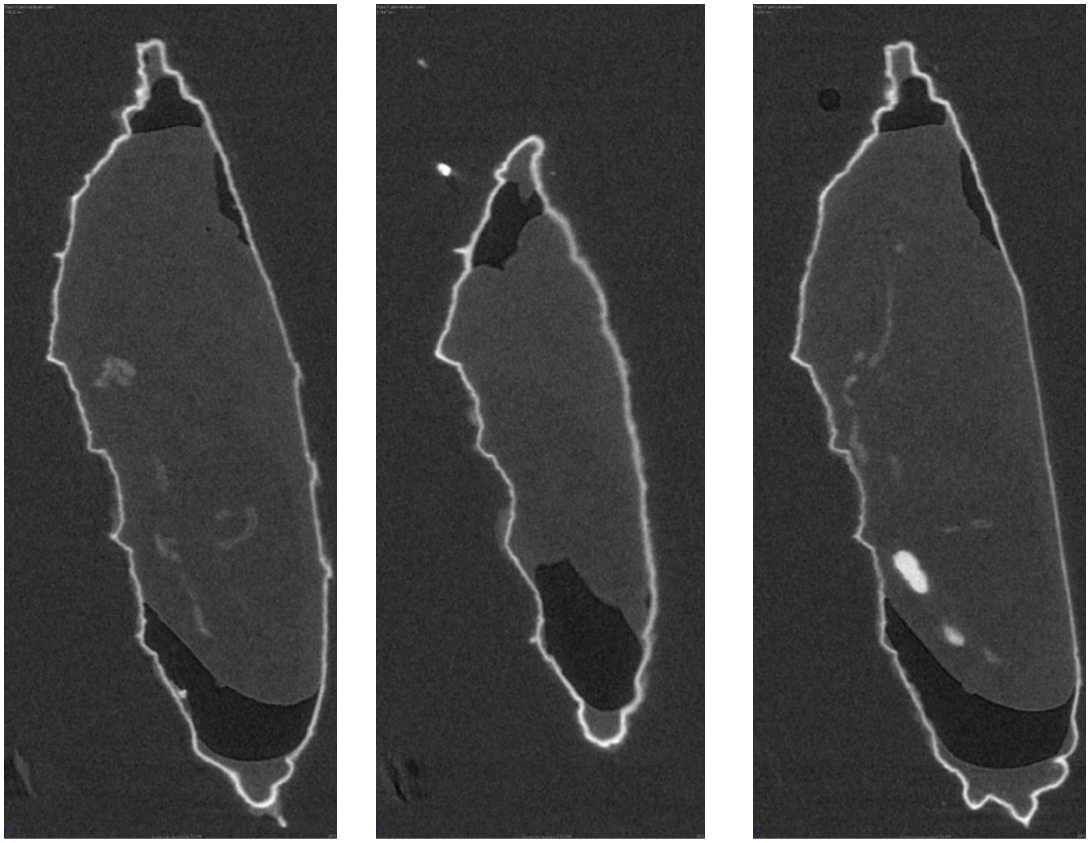


**Fig 29: Sagittal renderings of *Megaselia scalaris* pupa stained with KI for 24 hours.**

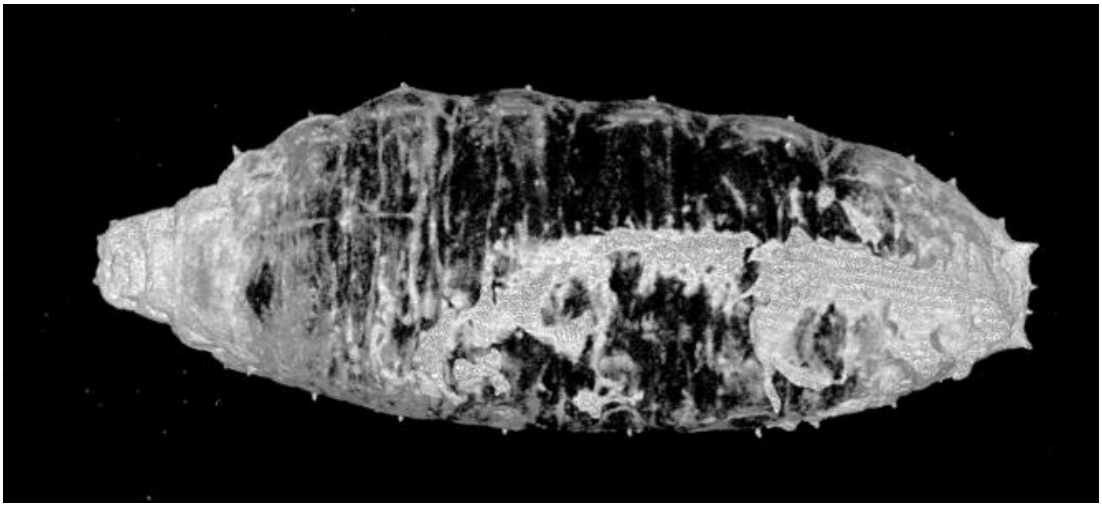


**Fig 30: Lateral surface rendering of *Megaselia scalaris* pupa stained with KI for 7 days.**

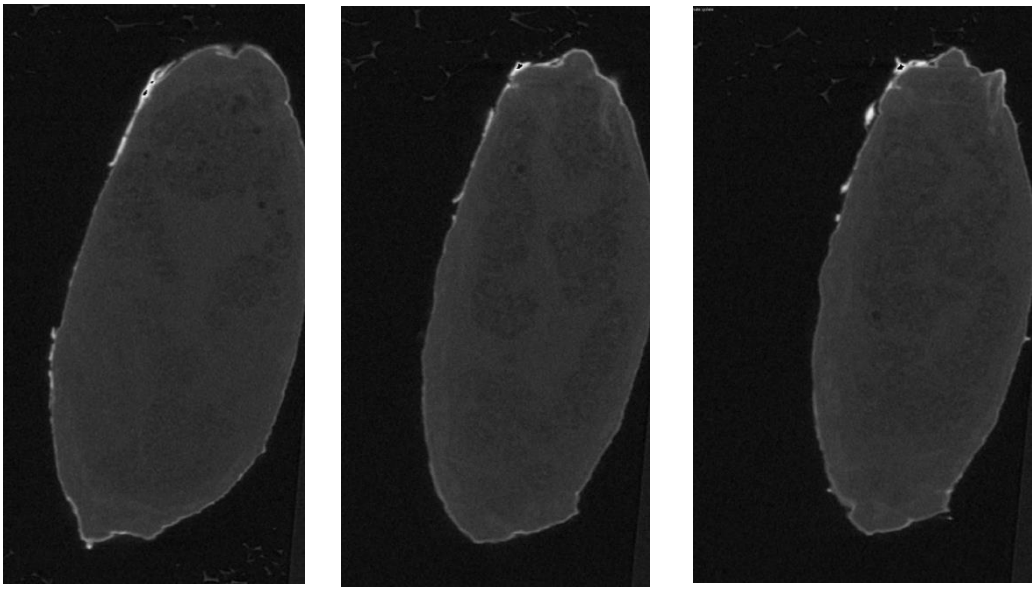


**Fig 31: Sagittal renderings of *Megaselia scalaris* pupa stained with KI for 7 days.**

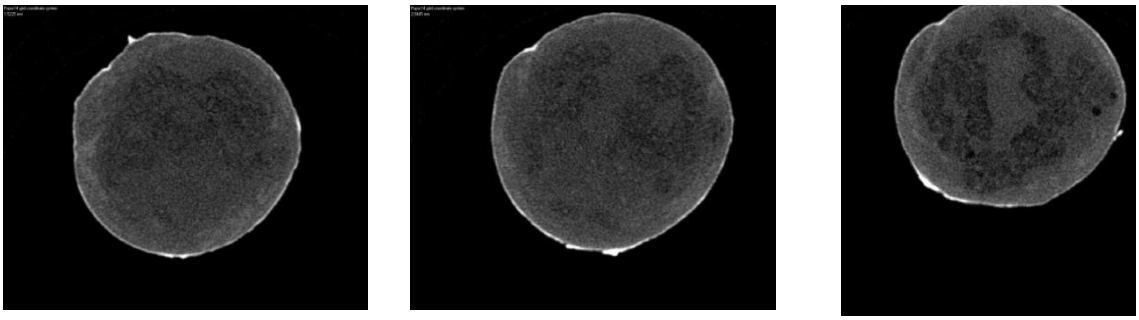


**Fig 32: Coronal renderings of *Megaselia scalaris* pupa stained with KI for 7 days.**

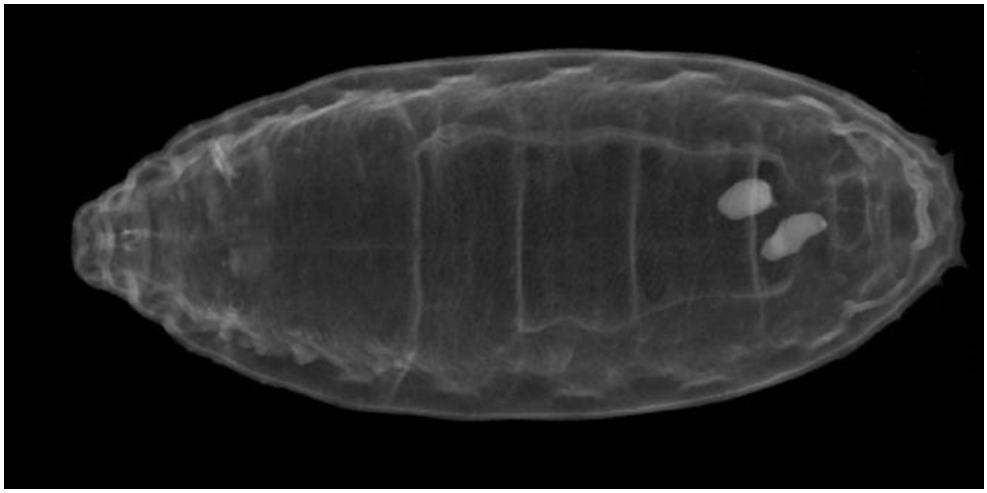


**Fig 33: Dorsal rendering of *Megaselia scalaris* pupa stained with KI for 3 days.**

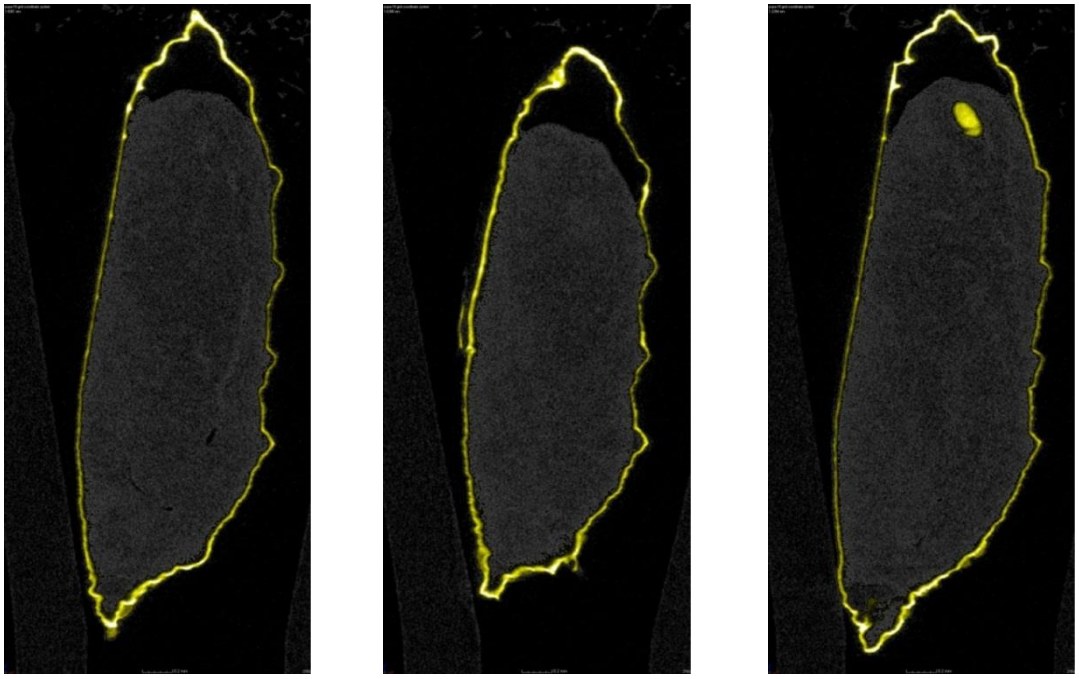


**Fig 34: Sagittal rendering of *Megaselia scalaris* pupa stained with KI for 3 days.**

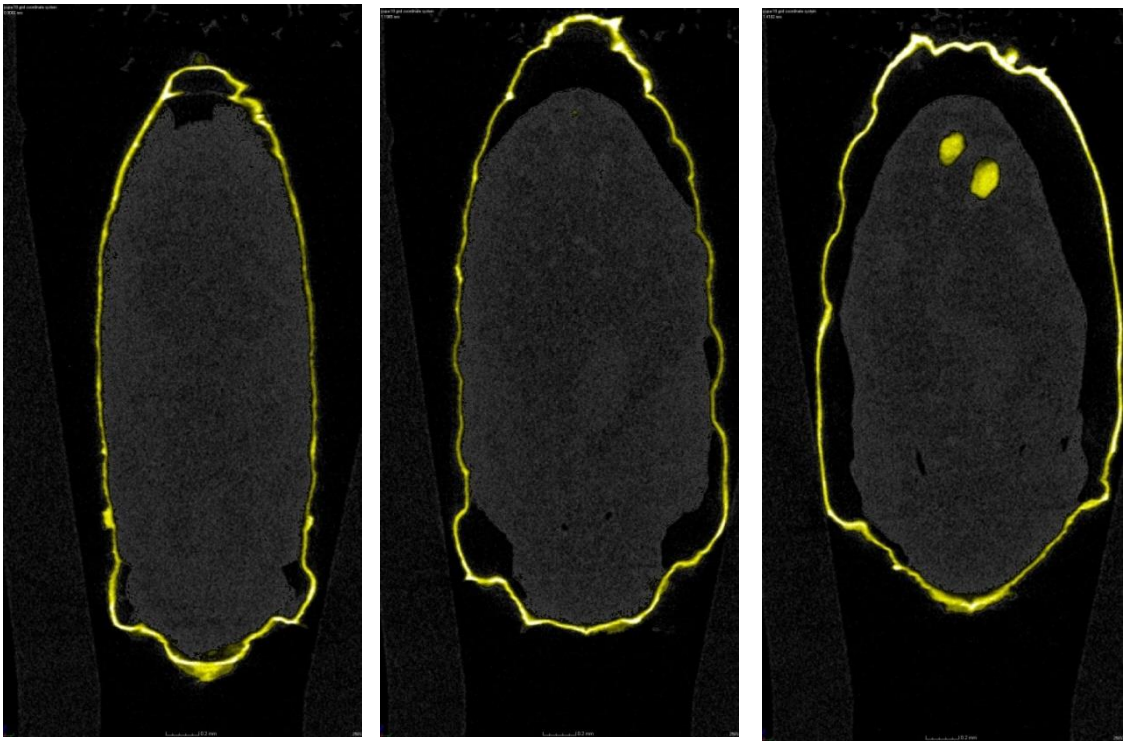


**Fig 35: Coronal rendering of *Megaselia scalaris* pupa stained with KI for 3 days.**

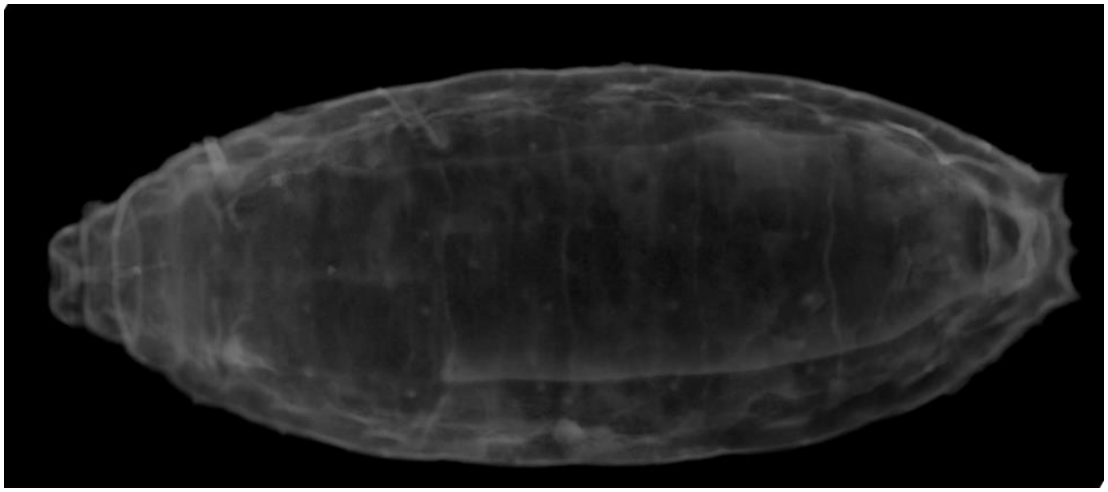


**Fig 36: Dorsal rendering of *Megaselia scalaris* pupa stained with KI for 5 days.**

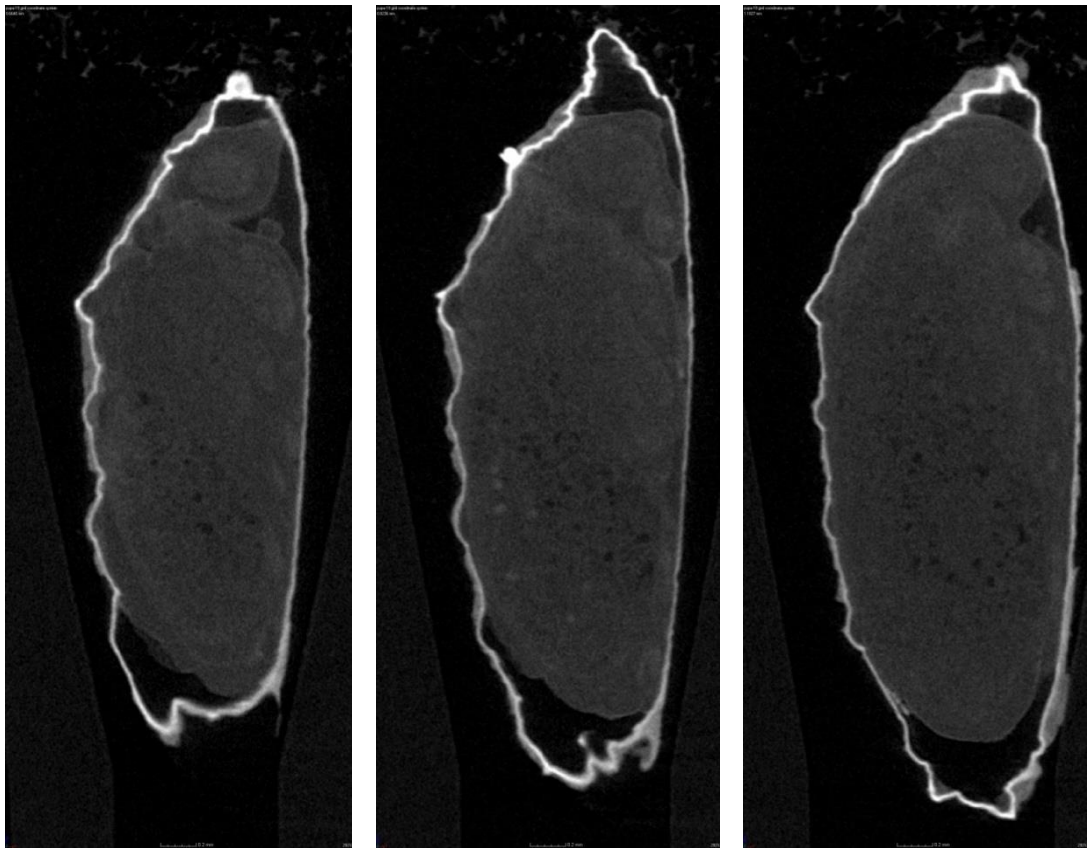


**Fig 37: Sagittal rendering of *Megaselia scalaris* pupa stained with KI for 5 days.**

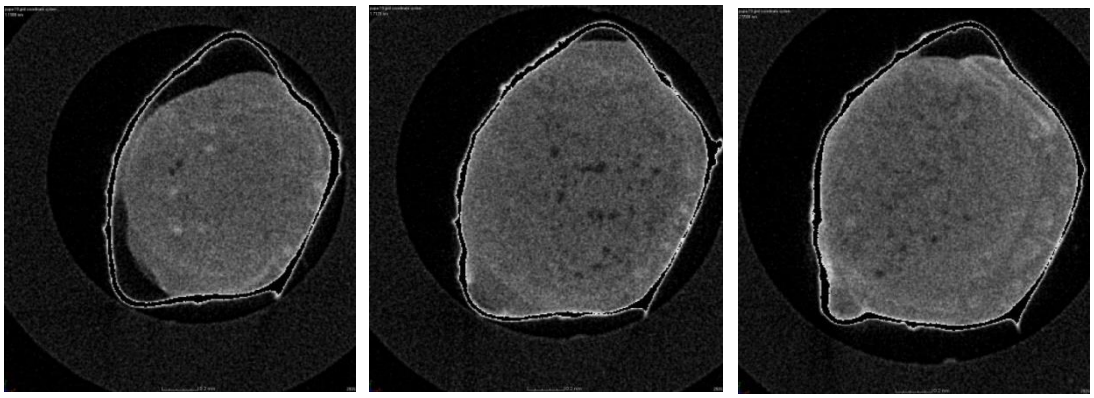


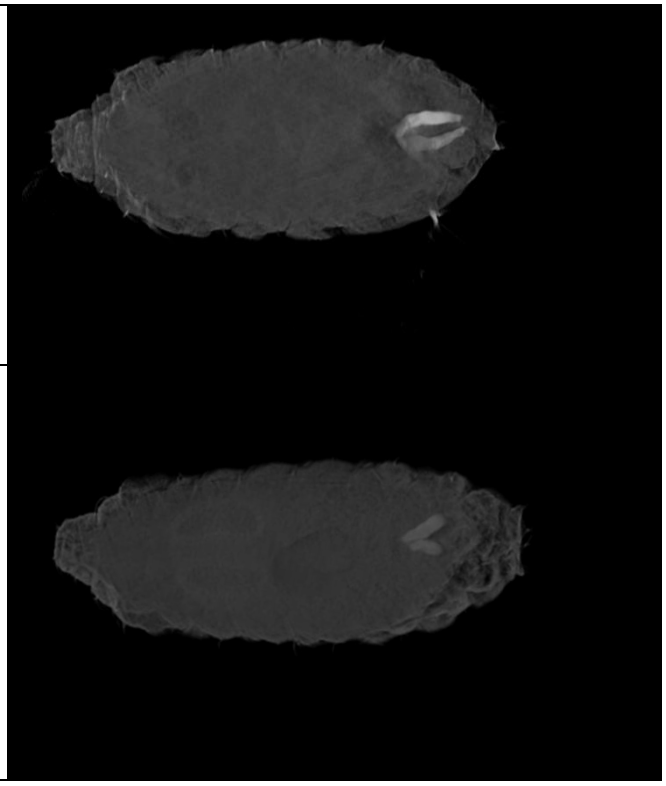
**Fig 38: Coronal rendering of *Megaselia scalaris* pupa stained with KI for 5 days.**

The images (*Fig 20* to *Fig 38*) show that the size of the specimen is of great importance as some X-ray and CT machines are unable to clearly image internal anatomical structures. Richards and colleagues (2012b) have shown that the Nikon XT 225 series works very well when used with a larger species such as *Calliphora vicina* (Diptera: Calliphoridae) however the Nikon XT 225 series does not work well with *Megaselia scalaris* due to the size of the specimens.

#### 2.2.3.5 Internal Imaging Collected At Elettra Synchrotron

In order to try to improve the resolution of the CT scan obtained with the Nikon, some samples were observed at the Elettra Synchrotron using monochromatic light and phase contrast. The puparia images were taken at different periods (*Table 21*) throughout the developmental stage from Time 0 % (Time 0 % was the initial pupation formation plus 30 min max to prevent disruption of the pupation process) to Time 100 %. The puparia were fixed using the hot water immersion method then stained with an aqueous potassium iodide, Metscher (2009) for 30 mins and then stored in 70% EtOH till imaging.

**Table 21: Developmental series of dorsal images displaying the morphology of pharate *Megaselia scalaris* female.**

| | |
|---|---|
| **0** |  |
| **10** | |

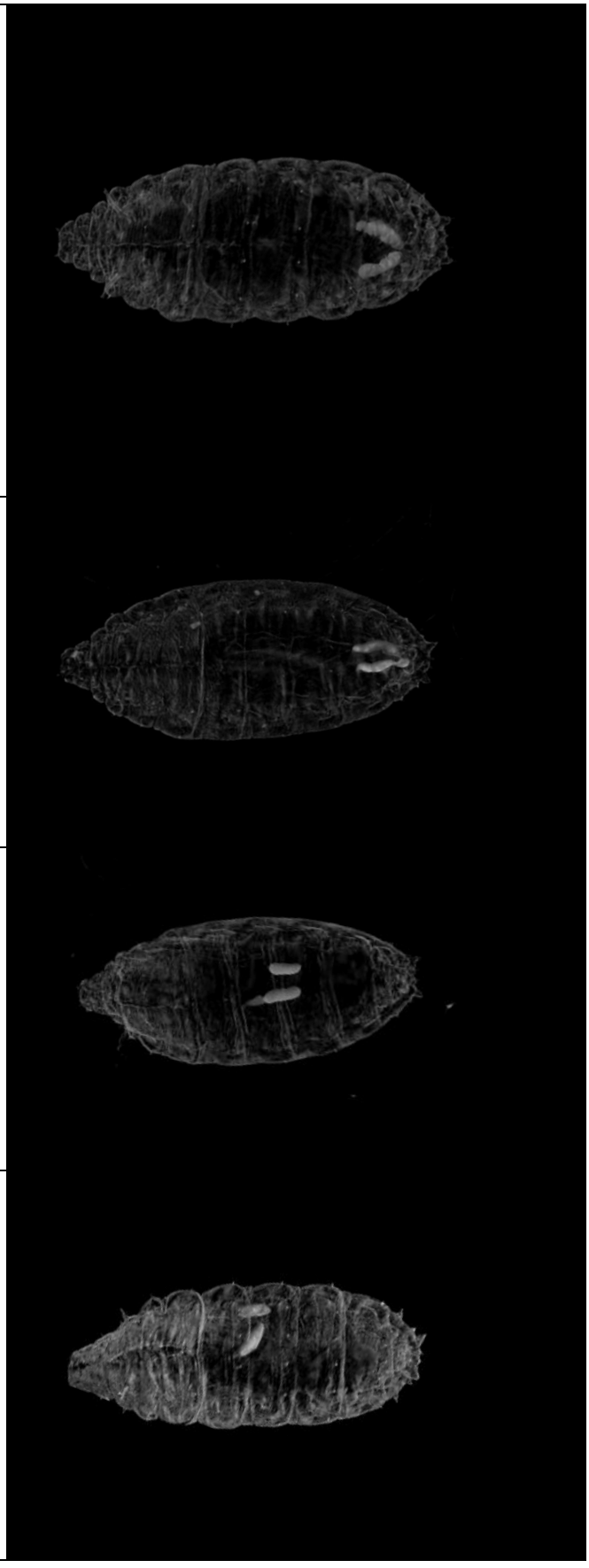
| | |
|---|---|
| **30** |  |
| **60** | |
| **80** | |
| **100** | |

**Table 22: Developmental series of dorsal images displaying the morphology of pharate *Megaselia scalaris* male.**

| | |
|---|---|
| **0** |  |
| **30** | |
| **60** | |
| **100** | |

In both the male and female images (*Table 21* and *Table 22*) we can see the reproductive organs. In the females the organs begin towards the rear of the puparia and they remain this way up to Time 60 % however when we look at Time 80 % we can see that the organs have moved towards the middle of the specimen and at 100 % we can see they have moved slightly passed the middle segment. When we compare this to the males we can see that again the organs again start at the rear of the abdomen however by Time 60 % they seem to have shifted towards the middle of the specimen however Time 100 % has conflicting results with the organs remaining towards the rear of the abdomen. A larger sample and a shorter time period have to be further analysed in order to better understand and describe the experiment.

### 2.2.4 Discussion

#### 2.2.4.1 Adult, Egg and Larval Imaging

Our morphological observations from the different development stages have the same findings to other researchers. The observations from the eggs are in agreement with previous work done by other researchers (Sukontason *et al*., 2004) who used potassium permanganate ($KMn0_4$) to view the eggs under a high powered microscope, however new technologies have become available that allow for materials to be viewed under the microscope with no preparation required. Our images show the morphology without stains and under high resolution.

#### 2.2.4.2 External Imaging of Pupa

Our results showed that the male pupa is smaller in size when compared to the female according to (Carareto and Mourão, 1988; Disney, 2008; Dian-Xing and Guang-Chun, 2014) and therefore this aided in sex determination of the pupa. The morphological characteristics of the male developmental stages are also observed in the female during the same stages and have therefore been recorded together.

Different killing and storing methods (i.e. HWI and stored in 70% EtOH) were used to determine the best method to dissect the pharate as some approaches were found to make the exoskeleton very brittle resulting in the pharate being easily destroyed. HWI was the best method as it also aided in softening the exoskeleton which made the dissection easier. Trying to remove the pharate prior to the respiratory horns being everted resulted in the pharate being easily damaged therefore the dissections took place after the respiratory horns everted which agreed with observations made by Dian-Xing and Guang-Chun (2014).

Colour differences were noted between our findings and those reported by Dian-Xing and Guang-Chun (2014), our wings were never viewed as black only, as white or grey, a yellow eye is never observed however we do see the changes in the colour of the abdomen at the same time the eye colour was reported by Dian-Xing and Guang-Chun (2014). Their newly emerged fly also differs in colour to ours; these variances may be put down to the differences in microscopes and lighting used as a Olympus BX41 stereomicroscope with lateral yellow illumination was used by Dian-Xing and Guang-

Chun (2014) whereas a Leica and Keyence digital microscope with white light were used to take our images.

By placing the pupal cases under the microscope allows the exoskeleton to become transparent which showed the external morphology of *M.scalaris.* The three main regions (head, thorax and abdomen) were easily identifiable from early on (14%) in the development process as were the mouth parts, legs, wings and antenna.

#### 2.2.4.3 Internal Imaging Collected Using Nikon XT H 225 series & Elettra Synchrotron

X-ray tomography is becoming increasingly used in the entomological field to help determine research from developmental rates, morphology of anatomical structures to estimating maggot mass volumes in bodies. Some technical problems were encountered during the collection of our initial images.

Dry imaging surface renderings of the dry female specimen was successful and external structures Including eyes, mouth parts and legs can be distinguished whilst the accessory glands related to the reproductive system can also be made out due to the density difference compared to the other internal organs. When looking at coronal and sagittal images, the dense areas can be made out clearly however there is difficulty when trying to identify further internal structures. The surface renderings of the male body show that the dense organs previously discussed are situated differently in the males' body and therefore it may be possible to use these organs as another sex determination tool. With the Nikon series, the male specimen is too small to be able to image further therefore no coronal or sagittal images were possible.

During the storage of specimens in 70% ethanol it was noted that the pupa cases became very brittle and would start to fall apart whilst in the ethanol. Trying to gently handle the specimens into the polyethylene mounting media would prove difficult due to the fragility of the cases. It was decided that ethanol was not a good storage medium for the *M.scalaris* when used with X-ray tomography. The imaging results were very similar to that of the dry specimens in that the dense internal organs can be seen along with the external structures as previously discussed. No coronal and sagittal images were taken.

The surface renderings of specimens stained with Potassium Iodide (KI) for 24 hours confirms that the Iodine has managed to enter the pupa case as there are a lot of

black areas on the rendering, this shows that the stain has begun to equilibrate the colour of the case and the internal structures. Due to the small amount of time in the stain the dense regions previously seen in the dry images are still present in this stained specimen. Observations were made that some of the specimens sunk to the bottom of the vial whilst in the stain whilst some floated. The specimens that sank were the ones imaged in all experiments.

The sagittal images show that the densest of regions can still be seen however no further structures can be observed.

Three day KI staining of specimens shows that the Iodine stain has managed to enter the pupa case as a lot of black areas are present on the rendering as observed in the 24 hour stain. The dense regions previously seen in the 24hr stain images are still present. The sagittal and coronal images show that the dense regions can still be seen whilst the other internal organs have started to darken in colour it is still impossible to be able to identify the relevant organs.

Surface renderings show that a 5 day staining in KI has made the dense organs seen previously in both the 24hr and 3 day stains fade as there is nothing visible in the puparia. Sagittal imaging shows some external features such as mouth parts and legs seen around the head and thorax region. The coronal images again show that some of the external structures can be made out however no clear internal structures are notable.

Seven day KI staining of specimens shows that staining for this length of time has had the maximum effect as the densest regions previously seen in both the 24hr and 3 day stains are no longer visible. Both the sagittal and coronal images again show that the dense regions are not visible however there are a lot of dark areas throughout the stack that are representing the internal structures, unfortunately they are not clear enough to be able to distinguish them.

Overall using the Nikon XT H 225 series did not give us the desired images due to the size of the samples. Whilst it has previously worked well in larger specimens e.g. *Calliphora,* for specimens as small as *M.scalaris* a lower kV is required for better images as the lowest the Nikon was only able to operate between 30 to 225 kV

Using Micro CT at Elettra in Trieste to continue work with pupa developmental studies using their monochromatic Synchrotron under 10 to 14 keV.

As a preliminary experiment it initially shows that we may be able to estimate the developmental stage of the females by determining the movement of the internal reproductive organs. Due to the conflicting image between Time 60 % and Time 100 % of the male images would suggest further imaging of the males needs to be completed to determine if the developmental stage can be estimated.

# 3: Effect of Diet on Development

## 3.1 Diets

### 3.1.1 Introduction

Collection and analysis of insect evidence from a forensic investigation and may be used to assist in forensic, legal or medico-legal cases by giving answers to important questions. Determining the period of insect activity on a cadaver is used to estimate the minimum post mortem interval (mPMI) or time since death, knowing the length of time required during each developmental stage of the insect at varying temperatures is imperative to a forensic case to determine the estimation of age of the insect (Varatharajan and Sen, 2000).

To be able to estimate the mPMI, it is of fundamental importance that the size and age of the insect is determined correctly when working with specimens taken from forensic investigations. Estimation of the development rate is completed in a laboratory by rearing the specimen on an artificial diet that mimics both human tissues along with the decomposition process. Factors that may affect development consist of geographical regions, high and low temperatures, direct access to the food source, indoor or outdoor death, clear or cloudy days, clothing, exposed or buried bodies, if the body was immersed in water, larval overcrowding, burnt and charred bodies (Campobasso *et al*., 2001).

For flies to oviposit, protein is an essential nutrient and can be sourced from numerous areas some of which include: animal and human excrement, animal carcasses, cadavers and food remains left around by humans. To precisely determine time since death, oviposition behaviour in necrophilous flies first needs to be established (Acikgoz *et al*., 2012). For successful development in eggs and larvae, females require moisture and therefore do not oviposit on either dehydrated or mummified tissue (Introna *et al*., 2001a).

Roberts *et al*. (1983) discusses that the larval development stage is the most sensitive phase and is most affected by lighting whilst the pupal stage responds more to temperature cycles than photoperiods.

Da Silva Mello *et al*. (2012), found that as the light phase increased the development time for all stages differed and was seen to be more pronounced for newly hatched larvae through to adult hood. Whilst much of the research looks into photo-period

and temperature growth rates, there is only a small amount of research looking into different pabulum having an effect on growth rates in relation to the *Megaselia scalaris*.

The development rate of *Calliphora vicina* when fed on decomposed liver was compared to those reared on frozen/thawed, minced and fresh whole liver, the research was carried out by Richards and colleagues (2012a). It was concluded that decomposed liver had a significantly adverse effect on the growth rates. Day and Wallman (2006) research the effect of frozen and thawed sheep liver on the development of *Calliphora augur*. It was concluded that there was no significant difference in larval development when fed on either frozen or thawed sheep liver when compared to larvae fed on fresh sheep liver.

Ireland and Turner (2006), found that when feeding *C.vomitoria* on three different pig tissues (liver, brain or muscle), that an increased rate of development was observed along with undersized larvae and adults, which may have been due to higher temperatures within the larval mass and an increased feeding regime.

Disney (2008), remarks that *M.scalaris* larvae require a liquefied sustenance to survive, to do this they secrete digestive enzymes on to a possible food source and have been documented surviving on a wide range of decomposing organic material such as curdled milk, paint, plants, animals and have also been reported breeding on boot polish. *Megaselia scalaris* flies have also been found to develop on ripe banana (Karunaweera *et al*., 2002) which aided in deciding the medium for the developmental studies.

There have been numerous studies (*Table 14*) of *Megaselia scalaris* development using a variety of temperatures ranging from 15 to 35 °C. Alongside the temperature studies using a wide range of foods (beef, *Drosophila* media, proteins etc.) were used. Overall findings demonstrated that larvae were longer at cooler temperatures and that development became stressful in the higher temperatures (> 30 °C). Few adults were observed emerging along with slow activity at 15 °C whilst the most rapid development was observed at 25 °C (Disney, 2008).

Larval diet influences the response to chemical cues therefore the diet that the larvae were fed on would be more attractive as an adult, this is called pre-imaginal conditioning (Tully *et al*., 1994; Barron and Corbet, 1999).

Trumble and Pienkowski (1979), researched temperature and photo-periods of *M.scalaris* using 26 specimens per temperature. The lower the temperature the longer the species survived. The flies were fed a *Drosophila* diet and a photo-period of LD 12:12 allowed for a more uniform survival at each temperature compared to LD 16:8. Larvae developed faster under LD 12:12 at 21 $^{o}$C and it was concluded that there were significant differences in growth rates at 21 and 27 $^{o}$C but no differences were observed at 32 $^{o}$C.

Five different diets and development rates of the *M. scalaris* were researched, the diets consisted of: nutrient agar (na), casein agar (ca), na + tissue extract from *Bradybaena similaris* (round snails), na + tissue extract from *Achatina fulica* (giant African snails), and na + tissue extract from *A.fulica* + cabbage leaves and which were determined to have an effect on the developmental rates (Idris *et al*., 2001).

Zuha and colleagues (2012) investigated the effect that different pabulum had on the development rates on post feeding larvae and pupa of *M. scalaris* by continual measuring and weighing of the specimens. It was determined that both temperature and pabulum types had an effect on puparia size in both the male and females. Pupal stages and emergence were also found to take longer the higher the temperature.

Baldridge *et al.* (2006) determined if forensically important flies had a preference to either bovine meat or universally prepared cat food (Whiskas$^{TM}$). It was determined the flies favoured bovine meat over cat-food for laying eggs or feeding. However for those who work in a laboratory environment the use of cat food is recommended as this would help to reduce the smell when the food dries up over time. Due to the *M.scalaris* larvae being able to consume anything that comes from either dead or alive organisms, makes rearing breeding colonies in a laboratory environment straightforward (Varney and Noor, 2010).

Different diets are seen to have an effect on the developmental data but how much of a difference is seen? Do different foods' affect the developmental rate or is that a temperature related parameter. This chapter is investigating two different diets that *M.scalaris* has been found breeding on to observe any differences in the developmental growth rate.

The objective of this study is to understand the developmental growth of this species using constant temperatures, time and size data as parameters.

### 3.1.2 Experimental Design

#### *3.1.2.1 Megaselia scalaris* Development on Sainsbury's smooth pate pet food

To test the effect of diet on growth rates, twenty vials, each containing 6 g of Sainsbury's smooth pate pet food were prepared; a small piece of filter paper was added to each of the vials, previous experiments showed the food would get very moist causing the eggs and larvae to drown, the addition of filter paper to the breeding vials prevented this.

Sainsburys smooth pate cat food is made up of: Meat and Animal Derivatives (Minimum 4% Turkey, Minimum 4% Chicken), Minerals, Various Sugars, Oils and Fats, Derivatives of Vegetable Origin.

Five male and female adult *M.scalaris* flies were added to the vials and left for 3 hours to oviposit. Ten vials were placed into a 20 ±0.5 °C incubator whilst the remaining vials were placed into a 25 ±0.5 °C incubator. Both incubators were programmed with a 12:12 photo-period. After 3 hours the adults were removed and this was called time zero. Thirty eggs from one of the ten vials were taken and measured whilst the remaining nine vials were left to allow the eggs to continue developing. After the initial egg measurements were taken, thirty measurements were taken from a vial every 24 hours until pupation. Due to the inability to sex the larvae, thirty of the largest larvae were selected for each experiment.

The HWI method (Adams and Hall, 2003) was used to kill the larvae, a kettle was used to boil the water, once boiled the water was left for thirty seconds and then poured over the larvae, the larvae were removed, placed on to a microscope slide then imaged/measured immediately afterwards. This method was used for all larval experiments unless otherwise stated.

One measurement was taken from the largest pupae which were not killed when measured and were placed back into the breeding vials after imaging/measuring and left to continue their development.

Once the flies began emerging, they were left for a total of three days to allow for maximum emergence. After three days the vials were placed into - 20 °C freezer and the

flies were killed. The left wing was removed from the females and 5 different measurements were taken (*Fig 39*).

Images/measurements were taken using a Leica M60 microscope and images taken using Leica software.

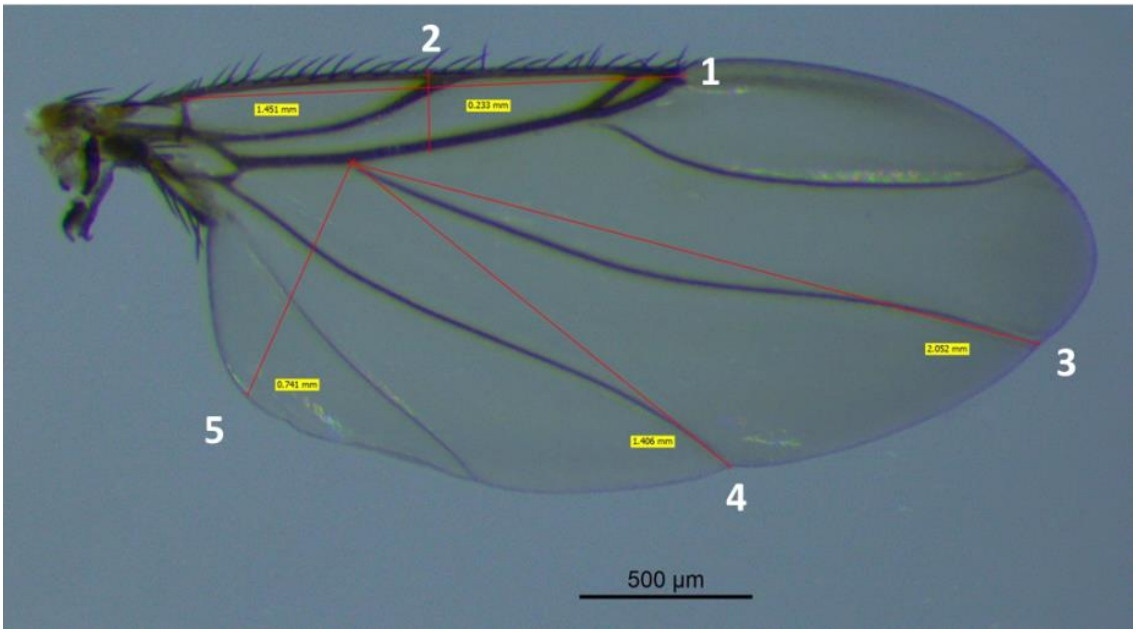



**Fig 39: Parameters of 5 different wing length measurements taken from *Megaselia scalaris* (red line), scale = 500µm, x40 magnification**

Statistical analysis was performed using Microsoft Excel and IBM SPSS independent sample t-test and ANOVA.

#### 3.1.2.2 *Megaselia scalaris* Development on Fresh Pork Liver and Banana

To test the effect of different diets on growth rates, twenty vials containing 8 g of homogenised fresh pork liver and 20 vials containing 6g fresh banana were prepared, a small piece of filter paper was added to each of the vials (to prevent drowning). Five male and female *M.scalaris* adults were added to each vial and left for 3 hours to lay eggs. Ten liver vials and 10 banana vials were placed in a 25 ±0.5 °C Incubator whilst the remaining vials were placed into a 20 ±0.5 °C incubator. Both incubators were programmed with a LD 12:12 photo-period.

After 3 hours the adults were removed and this was called 'time zero', the vials were left to allow continual development of the eggs.

The HWI method (Adams and Hall, 2003) was used to kill the larvae, a kettle was used to boil the water, once boiled the water was left for thirty seconds and then poured over the larvae which killed the larvae on contact, the larvae were removed, placed on to a microscope slide then imaged and measured immediately afterwards. This method was used for all larval experiments during this experiment unless otherwise stated.

Thirty larvae were taken from each food every 24 hours up to pupation. One length measurement was taken from the pupae a few days into pupation. The pupae were imaged and measured live and placed back into the breeding vials to continue their development.

Once the flies had emerged they were placed into - 20 °C and killed. The right wing was removed from the adult flies and measured (*Fig 39*) using a Leica M60 microscope.

### 3.1.2.3 Larval Food Preference

In order to investigate if larvae had a food preference as some literature reveal that *M.scalaris* larvae have been found eating and reproducing on strange foods such as boot polish, paint and banana (Lever, 1944; McCrae, 1967; Karunaweera *et al*., 2002).

Ten pet food bred third instar feeding larvae were placed into a 150 mm by 15 mm Petri dish. The dish had been divided into three compartments (*Fig 40*). A different food stimulus (*Table 23*) was placed into each of the compartments under light conditions. The experiment was performed with two or three food choices: during two compartmental experiments, larvae were placed into the bottom compartment (C), when all three compartments (A, B & C) each contained food stimuli, the larvae were placed in the middle of the three compartments. The larvae were monitored after fifteen and thirty minutes and their locations recorded. Each experiment had nine replicates. The stimuli were rotated in a random way in each of the compartments to avoid any location bias.

**Table 23: Different stimuli experiments used with *Megaselia scalaris* larvae.**

| | |
|---|---|
| Control (no stimuli) | Pet food vs. Liver |
| Sugar solution | Pet food vs. Liver vs. Sugar |
| Sugar solution vs. Pet food | Pet food vs. Liver vs. Banana |
| Sugar solution vs. Liver | Colgate Total Toothpaste |
| Honey | HP Brown Sauce |
| | |

NB: Colgate toothpaste is made up of: Aqua, Glycerin,Hydrated Silica,PVM/MA, Copolymer, Sodium Lauryl Sulfate, Cellulose Gum, Aroma, Sodium Hydroxide, Carrageenan,Sodium Fluoride, Triclosan,Sodium Saccharin, Mica, Cinnamal,Eugenol, Limonene, CI 77891, CI 42090, CI 47005, Contains: Sodium Fluoride (1450 ppm F).

HP brown sauce is made up of: Tomatoes, Malt Vinegar (from Barley), Molasses, Glucose-Fructose Syrup, Spirit Vinegar, Sugar, Dates, Modified Cornflour, Rye Flour, Salt, Spices, Flavourings, Tamarind.

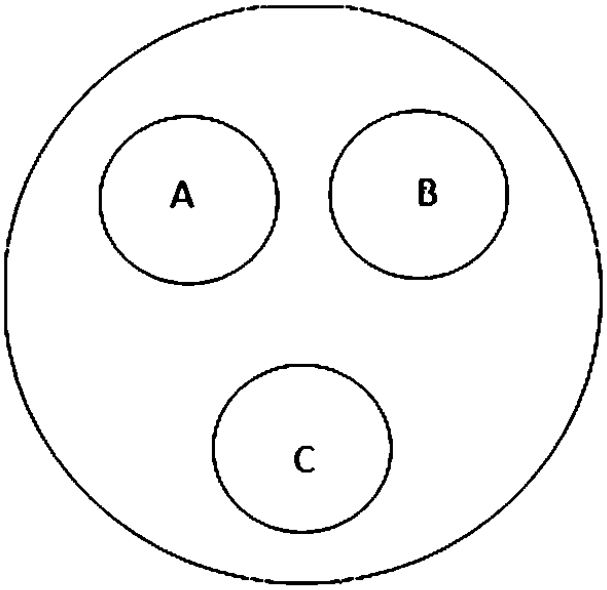



**Fig 40: Schematic of larvae maze representing compartments A to C.**

#### 3.1.2.4 Pre-imaginal Conditioning of *Megaselia scalaris*

To determine whether the larvae had been conditioned into a specific food preference from the rearing of the adult flies on a specific food i.e. pet food, further experiments were run using both liver and banana reared larvae.

### 3.1.3 Results

#### 3.1.3.1 *Megaselia scalaris* Development on Sainsbury's smooth pate pet food.

There has been much research done investigating development and growth rates of *M.scalaris*, it is essential to a forensic case to know at what temperature larvae were reared, as the length of larvae gives an estimation of age. The followings experimental results (*Fig 41*) show the larval, pupa and wing developmental differences between 20 °C and 25 °C from *M.scalaris* flies reared on fresh Sainsbury's smooth pate pet food. The pupa and wing measurements were taken from females whilst the larval data was taken from 30 random larvae.

30 measurements were taken from the vials every 24 hours until pupation.

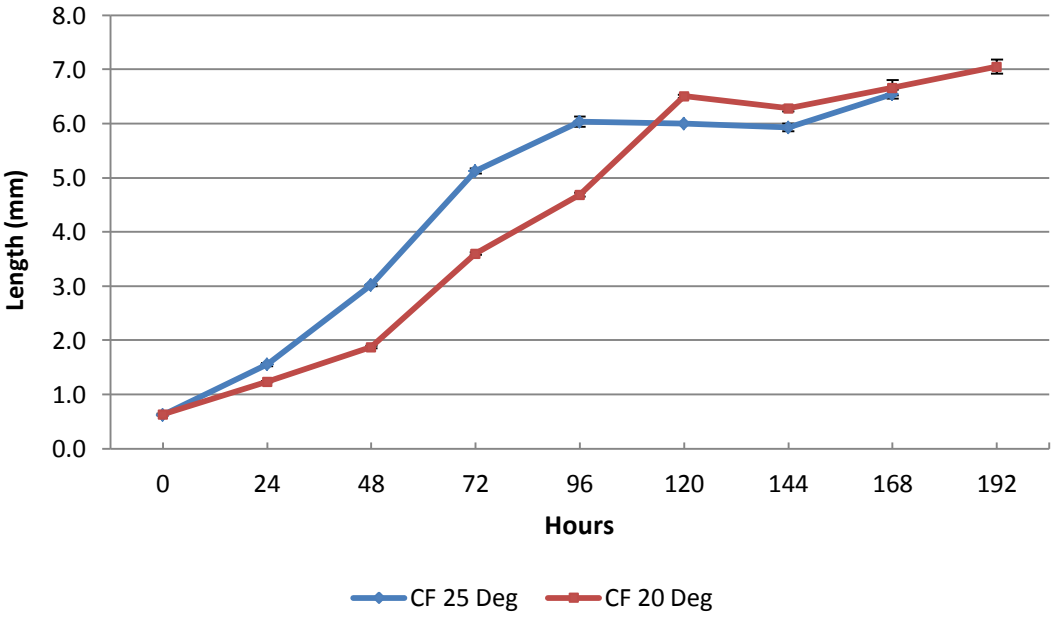



**Fig 41: Developmental data of *Megaselia scalaris* at 20 °C and 25 °C from egg to post feeding larvae reared on Sainsbury's smooth pate pet food**
**Measurements of length (mm) were recorded every 24 hours (n = 30). Error bars represent standard error of the mean.**

Overall development shows that the specimens under 25 °C developed quicker and faster when compared to those at 20 °C however the specimens at the lower temperature were longer towards the final stages of larval development. Larval measurements showed a significant difference between 24 and 144 hours (n=30, p=0.00)

however the last comparison measurement at 168 hour saw no significant difference (p=0.352).

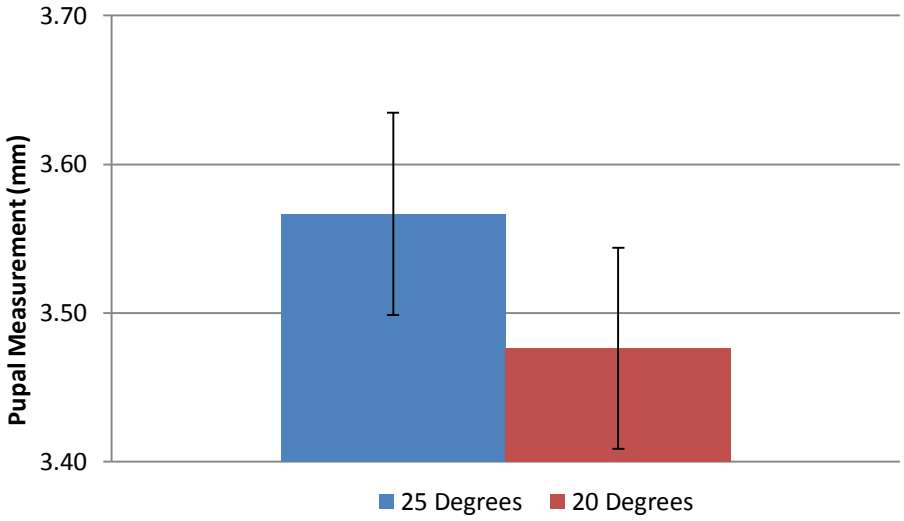



**Fig 42: Pupal length measurements of *Megaselia scalaris* at 20 °C and 25 °C reared on Sainsbury's smooth pate pet food.**
**Measurements of length (mm) were recorded half way through the pupation stage (n = 30, p=0.198). Error bars represent standard error of the mean.**

Pupal length was observed (*Fig 42*) to be longer at 25 °C however statistical analysis saw no significant differences (n=30, p=0.198) observed between the two temperatures.

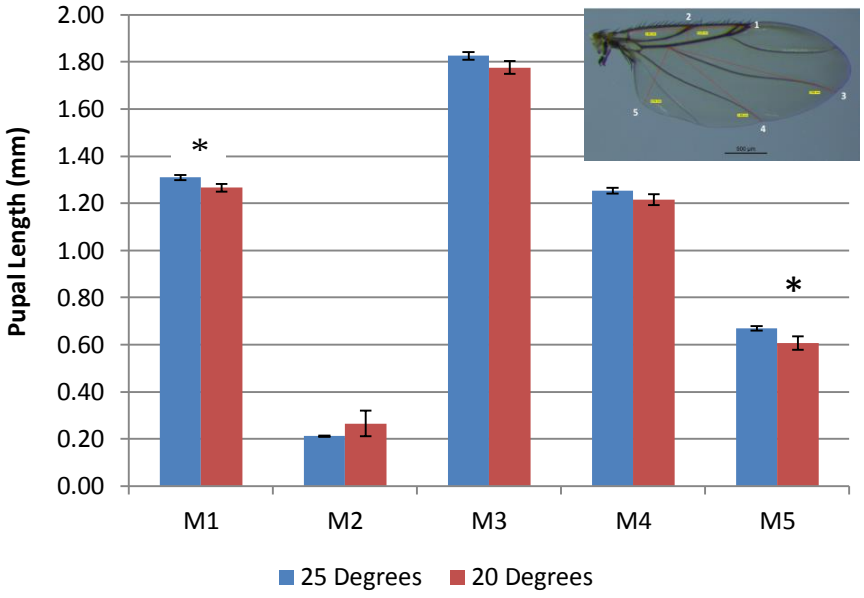



**Fig 43: Wing measurements from *Megaselia scalaris* females at 20 °C and 25 °C reared on Sainsbury's smooth pate pet food.**
**Measurements of length (mm) were taken from the left wing (n = 30). Error bars represent standard error of the mean, * represents significant differences.**

Wing measurements (*Fig 43*) taken from specimens at 25 $^{\circ}$C appear slightly longer with the exception of M2 which was shorter. The only measurements that showed a significant difference were M1 $p = 0.44$ (length from the subcostal to the end of the costa cilia) and M5 $p = 0.006$ (vein).

### 3.1.3.2 *Megaselia scalaris* Larval Development on Liver and Banana.

There has been much research investigating development and growth rates of *M.scalaris*, it is essential to a forensic case to know at what temperature larvae were reared, as the length of larvae gives an estimation of age. The followings experimental results and statistical analysis show if there are any differences in the larval, pupal and wing measurements taken when *M.scalaris* flies were reared on fresh banana, fresh pork liver and cat food at 20 and 25 °C.

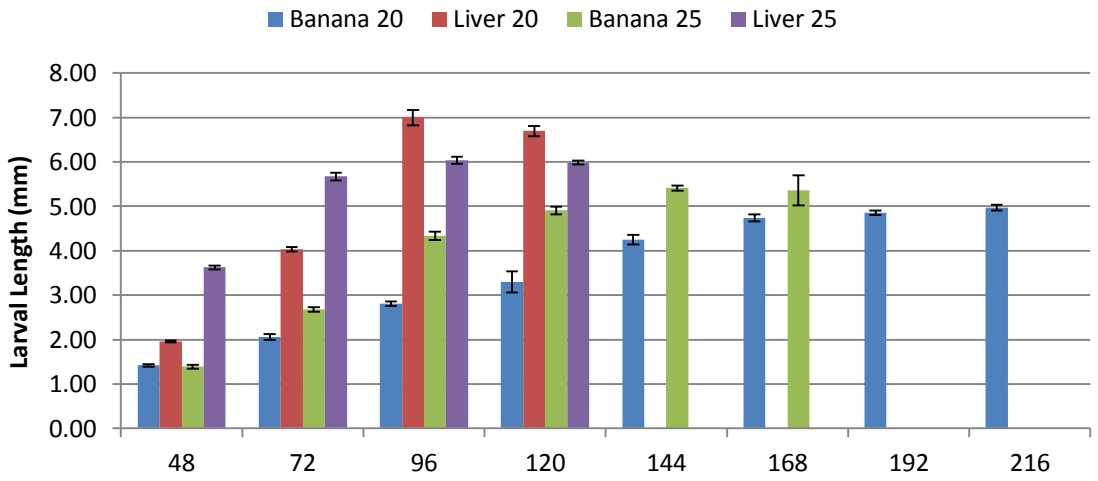



**Fig 44: Developmental data of *Megaselia scalaris larvae* reared on fresh banana and fresh pork liver at 20 and 25 °C from 48 hours to post feeding larvae.**
**Measurements of length (mm) were recorded every 24 hours (n = 240, p=0.00). Error bars represent standard error of the mean.**

*M.scalaris* larvae reared on fresh banana show that development at 20 °C took 48 hours longer to reach the post feeding stage when compared to those at 25 °C (*Fig 44*) however larval length was longer at the higher temperature. Statistical analysis (*Table 24*) showed significant differences in larval lengths from both temperatures (n=30, p=0.00) in all but the data from 48 hours (n=30, p=0.605). Overall when the data from both temperatures was analysed a statistical difference was observed (n= 150, p=0.00).

*M.scalaris* larvae reared on fresh pork liver show that at both temperatures (*Fig 44*), the post feeding stage was reached at the same time. Initially larval length was longer in the larvae reared at 25 °C up to 72 hours however at 96 hours larval length at 20 °C

exceed those at 25 °C and remained that way until post feeding. Statistical analysis (*Table 24*) showed significant differences (n=30, p=0.00) in all the length comparisons, the overall comparisons also showed a significant difference (n=120, p=0.00).

When the results are combined we can see that liver reared larvae at 20 °C have the longest length overall followed by liver reared larvae at 25°C, liver reared larvae was also quicker to reach the post feeding stage. Statistical analysis (*Table 24*) showed significant differences (n=30, p=0.00) in all the length comparisons when comparing banana and liver together. Comparison of the overall data also showed a significant difference (n=240, p=0.00).

**Table 24: p - values for independent t-test in which *Megaselia scalaris* larval lengths were reared under 20 °C and 25 °C and on either fresh banana or fresh pork liver were compared.**
**Measurements of length (mm) were recorded every 24 hours (n = 30) the experiment was run under a 12:12 photo-period. (B20= banana 20°C, B25 = banana 25 °C, L20=Liver 20°C, L25 = Liver 25°C). Significant differences have been underlined.**

| Conditions/Hours | 48 | 72 | 96 | 120 | 144 |
|---|---|---|---|---|---|
| B20 v B25 | 0.605 | 0.00 | 0.00 | 0.00 | 0.00 |
| B20 v L20 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| B25 v L25 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| L20 v L25 | 0.00 | 0.00 | 0.00 | 0.00 | - |

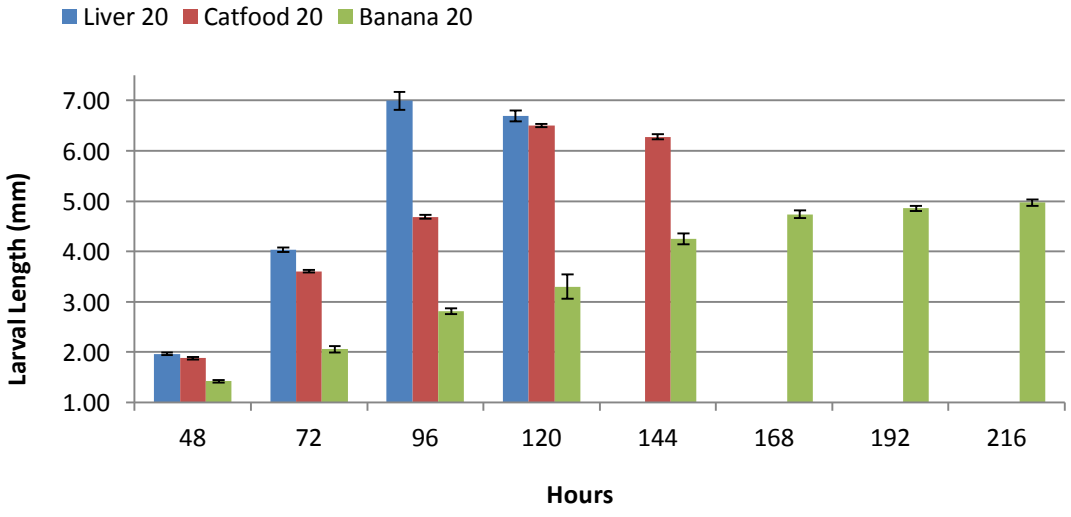



**Fig 45: Developmental data of *Megaselia scalaris larvae* at 20 °C from 48 hours to post feeding larvae reared on fresh pork liver, Sainsburys' pet food and fresh banana.**
**Measurements of length (mm) were recorded every 24 hours (n = 240, p=0.00). Error bars represent standard error of the mean.**

If we include the Sainsbury's smooth pate pet food results then the data at 20 °C shows us that liver is the first to reach the post feeding stage followed by cat food. Statistical analysis shows no significant difference when all three foods were compared at 20 °C (n=30, p=0.00) however there was a significant difference observed between liver and cat food at 48 hours (p=0.057) and 120 hours (n=30, p=0.153) (*Fig 45*). When the lengths were analysed together the data showed no significant difference (n=240, p=0.00) between the three foods.

At 25 °C significant differences were observed between banana and cat food (n=30, p=0.00) (*Fig 46 & Table 25*) however no statistical differences were seen between cat food and liver reared larvae during 96 (n=30, p=0.998) and 120 hours (n=30, p=0.057), the remaining hours showed significant differences (n=30, p=0.00).

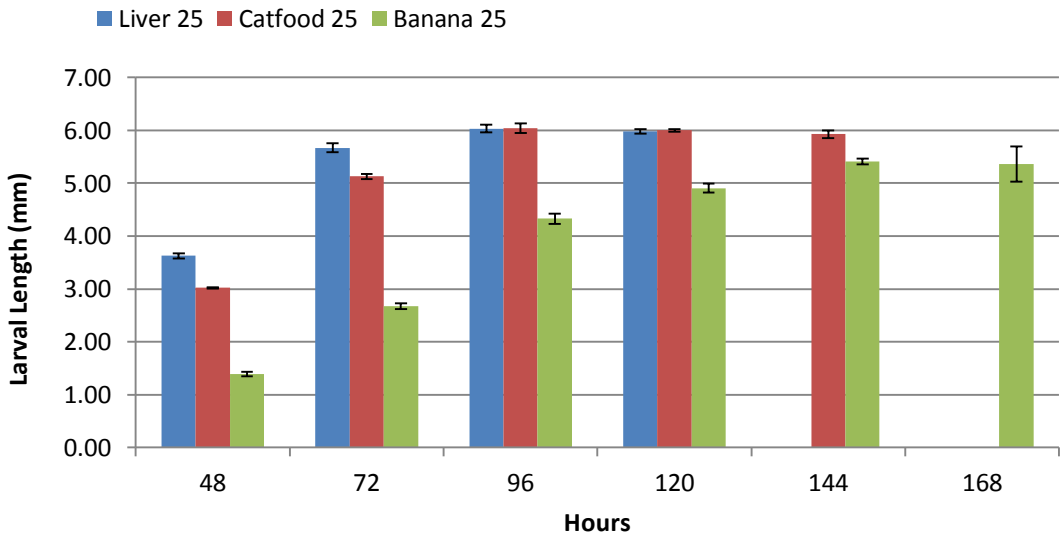



**Fig 46: Developmental data of *Megaselia scalaris* at 25 °C from 48 hours to post feeding larvae reared on fresh pork liver, Sainsburys' pet food and fresh banana.**
**Measurements of length (mm) were recorded every 24 hours (n = 30, p=0.00). Error bars represent standard error of the mean.**

**Table 25: p - values for independent t-test/ANOVA in which *Megaselia scalaris* larval lengths reared under temperatures 20 and 25 °C and reared under fresh banana, fresh pork liver and cat food are compared. Measurements of length (mm) were recorded every 24 hours (n = 30) the experiment was run under a 12:12 photo-period. (B20= banana 20°C, B25 = banana 25°C, L20=Liver 20°C, L25 = Liver 25°C, CF20= Cat food 20°C, CF25=Cat food 25°C). Significant differences have been underline.**

| Conditions/Hours | 48 | 72 | 96 | 120 | 144 |
|---|---|---|---|---|---|
| CF20 v B20 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| CF20 v L20 | 0.06 | 0.00 | 0.00 | 0.15 | - |
| CF25 v B25 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| CF 25 v L25 | 0.00 | 0.00 | 0.99 | 0.06 | - |
| CF/B/L 20 | 0.00 | 0.00 | 0.00 | 0.00 | - |
| CF/L/B 25 | 0.00 | 0.00 | 0.00 | 0.00 | - |

### 3.1.3.3 *Megaselia scalaris* Pupal Development on Liver and Banana.

The followings experimental results show the pupal differences between 20 °C and 25 °C from *M.scalaris* flies reared on fresh banana, fresh pork liver and Sainsbury's smooth pate pet food. The results from *M.scalaris* pupae reared on fresh pork liver show that the pupal length was longer at 20 °C when compared to the other specimens (*Fig 47*), statistical analysis (*Table 26*), shows significant differences (n= 30, p=0.00) when banana and liver reared specimens were compared and overall liver had longer pupae under both temperatures.

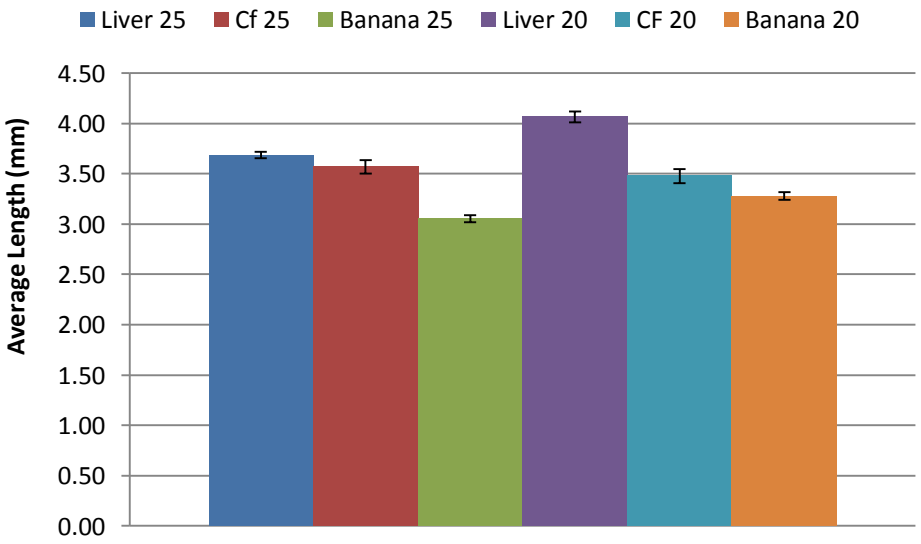



**Fig 47: Developmental data of *Megaselia scalaris* pupae *at* 20 °C and 25 °C reared on fresh pork liver, cat food and banana.**
**Measurements of length (mm) were recorded (n = 60, p=0.00). Error bars represent standard error of the mean.**

**Table 26: p - values from independent t-test in which *Megaselia scalaris* pupal lengths reared under temperatures 20 and 25 °C and reared under fresh banana and fresh pork liver are compared.**
**Measurements of length (mm) were recorded (n = 30) the experiment was run under a 12:12 photo-period. (B20= banana 20°C, B25 = banana 25°C, L20=Liver 20°C, L25 = Liver 25°C). Significant differences have been underlined.**

| Conditions | T-test p-value |
|---|---|
| B20 v L20 | 0.00 |
| B25 v L25 | 0.00 |
| B20 v B25 | 0.00 |
| L20 v L25 | 0.00 |

When we include the Sainsbury's smooth pate pet food result, the results from *M.scalaris* pupae reared on fresh banana, fresh pork liver and cat food show that overall liver still had the longer pupal length under both temperatures. There was very little difference observed in the pupal length of cat food pupae under both temperatures (n=30, p=0.198). Statistical analysis (*Table 27*), shows significant differences (p=0.00) when comparing each of the foods under a specific temperature, the same outcome was seen when comparing the foods under all temperatures (n=60, p=0.00).

**Table 27: p - values from independent t-test in which *Megaselia scalaris* pupal lengths reared under temperatures 20 and 25 °C and reared under fresh banana and fresh pork liver are compared.**
**Measurements of length (mm) were recorded (n = 30) the experiment was run under a 12:12 photo-period. (B20= banana 20°C, B25 = banana 25°C, L20=Liver 20°C, L25 = Liver 25°C). Significant differences have been underlined.**

| Conditions | T-test/Anova p-value |
|---|---|
| **CF20 v CF25** | 0.20 |
| **CF/B/L20** | 0.00 |
| **CF/B/L 25** | 0.00 |

### 3.1.3.4 *Megaselia scalaris* Adult Development on Liver and Banana

The following experimental results show the wing differences (*Fig 39*) between 20 and 25 °C from *M.scalaris* flies reared on fresh banana, fresh pork liver.

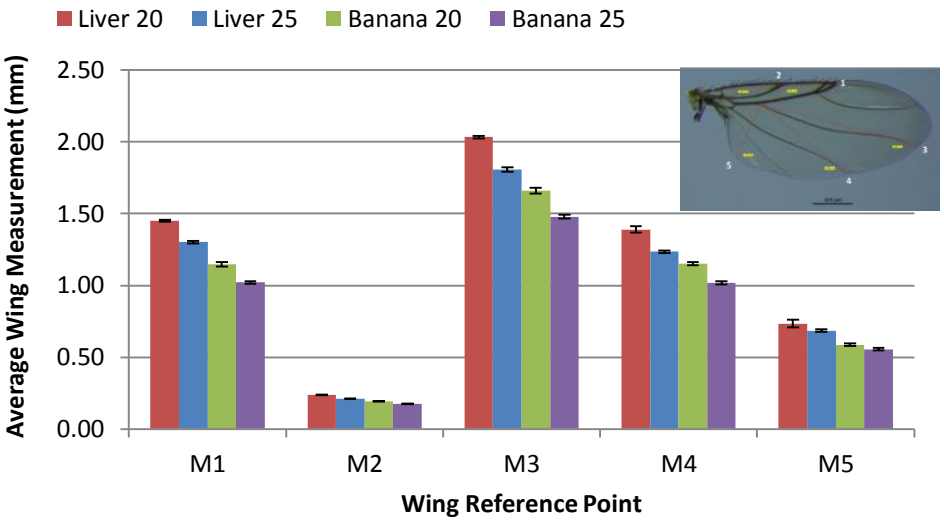



**Fig 48: Developmental data of *Megaselia scalaris* wing *at* 20 °C and 25 °C reared on fresh banana and fresh pork liver.**
**Measurements of length (mm) were taken from the left wing see (*Error! Reference source not found.*) for reference n = 30). Error bars represent standard error of the mean**

The results from the *M.scalaris* wing measurements from specimens reared on fresh banana show that the lengths 1, 3 and 4 were longer at 20 °C when compared to the other specimens (*Fig 48*), statistical analysis (*Table 28*), shows significant differences (n= 30, p=0.00/p=0.042) through all 5 measurements.

When banana and liver reared specimens were compared, liver was seen to have the largest wings at both 20 and 25 °C. A pattern can be observed showing that specimens at 20 °C had the largest wing measurements which was observed in all the reference points. Statistical analysis (*Table 29* and *Table 30*) showed significant differences when banana and liver were compared.

**Table 28: p - values from independent t-test in which *Megaselia scalaris* wing lengths reared under temperatures 20 and 25 °C and fed fresh banana are compared.**
**Measurements of length (mm) were recorded (n = 30) the experiment was run under a 12:12 photo-period. (B20= banana 20°C, B25 = banana 25°C). Significant differences have been underlined.**

| Parameter/WRP | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| B20 v B25 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 |

**Table 29: p - values from independent t-test in which *Megaselia scalaris* wing lengths reared under temperatures 20 and 25 °C and fed fresh pork liver are compared.**
**Measurements of length (mm) were recorded (n = 30) the experiment was run under a 12:12 photo-period. (L20= liver 20°C, L25 = liver 25°C). Significant differences have been underlined.**

| Parameter/WRP | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| L20 v L25 | 0.00 | 0.00 | 0.00 | 0.00 | 0.07 |

**Table 30: p - values from independent t-test in which *Megaselia scalaris* wing lengths reared under temperatures 20 and 25 °C and fed fresh banana and fresh pork liver are compared.**
**Measurements of length (mm) were recorded (n = 30) the experiment was run under a 12:12 photo-period. (B20= banana 20°C, B25 = banana 25°C, L20= liver 20°C and L25 = liver 25°C)**

| Parameter/WRP | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| B20 v L20 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| B25 v L25 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

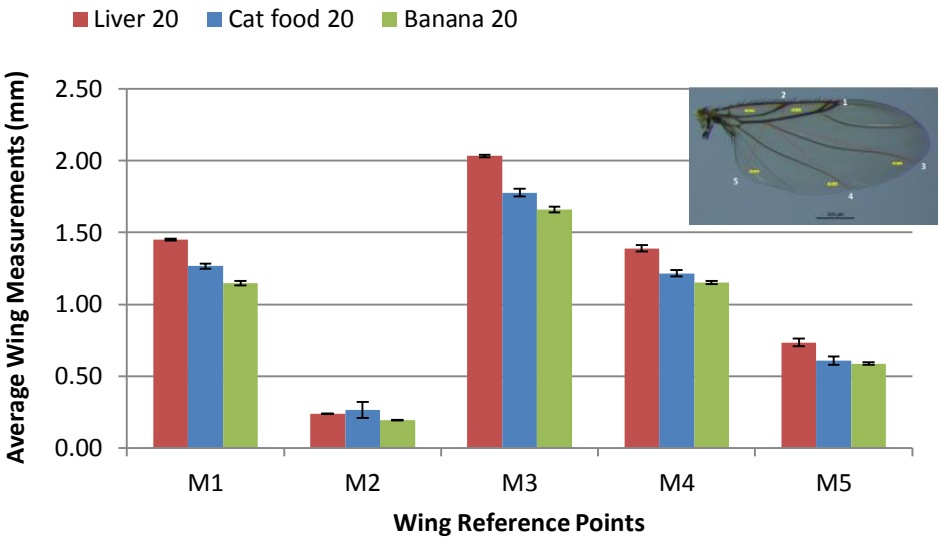



**Fig 49: Developmental data of *Megaselia scalaris* wing *at* 20 °C reared on, fresh pork liver, cat food and fresh banana.**
**Measurements of length (mm) were taken from the left wing (n = 30). Error bars represent standard error of the mean**

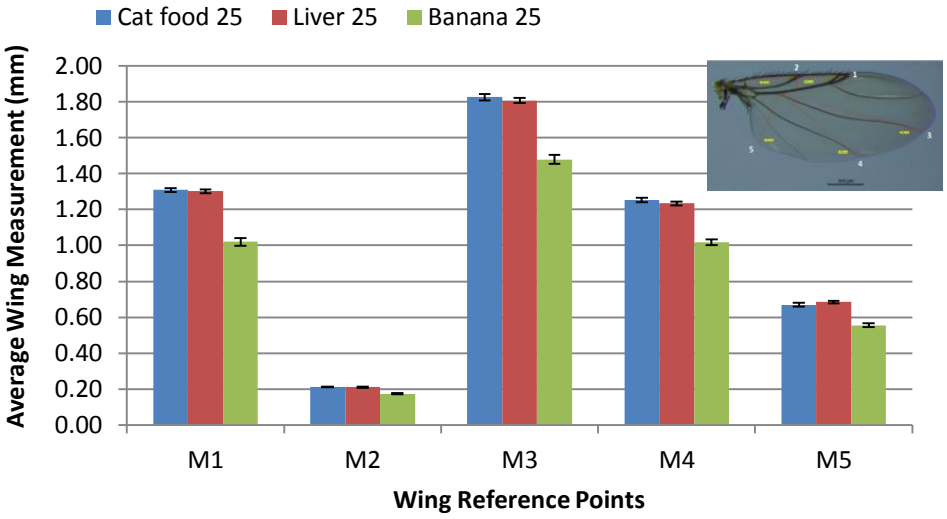



**Fig 50: Developmental data of *Megaselia scalaris* wing at 25 °C reared on fresh pork liver, cat food and fresh banana.**
**Measurements of length (mm) were taken from the left wing (n = 30). Error bars represent standard error of the mean**

When Sainsbury's smooth pate pet food is added to the data, at 20 °C we observe that liver has the longest wing measurements (*Fig 49*) followed by cat food and then banana however this changes at 25 °C and cat food wings measurements (*Fig 50*) are longer than the liver measurements. Statistical analysis (*Table 31*) shows that when banana and liver are compared to cat food individually a variety of differences are observed. Liver and cat food at 25 °C have no significant differences (n=30, p=>0.230).

**Table 31: p - values from independent t-test in which *Megaselia scalaris* wing lengths reared under temperatures 20 and 25 °C and fed on fresh pork liver, cat food and fresh banana are compared.**
**Measurements of length (mm) were recorded (n = 30) the experiment was run under a 12:12 photo-period. (B20= banana 20°C, B25 = banana 25°C, L20= liver 20°C and L25 = liver 25°C). Significant differences have been underlined.**

| Parameter/WRP | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| B20 v CF20 | 0.00 | 0.20 | 0.00 | 0.02 | 0.47 |
| B25 v CF25 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| L20 V CF20 | 0.00 | 0.63 | 0.00 | 0.00 | 0.00 |
| L25 v CF25 | 0.59 | 0.59 | 0.40 | 0.23 | 0.27 |
| B/L/CF20 | 0.00 | 0.28 | 0.00 | 0.00 | 0.00 |
| B/L/CF25 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

### 3.1.3.5 Larval Food Preference

In both two (*Fig 51*) and three well (*Fig 52*) experiments the larvae clearly show there is a significant difference between pet food and liver with pet food being the preference. As these larvae originated from cat food reared flies the same experiment was run using larvae originating from liver bred flies. These results (*Fig 55*) again show there is a significant difference between the foods with pet food again being the preference. A similar experiment was run with banana reared larvae (*Fig 56*), in which banana, cat food and liver were used. The results showed that banana was the preferred choice (p=0.000, N= 270).

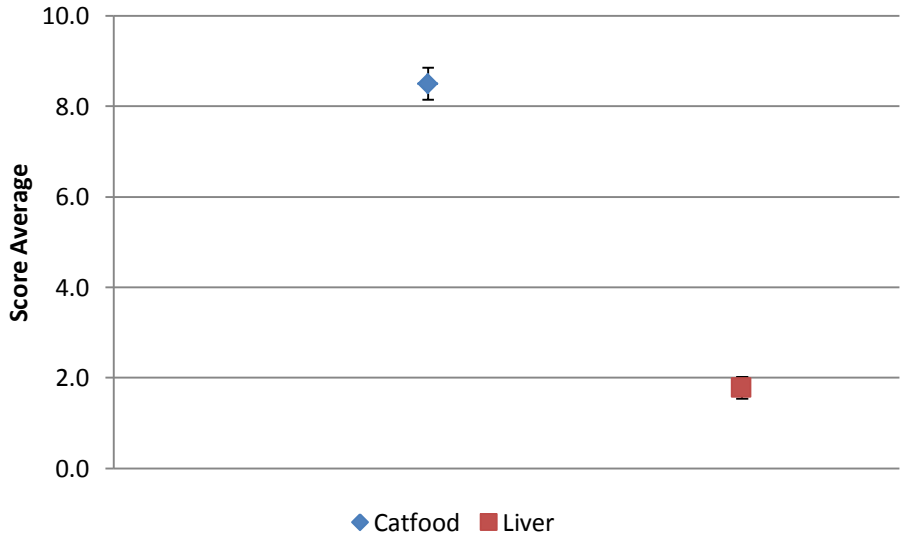



**Fig 51: Cat food reared *Megaselia scalaris* larvae food preference experiment, two well test: cat food vs liver. Error bars represent SEM, (p=0.00, N = 179).**

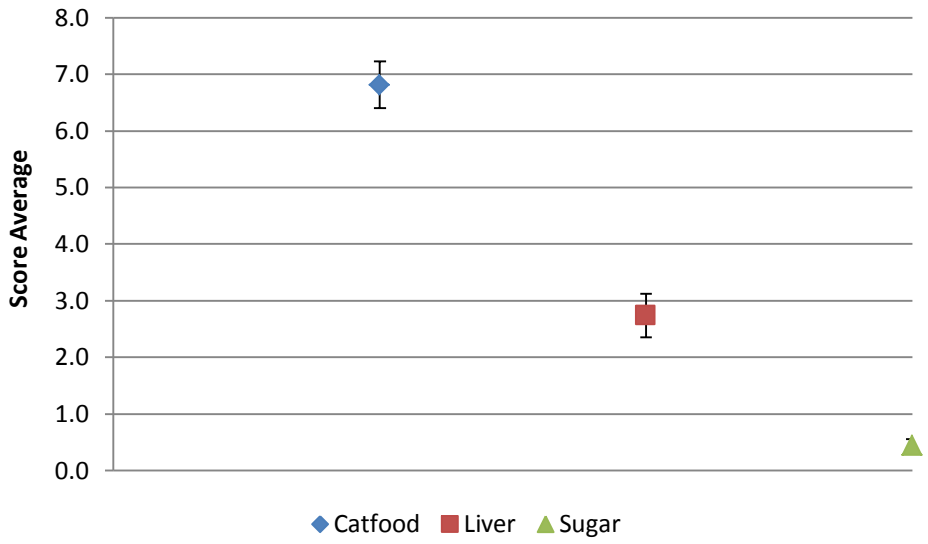



**Fig 52: Cat food reared *Megaselia scalaris* larvae food preference experiment, three well test: cat food, liver and sugar solution.**
**Error bars represent SEM, (p=0.018, N = 270).**

A further experiment was run using random ingredients. The first experiment showed that cat food was the preferred choice by the larvae followed by brown sauce and then honey (Fig 53), however there were no significant differences between the three (p=0.247, N=81). The second experiment showed that toothpaste was the preferred choice followed by cat food and then boot polish (Fig 54), there were significant differences observed between the three ingredients (p=0.000, N=81).

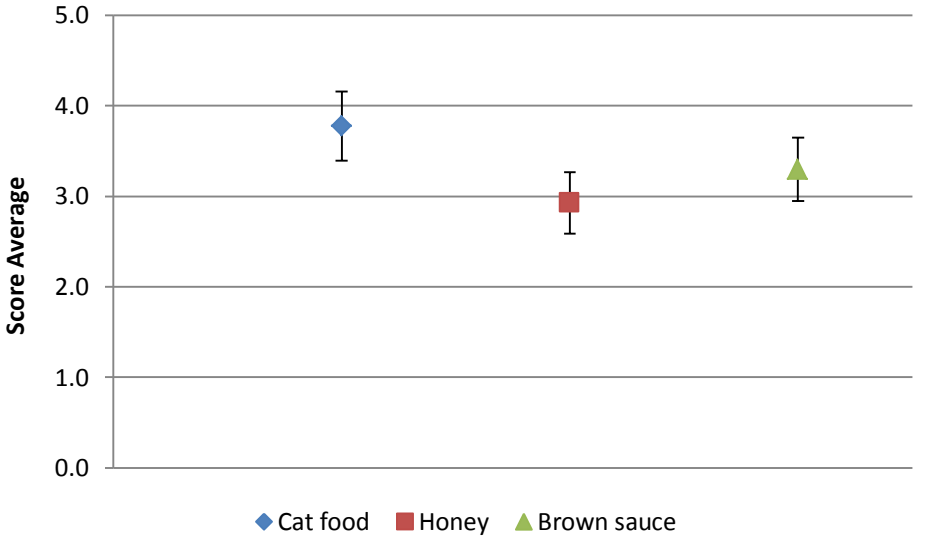



**Fig 53: Cat food reared *Megaselia scalaris* larvae food preference experiment, three well test: cat food, honey and banana.**
**Error bars represent SEM, (p=0.247, N = 81).**

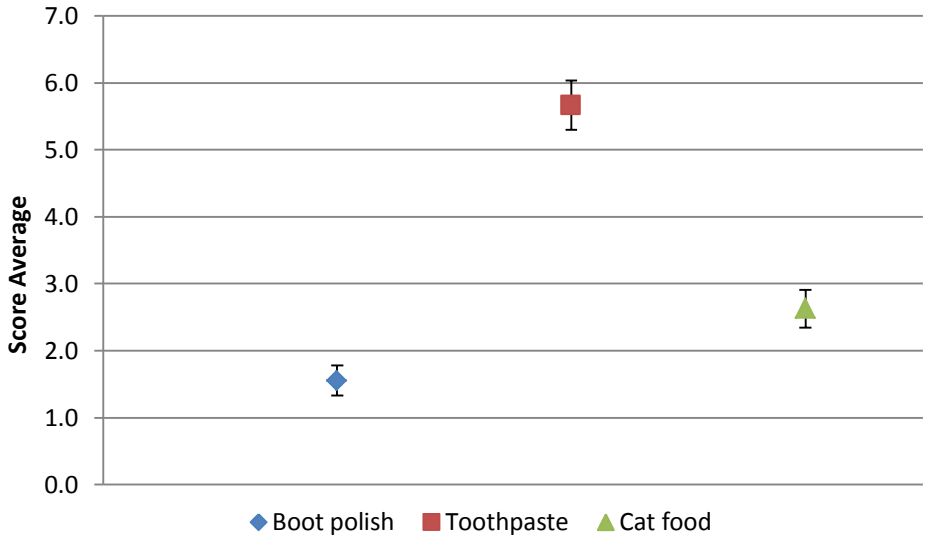



**Fig 54: Cat food reared *Megaselia scalaris* larvae food preference experiment, three well test: toothpaste, cat food and boot polish.**
**Error bars represent SEM, (p=0.000, N = 81).**

### 3.1.3.6 Pre-imaginal Conditioning of *Megaselia scalaris*

To determine if larvae showed a memory of preference for the food their parents were bred on. The experiments used cat food, liver and banana reared larvae.

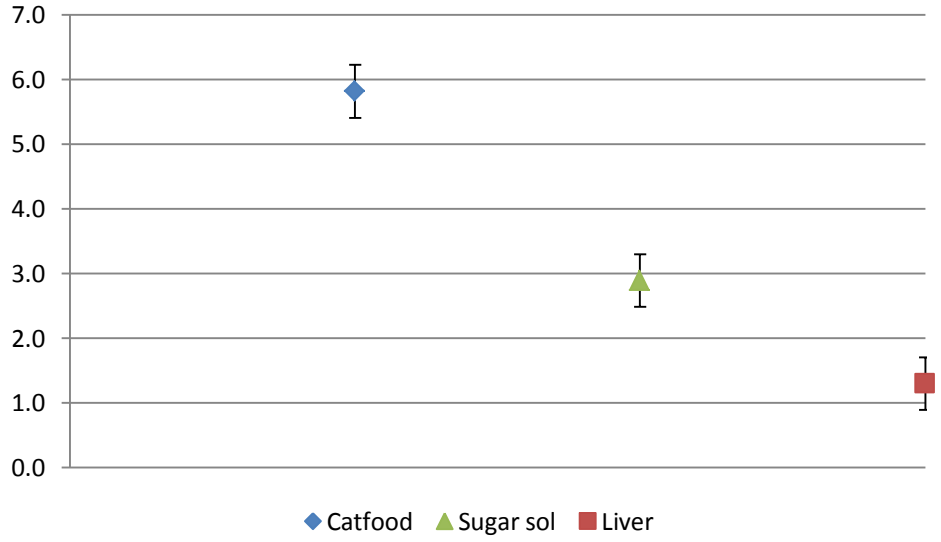



**Fig 55: Liver reared *Megaselia scalaris* larvae food preference experiment, three well test: Cat food, liver and sugar solution.**
**Error bars represent SEM, (p=0.000, N = 270).**

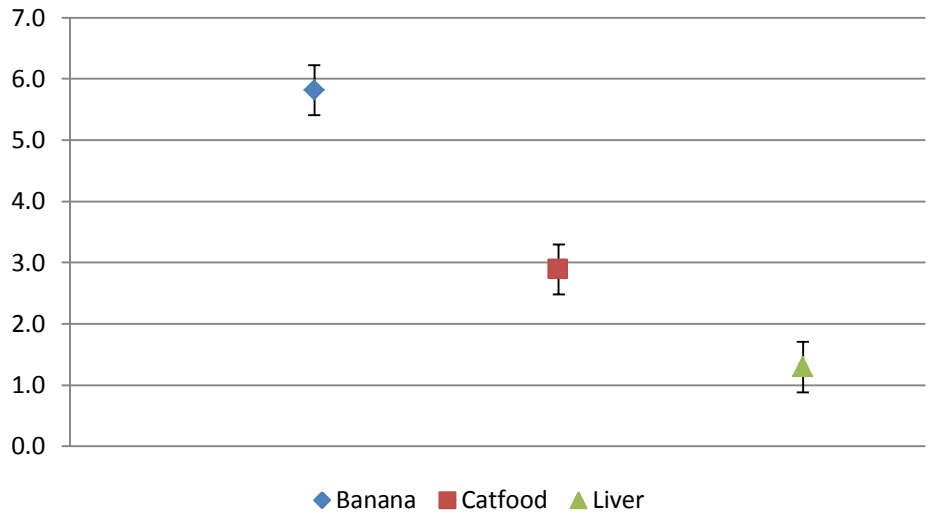



**Fig 56: Banana reared *Megaselia scalaris* larvae food preference experiment, three well test: cat food, liver and banana.**
**Error bars represent SEM, (p=0.000, N = 270).**

The results showed that the larvae from the liver reared colonies (*Fig 55*) did not have pre-imaginal memory of liver as the preference for these larvae was cat food followed by sugar solution with liver coming last. However the colonies reared on banana had a preference for banana followed by cat food and then liver (*Fig 56*).

### 3.1.4 Discussion

It is of fundamental importance that the size and age of the insect is correct when working with forensic cases. Development in the laboratory has to be completed on an artificial diet that mimics both human tissues alongside the decomposition process. Previous work has been completed on diet development data unfortunately numerous diets have been used and as seen in the standard developmental studies none of the parameters were the duplicated from previous research so the results are not comparable.

#### 3.1.4.1 *Megaselia scalaris* Development on Sainsbury's smooth pate pet food.

Statistical analysis (t-tests) were run on larval, pupal and wing measurements from *M.scalaris* reared on Sainsbury's smooth pate pet food to determine if any significant differences were observed between temperatures of 20 °C and 25 °C. Eggs were not measured as the flies that oviposited during the initial set up of the experiment originated from the same batch reared under the same conditions. Personal observations during all the developmental experiments saw the larvae decreasing in size whilst emptying the gut; they then extended themselves immediately prior to pupation which is observed with an increase in measurements. The reduction in size followed by an increase is also observed in Greenberg and Wells (1998) findings, along with the difference in size observed during rearing at cooler temperatures. Our findings showed that larvae reared at 25 °C were quicker to complete their developmental cycle however the larvae reared at 20 °C were longer in length from 120 hrs onwards.

The pupal length measurements showed no statistical difference between either temperatures (n=30, p=0.348). Zuha and Omar (2014) findings indicated that temperature significantly influence male and female puparia length with the lowest temperature having a significant difference when compared to the higher temperatures. It was also observed that the weight of the pupa at the lowest temperature had a significantly higher weight when compared to those reared at higher temperatures. Wing measurements M1 (p=0.038) and M5 (p=0.013) showed significant differences whilst the remaining measurements did not (M2: p=0.638, M3: p=0.307 and M4: p=0.449).

Findings showed that total development time of specimens reared at 20 °C from eggs to early pupation took a total of 8 days whilst specimens reared at 25 °C took 7 days. Pupation to adult emergence took 10 days at 20 °C whilst specimens at 25 °C took 9 days.

In total, at 20 ℃, it took a total of 18 days from egg to adult emergence whilst specimens reared at 25 ℃ took 16 days.

#### 3.1.4.2 *Megaselia scalaris* Development on Fresh Liver and Banana.

Richards and colleagues (2012a) studying *C. vicina,* found that the immature stages reared on fresh liver required the least amount of time to reach maximum larval length, pupariation when compared to those fed on other diets which is what was observed during our development times experiments followed by those reared on Sainsbury's smooth pate pet food and then banana.

Zuha and co-workers (2012) used a diet of either beef liver tissue or liver agar and they discovered that there was no difference in larval sizes reared on either diet however different weights of both pupae and larvae were detected on the liver agar diet at 27 ℃. It was concluded that as growth could be influenced by different temperature and food types it could possibly lead to the wrong age estimation of this species, which agrees with our findings which showed that larvae reared at 25 ℃ were quicker to complete their developmental cycle however the larvae reared at 20 ℃ were longer in length.

The pupal length measurements showed that liver 20 ℃ and 25 ℃ had the longest pupal lengths when compared to those reared on Sainsbury's smooth pate pet food and then banana.

Idris and colleagues (2001) concluded that diets did have an effect on the developmental rate of the *M.scalaris* larvae and pupa but no temperature parameter was discussed, our experiments have demonstrated that different foods do have an effect on developmental rate and growth and that the rearing temperature also has an impact.

Ideally developmental studies should be completed and the results compared to developmental studies using cadavers to determine the authenticity of the developmental data. This may also help towards determining what diets are suitable for determining developmental rates in forensic cases when reared in a laboratory environment. Unfortunately there is much controversy around this area especially in the UK where there are no 'human body farm' facilities and this nature of research. Till now the pig

model has provided useful information by providing answers to the main questions about decomposition, insect development and PMI estimation.

#### 3.1.4.2 Larval Food Preference and Pre-imaginal Conditioning.

During the experiments it was observed that Sainsbury's smooth pate pet food has a stronger smell than liver and therefore if flies detect food primarily by odour as commented by Erzinclioglu (1996) then this could be the reason for the attractiveness of pet food.

The larvae have shown to be attracted to some unusual ingredients such as brown sauce, honey and toothpaste which would suggest they are attracted to a wider range of materials than suggested.

Pre-imaginal conditioning (Tully *et al*., 1994; Barron and Corbet, 1999) experiment showed this theory to be incorrect as both liver reared adult flies and larvae showed that they had a preference for pet food rather than liver.

## 3.2 Entomotoxicology

### 3.2.1 Introduction

Toxicology is a scientific field that uses quantitative and qualitative analysis to identify illegal substances and their metabolic products. Drug related deaths have increased over the recent years and in some cases the body of the victim may often remain undiscovered for a period of time which may find the body in a greatly decomposed state or where there is no blood or urine left for samples to be taken (Tanaka *et al*., 1994; Goff and Lord, 1994). Entomotoxicology is the application of toxicological analysis to determine the qualitative and/or quantitative quantities of toxic substances found on insects feeding from human remains (Gagliano-Candela and Aventaggiato, 2001).

Drugs of abuse or psychotropics are detectable in carrion feeding larvae (*Table 32*). The drugs detected in the larvae specimens were also identified in the tissues from the cadaver however the concentrations found in the larvae were much lower than those in cadaveric samples therefore giving the conclusion that determining drug concentrations in insects for entomotoxicological purposes may have almost no interest in practical forensic casework unless research is actively advanced to understand the factors that that affect the drug concentrations in insects is further understood (Tracqui *et al*., 2004). The above statement would suggest that further research is required before the full potential of entomotoxicology is taken seriously (Murthy and Mohanty, 2010).

**Table 32: Drugs detected in different developmental stages of arthropods.**

| Substance | Sample | Reference |
|---|---|---|
| Copper, iron, zInc. | Housefly (adult) | (Sohal and Lamb, 1979) |
| Phenobarbital | Blowfly (larvae) | (Beyer *et al*., 1980) |
| Mercury | Blowfly (larvae, puparia, adult) and beetles | (Nuorteva and Nuorteva, 1982) |
| Arsenic | Piophilidae, Psychodidae and Muscidae | (Leclercq, 1978) |
| Selenium | Adult house fly | (Simmons *et al*., 1988) |
| Malathion | Fly larvae | (Gunatilake and Goff, 1989) |
| Cocaine | Fly larvae | (Goff *et al*., 1989) |
| Bromazepam, levomepromazine,morphine, phenobarbital, triazolam,oxazepam, phenobarbital, alimemazine, clomipramine | Fly larvae | (Kintz *et al*., 1990a, Kintz *et al*., 1990b, Kintz *et al*., 1990c) |
| Opiates | Fly larvae | (Introna Jr *et al*., 1990) |

| | | |
|---|---|---|
| Heroin | Fly larvae | (Goff *et al*., 1991) |
| Cocaine | Fly larvae and beetle faecal material | (Manhoff *et al*., 1991) |
| Cocaine | Fly larvae and puparia | (Nolte *et al*., 1992) |
| Amitriptyline, propoxyphene and acetaminophen | Fly larvae | (Wilson *et al*., 1993) |
| Opiates | Fly larvae | (Kintz *et al*., 1994) |
| Amitriptyline and nortriptyline | Empty fly puparia and beetle exuvie | (Miller *et al*., 1994) |
| Phenobarbital, paracetamol | Fly larvae | (Sadler *et al*., 1997a) |
| 3,4 methylenedioxy methamphetamine | Fleshfly (larvae and puparia) | (Goff *et al*., 1997) |
| Morphine | Fly larvae | (Hédouin *et al*., 1999) |
| Secobarbital | Fly larvae | (Levine *et al*., 2000) |
| Diazepam | Blowfly (larvae, puparia and adult) | (Carvalho *et al*., 2001) |
| Morphine | Fly larvae | (Bourel *et al*., 2001a, Bourel *et al*., 2001b, Bourel *et al*., 2001c) |
| Cocaine, opiates, phenobarbital, levomepromezine, amitriptyline, nortriptyline, tioridazine and clomipramine | Blowfly(larvae) and human tissues | (Campobasso *et al*., 2004b) |
| Parathion | Diptera,Coleoptera, Hymenoptera, Isopoda and Acari | (Wolff *et al*., 2001) |
| Nordiazepam and oxazepam | Fly larvae and puparia | (Pien *et al*., 2004) |
| Cannabis sativa, cocaine and dietilpropione (amphepramone) | Blowfly(larvae, puparia and adult) | (Carvalho *et al*., 2004) |
| Paracetamol | Blowfly larvae | (O'Brien and Turner, 2004) |
| Amphetamines and Barbiturates | Blowfly larvae | (Peace, 2005) |
| Morphine | Blowfly larvae | (Gunn *et al*., 2006) |
| Amphetamine | Blowfly larvae | (Definis-Gojanović *et al*., 2007) |
| Diazepam | Blowfly larvae | (Beauchamp *et al*., 2007) |
| Codeine | Larvae, Pupa and imago | (Kharbouche *et al*., 2008) |
| Methadone | Blowfly larvae | (Gosselin *et al*., 2010) |
| Methadone | Blowfly larvae | (Gosselin *et al*., 2011a) |
| Malathion | Blowfly larvae | (Mahat *et al*., 2012) |
| Methylphenidate | Blowfly larvae | (Bushby *et al*., 2012) |
| Ketamine | Blowfly larvae | (Zou *et al*., 2013) |
| Codeine, Sodium Pentothal | Blowfly larvae | (Gui *et al*., 2010) |
| Methamphetamine | Blowfly larvae | (Mullany *et al*., 2014) |
| Ketamine | Blowfly larvae | (Lu *et al*., 2014) |
| Methamphetamine | Blowfly larvae | (Magni *et al*., 2014) |
| Ketamine Hydrochloride, Xylazine | Blowfly larvae | (Singh *et al*., 2014) |
| Methylphenidate hydrochloride, phenobarbital, and methylphenidate hydrochloride associated with phenobarbital | Larval and pupa | (Rezende *et al*., 2014) |

Decomposing tissues containing drugs or toxins have been shown to affect the rate of development of larvae and oviposition of adult flies (Voss *et al*., 2008). Puparia of *M.scalaris* was recovered from the remains of a female drug addict that had died in her home. Both Amitriptyline and Nortriptyline were found to have greater concentrations in the puparia than in either the excrement or exuviae of the insect. Due to Phorids having a preference for soft tissues, where the drug concentrations may be higher would reflect the high density of larvae that were collected in these areas (Miller *et al*., 1994).

Due to the limited entomotoxicological research on *M.scalaris*, many questions are left unanswered. Some of these questions may include: which developmental stage allows for the best toxicological analysis and does insect growth suggest larger drug concentrations?

### 3.2.2 Experimental Design

Three concentrations of Amitriptyline were made up in a saline solution along with a fourth blank solution which consisted of solely saline solution. The experiment was run blind without the knowledge of the drug concentrations until after the experiment was completed. The toxicological analysis was completed in collaboration with Dr Peter Maskill and Emma Lomas (PhD Research student).

The different drug concentrations were labelled A to D. Four repetitions of each drug concentration was set up and labelled 1A, 2A etc.

- A = Control 0mg/kg (0.9% salt solution/ saline)
- B = Low Conc. (6mg/kg)
- C = Middle Conc. (12mg/kg)
- D = High Conc. (41mg/kg)

A small amount of tissue was placed into the bottom of the vials to collect any liquids which would prevent larval drowning. One hundred grams of fresh pork liver was weighed out and placed into a blender along with Drug A. The samples were then homogenised over 1 minute, 5 g of liver/drug mixture were weighed out into 20 vials. A small piece of filter paper was added to the top of the liver this prevented the sleeping flies from landing directly on to the meat and dying, it also provided a platform for the flies to lay their eggs. Five flies of both sexes were added to each vial and left for a few hours to lay eggs, once enough eggs were laid the adults were removed and this period was labelled 'Time Zero'. The first measurement would be taken 48 hour from this point and then every 24 hours until pupation. The vials were placed into a 25 °C incubator programmed with a LD12:12 photo-period.

This method was also used to set up Drugs B, C and D.

Qualitative analysis of the presence of amitriptyline in flies and liver was carried out using (high performance liquid chromatography with diode array detection) HPLC-DAD.

The flies and larvae were homogenised, 800 µl of deionised were added to each sample and vortexed for 30 seconds. The vials were left at room temperature for one hour and then placed into a -20 °C freezer. Each sample tube had 0.5 ml removed and placed into a separate plastic 15 ml centrifuge tube in which 0.5 ml 0.2M Sodium carbonate (Na2CO3) and 5ml 1-chlorobutane was added. The samples were mixed for 30 minutes to extract the drugs from the homogenised samples. The top layer (1-chlorobutane layer) was removed and placed in a separate tube; the solutions were dried down using a TurboVap at 40 °C for 30 minutes. The samples were then reconstituted in 100µl methanol and transferred to a HPLC vial.

The HPLC was a Dionex Ultimate 3000 and the method conditions were:

Column: Waters Spherisorb 5µm OD/CN, 4.6 x 150 mm Analytical Cartridge
Column Thermostat: 25 °C
Mobile Phase A: 57 %
Mobile Phase C: 43 %.
Flow Rate: 2.0 mL/min
Injection Volume: 40 µL
Run time: 7.0 min
Gradient: Isocratic
Wavelength: 210 nm and 220 nm
Retention Times: Amitriptyline: Approx. *3.8* min
Clomipramine: Approx. *4.9* min

### 3.2.3 Results

#### 3.2.3.1 Insect Data

There has been little research done investigating development and growth rates of *M.scalaris* when reared on drugged pabulum. It is essential to a forensic case to know what effect drugs will have on the rate of insect development. The followings experimental results show the larval (*Fig 57*), pupa (*Fig 58*) and adult developmental differences at 25 °C from flies reared on fresh pork liver that had been laced with amitriptyline. There were four repetitions of each concentration in which 30 measurements were taken from each therefore n = 120 measurements per concentration.

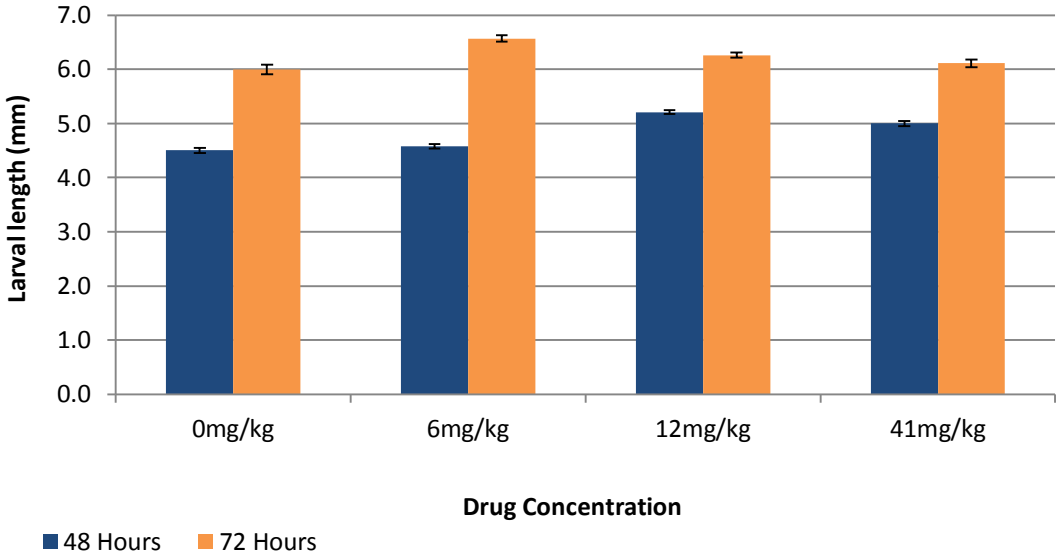



**Fig 57: Larval length measurements (mm) with standard error of the mean bars from *Megaselia scalaris* reared on fresh pork liver.**
**Liver contains increasing amitriptyline concentrations at a constant temperature of 25 °C under a LD 12:12 photo-period. n=120 per concentration.**

**Table 33: p - values for ANOVA Tukey *post hoc* multiple comparison test.**
**Each concentration is compared to all others from *Megaselia scalaris* larval length measurements (mm) reared on fresh pork liver containing increasing amitriptyline concentrations at a constant temperature of 25 °C under a 12:12 photo-period taken after 48 and 72 hours.**

| Concentration (48 hours) | A | | | B | | | C | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B | C | D | A | C | D | A | B | D | A | B | C |
| Sig. | .622 | .000 | .000 | .622 | .000 | .000 | .000 | .000 | .003 | .000 | .000 | .003 |

| Concentration (72 hours) | A | | | B | | | C | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B | C | D | A | C | D | A | B | D | A | B | C |
| Sig. | .000 | .026 | .599 | .000 | .007 | .000 | .026 | .007 | .399 | .599 | .000 | .399 |

The larval lengths show that at 48 hours, larvae at concentrations C and D are developing more rapidly when compared to A and B as shown by the statistical analyses (*Table 33 to Table 35*). Overall there are no differences seen between concentrations A and B (p = 0.622) whilst the rest of the concentration comparisons are significantly different ranging from p = 0.00 to 0.03. However this changes at the 72 hour measurements in which A and B becomes significantly different whilst C and D are not significantly different.

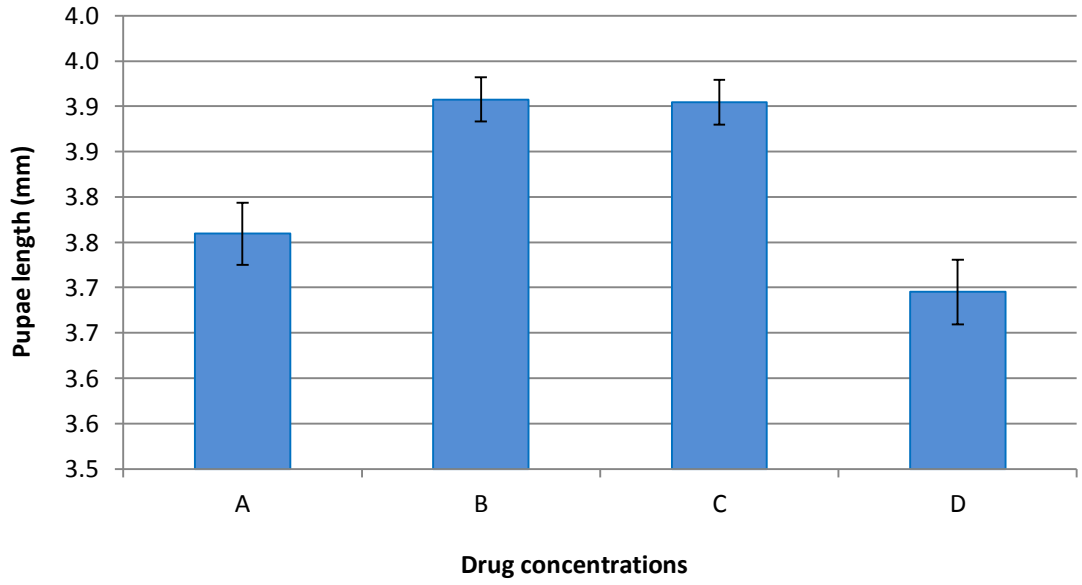



**Fig 58: Pupal length measurements (mm) of *Megaselia scalaris* reared on fresh pork liver.**
**Liver contains increasing amitriptyline concentrations at a constant temperature of 25 °C under a 12:12 photo-period. N= 120.**

**Table 34: p- values for ANOVA Tukey *post hoc* multiple comparison test.**
**Each concentration is compared to all others from *Megaselia scalaris* pupal length measurements (mm) reared on fresh pork liver containing increasing amitriptyline concentrations at a constant temperature of 25 °C under a 12:12 photo-period.**

| Concentration | A | | | B | | | C | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B | C | D | A | C | D | A | B | D | A | B | C |
| Sig. | .003 | .004 | .436 | .003 | 1.00 | .000 | .004 | 1.000 | .000 | .436 | .000 | .000 |

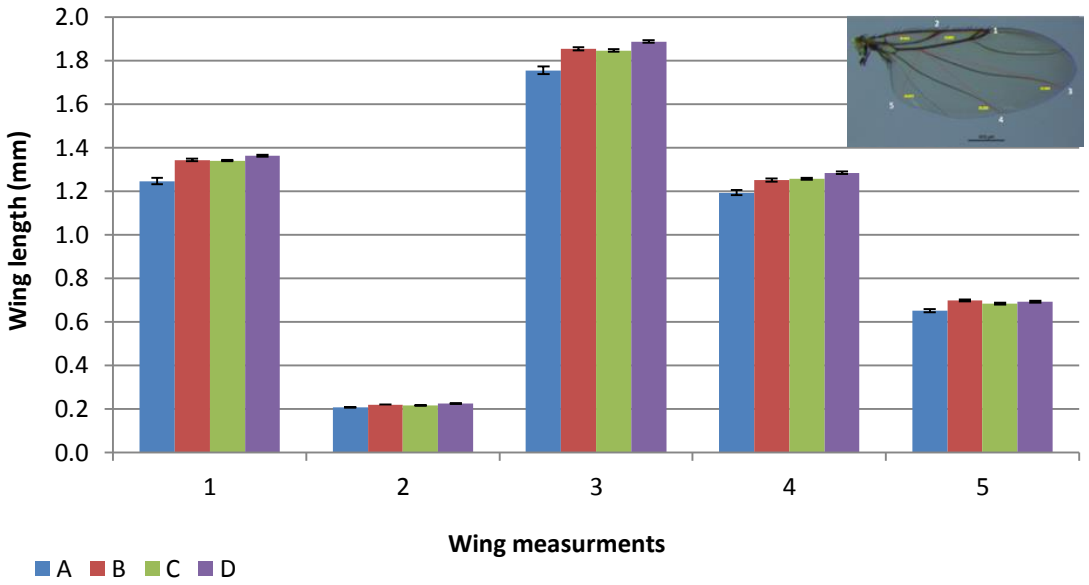



**Fig 59: Wing measurements (mm) from five different points (*shown in Figure 5*) of *Megaselia scalaris* reared on fresh pork liver.**
**Liver contains increasing amitriptyline concentrations at a constant temperature of 25 °C under a 12:12 photo-period. (n =120 per concentration).**

**Table 35: p- values for ANOVA Tukey *post hoc* multiple comparison test.**
**Each concentration is compared to all others from *Megaselia scalaris* wing measurements (mm) reared on fresh pork liver containing increasing amitriptyline concentrations at a constant temperature of 25 °C under a 12:12 photo-period.**

| Concentration | A | | | B | | | C | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Measurement 1 | B | C | D | A | C | D | A | B | D | A | B | C |
| Sig. | .000 | .000 | .000 | .000 | .995 | .323 | .000 | .995 | .210 | .000 | .323 | .210 |
| Measurement 2 | | | | | | | | | | | | |
| Sig. | .000 | .000 | .000 | .000 | .216 | .216 | .000 | .216 | .001 | .000 | .216 | .001 |
| Measurement 3 | | | | | | | | | | | | |
| Sig. | .000 | .000 | .000 | .000 | .952 | .122 | .000 | .952 | .032 | .000 | .122 | .032 |
| Measurement 4 | | | | | | | | | | | | |
| Sig. | .000 | .000 | .000 | .000 | .922 | .011 | .000 | .922 | .068 | .000 | .011 | .068 |
| Measurement 5 | | | | | | | | | | | | |
| Sig. | .000 | .000 | .000 | .000 | .283 | .910 | .000 | .283 | .678 | .000 | .910 | .678 |

#### 3.2.3.2 Entomotoxicology Data

There has been limited research done investigating toxicological sampling of *M.scalaris* when reared on drugged pabulum. The research that has been done used different toxicological sampling methods such as GCMS so comparisons are not possible. This preliminary entomotoxicological study was to try and determine from which development stage we could detect the drug.

**Table 36: Calibration and quality control data for Amitriptyline detection on entomotoxicological analysis.**

| Calibration | Amitriptyline Detected | Quality Control | Amitriptyline Detected |
|---|---|---|---|
| 0.16mg/L | Yes | Low QC (0.5mg/L) 1 | Yes |
| 0.31mg/L | Yes | Low QC (0.5mg/L) 2 | Yes |
| 0.62mg/L | Yes | High QC (5mg/L) 1 | Yes |
| 1.25mg/L | Yes | High QC (5mg/L) 2 | NOT RUN |
| 2.50mg/L | NOT RUN | | |
| 5.00mg/L | Yes | | |
| 10.00mg/L | Yes | | |
| 20.00mg/L | Yes | | |

**Table 37: Amitriptyline detection for *Megaselia scalaris* specimens at different drug concentration. Thirty specimens were analysed from each concentration.**

| Specimen | Drug Concentration | | | |
|---|---|---|---|---|
| **Larvae (48 hours)** | **A1** | **A2** | **A3** | **A4** |
| Amitriptyline Detected | No | No | No | No |
| **Larvae** | **B1** | **B2** | **B3** | **B4** |
| Amitriptyline Detected | Yes | Yes | Yes | Yes |
| **Larvae** | **C1** | **C2** | **C3** | **C4** |
| Amitriptyline Detected | No | No | No | No |
| **Larvae** | **D1** | **D2** | **D3** | **D4** |
| Amitriptyline Detected | Yes | Yes | Yes | No |
| | | | | |
| **Larvae (72 hours)** | **A1** | **A2** | **A3** | **A4** |
| Amitriptyline Detected | No | No | No | No |
| **Larvae** | **B1** | **B2** | **B3** | **B4** |
| Amitriptyline Detected | No | No | No | No |
| **Larvae** | **C1** | **C2** | **C3** | **C4** |
| Amitriptyline Detected | No | No | No | No |

| Larvae | D1 | D2 | D3 | D4 |
|---|---|---|---|---|
| Amitriptyline Detected | No | No | No | No |
| | | | | |
| **Pupa** | **A1** | **A2** | **A3** | **A4** |
| Amitriptyline Detected | No | No | No | No |
| **Pupa** | **B1** | **B2** | **B3** | **B4** |
| Amitriptyline Detected | No | No | No | No |
| **Pupa** | **C1** | **C2** | **C3** | **C4** |
| Amitriptyline Detected | No | No | No | No |
| **Pupa** | **D1** | **D2** | **D3** | **D4** |
| Amitriptyline Detected | No | No | No | No |
| | | | | |

Amitriptyline was detected in specimens B (low concentration) in the 48 hours specimens and also in D (high concentration) in the 48 hours specimens (*Table 36* and

*Table* 37). The drug was not detected in any other *M.scalaris* specimens including the pupa in which previous research revealed that this was the most reliable development stage for drug detection. It was observed that the Amitriptyline detected in the specimens was below the limit of quantitation of 0.31 mg/L.

### 3.2.4 Discussion

When the data from larvae bred on food added with different amounts of Amitriptyline is compared to that from the control that had no drug (Drug A), the insects were observed to develop rapidly during the larval stage. The control larvae took 7 days from egg to pupation whilst the drugged and blank saline larvae took 4 days from egg to pupation, the pupal period between the no drug control and the drugged specimens had a two day difference in emergence (CND = 9 days, Drugged and blank saline = 11 days). A difference in pupal period was also observed by Goff and Lord (1994) who used cocaine in rabbits to determine the effects that drugs may have on the development of *Boettcherisca peregrina*. When the same experiment was run using Amitriptyline and *Parasarcophaga ruficornis* no differences were observed until the larvae reached maximum size and a prolonged period of post feeding stage was recorded, however a prolonged period of post feeding was not observed during our experiment.

In general research carried out by Nazari (2011) observed that Amitriptyline had an effect on the viability, eclosion and developmental time of *Drosophila melanogaster* however it had no effect on the sex ratio. The difference in development time and eclosion was also observed during our experiment. Due to the experiment being a preliminary test we did not research the viability or sex ratio data.

There has been much entomotoxicological analysis completed on the larger Diptera flies e.g. *Calliphora* spp. (Sadler *et al*., 1995; Sadler *et al*., 1997c; Sadler *et al*., 1997b) and *Lucilia* spp. (Hédouin *et al*., 1999; Bourel *et al*., 1999; Kharbouche *et al*., 2008) however quite limited research was found on *Megaselia scalaris.* The methods of other researchers have involved injecting live specimens with drugs of different concentrations and feeding the deceased tissues to fly colonies, the vast difference in methods used may explain our results.

The only work citing the effect of Amitriptyline on *M.scalaris* was done by Miller and colleagues (1994) who collected empty fly puparia, beetle faecal material (frass) and cast beetle skins from a case where mummified remains of a middle-aged

white female were discovered at her residence. The body was extremely mummified with some loss of tissues and considerable insect activity including a combination of mummified integument, adipocere, and filamentous insect frass. Numerous prescription vials, most of which were empty were found near the remains. Empty fly puparia, beetle faecal material (frass) and cast beetle skins were collected from the scene and sent for extraction and analysis of both amitriptyline and nortriptyline. The results showed that Amitriptyline concentrations were greater in puparia than in exuviae or frass. This may be explained by the food source preferences characteristic as skin beetles (Dermestidae) feed primarily on dried integument whereas scuttle flies (Phoridae) have a preference for soft tissues where acute drug concentrations are likely to be higher. Miller and colleagues (1994) also comment that during analysis, for the drugs to be released the protein/chitin matrix needs to be broke down, which requires strong acids or bases to be used for the breakdown of the protein/chitin. Chitin remains are discarded during the metamorphosis stage from larvae to adult. Once the acid/base analysis has been completed then more routine toxicological analysis can be undertaken for analytical isolation.

Our limited results in which the puparia did not show any drug present may suggest that further research into the toxicological analysis from *M.scalaris* is required. The liver and the drug may have not mixed together correctly in which the flies may not have ingested the drug as readily, as other experiments commented that live specimens were injected with the fatal levels of a drug and then fed to the fly colonies. We may also need to investigate completing the toxicological analysis using different analytical equipment e.g. GCMS so that we can compare results to those previously published. In addition, a wider spectrum of concentrations needs to be analysed in order to identify the minimal amount of drug on the pabulum that can be detected in the puparia.

# 4: Chronobiology of *Megaselia scalaris*

## 4.1 Locomotor Activity

### 4.1.1 Introduction

Clock, controls the majority of the phases of the life cycle of an insect, such as eclosion and emergence. In addition the most important activity of an insect is under clock control like mating feeding, walking and flying. The understanding of the circadian clock mechanism is a crucial element in different disciplines from human health to pest control. The improvement in the knowledge of this field plays an important role in forensic entomology. In fact in several insects, routines such as feeding, mating, ovipositing or emergence times have been demonstrated to be under clock control (Danks, 2003; Vanin *et al*., 2012a).

The clock is synchronised by external stimuli such as temperature and light. Light stimuli research in Phlebotominae (Diptera: Psychodidae), *Lucilia sericata* (Diptera: Calliphoridae) and *Drosophila melanogaster* (Diptera: Drosophilidae) was undertaken.

Locomotor activity is one of the clock outputs as demonstrated by several authors such in *Drosophila melanogaster* (Helfrich-Förster, 2005). Research has been undertaken in different fly species e.g. *Drosophila pseudoobscura* to look into whether adult eclosion patterns are affected by a change in rhythm of the circadian clock along with adult locomotor activities (Engelmann and Mack, 1978). Observations showed that *L.sericata* had a different response to colours (blue, black, green, dark blue and red) under laboratory conditions (Wall and Smith, 1996). Males and females were shown to be more drawn to yellow and least attracted to red. The experiment was then moved outside, however there were no differences observed and further analysis showed that any differences seen may be due to the experimental site being in either a shaded or a sunlit area.

There are many ommatidia that make up an insect compound eye which collect optical information. Each ommatidium is composed of a cornea, cone (crystalline), pigment cells and photoreceptors. Each ommatidia has a number of rhabdomeres (different species have different amounts of rhabdomeres) which run centrally and vertically through the cone. The rhabdomere is where light detection takes place (Stavenga, 2002). Dark coloured eyes are common and observed in many insect species and personal observations include *Megaselia scalaris*. Colour vision has been

investigated by Fukushi (1989); Bernard and Remington (1991); Arikawa and Stavenga (1997) and Stavenga (2002) are found to be common amongst different insect species.

Hoel and colleagues (2007) comment that differing intensities of light are an attractant to phlebotomine sand flies. Blue, green, red, orange, yellow, infrared LEDS, no light and incandescent light were used. It was observed that blue or green light was preferred, but light colours were also popular. Incandescent light performed better than red, orange and yellow and was nearly as popular as the green and blue lights. A second experiment was run in which sand flies preferred red to all other colours.

LEDs are highly monochromatic, emitting a pure colour in a narrow frequency range. The colour emitted from an LED is identified by peak wavelength and measured in nanometres (nm). Wavelengths of LED colours (*Table 38*) used above range from between the broad spectrum (white) and $\lambda < 760$ (red). Visible light falls in the range 400 to 760 nm whilst infrared is in the range of 760 to 3000 nm (Da Silva and Simonis, 2005).

The objective of this chapter is to attempt to determine the periods in which *M.scalaris* are able to search for food, their locomotor activities, pupal emergence times, oviposition preference and to determine if the different sexes had an attractiveness to light.

### 4.1.2 Experimental Design

#### 4.1.2.1 General Maze Preparation

The adult flies were sedated using the icing method which involved placing the breeding chambers on to ice for a few minutes whilst rotating the jar halfway through the process; this allows the flies to collect at the bottom of the chamber for easier collection. Once asleep the flies were sexed using both size and observations of the flies' abdomens (*Fig 10 and Fig 11*).

The females had large rotund white abdomens whilst the males were observed to be a lot smaller in size and had much smaller abdomens (*Fig 10 and Fig 11*). The males and females were placed into individual tubes, in which each contained ten to thirteen adult flies which allowed for any losses.

Flies were placed into the starting tube the day prior to the experiments being run in order to avoid any manipulation that could have any effect on the flies' behaviour.

The starting tubes contained a small piece of absorbent paper which was placed at one end of the tube and a freshly prepared sugar solution was injected on to it to allow the flies some nourishment whilst housed in the tubes. The other end of the tube was sealed using clips which allowed quick attachment and removal to the maze.

#### 4.1.2.2 Maze Activity Monitoring

To test the adult preference of either food or different coloured lights as attractants, a plastic maze was used (as described in a previously used in a behavioural study in *Drosophila* (Hay and Crossley, 1977). Schematic representation of the maze can be seen in (*Fig 60*). The maze experiment was conducted using both males and females separately under different conditions (i.e. dark/light conditions, AM/PM time periods).

Between 7 am and 1 pm were termed (AM) and the afternoon between 1 pm and 7 pm were termed (PM).

Light conditions, the maze was placed on a work bench in front of a window allowing the maze to be positioned in natural light, the laboratory lights were also switched on during the experiments'. Dark conditions were imitated by placing the maze into a black box; all edges were sealed to prevent any light seeping through into the box.

Dark and light condition experiments were run in both the AM and PM periods using both male and female adults to determine if there were any differences observed in activity between the two time periods. Each experiment had three replicates.

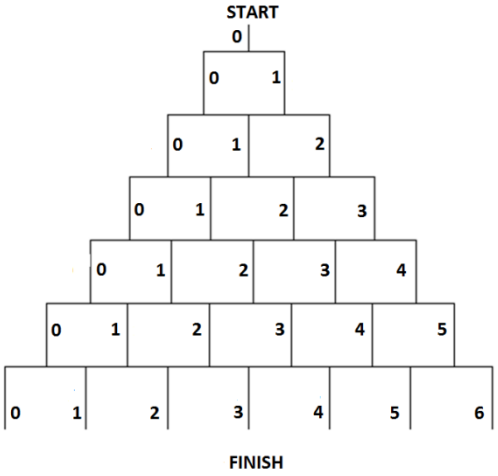



**Fig 60: Schematic of control maze with scores.**

#### 4.1.2.3 Control Maze

The control maze had empty vials at the finish points of the maze i.e. no stimuli were present. The flies housed in the starting tubes were connected to the start area of the maze as shown in (*Fig 60*). The flies were released into the maze and the experiment ran for a total of sixty minutes, the timer was stopped at thirty minutes and the flies' positions were recorded, this method was repeated again at sixty minutes. Once completed the scores were determined and analysed.

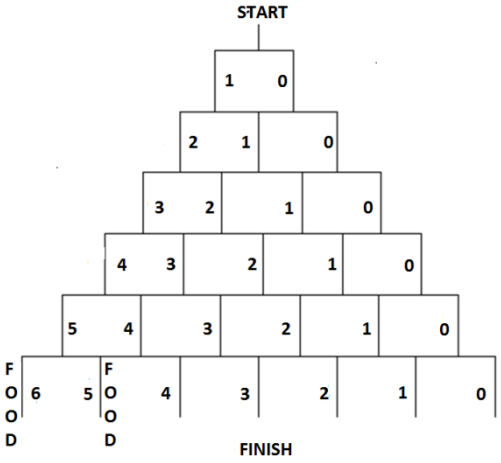



**Fig 61: Schematic showing position of food with scores.**
**The food could move from right to left with the scores being altered accordingly.**

#### 4.1.2.4 Food Stimulus

Using the same parameters (i.e. males/females, dark/light conditions, AM/PM time periods) as the control maze, experiments were run to determine if a food stimulus would affect the locomotor activity of the adult flies. The first stimulus used in the maze was pet food (Sainsbury's pate cat food). The stimulus was placed on to either the right or left side of the maze (*Fig 61*), the vials with the stimuli had the highest score. Food used as stimulus weighed ~ 0.5 g.

A second food stimuli used was fresh pig liver collected from the local supermarket. The same method and parameters as the pet food experiment were used. Food used as stimulus weighed ~ 0.8 g.

#### 4.1.2.5 Light Stimulus

To run the coloured light experiment, a method similar to that of the dark condition control experiments was implemented. These experiments involved a small Light Emitting Diode (LED) being fixed into the corner of the dark box.

The lights were run off a single electrical board in which each light had the same electrical output. The colours used were white (W), red (R), blue (B), green (G) and orange (O).

**Table 38: LED colours and wavelengths (Da Silva and Simonis, 2005)**

| Colour | Wavelength (λ) |
|---|---|
| **White** | Broad Spectrum |
| **Blue** | 450 < - < 500 |
| **Green** | 500 < - < 570 |
| **Orange** | 590 < - < 610 |
| **Red** | 610 < - < 760 |

The lights were placed to the right or left hand side of the maze, no other stimuli was used, and the highest scores were positioned with the stimuli (*Fig 62*). The flies were released into the maze and recorded after thirty and sixty minutes as per previous methods. Experiments were run in triplicate.

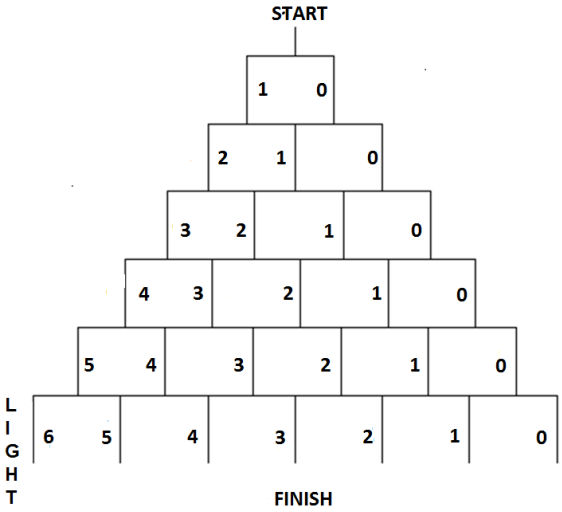



**Fig 62: Schematic showing LED positions with scores.**

In order to investigate the strength of the stimuli, a further experiment was run in which a food stimulus was added to the opposite end of the maze from where the light was placed (*Fig 63*). Pet food was used as the stimulus in this experiment due to earlier positive results. Experiments were again run in triplicate, once completed the food, light and scores were rotated as previously seen in both the food or light experiments.

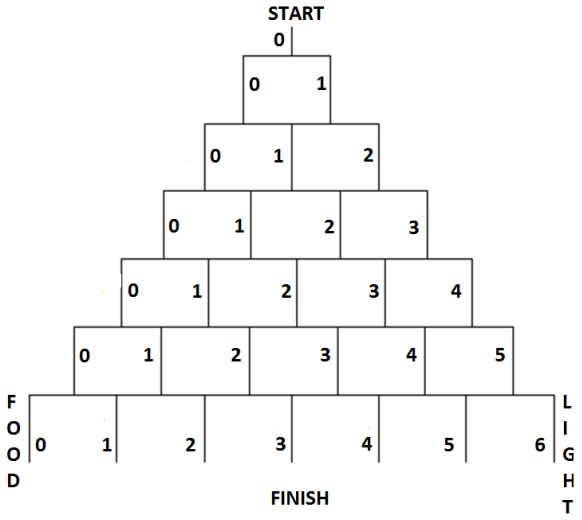



**Fig 63: Schematic showing food and LED positions.**

#### 4.1.2.8 Statistical Analysis

Mann-Whitney non-parametric tests were carried out on the maze results to determine effects of different parameters had on the attractiveness of food stimuli. Score was used as the test variable and gender, time and condition were used as the grouping variable.

### 4.1.3 Results

#### 4.1.3.1 Food Stimuli in Adult Maze

Activity monitoring was carried out to determine the effect of either food or lights from different colours as attractants and to observe locomotor activities of *M.scalaris* to determine if these parameters had an effect during both light and dark periods using different foods. Experiments were carried out in both natural light and in an incubator.

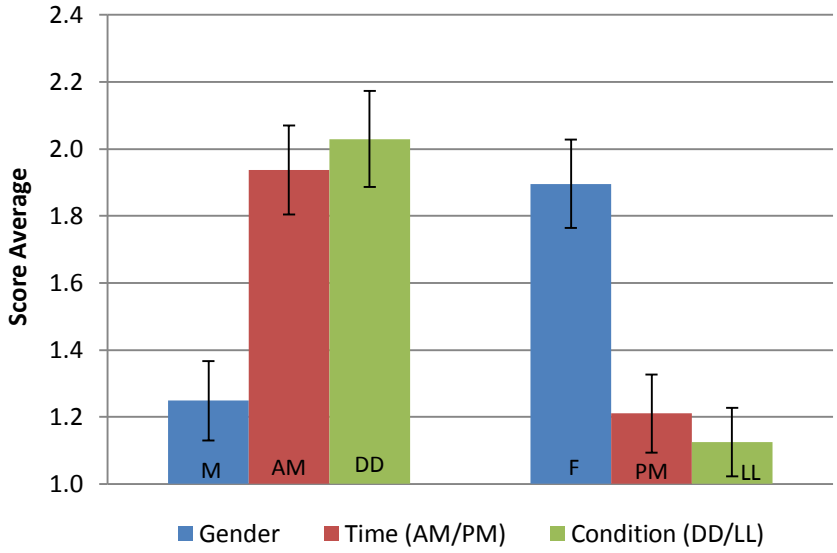



**Fig 64: Maze results for *Megaselia scalaris* flies under control conditions, no stimulus was used. Error bars represent SEM.**
**(DD = dark, LL= Light), N=477, Gender p=0.00, Time p=0.00, Condition p=0.00**

The comparison of the control results indicates a significant difference in male and female activity, dark/light phases and AM/PM time periods (*Fig 64*). Females show a significant difference in activity when compared to the males (p = 0.00) and the most activity was observed during the dark in the AM time period (p = 0.00).

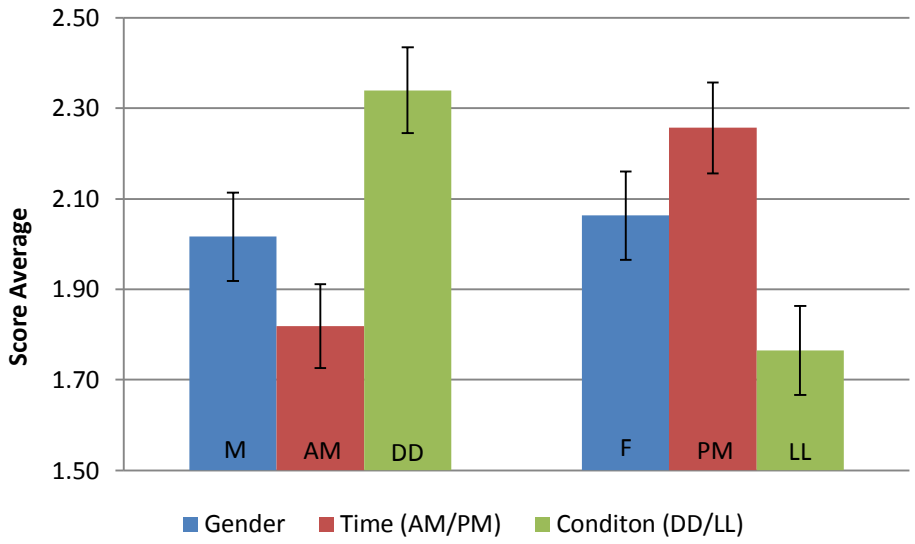



**Fig 65: Maze results for *Megaselia scalaris* flies, pet food stimulus was used. Error bars represent SEM. (DD = dark, LL= Light), N=995, Gender p=0.97, Time p=0.01, Condition p=0.00**

In the presence of pet food (*Fig 65*), both male and female activity has no significant difference (p=0.97). In contrast, activity during the dark phase differs from the light phase (p=0.00) along with the activity in the time period (p=0.01).

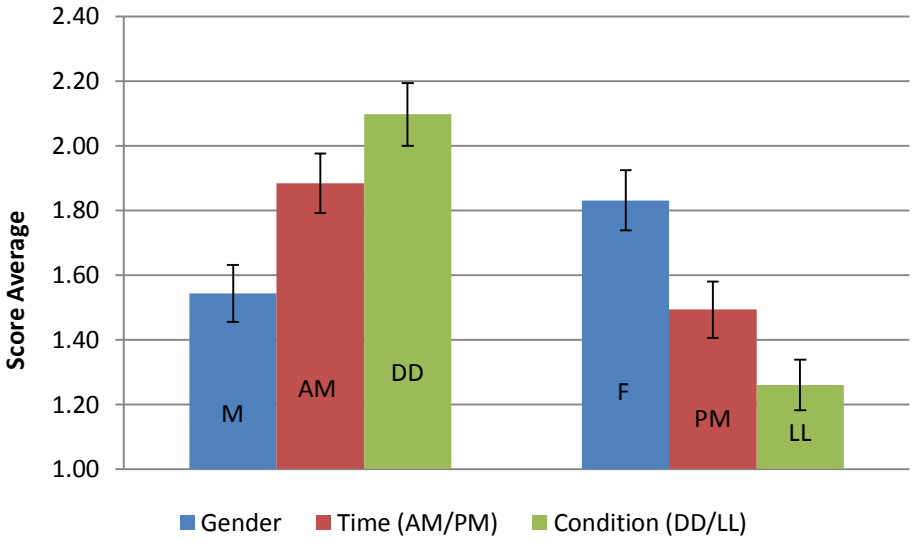



**Fig 66: Maze results for *Megaselia scalaris* flies, liver stimulus was used. Error bars represent SEM. (DD = dark, LL= Light), N=1036, Gender p=0.01, Time p=0.00, Condition p=0.00**

In the presence of liver (*Fig 66*), male and female activities differ (p=0.01). Females show the most activity compared to the males. Most activity was observed with during the dark period (p=0.00) along with the AM time period (p=0.00).

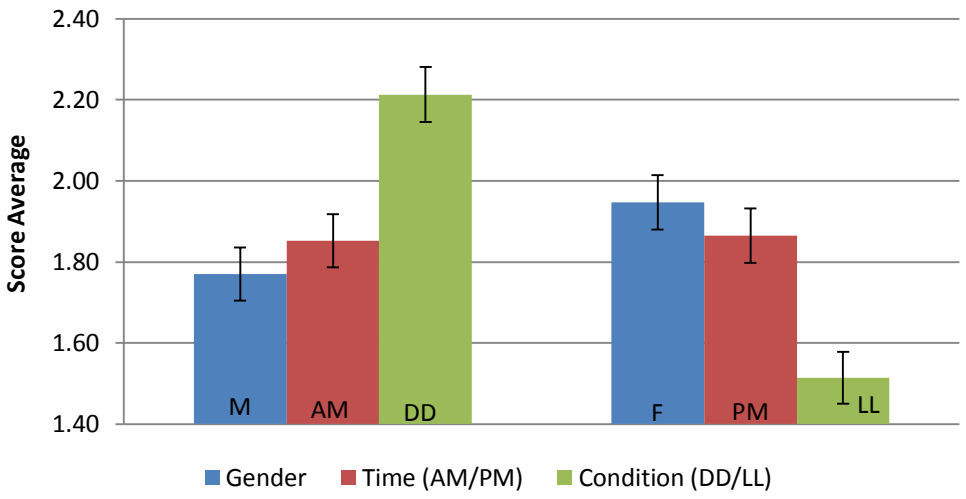



**Fig 67: Maze results for *Megaselia scalaris* flies, pet food and liver stimulus was used. Error bars represent SEM.**
**(DD = dark, LL= Light), N=2031, Gender p=0.06, Time p=0.88, Condition p=0.000**

When comparing the results between pet food and liver (*Fig 67*), both male and female activity does not have any difference (p=0.06). No difference in activity was observed in the AM or PM time period (p=0.88). However a significant difference in activity was observed during the dark phase (p=0.00) when compared to the light.

#### 4.1.3.2 Food Preference Analysis

Preference experiments comparing pet food vs. liver (*Fig 68*) clearly reveals a preference for pet food and not liver. This behaviour has been dissected considerably by carrying out separate experiments in both dark and light conditions (*Fig 69*). In both of the experiments the average indicates a cat food preference (dark p=0.023, light p=0.002).

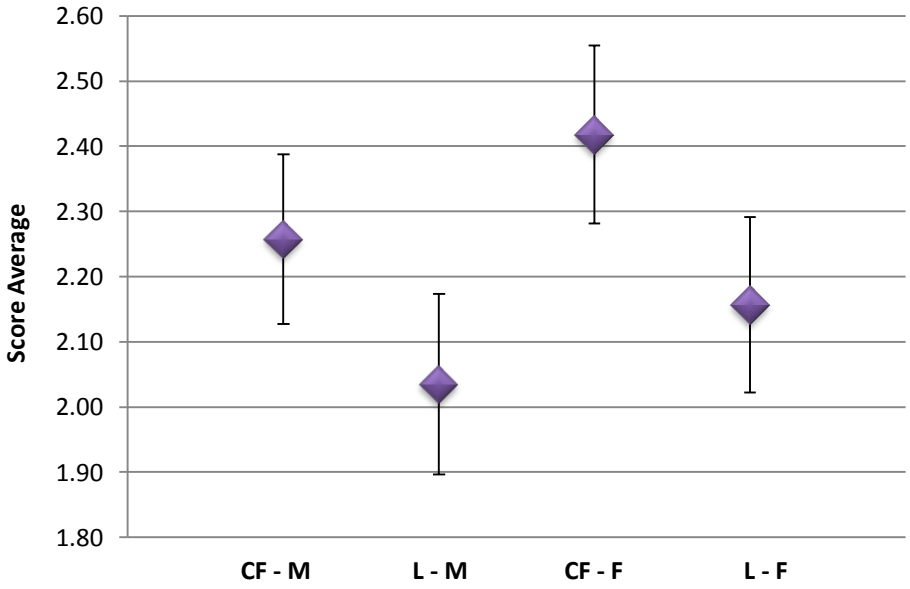



**Fig 68: Maze results for *Megaselia scalaris* flies, pet food and liver stimulus was used. Error bars represent SEM Average score ± SEM of food maze performed in dark condition.**
**(CF=Cat food, L=liver, M = Male, F = Female), (N=1002, p=0.02).**

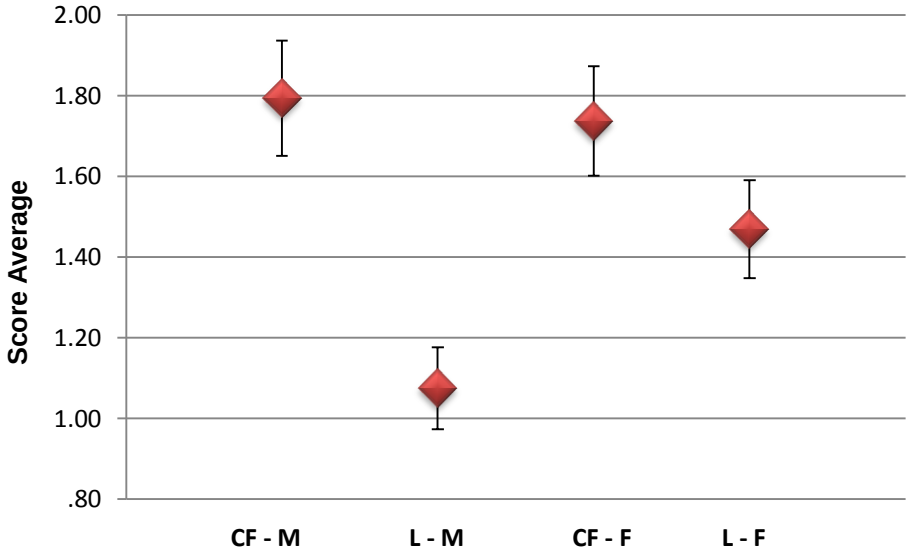



**Fig 69: Average score ± SEM of food maze performed in light condition.**
**(CF=Cat food, L=liver, M = Male, F = Female), (N=1029, p=0.00).**

#### 4.1.3.3 Overall Food Preference in Dark and Light Conditions

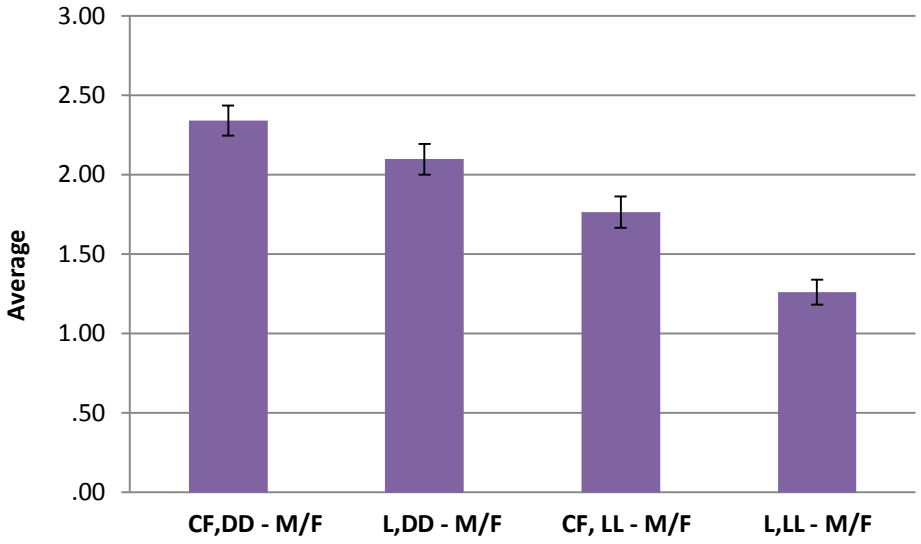



**Fig 70: Average score ± SEM of food maze performed in light/dark condition. (CF=Cat food, L=Liver, LL= Light, DD = Dark), (N=2031, p=0.000).**

Overall the results (*Fig 70*) show that the adults preferred the cat food in dark conditions followed closely by the liver also in dark conditions. Significant differences were observed when all four experiments were analysed (p=0.00).

#### 4.1.3.4 Light Stimuli

To determine the preference of different coloured lights and the effect that the different parameters had on the attractiveness of the light stimuli in both sexes the maze was used. The results were determined by the range being calculated using a stochastic method using high and low scores from the maze results.

Cold white (CW), Blue (B), Green (G) Red (R) and Orange (O) lights were used to test the attractiveness of light.

Males and females show different behaviour (*Fig 71*), males clearly prefer cold white, blue, green and orange lights and they seem not to see the red light according to other species behaviour (p=<0.00, N=1500).

In contrast females seem to be attracted by the red light and they do not present any directional behaviour under other light colours (*Fig 71*). A statistical difference in behaviour towards the red light is observed (p=0.126, N=1940).

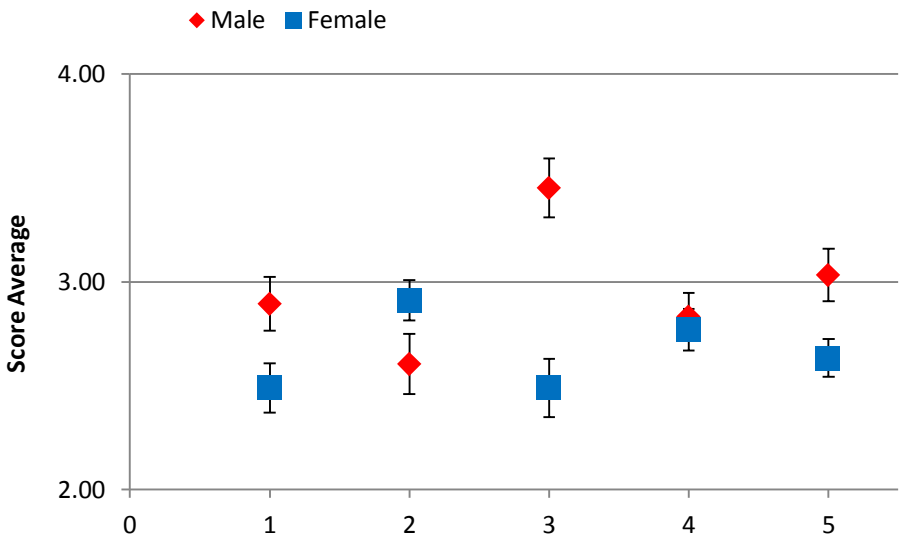



**Fig 71: Combination of left and right light stimulus for *Megaselia scalaris* males (N=1500) and females (N=1940). Error bars represent SEM. 1=White, 2=Red, 3=Blue, 4=Green, 5=Orange.**

#### 4.1.3.5 Light and Food Stimuli

The maze was used in this experiment to determine the strength of coloured lights or food as attracting stimuli. The results were determined using the same approach in the previous experiments and the same colours were used as in the light stimuli experiments.

Male and females (*Fig 72*) show no preference for the food and prefer to move towards the light. Males show a significant difference in behaviour (p=0.00, n=1411).

Females (p=0.00, n=2885) show a preference for cold white whilst the males show a preference for all colours with blue having the highest score followed by cold white.

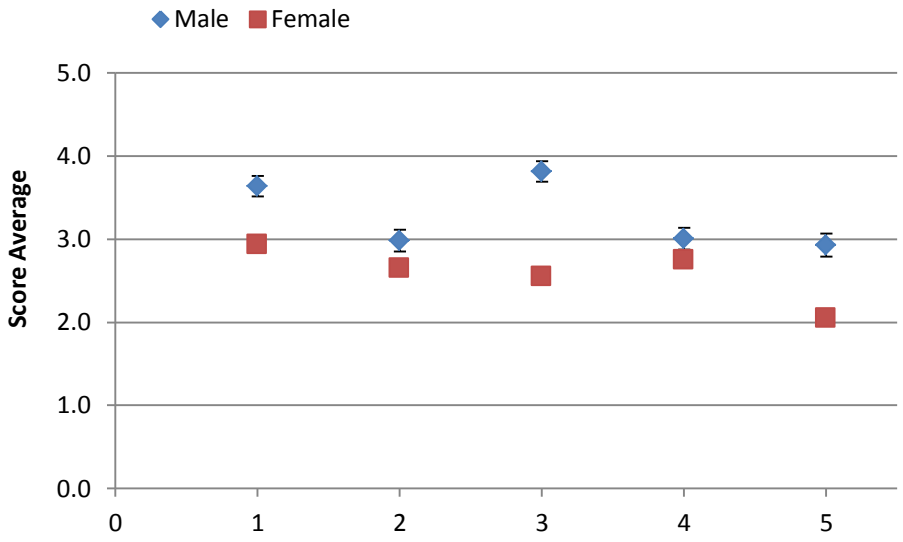



**Fig 72: Combination of left and right light/food stimulus for *Megaselia scalaris* males (N=1411) and females (N=2885). Error bars represent SEM.**
**1=White, 2=Red, 3=Blue, 4=Green, 5=Orange.**

### 4.1.4 Discussion

*Megaselia scalaris* males and females show different behaviour, males clearly prefer cold white, blue, green and orange and they seem not to see red light, in contrast females seem to be attracted by red light and they do not present any directional behaviour under other light colours. Disney *et al*. (1982) found that by using different coloured water traps showed that flies in general have different colour preferences which has been shown by the results of the light maze.

Hardie (1986) discusses that many insect species have darkly coloured eyes, but distinct colours or patterns are frequently featured. The screening pigments in the pigment cells commonly determine the eye colour. The red screening pigments of fly eyes and the dorsal eye regions allow stray light to photo-chemically restore photo-converted visual pigments. A similar role is played by yellow pigment granules inside the photoreceptor cells which function as a light-controlling pupil. Most insect eyes contain black screening pigments which prevent stray light to produce background noise in the photoreceptors. The dipteran compound eye conceals an equally regular array of photoreceptors and interneurons that constitutes one of the most thoroughly analysed visual systems. One of these regions appears specialized for the analysis of polarized light patterns in the sky, whilst the other which is found only in males is apparently devoted to the task of tracking females in flight. This may explain the difference between the male and female results if the same is true for the *Megaselia* species.

Research was undertaken by Wooldridge and colleagues (2007) in which carcasses were placed outside either raised off the ground or placed on to the ground, results showed that during the dark and also in artificially lighted areas that no evidence was found of nocturnal oviposition. Using *L.sericata* and *C. vomitoria* further research was done determine the attractiveness of odours; this concluded that whilst flies may be stimulated by the odours they seem unable to bring themselves to the source in the dark (Green, 1951a; Faucherre *et al*., 1999).

During the experiments eggs were observed in the tubes at either AM or PM time periods however a more detailed study on oviposition can be read in Chapter 4.2.

## 4.2 Diurnal/Nocturnal Activity and Oviposition

### 4.2.1 Introduction

Flight activity by insects is thought to occur during a portion of the 24 hour cycle and is therefore periodic. Insect flight has many parameters surrounding it such as time of day, light intensity, amplitude may be affected by temperature, some insects are only able to fly in direct sunlight and this may explain the reason behind short flight periods. Adult feeding habits were also found to have an effect on the time of flight. Feeders of decaying organic matter were seen to have higher presence in the dusk & dawn periods followed by high numbers in the day time, night time saw a very small presence (Lewis and Taylor, 1965).

It has long been thought by some entomologists that forensically important flies are unable to fly, be active or to lay their eggs in the night time period (Faucherre *et al*., 1999). Over the years there has been much research (*Table 7*) investigating whether flies are able to oviposit during the night i.e. after sunset and before sunrise, by using many different parameters. Much of the research carried out has received contradictory results or criticism from other researchers on the methods used to determine nocturnal oviposition.

Erzincliogl (1996) remarks that it is incorrect to believe that flies are unable to oviposit in dark conditions as they are known to be able to oviposit in dark areas. Dark conditions may entail; darkness in daylight (e.g. cave, coffins, dark rooms) as well as dark during the night period. This theory would be relevant to flies such as *M.scalaris* that are reported to be able to burrow down into the soil to lay their eggs on to buried cadavers (Smith, 1986).

Acikgoz and colleagues (2012) discuss the requirement of protein by flies in order to lay their eggs, protein is sourced from numerous areas some of which include: animal and human excrement, animal carcasses, cadavers and food remains left around by humans. To precisely determine time since death, ovipositing behaviour in necrophilous flies first needs to be established.

Colonisation of carrion by insects allow for the post mortem interval (PMI) to be estimated. However it is thought by some, that flies are not active during the night time

period and therefore are not able to oviposit during this time (Barnes *et al*., 2014). To put that into a forensic context, if eggs were located on a cadaver, the conclusion would be that death occurred during the previous day or before. Determining nocturnal oviposition in forensically important flies is of fundamental importance so that the PMI can be estimated correctly by the forensic entomologist. Understanding the allure of food and light stimuli for *M. scalaris,* in both dark and light conditions, are important for the evaluation of the eggs' laying time, with consequences in the mPMI estimation.

In order to understand the ability of the flies to colonise a body we need to understand when individuals of both sexes are active. To describe the behaviour and the potential role that the circadian clock may have on both the locomotor activity of *M.scalaris,* TriKinetics Inc. technology used previously in *Drosophila* studies which allows for factual data rather than observational data whist determining nocturnal oviposition in forensically important flies is of fundamental importance so that the PMI can be determined with more precision by the forensic entomologist.

One of the main questions asked by entomologists is whether oviposition was at any point delayed as this may have an overall effect on the PMI estimation possibly causing discrepancies of up to 12 hours (Amendt *et al*., 2008). To be able to determine this first we must understand when *M.scalaris* is able to lay eggs and in what conditions.

### 4.2.2 Experimental Design

#### 4.2.2.1 Diurnal/Nocturnal Activity of Megaselia scalaris

Locomotor activity of *M.scalaris* can be measured using TriKinetics Inc. *Drosophila* activity monitors (DAM) (*Fig 73 and Fig 74*). The TriKinetics Inc. *Drosophila* Activity Monitoring System enables a biologist to accurately characterize the locomotor and eclosion behaviour rhythms. The monitor contains 32 tubes, each tube is made from Pyrex glass with a diameter of 5 mm with a length of 65 mm; each tube houses an individual fly. An infrared beam (IR) is present across the tubes midpoint, so when the fly travels through the middle of the tube, the IR beam is interrupted and an activity count is recorded by the systems host computer using the DAM system date acquisition software package (*Trikinetics*).

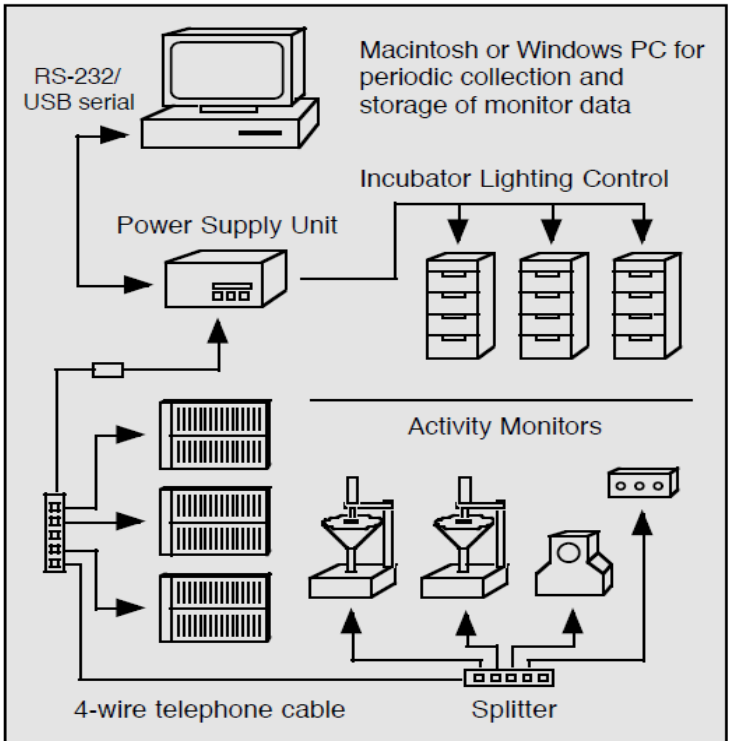



**Fig 73: Schematic of TriKinetics Inc. equipment set up (Trikinetics).**

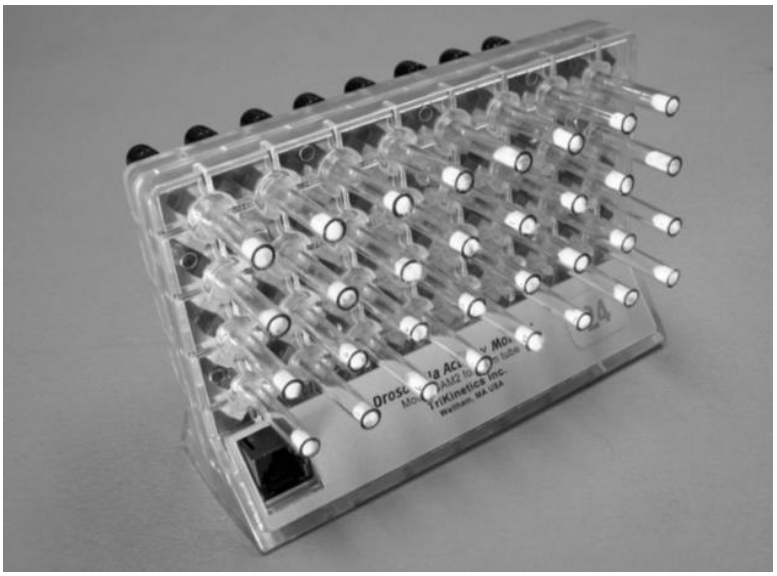


**Fig 74: TriKinetics Inc. Drosophila Activity Monitor (Trikinetics).**

*Drosophila* fly medium (Toivonen *et al*., 2007) was placed into one end of the tube which was sealed with wax. The opposite end of the tube had a cotton wool plug allowing for air flux. The tubes were placed into the DAM and the preliminary experiment was left to run for 7 days. Temperature, humidity, light intensity was recorded by a *Drosophila* environmental monitor (EM) (*Fig 75*).


**Fig 75: TriKinetics Inc. environmental monitor (Trikinetics).**

The LAM (Locomotor activity monitoring) recording was set up in an incubator with a temperature of 21 $^{o}$C and a photo-period of 12:12 LD.

Puparia sexing was undertaken so that virgin females could be used for the female experiments. The experiment was carried out with virginal females to prevent false recordings of activity from any larval hatching from the eggs laid by gravid females during the experiment. Flies were entrained under LD 12:12 conditions for at least three days prior to the experiment.

#### 4.2.2.2 Oviposition

##### 4.2.2.2.1 Oviposition in a Light Environment

To investigate if oviposition occurred during day time hours between the hours of 7 am and 7 pm, ninety glass tubes were prepared; pet food was positioned into one end of the tube and sealed with wax, this allowed the food to remain fresh for the short period of the experiment. Pet food was used as flies have shown a preference for ovipositing on this media when compared to liver. Female *M.scalaris* flies, previously entrained to LD 12:12 were individually placed into glass tubes and sealed with a cotton wool plug which would allow a sufficient air flux into the tube to prevent suffocation. The tubes were then placed inside an LMS cooled incubator 410XAL. The incubator was programmed to 21 ± 0.5 $^{o}$C with a LD 12:12 photo-period condition in which the incubator lights were programmed to switch on at 7am and switch off at 7 pm. Once the experiment was completed the flies were removed from the incubator and immediately placed on to ice so that no further ovipositing would occur. The flies were removed from the tubes to prevent any obstruction when looking for eggs. The tubes were examined under a Leica M60 microscope to determine if any oviposition activity had occurred and a count of how many eggs were taken.

##### 4.2.2.2.2 Ovipositing in a Dark Condition in a Light Environment

To determine if oviposition occurred during the day (7 am to 7 pm) in a dark environment to imitate environments such as coffins, caves, chimneys etc. The same general method as seen in the light environment experiment was used however this time the tubes were placed into a black box. The box was then placed into an additional black box so that no light penetration could occur. The boxes were placed into the incubator and once was completed the same removal and examination method was used.

##### 4.2.2.2.3 Ovipositing In Dark Condition

To determine whether oviposition could occur during the night time period (i.e. after 7 pm); the tubes were placed into the Incubator at 7 pm and were removed from the incubator at 7 am. Once completed the same removal and examination method was used as previously mentioned.

##### 4.2.2.2.4 Statistical analysis

Statistical analysis was performed using Microsoft Excel and IBM SPSS. Analysis includes chi square test, Anova and averages.

### 4.2.3 Results

#### 4.2.3.1 Diurnal/Nocturnal Activity of *Megaselia scalaris*

Locomotor activity results using the *Drosophila* activity monitor indicates that the male flies show both diurnal and nocturnal activity and a bimodal behaviour represented by the two peaks which corresponded to the transition between dark to light and light to dark.

The transition from dark to light clearly shows a response to the light being off (*Fig 76*, *Fig 77* and *Fig 78*); in fact from the data it is not possible to see any anticipation. In contrast the evening the peak seems to indicate a weak anticipation to the light switching off but further investigation is required to solve this point. The activity during the diurnal phase is significantly different (lower than 0.05) to the nocturnal activity.

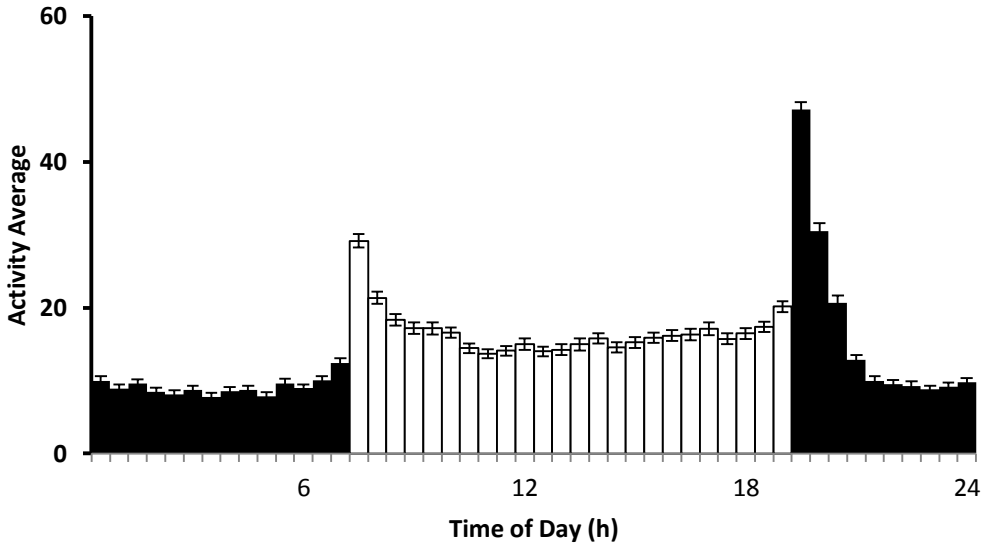



**Fig 76: Locomotor activity profiles recorded (±SEM) with DAM Trikinetics show male *Megaselia scalaris* activity.**
**(N=593, LD 12:12, T = 21°C, p=0.00), the activity profiles (white represents daytime and black represents night) showed bimodal peaks at morning and at evening.**

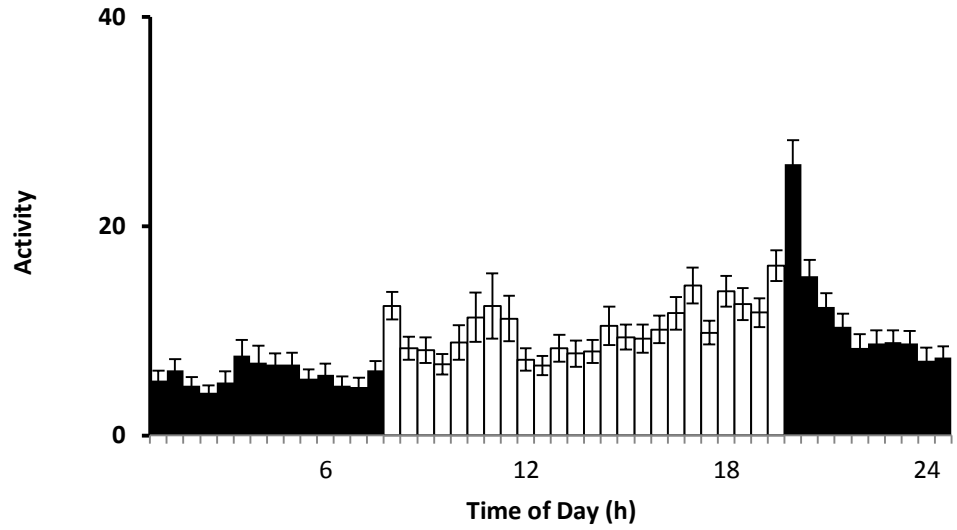



**Fig 77: Locomotor activity profiles recorded (±SEM) with DAM Trikinetics show female *Megaselia scalaris* activity.**
**(N=205, LD 12:12, T = 21oC, p=0.00), the activity profiles (white represents daytime and black represents night) showed bimodal peaks at morning and at evening.**

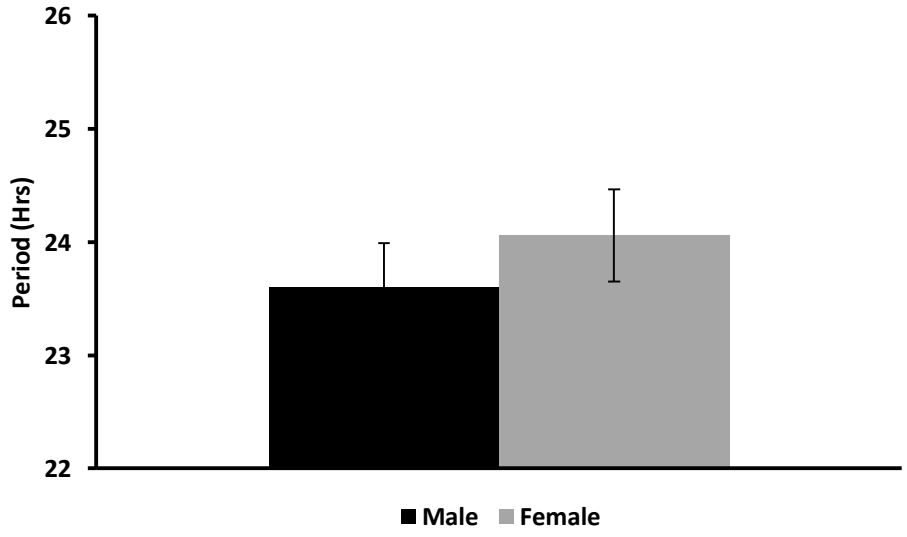



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**Fig 78: Average period of activity (±SEM) of *Megaselia scalaris* males and females.**
**Derived from the experiments in Fig 79 & 80, both sexes have a period of circa 24 hours with no statistical differences (N=69, df 68, p=0.22).**

In order to understand if *M.scalaris* behaviour was under (circadian) clock regulation we performed an experiment run over eight days in which flies were entrained under LD 12:12 conditions for 3 days and then maintained in complete dark for five days

(*Fig 79* and *Fig 80*). The activity pattern show the presence of a morning and evening peak in both male and females along with a drastic reduction of the burst of activity related with the switching on and off of the light. During the period of complete darkness the activity peak is still visible in both the sexes showing that they are still active in a completely dark environment. As the days continue a gradual decrease in activity can be seen daily as the circadian clock is disrupted by the absence of light.

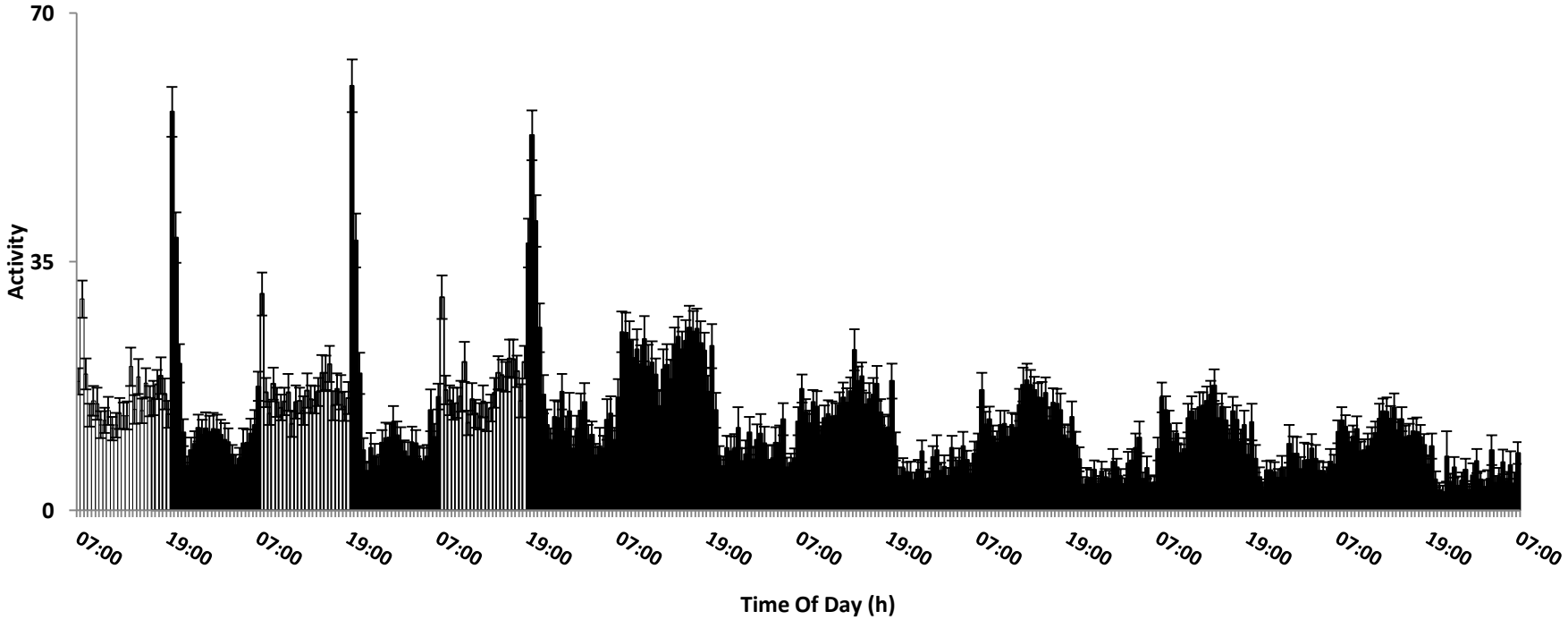


**Fig 79: Average activity profiles (±SEM) (number of counts 30mins-1) of *Megaselia scalaris* male flies (N=97) under three days of LD 12:12 and five days in complete darkness under constant temperature (20 °C).**
**The activity bimodal profile (Morning and Evening peaks) are maintained in complete darkness conditions.**

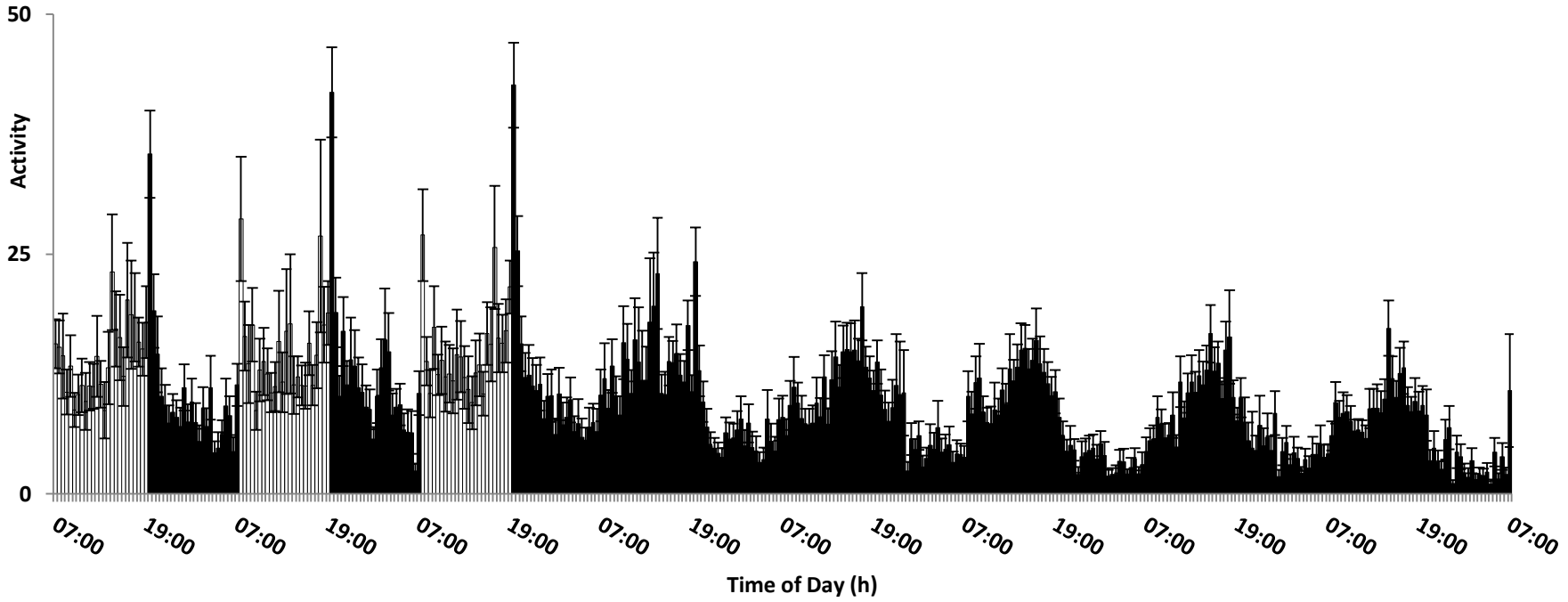


**Fig 80: Average activity profiles (±SEM) (number of counts 30mins-1) of *Megaselia scalaris* female flies (N= 72) under three days of LD 12:12 and five days in complete darkness under constant temperature (20 °C).**
**The activity bimodal profile (Morning and Evening peaks) are maintained in complete darkness conditions.**

#### 4.2.3.2 Oviposition

It is common knowledge that flies are able to lay eggs during the day however *Megaselia scalaris* is also referred to as a 'coffin fly' and is therefore often found in dark environments. It was undetermined whether *M.scalaris* were able to lay their eggs in either a dark condition during the day (dark in daylight) or during the night.

The results show that 35% of females were able to oviposit during the night compared with 37% during the day (*Fig 81*).

When flies were left to oviposit during their subjective day in DD only 14% did so. There were no significant differences in the percentage of oviposition when comparing night, day, darkness during daylight ($\chi^2$ test, df 2, p=0.90).

Overall the number of eggs per tube showed a significant difference in the three conditions (p=0.00). In light conditions flies laid more eggs (17.5+/- SEMS egg per tube) than in dark conditions (p=0.013). No difference was observed when comparing the dark conditions during the day or night.

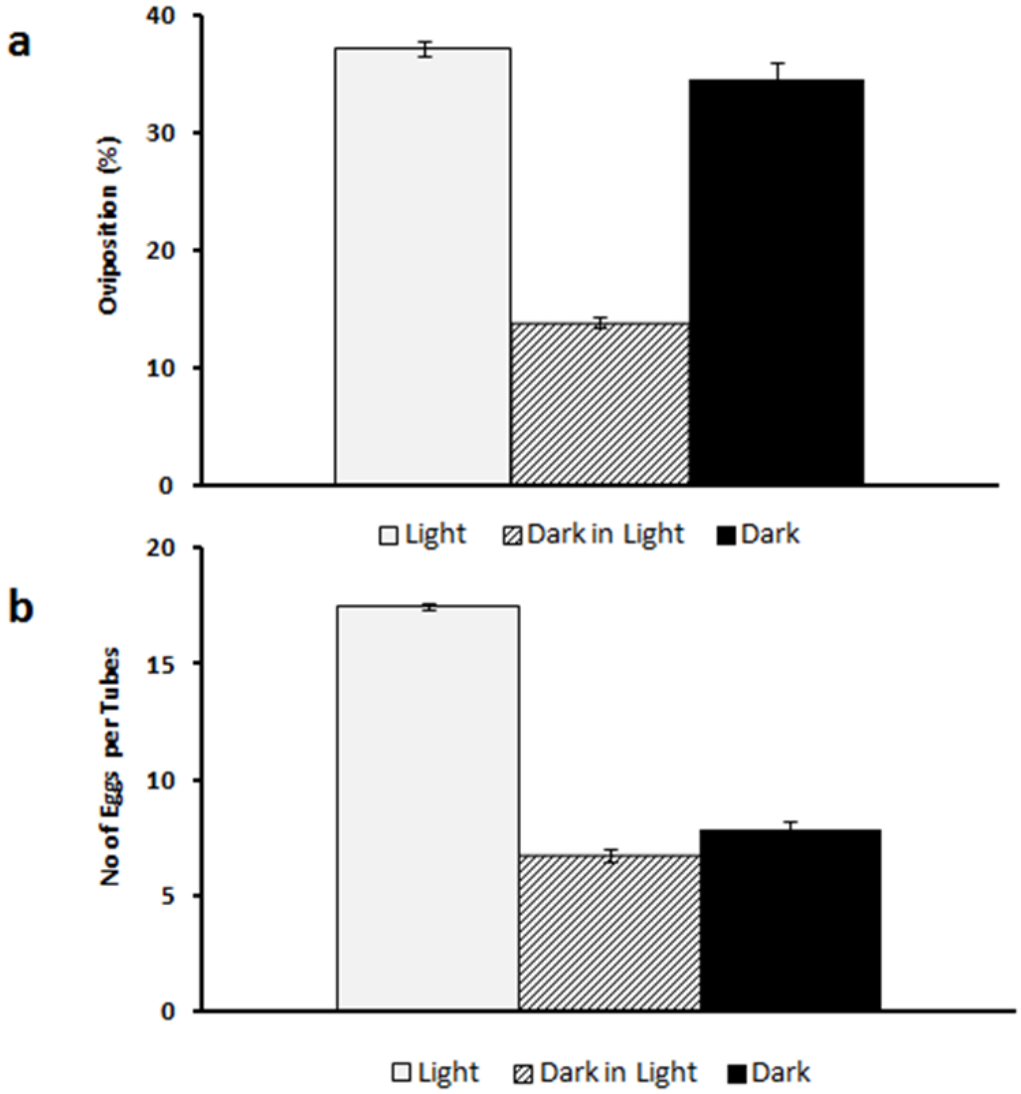


**Fig 81: a) Average percentage of tube with eggs. *Megaselia scalaris* flies laid eggs in all the tested conditions, no statistical difference was observed comparing the number of ovipositions ($\chi$2 test, df 2, p=0.90).**
**b) Number of eggs per tube, flies laid eggs in all the conditions but with a statistical significant difference comparing the number of eggs laid in light and dark conditions (p=0.013).**

### 4.2.4 Discussion

#### 4.2.4.1 Diurnal/Nocturnal Activity of *Megaselia scalaris*

Diurnal and nocturnal activity patterns of flies are important aspects that are currently being researched as currently necrophagous flies are thought to be inactive during the night however if this is found to not be the case then the mPMI could be out by a period of up to 12 hours. The present knowledge of nocturnal activity needs to be improved in order to further understand the environmental parameters that may further affect nocturnal activity (Amendt *et al*., 2008). Flies are thought to active through only a portion of the 24 hour period which may suggest that insect activity is periodic (Lewis and Taylor, 1965).

It was observed by Payne (1965) that there was a rapid decrease in the activity of blow flies after sunset. Baldridge *et al*. (2006) saw fly activity cease within an hour after sunset however it was suggested that nocturnal environmental conditions e.g. air temperature, wind speed and humidity should be researched further. Artificial illumination as an exogenous activity stimulant in the presence of an odour cue showed nocturnal activity when researched by (Greenberg, 1990b; Wooldridge *et al*., 2007).

When an insect is transferred from a LD environment to a continuous DD environment, if the parameters (e.g. temperature) remain the same then the rhythmic activity becomes 'free running' and this will reveal the natural periodicity. When cockroaches *Leucophaea maderae, Byrsotria fumigata* and *Periplaneta America* (Blattodea, Blattidae) were transferred into complete DD the rhythm continued for up to 3 months (Saunders, 2002).

The burst at the switch on/switch off of the lights are a clear response to the change in light conditions which are observed during all three activity experiments. Under LD 12: 12 (*Fig 76* and *Fig 77*), *M.scalaris* males and females both demonstrate that there are significant differences in their locomotor activities between dark and light conditions and further results establish that the flies are both diurnal and nocturnal in activity; the general pattern of activity for both sexes indicates a persistent nocturnal activity. The locomotor activity for 3 days LD 12:12/ 5 day LD 00:24 (*Fig 79* and *Fig 80*)

show that there is a clear rhythmic pattern in the levels of diurnal activity as well as nocturnal activity.

The only other locomotor activity research done on *Megaselia* species was completed by (Lewis and Taylor, 1965) in which a few different *Megaselia* species had their flight distribution recorded (*Fig 82* to *Fig 84*).

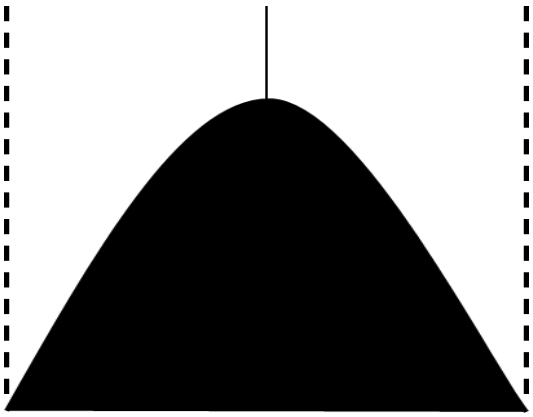

**Fig 82: Flight distribution curve of undetermined *Megaselia* species and undetermined sexes. (Lewis and Taylor, 1965)**

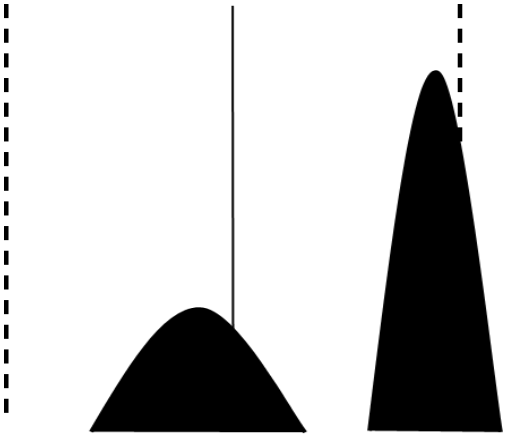

**Fig 83: Flight distribution of curve of undetermined *Megaselia* species and undetermined sexes. (Lewis and Taylor, 1965)**

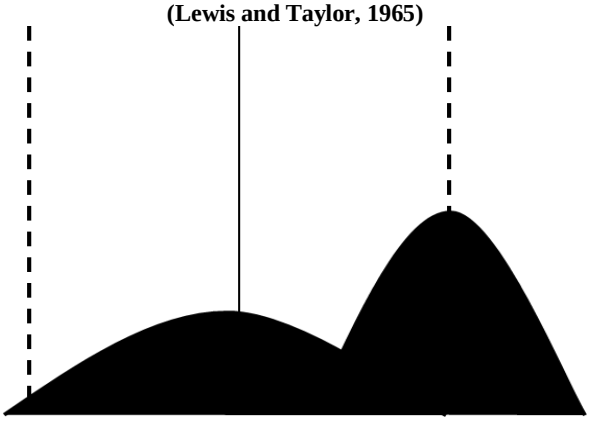

**Fig 84: Flight distribution curve of *Megaselia halterata* of both male and female sexes. (Lewis and Taylor, 1965)**

The curves demonstrate that the majority of *Megaselia* species have a bimodal activity and during the day and also at the end of the day and during the night which supports our findings. The second peak, the evening burst is common as well in *Drosophila melanogaster* in both natural and laboratory conditions (Vanin et al., 2012a).

#### 4.2.4.2 Oviposition

The factors that may delay the oviposition on a dead body are of critical importance for mPMI estimation. Numerous researchers, state that blowflies are considered to be diurnal and not very active during the nocturnal period, therefore this may affect the oviposition on an exposed cadaver at night. Often authors pointed the attention on the ability of blowflies to lay eggs during the day in dark environments (Nuorteva, 1977; Erzinclioglu, 1996; Wooldridge *et al*., 2007).

*Megaselia scalaris* is not only able to oviposit during daylight hours in either light or dark conditions but that they are able to oviposit during the night time period which supports both Erzinclioglu (1996) and Smith (1986) discussions completed with other species, mainly Calliphoridae and Muscidae.

There are other parameters that need to be considered such as would a full moon or artificial lighting provide enough light to allow for oviposition in the dark (Kempinger *et al*., 2009). However despite these comments some blowflies have been caught ovipositing during the night (Green, 1951a; Faucherre *et al*., 1999).

It has been suggested by Saunders (2002) that adults entrained to a LD 12:12 photo-period would oviposit during the early part of the night and at dusk.

A circadian pattern was observed in oviposition in a variety of insect species, it is thought by Howlader and Sharma (2006) that eggs laid during the night time period would elude desiccation or parasitic infestation.

In a normal light condition, i.e. during the daytime hours, 37.2 % of *M.scalaris* females oviposited. During the light phase but maintained in a completely dark environment showed that 13.9 % of females were able to oviposit in this condition whilst the night time period showed that overall 34.5 % of females were able to oviposit.

Our experiments have demonstrated that *M.scalaris* is able to oviposit in dark conditions during the night. Further work is required to determine the oviposition rates on different media as well as determining the lowest temperature at which oviposition may occur, knowing that the lowest daily temperature occurs during the night before sunrise.

## 4.3 Emergence

### 4.3.1 Introduction

Evolution of the circadian clock has allowed synchronization of behaviour, physiology and metabolism to the 24h geophysical cycles of the Earth. The understanding of the circadian clock mechanism is a crucial element of forensic entomology as it is able to control routines such as feeding, mating, ovipositing or emergence times (Vanin *et al*., 2012a).

Studies on different photo-periods affecting the immature development of *Chrysomya albiceps* were conducted by da Silva Mello and colleagues (2012). By using different dark and light cycles LD 12:12, photophase (LD 24:00) and scotophase (LD 00:24), they researched how different photo-periods may influence larval body weight and the viability of the species. It was shown that the different photo-periods had an effect on the rhythm of emergence with shorter light conditions (LD 00:24) showing a more homogenous adult emergence pattern whilst under longer day conditions the rhythm was slower.

Research has been undertaken in different fly species e.g. *Drosophila pseudoobscura* to look into whether adult eclosion patterns are affected by a change in rhythm of the circadian clock along with adult locomotor activities (Engelmann and Mack, 1978).

Females have been observed by Benner and Ostermeyer (1980) emerging from the pupa later than males, this allows the males to feed and for their sperm to mature prior to the females emergence. Disney (2008), comments that other researchers have been unable to conclude this fact.

This is the case with the parasitic wasp *Nasonia vitripennis* (Hymenoptera, Pteromalidae) in which the males emerge first after chewing a hole in the host puparia, the males then wait at the exit hole for the females to emerge so they can mate straightaway (Bertossa *et al*., 2010)

To describe the behaviour and the potential role that the circadian clock may have on both the emergence times of the *M.scalaris,* Trikinetics technology used previously in *Drosophila* studies which allows for factual data rather than observational data.

### 4.3.2 Experimental Design

#### 4.3.2.1 Pupa Emergence in a LD 12:12 Environment

To investigate periods of pharate emergence from puparia, the Trikinetics locomotor activity monitor was used with glass tubes measuring 3 cm in length and 5 mm in diameter. The same method regards food and air flux were used as referred to in the previously completed activity monitoring experiments. The specimens had been reared under 20 ± 0.5 $^{o}$C in LD 12:12 conditions up to the pupal stage.

Pupa were individually placed into the midpoint of the tubes and placed into the activity monitor. The pupa resided in the middle of the monitor beside the IR beam. Recordings were performed in an Incubator with a temperature of 21 ± 0.5 $^{o}$C and a photo-period of LD 12:12.

Once the experiment was completed both pupa and emerged flies were removed. The flies were sexed to determine if any emergence pattern was observed between the two sexes and the pupa were measured to further understand the differences seen between the sexes.

#### 4.3.2.2 Pupa Emergence in LD (00:24) Environment

The same method was used as seen in the LD 12:12 experiments, however to imitate constant darkness, the monitors were placed into a black box and sealed; the box was then placed into a larger black box to prevent any light from penetrating in.

#### 4.4.2.3 Pupa Emergence in LD (24:00) Environment

The same method was used as seen in the LD 00:24 experiment; the black box used had white LED lights mounted into it; the lights which were connected to the mains and remained switched on constantly till the experiment was completed. The monitors including the environmental monitor were placed into the box, sealed and the experiment was left to run. The results were monitored daily to confirm the light remained static during the experiment.

### 4.3.3 Results

Pupa emergence experiment run in LD 12:12 photo-period (N=67, p=0.00) determines that there is a significant difference between the emergence in both light and dark conditions whilst pupa emergence which was run in complete darkness (N=46, p=0.069) show no significant differences. The emergence results showed that *M.scalaris* males pupated prior to females (*Fig 85*).

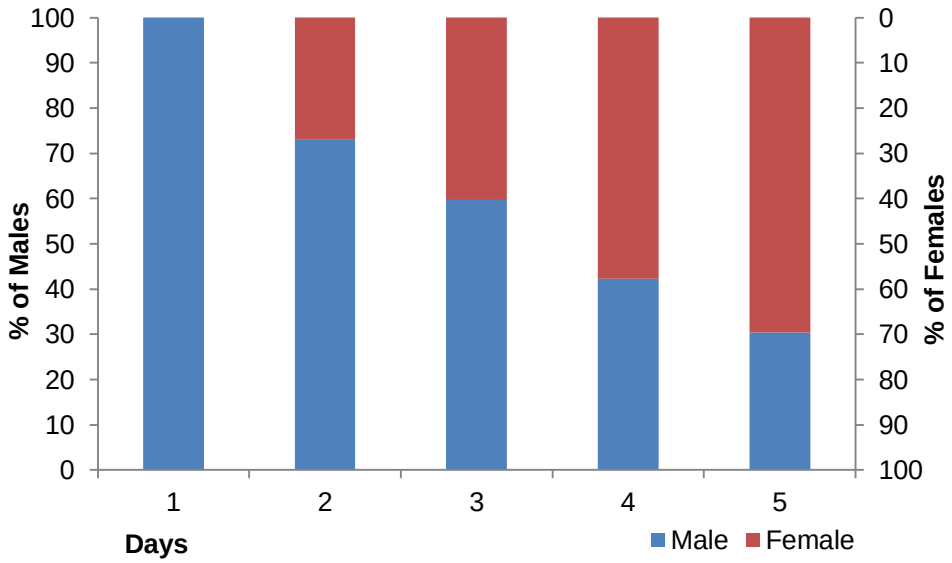


**Fig 85: Emergence pattern from both sexes of *Megaselia scalaris* adult flies at 20°C.**

In order to evaluate if the emergence is controlled by an internal clock, pupae were placed under different lighting conditions (LD 12:12, 00:24 and 24:00) which when compared showed a difference between the three different conditions. Calculating the degree of rhythmicity (*R*) is determined by identifying the highest number of eclosions within an 8 hour period. *R* is then calculated by dividing the number of eclosions outside this eight hour period by the number within the 8 hour period and multiplying the answer by 100 as used by Goto and colleagues (2006). If the pattern of emergence is completely uniform this would be represented by *R*=0. Values of *R* greater than 90 show statistically uniform emergence, values less than 60 represent a rhythmic pattern and those between 60 and 90 represent a weak rhythmicity.

#### 4.3.3.1 LD 12:12 Pupa and Sex emergence Patterns

A clear unimodal pattern is observed during the second half of the day, the pattern is the same for both sexes (*Fig 86*, *Fig 87* and *Fig 88*) showing a rhythmic pattern of emergence. In contrast when data from LD 12:12 is compared to both LD 00:24 and LD 24:00 (*Fig 89* to *Fig 92*) the patterns appear to shift showing both a statistically uniform and an arrhythmic pattern of emergence.

In LD 12:12 conditions, 80 % of the flies emerged during daylight hours and emergence of all flies took 6 days with 1 female fly emerging on day 8.

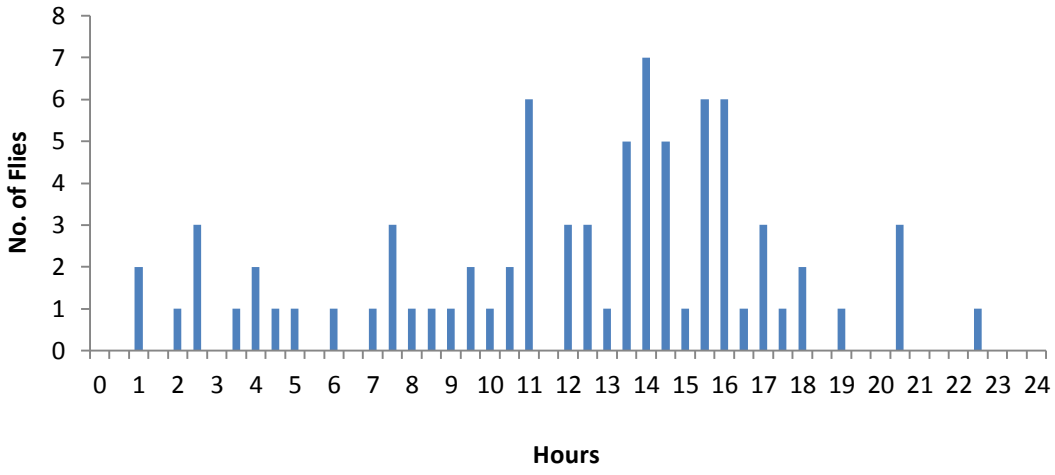


**Fig 86: LD 12:12 – Emergence Data of males and females of *Megaselia scalaris* adult flies at 20 °C.**

A rhythmic pattern is observed under LD 12:12 with R = 24 which is below the threshold of 60 (highest number of emergence within an eight hour period = 58; Number of emergence outside this eight hour period = 28; R (Rhythmicity) = 28/58 * 100 = 24).

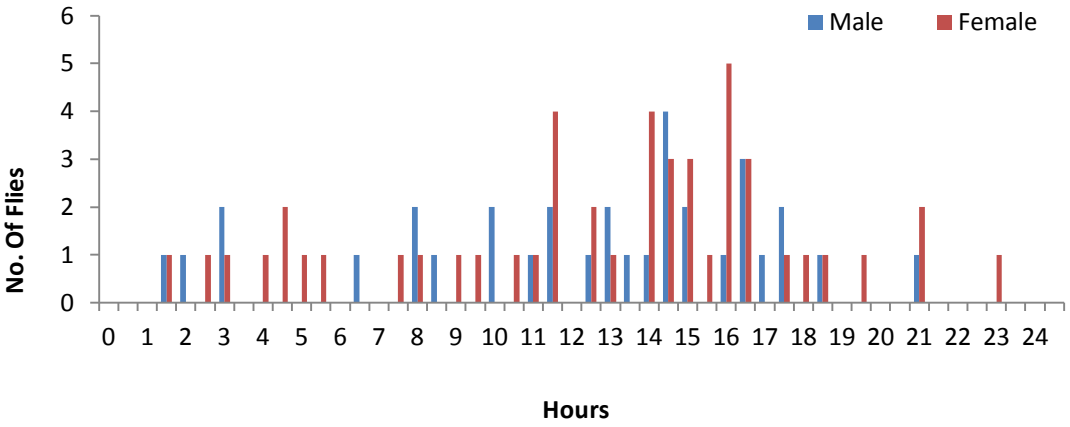


**Fig 87: LD 12:12 – Sex Emergence Pattern of *Megaselia scalaris* adult flies at 20 °C.**

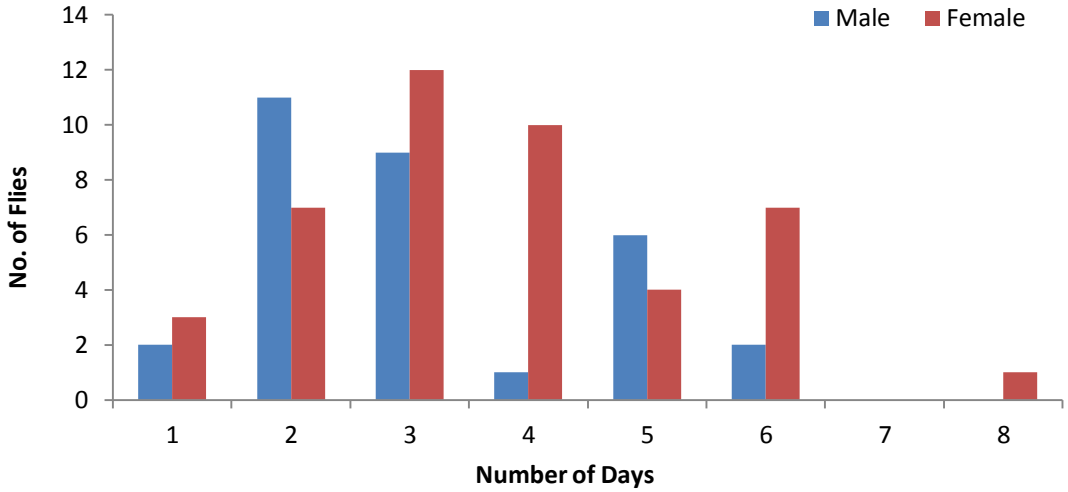


**Fig 88: LD 12:12 – Number of days taken by *Megaselia scalaris* adult flies to emerge from puparia at 20 °C.**

#### 4.3.3.2 LD 00:24 Pupa and Sex emergence Patterns

LD 00:24 shows the pattern shifting with only 56% of flies emerging from the pupa during the daylight hours and the emergence of all flies took 6 days.

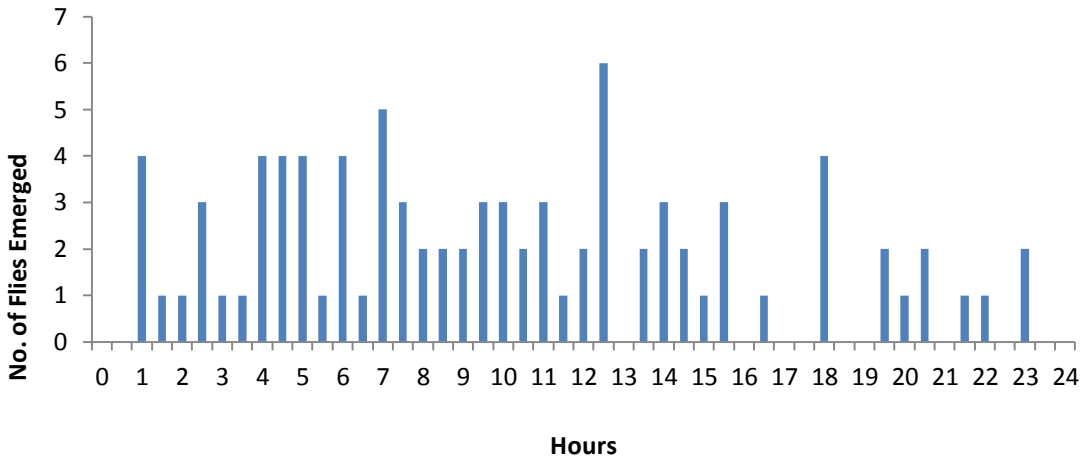


**Fig 89: LD 00:24 – Emergence Data of males and females of *Megaselia scalaris* adult flies at 20 °C.**

A statistically uniform emergence pattern is observed under LD 00:24 as R = 100 which is above the threshold of 90 (highest number of emergence within an eight hour period = 44; Number of emergence outside this eight hour period = 44; R (Rhythmicity) = 44/44 * 100 = 100).

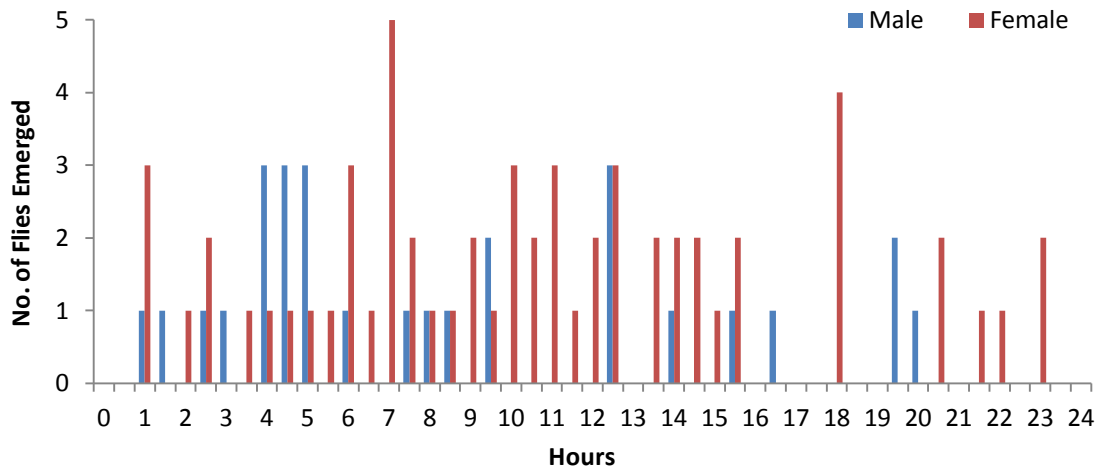


**Fig 90: LD 00:24 – Sex Emergence Pattern of *Megaselia scalaris* adult flies at 20 °C.**

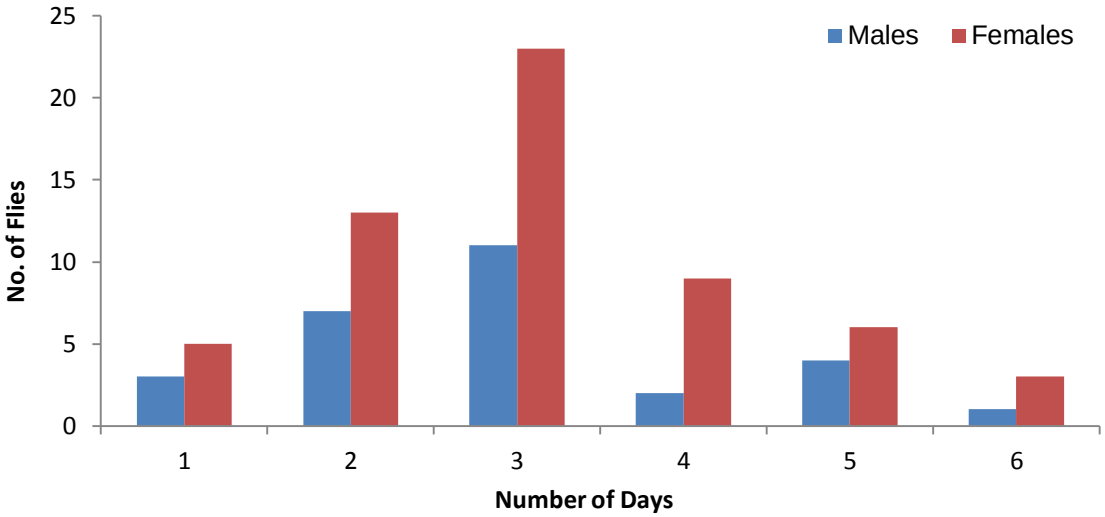


**Fig 91: LD 00:24 – Number of days taken for *Megaselia scalaris* adult flies to emerge from puparia at 20 °C.**

#### 4.3.3.3 LD 24:00 Pupa and Sex Emergence Patterns

LD 24:00 conditions showed 57% of flies emerged from the pupa during the daylight hours and the number of days it took for the flies to emerge fell by 2 days when compared to LD 00:24 (*Fig 92* to *Fig 94*).

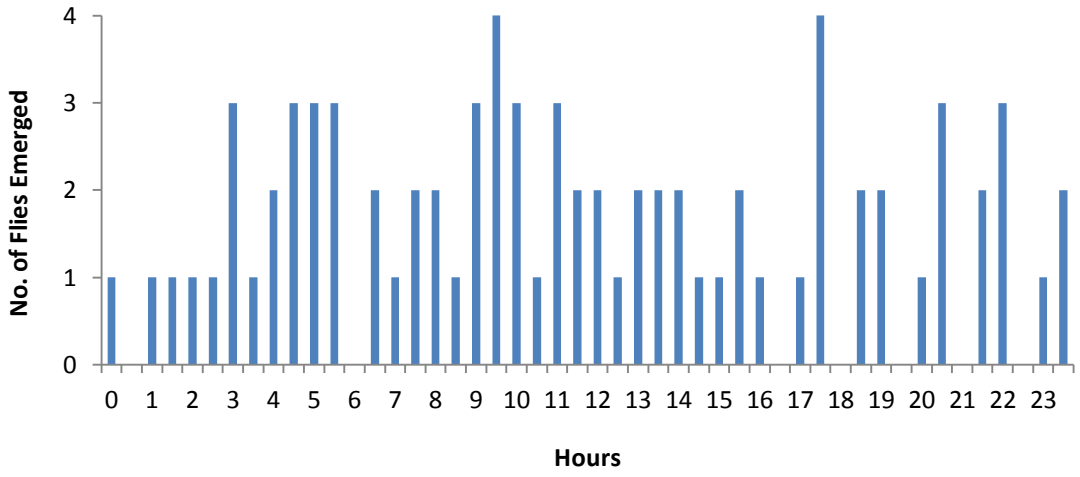


**Fig 92: LD 24:00 – Emergence Data of males and females of *Megaselia scalaris* adult flies at 20 °C.**

An arrhythmic pattern is observed under LD 00:24 as R = 79 which is between the threshold of 60 and 90 (highest number of emergence within an eight hour period = 35; Number of emergence outside this eight hour period = 44; R (Rhythmicity) = 35/44 * 100 = 79)

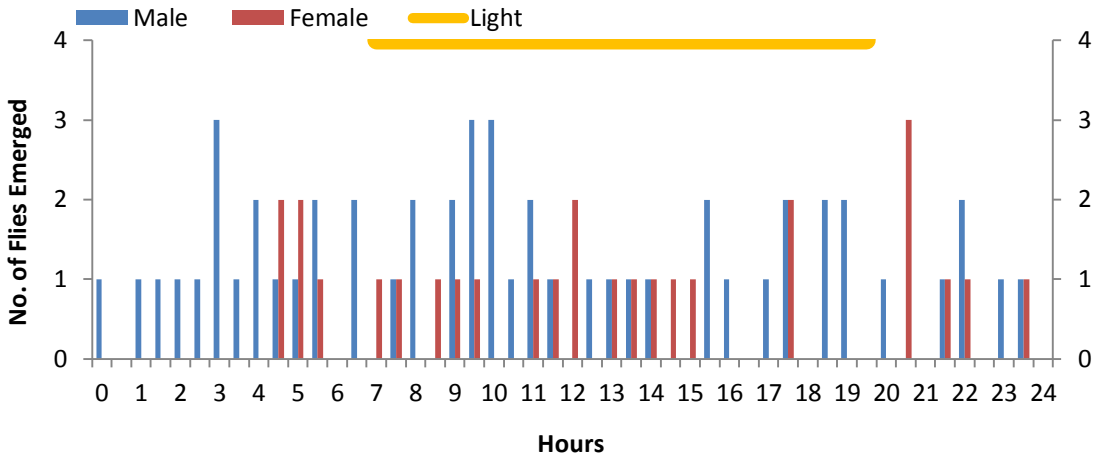


**Fig 93: LD 24:00 – Sex Emergence Pattern of *Megaselia scalaris* adult flies at 20 °C.**

**(Light symbol represents when the light would normally illuminate)**

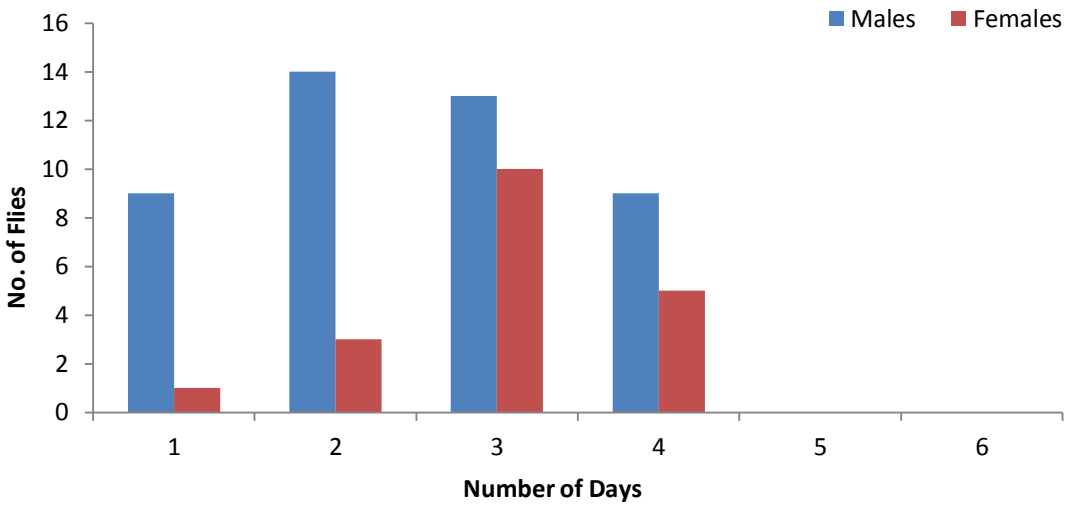


**Fig 94: LD 24:00 – Number of days taken for *Megaselia scalaris* adult flies to emerge from puparia at 20 °C.**

#### 4.3.3.4 Summary of emergence results

The graph (*Fig 95*) summarises the emergence results showing the significant difference of emergence in photo-phase when compared to scoto-phase.

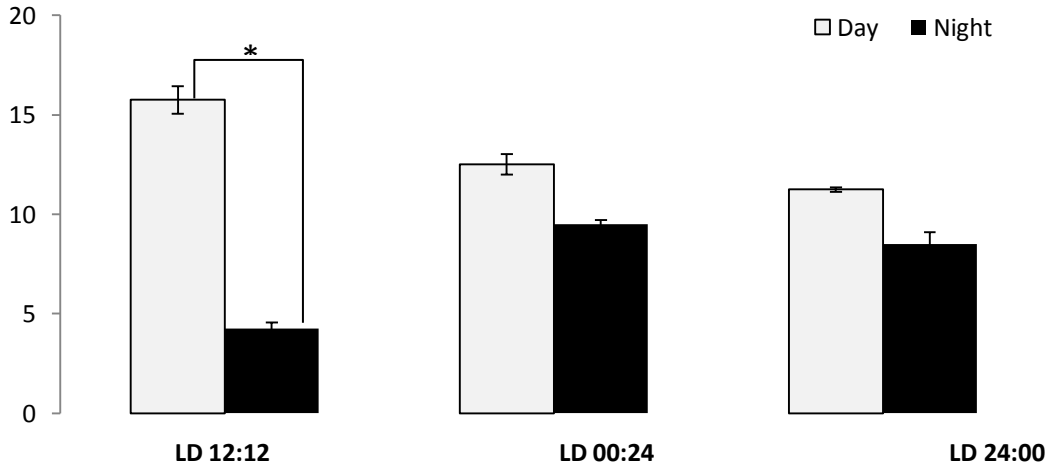


**Fig 95: Day & night emergence average with SEM at 20 °C. LD 12:12 N=80, p=0.00, LD 00:24 N=88, p=0.24, LD 24:00 N= 79, p=0.26.**

*** represents significant difference at 0.05 level**

### 4.3.4 Discussion

Time of emergence plays an important role in the understanding of the organism biology and behaviour and a fundamental role in Forensic Entomology when mPMI is requested. The pattern of emergence and flight periodicity is discussed by Lewis and Taylor (1965) in which it is thought that the emergence rhythm may influence the time of flight in the insect. In the majority of fly species both sexes fly together as both sexes respond to the same light intensities however this is not the case for all species (Lewis and Taylor, 1965).

Benner and Ostermeyer (1980) comment on *M.scalaris* males pupating prior to females, our observations are in agreement with this research as our males pupated one day prior to the females (*Fig 85*). We also saw that males emerged prior to females, it is understood that this enables the males to feed and allows their sperm to mature prior to meeting the females, also the number of males observed were greater than that of females (Disney, 2008).

Pupal emergence experiments were carried out in three different conditions: LD 12:12, 00:24 and 24:00. Pupal emergence experiments in LD 12:12 showed a significant difference between the light and dark phases (*Fig 86*) (N=80, p=0.00) with the majority of the flies emerging in the light. In contrast pupa emergence experiments in LD 00:24 or 24:00 do not show any significant differences (N=88, p=0.24 and N= 79, p=0.26 respectively).

The observations regarding the pupa emergence agree with the results reported by da Silva Mello and colleagues (2012).They showed that LD 00:24 had a quick emergence in which their flies appeared within a day whilst their LD 12:12 took 5 days for all flies to emerge. The results seen for LD 00:24 and 24:00 showed that the emergence pattern was different to that of LD 12:12. Using the rhythmicity determination method as reported by (Goto *et al.*, 2006) we were able to determine the rhythmicity data for *M.scalaris* eclosions.

- **LD 12:12** = Rhythmic pattern

  The majority of eclosions occurring between 10.30 to 18:00.
- **LD 00:24** = Uniform pattern

The majority of eclosions occurring between 05:00 to 12:30.

- **LD 24:00** = Arrhythmic

  The majority of eclosions occurring between 04:30 to 12:00

Benner and Ostermeyer (1980) comment on *M.scalaris* males pupating prior to females, our observations are in agreement with this research as our males pupated one day prior to the females (*Fig 85*).

The pupa emergence determines that there are different rhythms during full darkness conditions and light/dark conditions. In addition our experiments demonstrated that emergence in this species is light/dark regulated and that in continuous dark it can happen both during the dark or the light subjective phase. The LD 12:12 findings are in agreement with the findings of *Drosophila pseudoobscura* (Diptera, Drosophilidae) by Saunders (2002) and LD 24:00/00:24 results are in agreement with the research by Truman (1971) on *Antheraea pernyi* (Lepidoptera, Saturniidae) which saw the emergence rhythm begin to break down over longer photo-periods.

# 5: Burial Behaviour in Soil and Penetration through Bandage and Fabrics

## 5.1 Burial behaviour in soil

### 5.1.1 Introduction

Carrion throughout the decomposition process undergoes a series of changes from: fresh, bloated, active decay, advanced decay, dry and finally skeletisation. During each of these stages, many different species of insect will be attracted to the carrion. Insects colonise the carrion in a predictable pattern otherwise known as the succession pattern and this is one of two methods used to estimate PMI, however the succession pattern may vary greatly due to location of the cadaver, environmental temperature, habitat and whether the cadaver is buried or above ground (Smith, 1986). The second method of PMI is by analysing developmental data taken from either larvae or pupa collected at the scene (Payne, 1965; VanLaerhoven and Anderson, 1999).

Temperature and humidity plays an important role in the decomposition process, as very high or very low temperatures may affect the organic matter decay whilst polar temperature can preserve the body for many years. Insect activity is also affected by temperature as is the rate of insect development which in turn has a relative impact to the decomposition of a cadaver (Campobasso *et al*., 2001). Other factors that may have an effect on decomposition and colonisation may include outdoor locations, indoor location burial and submerged in water (Amendt *et al*., 2004).

For a cadaver that has been colonised on the ground surface, a PMI may be estimated by collecting temperature comparisons from both the crime scene and from the nearest weather station and then using a regression equation from the relationship between the internal and external data (Pastula and Merritt, 2013; García-Rojo *et al*., 2013). Whilst cadavers that are buried, the equation does not give realistic response therefore a Post Burial Interval (PBI) was introduced by VanLaerhoven and Anderson (1999) which involves measuring the soil temperature as this is the best prediction of internal buried carcass temperatures. Different studies have shown that bodies that have been buried decompose at a slower rate than those bodies that are left exposed to the elements (Lundt, 1964; Payne, 1965; Payne and King, 1968). Manhein (1996) previously showed that 79 % of cadavers had been located at depths of between 30 and 90 cm.

To determine the entomofauna of buried bodies, skeleton studies were conducted. Unclaimed bodies from Municipal Cemetery of La Plata were donated to School of Medical Sciences, National University at La Planta for research and teaching purposes. The skeletons were received in numbered plastic bags which included sediments and external wrappings and death records (age, sex, nationality, date and cause of death, location at cemetery and date of exhumation) were also included. Insect remains were collected from and exhumed bundle of a 5 month male who cause of death was non-traumatic cardiorespiratory arrest. The body had been buried into a 40cm deep grave underground in a soft wooden coffin which was made for rapid decomposition; the body had been clothed in woollens and a disposable nappy as well as wrapped in two woollen blankets. The exhumed bundle revealed insect species of forensic importance (Mariani *et al*., 2014).

*Megaselia scalaris* were amongst the first taxa to colonise the buried remains along with *Fannia canicularis* (Diptera, Fanniidae), *Muscina stabulans* (Diptera, Muscidae) and *Ophyra aenesens* (Diptera, Muscidae). In Buenos Aires, *M.scalaris* is frequently found in exhumed bodies and soils (Mariani *et al*., 2014).

Fauna of the grave was researched in which 100 graves were exhumed over different time periods. In 37 of the burials, Phoridae puparia were identified but not the species (Motter, 1898).

It is important to understand insect burial behaviour to further comprehend the environmental parameters which may affect larval behaviour, this information may help to assist the entomologist in locating larvae which have buried down below the surface (Gomes *et al*., 2009).

The question surrounding *Megaselia scalaris* are whether the larvae or the adults of this species are able to reach a buried body and are the larvae/adults able to return to the outside world after completing development on/in the body?

### 5.1.2 Experimental Design

To determine the depth of soil that *M.scalaris* adult flies are able to burrow down to oviposit on carrion, two different materials was used: sand (*Fig 96*) and clay soil (*Fig 97*).

1 L cylinders were used to determine depth of soil. To determine water content of soils, a 200ml sample was weighed and then placed into an oven at a temperature of 75 $^{o}$C. The soil was removed from the oven a few days later and re-weighed. The difference was calculated and water content determined.

Soil characterisation was carried out by determining structure, colour consistency and texture of the soil using Munsell soil identification protocols (Munsell and Color, 2000).


**Fig 96: Microscopy image of sand particles used in the burial experiment. Scale = 0.12mm, x26 magnification**


**Fig 97: Microscopy image of soil particles used in the burial experiment. Scale = 0.12mm, x26 magnification**

**Table 39: Initial soil experiment to determine burial depth capability for *Megaselia scalaris*.**

| Sample Reference | Soil Type | Soil Depth in millilitres (ml) | | |
|---|---|---|---|---|
| A | Sand | 125 | 250 | 500 |
| B | Soil | 125 | 250 | 500 |
| C | Sand/Soil | 125/125 | 250/250 | - |
| D | Soil/Sand | 125/125 | 250/250 | - |

Thermocron data loggers (- 40 to + 85 °C) were placed into the control and soil experiment and programmed to take readings every 30 mins over the period of the experiments.

A small layer of soil was placed on the bottom of the 1 L cylinder, a thermocron data logger was placed on to the soil and ~18 g of pet food was placed on the top of the soil and data logger. The data logger was protected by a plastic covering to prevent damage during the different decomposition stages of the meat. Soil was then added to the cylinder to make up the correct soil depth (*Table 39*). Initially sand and soil were measured to different depths from 125 ml up to 500 ml and a further data logger placed on to the top of the soil. A second experiment was run in which a mixture of sand and soil were measured to different depths i.e. 125 ml of soil and 125 ml sand was placed on top.

Ten male and female adult *M.scalaris* flies were added to the cylinder and the top was sealed with blue roll to allow for ventilation. The same set up was used for both the sand and sand/soil (*Fig 98* and *Fig 99*) experiments.

The cylinders were left for 1 month at room temperature to determine if any activity would be observed.

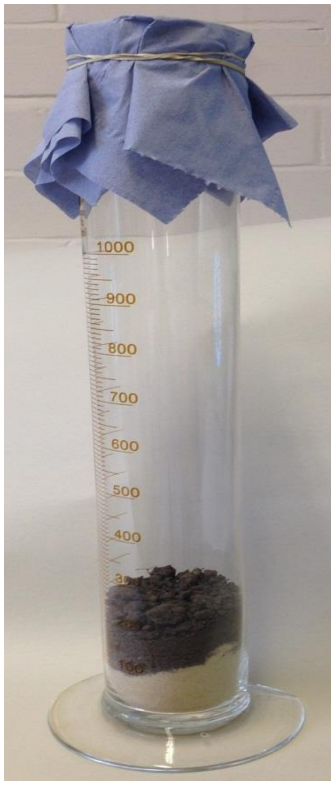


**Fig 98: Initial burial experiment with 125ml Soil and 125ml sand.**


**Fig 99: *M.scalaris* adults in cylinder containing 125ml Soil and 125ml sand.**

A further experiment was set up to determine if there was a minimum particle size that *M.scalaris* would not be able to burrow through after observing the issues with sand. Soil was sieved (W.S Tyler testing sieves) in to different particle sizes to determine the adults digging capability (*Table 40*) (*Fig 100* to *Fig 104*) and the same method used as previously explained.

**Table 40: Soil burial experiment to determine particle size capability for *Megaselia scalaris*.**

| Soil Type | Particle size (mm) | Soil Depth (ml) |
|---|---|---|
| Soil | 4.0 | 250 |
| Soil | 2.8 | 250 |
| Soil | 2.0 | 250 |
| Soil | 1.0 | 250 |


**Fig 100: Not sieved soil**

**Fig 101: 4.0 mm soil particles**

**Fig 102: 2.8mm soil particles**

**Fig 103: 2.0mm soil particles**


**Fig 104: 1.0mm soil particles**

### 5.1.3 Results

The phorid *Megaselia scalaris* has been reported as being able to burrow down up to 6 feet, as they are commonly found amongst the entomofauna of exhumed bodies or coffins (Mariani *et al*., 2014). Little information is available about the kind of soil this fly is able to dig through to reach a cadaver. How far the fly is able bury and oviposit has yet to be determined therefore to be able to determine burial depths in different soil environments of the fly two different experiments were set up using both sand and sandy loam garden soil. The average temperature recorded throughout the experiments was 16.0 + 1.0 °C.

To determine the water content of the soil, 3 x 200ml soil were placed into 75 °C oven and left for a three days to dry. The results showed that there was an average of 16 % ± 1.73 SD of water in soil (*Table 41*) with no statistical difference between the samples.

**Table 41: Weight of water content from soil taken after three days of drying in 75 °C oven.**

| Item | 1 | 2 | 3 |
|---|---|---|---|
| **Boat** | 7.24g | 7.14g | 7.10g |
| **Boat and Soil** | 203.69g | 196.38g | 187.92g |
| **Boat and Dry Soil** | 161.89g | 156.53g | 149.72g |
| **Water Content** | 34.56g | 32.71g | 31.10g |

During the course of the experiment condensation was observed from the bottom of the cylinder making its way towards the top of the soil.

Upon completion of the experiments, the soil was removed and separated into five layers to determine the pupal count throughout the cylinder (*Table 42*).

**Table 42: Results of pupae found at 50ml increments throughout 1 litre cylinder filled with sandy loam garden soil and sand.**
**1 represents top layer, 4 represents area containing the bait, 5 represents bottom of cylinder underneath bait.**

| Depth of soil (ml) | Soil | | | | | Total | Sand | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Layer | 1 | 2 | 3 | 4 | 5 | | 1 | 2 | 3 | 4 | 5 | |
| 125 | 14 | 26 | 47 | 459 | 106 | 652 | 0 | 0 | 0 | 0 | 0 | 0 |
| 250 | 2 | 8 | 81 | 222 | 91 | 404 | 0 | 0 | 0 | 0 | 0 | 0 |
| 500 | 0 | 0 | 0 | 17 | 72 | 89 | 0 | 0 | 0 | 0 | 0 | 0 |

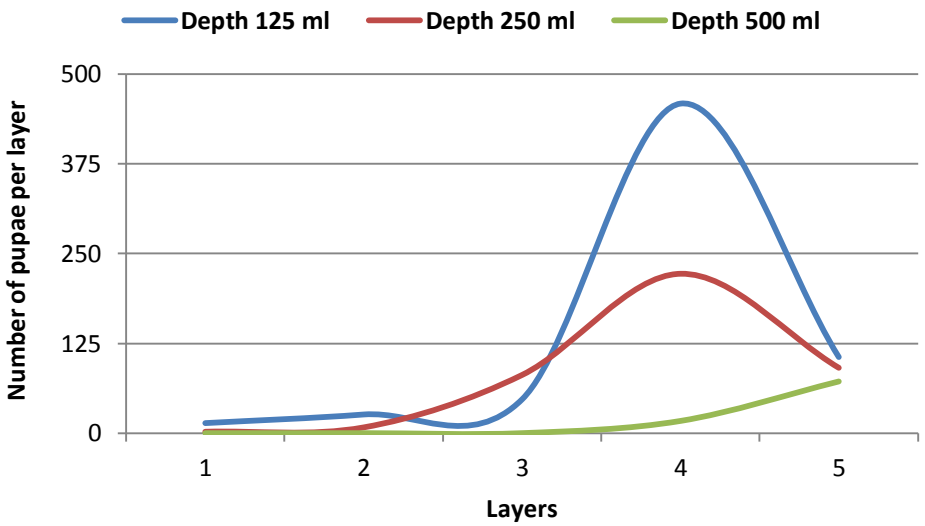


**Fig 105: Number of pupae found at 50ml increments throughout 1 litre cylinder filled with either sandy loam garden soil.**

**Table 43: Percentage of pupae located at different layers throughout the sandy loam garden soil.**
**1 represents top layer, 4 represents area containing the bait, 5 represents bottom of cylinder underneath bait.**

| Depth of soil (ml) | Percentage of Pupae Located in Soil | | | | |
|---|---|---|---|---|---|
| Layer | 1 | 2 | 3 | 4 | 5 |
| 125 | 2.15 | 3.99 | 7.21 | 70.40 | 16.26 |
| 250 | 0.50 | 1.98 | 20.05 | 54.95 | 22.52 |
| 500 | 0.00 | 0.00 | 0.00 | 19.10 | 80.90 |

The results from the soil burial experiment (*Table 42*,*Table* 43 and *Fig 105*) demonstrate that *M.scalaris* flies were able to bury through the soil to the bait and continue their development stages i.e. larvae, pupa. The graph shows that the majority of pupae were located on/near the bait the deeper the bait is buried into the soil.

Adults were not able to penetrate the sand experiments and were found dead on top of the sand after a couple of days without laying any eggs. The sand/soil experiments showed similar results, the flies were not able to penetrate the sand to reach the soil and therefore died on top of the sand whilst the soil/sand experiments found the flies able to burrow down a short way before reaching the sand, the flies did not lay any eggs and were found dead on top of the sand half way down the experiment.

A further experiment (*Table 40*) was set up to determine if there was a minimum particle size that *M.scalaris* would not be able to burrow through. Numbers were not collected for this experiment as observations (*Table 44*) on burial behaviour in different soil sizes were the main point of this experiment. The adult flies noticeably vanished after the first day within the general soil cylinders; whilst the rest of the experiments saw the adults remain on the surface of the soil. The egg activity was not observed in the general soil experiment due to the flies being below the surface or the 1.0 mm particle size as the eggs. The male and female adult flies re-emerged from the soil 27 - 28 days (possibly for mating purposes), two days later the flies burrowed down into the soil.

Cylinder 1 and 2 showed no re-emergence of flies over the course of the experiment.

**Table 44: Observation of *Megaselia scalaris* burial activity through different particle sizes over time.**

| Observations/ Soil size | 1 Day | 3 Days | 8 days | 14 days | 27-28 days |
|---|---|---|---|---|---|
| **General Soil (not sieved)** | Flies were not visible after first day | No activity | Many larvae seen | Pupa and larvae present | Flies emerged from pupa |
| **4.0mm** | Flies still present. Egg clusters present on soil surface | Eggs present | Flies still present along with larvae | Pupa present amongst soil. | Flies emerged from pupa |
| **2.8mm** | Flies still present. Egg clusters present on soil surface | First instar larvae present | Many larvae on glass walls | Many pupae present | Flies emerged from pupa . |
| **2.0mm** | Flies still present. Egg clusters present on soil surface | First instar larvae present | Many larvae present | Many pupae present | Flies emerged from pupa |
| **1.0mm** | Flies still present. Egg clusters present on soil surface | No activity seen | No activity seen | Pupa present in soil | Flies emerged from pupa |

### 5.1.4 Discussion

Our experiments showed that *Megaselia scalaris* adults were able to burrow down to reach the bait and continue their reproduction through all developmental stages, however the flies were noted to return to the surface after emerging from the puparia possibly to mate as after a couple of days they buried back down into the soil. The sand was found to be a very dry environment resulting in the death of all the adults a few days after the experiments were set up.

A post mortem performed on a 15 year old female Indian python weighing 70kg found the snake had *Megaselia scalaris* larvae in the lungs. Pupae and adults were located in the tracheal lumen which would point to the post feeding larvae moving away from the pabula to pupate, this behaviour may prevent developmental problems in the pupae caused by bacteria or other organisms found in the pabulum and increased humidity (Vanin *et al*., 2012b). Had the snake been left to observe *M.scalaris* development it may have been of interest to determine whether the adults left the snake to mate and colonise other food resources elsewhere or whether they would have stayed inside the snake to continue breeding and feeding.

Hutchet and Greenberg (2010) discuss that many fly remains are collected from the buried corpses on archaeological digs. It is not known as to whether the flies colonised the body prior to burial or whilst the body was in an unfilled grave along with the length taken between death and burial or whether the fly was able to burrow down into the soil to reach the buried corpse to colonise. It was confirmed that the blowflies *Cochliomyia macellaria* and *Compsomyiops verena* (Diptera, Calliphoridae) found on this dig were unable to reach a cadaver buried beyond 1 metre and therefore the colonisation must have taken place prior to burial.

VanLaerhoven and Anderson (1999) researched the insect succession from buried carrion in British Columbia, their findings showed that numerous species of Diptera were the most common inhabitants of buried carcasses however the depth of burial was not studied which would have a significant effect on the insect succession.

Gunn and Bird (2011) investigated the ability of blowflies *Calliphora vomitoria, Calliphora vicina, Lucilia sericata, Muscina stabulans* and *Muscina prolapsa* to colonise buried remains. Their data suggests that it is unlikely that *C.vomitoria* would colonise a buried cadaver unless there were large enough channels allowing access, however if the

eggs had been laid on the cadaver prior to burial and then buried, the eggs would continue to develop through all the developmental stages normally. *Calliphora vicina* may be more successful in colonising buried cadavers but further work is needed to verify this. *Lucillia sericata* may reach cadavers up to a depth of 10 cm but a variable success rate was observed. Both *M.prolapsa* and *M.stabulans* were able detect and colonise cadavers buried in loose soil at least 40 cm in depth which supports our experiment using different soil particles as they proved difficult for *M.scalaris* to penetrate as they were unable to burrow down into the soil and instead were observed laying their eggs on the soil surface, observations showed the larvae were also unable to burrow down as the pupae were located on the top of the soil.

Szpila and colleagues (2010) investigated species *Eumacronychia persolla* and *Phylloteles pictipennis* (Diptera, Sarcophagidae) and their results showed that they were able to penetrate dry loose soil to reach a buried cadaver which may make this species a useful forensic indicator when dealing with buried bodies in dry environments. However whilst a few insects are able to reach a buried cadaver, it does restrict access to many of the other carrion insects (Campobasso *et al*., 2001).

## 5.2 Penetration through bandages and fabrics

### 5.2.1 Introduction

Numerous adult fly species, for example blowflies, housefly and stable fly, may hold over 100 species of pathogenic microorganisms, which are associated with more than 65 diseases of humans and animals (Greenberg *et al*., 1970; Greenberg, 1971; Greenberg, 1973). It has been accepted for a long time that flies are transmitters of infectious and parasitic diseases of both man and animal along with the importance and knowledge of sanitary conditions. When a fly lands on a source of contamination (i.e. a cadaver, faeces, open wounds, contaminated food, etc.) it is possible for the fly to then transmit through direct or indirect contact an infectious agent to man or animal (Fischer, 2007).

Myiasis refers to the infestation of living tissue from either animals or humans by Diptera larvae. In the veterinary field, myiasis is often referred to by other names e.g. fly-blown, fly strike (Rossi-Schneider *et al*., 2007). In certain conditions involving cases of neglect, myiasis may be evident and used as evidence which could be presented in a court of law. Neglect covers a wide variety of cases ranging from the elderly (Benecke *et al*., 2004), children (Benecke and Lessig, 2001), adults (Nazni *et al*., 2011) and also non-domesticated and domesticated animals (Hall and Wall, 1995) and (Anderson and Huitson, 2004).

Myiasis caused by Phoridae have been reported in non-wound myiasis such as nasopharyngeal (Carpenter and Chastain, 1992), urogenital (Singh and Rana, 1989), intestinal (Singh *et al*., 1988) and ocular (Wright, 1927). Nosocomial (within a hospital type environment) myiasis infections by Phoridae have also been reported by Hira *et al.* (2004).

Five cases of wound myiasis were reported by Patton and Evans (1929) in which *M.scalaris* was involved. In 2010, New Zealand reported that *M.scalaris* was a new introduced species and had been reported in a number of human myiasis cases (Derraik *et al*., 2010).

Two cases of wound myiasis by *Megaselia* species in USA were reported (Sherman, 2000). Many cases of myiasis acquired in a hospital environment are often

under reported or not reported at all, Joo and Kim (2001) discuss the reported cases of hospital myiasis. The patients reported age ranged from 8 to 82 years of age under a variety of different mental states from alert to comatose or anaesthetised. The sites the larvae reported were either wounds, ulcers, oral cavities and the nose.

The objective of this study is to understand the conditions in which *M.scalaris* is able to penetrate material i.e. bandages or fabrics such as cotton, to either oviposit on to flesh or for larvae to work their way through the dressings to reach the flesh.

### 5.2.2 Experimental Design

In both the human and animal kingdom parasitic infections are extremely common. There have been numerous reports of Phoridae myiasis, however there is limited information surrounding the conditions. This research is investigating different materials in which *M.scalaris* may penetrate to oviposit on to flesh.

#### 5.2.2.1 Fabric Penetration

To determine whether the females were able to place their ovipositors through the weaves of the material and lay eggs underneath the material or whether if laid on top of the material whether the first instar larvae were able penetrate through either the cotton (*Fig 106*) or the polyester (*Fig 107*) the following experiment has been set up. Ten ml of cat food was placed into a screw top tube. The tube lid had a hole pre-made into it to allow access for eggs/larvae. Material measured 5 cm x 9 cm which was placed over the top of the tube and the lid screwed on over the material. The control tube contained 10 ml of cat food with no material placed across the lid. The cat food does not come into contact with the fabric and is approximately 10 cm away from the fabric. The tubes were then placed into individual glass jars, 5 adult male and female *M.scalaris* were added to the jars which were sealed with paper roll to allow for air flux. The experiment was run for 2 weeks however the flies were removed after 1 week. Once the experiment was completed, the material and meat were removed and examined macroscopically and microscopically for the presence of eggs and larvae. Three replicates were set up.

The fabric structures and weave design are reported in (*Fig 106* and *Fig 107*).

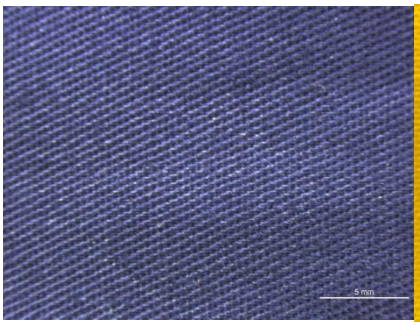


**Fig 106: Cotton material (scale=5mm) x6.3 magnification**

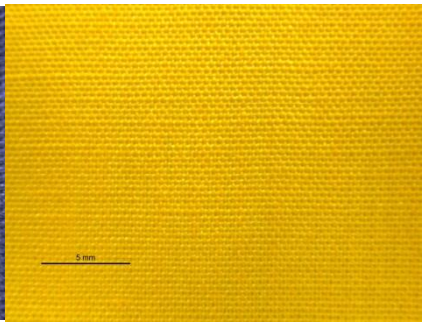


**Fig 107: Polyester material (scale=5mm) x6.3 magnification**

Initial observations found that the pet food dried out too quickly and the flies died prior to oviposition occurring, to resolve this, two different methods were used. The first method involved placing a small damp sponge under the plastic tubes whilst the second method was to place a small damp sponge in the tube underneath the cat food. Either method worked in decreasing the time of the drying out period which in turn allowed for oviposition to occur. The results reported in this chapter are from the damp sponge being placed underneath the food.

#### 5.2.2.2 Bandage penetration

##### 5.2.2.2.1 One layer

In order to verify if penetration through the bandage was achievable, three small pieces of fresh pork belly 3 x 2 cm were wrapped in one layer of 10 $cm^2$ cotton cohesive bandage (Ko-flex) (*Fig 108*), Polymer dressing (*Fig 109*), cotton bandage (Ko-flex compression bandage) (*Fig 110*), Cotton compression bandage (K-plus compression bandage), soft-absorbent sub compression bandage (K-soft) (*Fig 111*), cotton tubular bandage (Comfinette) (*Fig 112*) and cling film. The control had no wrappings around the meat. The parcels were sealed with an elastic band to prevent penetration occurring at the opening. The bandages used in this research were not used for their primary role but in a general role to determine if *M.scalaris* immature stages were able to penetrate through this barrier.

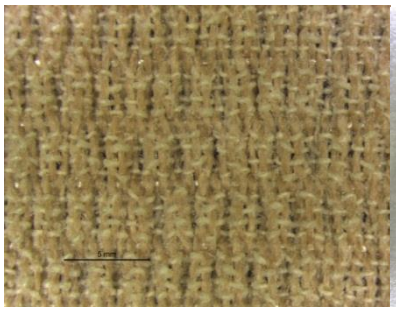

**Fig 108: Cohesive bandage (scale=5mm) x6.3 magnification**

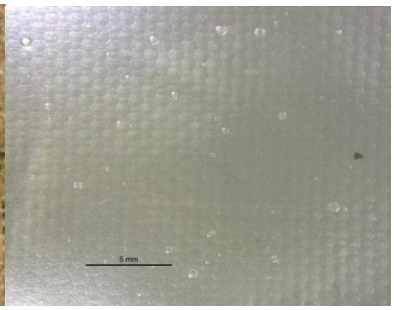

**Fig 109: Polymer dressing (scale=5mm) x6.3 magnification**

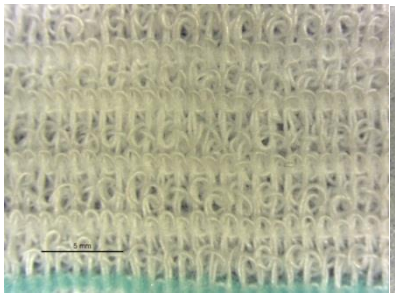

**Fig 110: Cotton compression bandage (scale=5mm) x6.3 magnification**

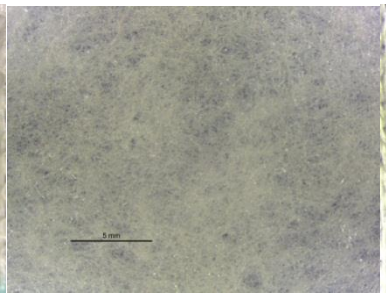

**Fig 111: Soft sub compression bandage (scale=5mm) x6.3 magnification**

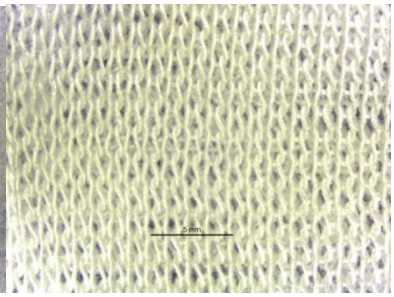

**Fig 112: Cotton stockinette (scale=5mm) x6.3 magnification**

The wrapped meats were then placed into individual breeding chambers, 5 male and female adult *M.scalaris* flies were added to the chambers and left for 24 hours to allow for oviposition. The adults were removed and the jars were left for 1 week at room temperature (~19 $^{o}$C) to allow for larval development if oviposition had occurred after which the presence of larvae was determined. Two repeats were performed.

The meats used in the experiment were becoming too dry too quickly during the week and therefore the larvae were not able to complete their development and died. To rectify this, a damp sponge was placed into the jars to allow sufficient moisture for the flies to continue their development.

#### 5.2.2.2.2 Two layers

Pork belly measuring 3 x 15 cm was wrapped up using same materials (*Fig 108* to *Fig 112*) used in the one layer experiments. Two layers of material were wrapped around the meat and tightly sealed at each end using elastic bands. Some of the dressings were found not to be as pliable as the cotton bandages and when wrapped/folded, left small gaps around the meat, this was rectified by placing surgical tape over the gaps to prevent easy access to the flies. Surgical tape was chosen as this is a common material seen around bandages in hospitals. A control with no wrappings was also set up. Three replicates were set up.

The samples were placed into containers; ten male and female flies were added to the containers and sealed. Duct tape was placed around the sealed box to prevent any possible escapes.

The experiment was left for two weeks at room temperature (~19 $^{o}$C). After the first week all flies were removed and the boxes left for a further week to allow for development of any eggs. After the second week the wrappings and meats are examined under a stereomicroscope and any eggs or larvae seen are reported.

#### 5.2.2.2.3 Five layers

Pork belly measuring 3 x 15 cm was wrapped using same materials (*Fig 108* to *Fig 112*) that were used in the previous experiments. Five layers of material were wrapped around the meat and tightly sealed at each end using elastic bands. Some of the dressings were found not to be as pliable as the cotton bandages and left small gaps when folded around the meat, this was rectified by placing surgical tape over the gaps to prevent easy access to the flies. A control with no wrappings was also set up. Three replicates were set up.

### 5.2.3 Results

#### 5.2.3.1 Material Penetration

*Megaselia scalaris* is well related to cases of human myiasis within nosocomial environments therefore to investigate if flies required direct access to flesh for myiasis to occur or whether they were able to lay eggs on to the surface of either a bandage or clothing material or whether the first instar larvae would be able to work their way through the material was researched.

**Table 45: Average number of eggs on material after two weeks.**
**Ten adult *Megaselia scalaris* flies (5 male, 5 female) were added and removed one week later.**

| Development stage | Material | | |
|---|---|---|---|
| | Control | Cotton | Polyester |
| Eggs | 0 | 59 ± 19.2 | 68 ± 46 |
| Larvae | > 200 | 0 | 0 |
| Pupae | 0 | 0 | 0 |

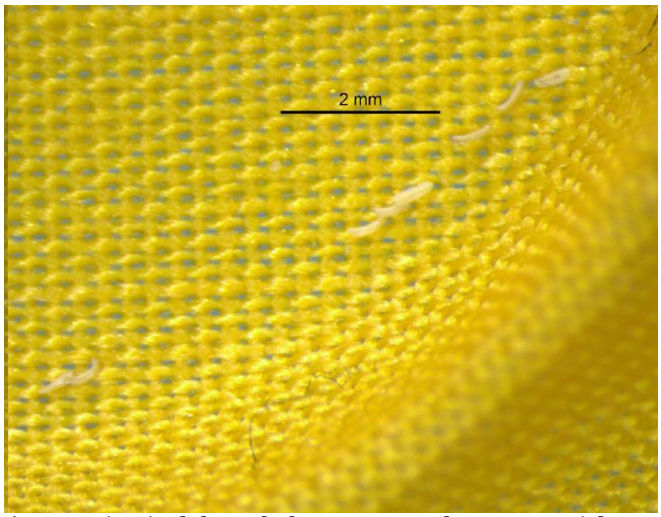


**Fig 113: *Megaselia scalaris* eggs oviposited through the weave on polyester material. Images taken using a Leica microscope.**
**Scale bar = 2 mm, x26 magnification.**

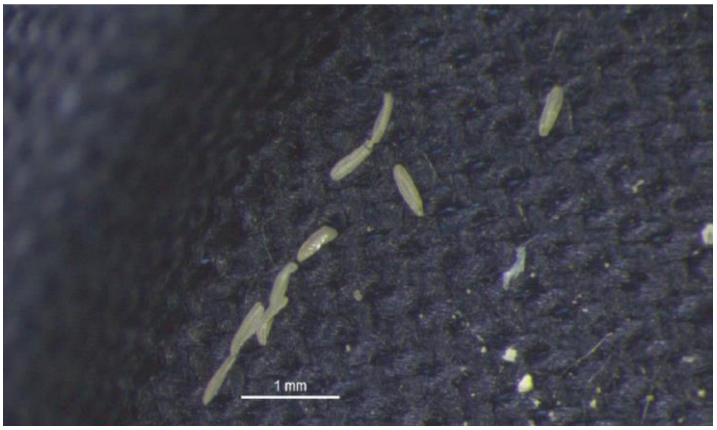


**Fig 114: Number of *Megaselia scalaris* eggs deposited through the weave on to cotton material. Images taken using a Leica microscope of using.**
**Scale bar = 1 mm, x32 magnification.**

The control observed the flies entering the tubes and laying a vast amount of eggs on to the media. The experiments using cotton and polyester showed that the flies were able to penetrate the material with their ovipositors as the eggs were laid on the underside of the material (*Table 45*); however the majority of the eggs seen were laid on top of the material (*Fig 113 & Fig 114*). The average number of eggs oviposited on the polyester fabric was 68 ± SD 46 which had the most eggs oviposited whilst cotton had an average of 59 ± 19.2 eggs. None of the eggs that were laid developed, this may have been due to the distance between the media and the egg and the environment may have been too dry to allow for further development.

#### 5.2.3.2 Bandage Penetration

##### 5.2.3.2.1 One Layer

It was only the presence of the larvae within the meat that showed the experiment to be successful. On microscopic examination eggs were seen on the outside surface of the bandage however no larvae were present on the outside, when opened up the larvae were present on the meat inside the bandage. Eggs were present on the outside on all material (*Fig 115* and *Fig 116*); larvae were present on the outside of the material on 6 out of 7 of the materials (including control), pupa was present in 2 of the 7 experiments with the control having the majority. When the bandage was opened up eggs were found on 2 of the 6 experiments whereas larvae were seen on 5 of the 6 experiments, no pupa was present inside the bandage. The only bandage wrapping in this experiment to prevent myiasis was the cling film wrap (*Table 46*).

**Table 46: Developmental stage averages with ± SD from one layer of bandage wrapping.**
**Ten adults *Megaselia scalaris* flies (5 male, 5 female) were added for 7 days then removed and the experiment left for a further 7 days to allow development.**

| Bandage Material | Surface of bandage | | | Surface of meat | | |
|---|---|---|---|---|---|---|
| | Eggs | Larvae | Pupa | Eggs | Larvae | Pupa |
| Control | N/A | N/A | N/A | 47 ± 66 | 35 ± 1.4 | 0 |
| Cotton compression | 10.5 ± 15 | 28.5 ± 11 | 0 | 0 | 13 ± 7 | 0 |
| Soft sub compression | 66 ± 88 | 0 | 0 | 0 | 32 ± 0.7 | 0 |
| Polymer | 20 ± 3 | 8.5 ± 12 | 0 | 0 | 0 | 0 |
| Cohesive | 0 | 9 ± 6 | 0 | 0 | 53 ± 35 | 0 |
| Cotton stockinette | 10 ± 14 | 20.5 ± 15 | 0 | 4 ± 6 | 7 ± 10 | 0 |
| Cling film | 8.5 ± 12 | 0 | 0 | 0 | 0 | 0 |

**Table 47: Anova (P value) results from positions of development stage located on one layer of bandage wrapping.**

| Position | P value |
|---|---|
| Eggs on Outer Layer | 0.549 |
| Eggs on Pork | 0.506 |
| Larvae on Outer Layer | 0.063 |
| Larvae on Pork | 0.043 |

**Table 48: Tukey *post hoc* (P value) results from positions of larvae located on one layer of bandage wrapping.**

| Dressing | Comparison | P Value |
|---|---|---|
| | Cohesive | .845 |
| | Sub soft compression | 1.000 |
| Control | Cotton Compress | .711 |
| | Cotton Stockinette | .495 |
| | Polymer | .292 |
| | Cling film | .292 |
| | Control | .845 |
| | Sub soft compression | .729 |
| Cohesive | Cotton Compress | .194 |
| | Cotton Stockinette | .117 |
| | Polymer | .065 |
| | Cling film | .065 |
| | Control | 1.000 |
| | Cohesive | .729 |
| Sub soft compression | Cotton Compress | .830 |
| | Cotton Stockinette | .619 |
| | Polymer | .384 |
| | Cling film | .384 |
| | Control | .711 |
| | Cohesive | .194 |
| Cotton Compress | Sub soft compression | .830 |
| | Cotton Stockinette | .999 |
| | Polymer | .957 |
| | Cling film | .957 |
| | Control | .495 |
| | Cohesive | .117 |

| | | |
|---|---|---|
| **Cotton Stockinette** | **Sub soft compression** | **.619** |
| | **Cotton Compress** | **.999** |
| | **Polymer** | **.998** |
| | **Cling film** | **.998** |
| | **Control** | **.292** |
| | **Cohesive** | **.065** |
| **Polymer** | **Sub soft compression** | **.384** |
| | **Cotton Compress** | **.957** |
| | **Cotton Stockinette** | **.998** |
| | **Cling film** | **1.000** |
| | **Control** | **.292** |
| | **Cohesive** | **.065** |
| **Cling Film** | **Sub soft compression** | **.384** |
| | **Cotton Compress** | **.957** |
| | **Cotton Stockinette** | **.998** |
| | **Polymer** | **1.000** |

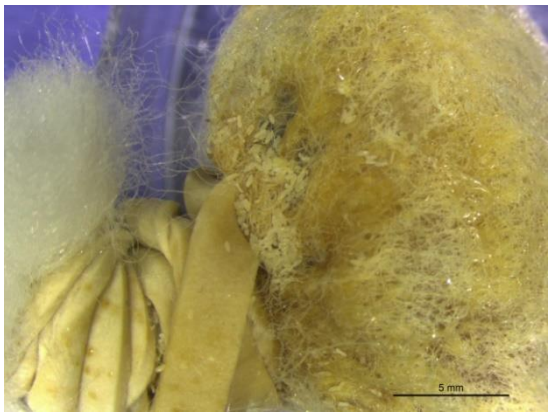


**Fig 115: Eggs found on the surface of the soft sub compression bandage wrapped around a piece of pork, left for 7 days at room temperature whilst exposed to 5 male and 5 female adult *Megaselia scalaris* flies (Scale = 5 mm).**

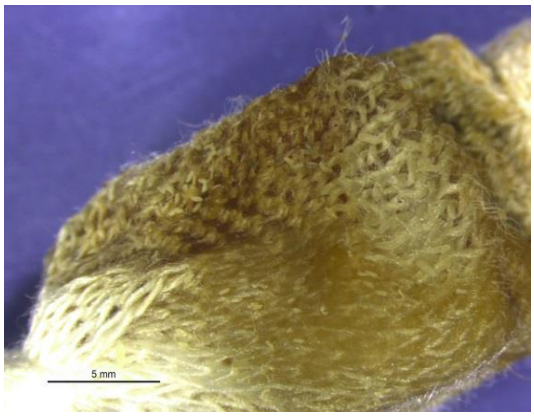


**Fig 116: Eggs found on the surface of the cotton stockinette wrapped around a piece of pork, left for 7 days at room temperature whilst exposed to 5 male and 5 female adult *Megaselia scalaris* flies (Scale = 5 mm).**

The developmental stages present through the one layer of bandage showed that *M.scalaris* were able to penetrate all the bandages where large amounts of eggs were observed (*Fig 115* and *Fig 116*). The one material which was able to prove resistant was cling film, whilst eggs were laid on the surface there was no penetration through the film to the flesh (*Table 46*). Anova statistical analysis (*Table 47*) showed significant differences between the materials (p=0.043), Tukey *post hoc* results (*Table 48*) showed no significant difference between the dressings.

### 5.2.3.2.2 Two Layers

**Table 49: Average developmental data with ± SD from two layers of bandage wrappings. Twenty adult *Megaselia scalaris* flies (10 male, 10 female) were added for 7 days then removed and the experiments were left for a further 7 days to allow development. E=Eggs, L=Larvae, P=Pupae.**

| Bandage | Surface of bandage (1st Layer) | | | Second layer of bandage (2nd Layer) | | | Surface of meat | | |
|---|---|---|---|---|---|---|---|---|---|
| | E | L | P | E | L | P | E | L | P |
| Control | N/A | N/A | N/A | N/A | N/A | N/A | 0 | 171 ± 74 | N/A |
| Cotton compression | 0 | 97 ± 140 | 0.7 ± 1.2 | 0 | 7 ± 10 | 0 | 0 | 12 ± 17 | 0 |
| Soft sub compression | 0 | 27 ± 12 | 26 ± 46 | 0 | 12 ± 10 | 0 | 2 ± 3 | 5 ± 6 | 0 |
| Polymer | 0 | 10 ± 7 | 0.6 ± 1.2 | 9 ± 16 | 10 ± 18 | 0 | 0 | 8 ± 12 | 0 |
| Cohesive | 0 | 122 ± 133 | 7± 13 | 0 | 6 ± 6 | 0 | 0 | 26 ± 13 | 0 |
| Cling film | 3 ± 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 50: Anova (P value) results from positions of development stage located on two layers of bandage wrapping**

| Position | P value |
|---|---|
| Eggs on Outer Layer | 0.458 |
| Eggs on 1st Layer | 0.458 |
| Eggs on Pork | 0.458 |
| Larvae on Outer Layer | 0.107 |
| Larvae on 1st Layer | 0.566 |
| Larvae on Pork | 0.000 |
| Pupa on Outer Layer | 0.477 |
| Pupa on 1st Layer | - |
| Pupa on Pork | - |

**Table 51: Tukey *post hoc* (P value) results from positions of larvae located on two layers of bandage wrapping**

| Dressing | Comparison | P value |
|---|---|---|
| | Cohesive | .001 |
| | Sub soft compression | .000 |
| **Control** | Cotton Compress | .001 |
| | Polymer | .000 |
| | Cling film | .000 |
| | Control | .001 |
| | Sub soft compression | .963 |
| **Cohesive** | Cotton Compress | .993 |
| | Polymer | .982 |
| | Cling film | .913 |
| | Control | .000 |
| | Cohesive | .963 |
| **Sub soft compression** | Cotton Compress | 1.000 |
| | Polymer | 1.000 |
| | Cling film | 1.000 |
| | Control | .001 |
| | Cohesive | .993 |
| **Cotton Compress** | Sub soft compression | 1.000 |
| | Polymer | 1.000 |
| | Cling film | .997 |
| | Control | .000 |
| | Cohesive | .982 |
| **Polymer** | Sub soft compression | 1.000 |
| | Cotton Compress | 1.000 |
| | Cling film | .999 |
| | Control | .000 |
| | Cohesive | .913 |
| **Cling Film** | Sub soft compression | 1.000 |
| | Cotton Compress | .997 |
| | Polymer | .999 |

**Fig 117: Cotton compression bandage wrapped around a piece of thick cut pork belly.
Left in a 1.1L plastic airlock container after being exposed to 20 adult *Megaselia scalaris* flies for 7 days and the offspring left for a further 7 days at room temperature (~ 19oC). Image shows large amounts of larvae found on the surface that appears to have gained access to the pork via a gap by the first blue line.**

Two layered bandage wrappings experiment showed that eggs, larva and pupae were all observed, larvae was located in relatively high numbers when compared to the numbers of eggs and pupae found. The largest numbers of larvae were located on the control sample where there was no protection against the larvae which is what would be expected.

All materials had eggs oviposited on to their surfaces (*Table 49*). Further development up to pupation was observed in all bandage materials used except one. Cling film again showed no further development after oviposition. Anova statistical analysis (*Table 50*) showed significant differences (p=0.00) between the number of larvae observed on all materials whilst the Tukey *post hoc* results (*Table 51*) showed significant differences between the control and the other dressings.

### 5.2.3.2.3 Five Layers

**Table 52: Developmental stage data averages with ± SD from five layer bandage wrappings.**
**Twenty adult *Megaselia scalaris* flies (10 male, 10 female) were added for 7 days then removed and the experiment left for a further 7 days to allow development.**

| Bandage | Egg | | | | | | Larvae | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5th | 4th | 3rd | 2nd | 1st | Pork | 5th | 4th | 3rd | 2nd | 1st | Pork |
| Control | N/A | N/A | N/A | N/A | N/A | 0 | N/A | N/A | N/A | N/A | N/A | 186 ± 55 |
| Cohesive | 0 | 0 | 0 | 0 | 0 | 0 | 63 ± 65 | 1 ± 1 | 1 ± 2 | 7 ± 6 | 14 ± 20 | 27 ± 18 |
| Soft sub compression | 0 | 0 | 0 | 0 | 0 | 0 | 40 ±38 | 4 ±7 | 0 | 1 ±2 | 3 ±5 | 20 ±35 |
| Polymer | 0 | 0 | 0 | 0 | 0 | 0 | 14 ±17 | 2 ±2 | 10 ±17 | 0 | 0.3 ±0.6 | 5 ±6 |
| Cotton compression | 0 | 2 ± 3 | 0 | 0 | 0 | 0 | 105 ±151 | 4 ±4 | 5 ±8 | 1 ±2 | 3 ±4 | 40 ±39 |
| Cling film | 0 | 0 | 0 | 0 | 0 | 0 | 29 ±44 | 0 | 0 | 0 | 0 | 0 |

**Table 53: Developmental stage data averages with ± SD from five layer bandage wrappings. Twenty adult *Megaselia scalaris* flies (10 male, 10 female) were added for 7 days then removed and the experiment left for a further 7 days to allow development.**

| Bandage | Pupae | | | | | |
|---|---|---|---|---|---|---|
| | 5th | 4th | 3rd | 2nd | 1st | Pork |
| Control | N/A | N/A | N/A | N/A | N/A | 5 ± 8 |
| Cohesive | 2 ± 3 | 0 | 0 | 0 | 0 | 0 |
| Soft sub comp | 0 | 0 | 0 | 0 | 0 | 0 |
| Polymer | 0 | 0 | 0 | 0 | 0 | 0 |
| Cotton compression | 7 ±12 | 0 | 0 | 0 | 0 | 0 |
| Cling film | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 54: Anova (P value) results from positions of development stage located on five layers of bandage wrapping**

| Position | P value | Position | P value |
|---|---|---|---|
| Eggs on Outer Layer | - | Pupae on Outer Layer | 0.458 |
| Eggs on 4th Layer | 0.458 | Pupae on 4th Layer | - |
| Eggs on 3rd Layer | - | Pupae on 3rd Layer | - |
| Eggs on 2nd Layer | - | Pupae on 2nd Layer | - |
| Eggs on 1st Layer | - | Pupae on 1st Layer | - |
| Eggs on Pork | - | Pupae on Pork | 0.458 |
| | | | |
| Larvae on Outer Layer | 0.428 | | |
| Larvae on 4th Layer | 0.450 | | |
| Larvae on 3rd Layer | 0.504 | | |
| Larvae on 2nd Layer | 0.066 | | |
| Larvae on 1st Layer | 0.368 | | |
| Larvae on Pork | 0.000 | | |

**Table 55: Tukey *post hoc* (P value) results from positions of larvae located on five layers of bandage wrapping.**

| Dressing | Comparison | P value |
|---|---|---|
| | Cohesive | .000 |
| | Sub soft compression | .001 |
| **Control** | Cotton Compress | .000 |
| | Polymer | .000 |
| | Cling film | .000 |
| | Control | .000 |
| | Sub soft compression | .995 |
| **Cohesive** | Cotton Compress | 1.000 |
| | Polymer | .945 |
| | Cling film | .887 |
| | Control | .001 |
| | Cohesive | .995 |
| **Sub soft compression** | Cotton Compress | .993 |
| | Polymer | .732 |
| | Cling film | .629 |
| | Control | .000 |
| | Cohesive | 1.000 |
| **Cotton Compress** | Sub soft compression | .993 |
| | Polymer | .951 |
| | Cling film | .897 |
| | Control | .000 |
| | Cohesive | .945 |
| **Polymer** | Sub soft compression | .732 |
| | Cotton Compress | .951 |
| | Cling film | 1.000 |
| | Control | .000 |
| | Cohesive | .887 |
| **Cling Film** | Sub soft compression | .629 |
| | Cotton Compress | .897 |
| | Polymer | 1.000 |

**Fig 118: Polymer bandage wrapped around a piece of thick cut pork belly.**
**Left in a 1.1L plastic airlock container after being exposed to 20 adult *Megaselia scalaris* flies for 7 days and the offspring left for a further 7 days at room temperature (~19 °C). Image shows large amounts of mould found on the 5th layer of the polymer bandage.**

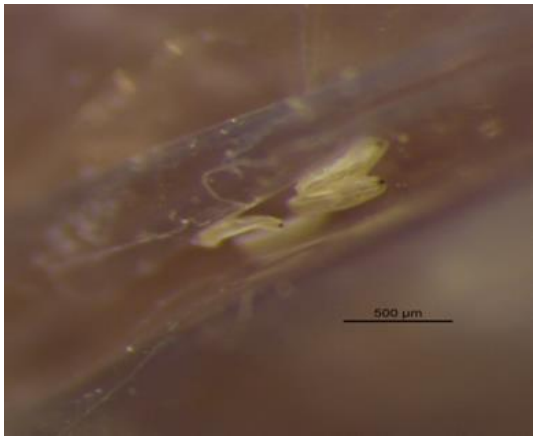


**Fig 119: Cling film bandage wrapped around a piece of thick cut pork belly.**
**Left in a 1.1L plastic airlock container after being exposed to 20 adult *Megaselia scalaris* flies for 7 days and the offspring left for a further 7 days at room temperature (~ 19 °C).**

Observations during the experiment saw eggs, larva and pupae were all present during the experiment (*Fig 118* and *Fig 119*).

The largest numbers of larvae were located on the fifth layers of the material and on the pork with a small number of larvae found between these layers. Only one material showed that larvae were not able to penetrate through its layers and this was cling film. A large number of larvae were observed on the $5^{th}$ layer but no other layers.

Larvae were located in relatively high numbers when compared to the numbers of eggs and pupae found. The largest numbers of larvae were located on the control sample where there was no protection against the larvae, which is what would be expected (*Table 52* and *Table 53*).

Anova statistical analysis (*Table 54*) showed significant differences (p=0.00) on the number of larvae observed on the pork from each bandage type, the Tukey *post hoc* analysis (*Table 55*) shows a statistical difference between the larvae on the control and the other dressings.

### 5.2.4 Discussion

Overall our experiments showed that there were differences between the control and the remaining dressings. Different developmental stages were found throughout the different layers of dressings in all but one dressing. The most successful covering in all wrappings used was cling film which found numerous eggs laid on the surface of the film but neither adults or larvae were able to penetrate the film to reach the meat within. The research conducted shows that *M.scalaris* are able to lay eggs on the surface of the bandage and the 1$^{st}$ instar larvae were then able to work their way towards the flesh.

In Forensic Entomology myiasis has been used to provide useful evidence in cases such as abuse and neglect. Whilst there are some reports of nosocomial myiasis some of these reports discuss the reasoning that flies were able to lay directly on to flesh before the damaged flesh was enclosed in bandages rather than them laying on the bandage surface for the larvae to burrow down on to.

Myiasis is a topic that has been discussed for many years with the first discussion being printed by Hope (1840).

Huntington and colleagues (2008) discuss the wound myiasis of a gentleman with long standing venous stasis ulcers. He had to attend surgery every week for dressing changes however he would occasionally miss an appointment and turn up a few days later. At one follow up appointment to re-dress his four layer compressive dressing, maggots were found under the dressing. An antimicrobial barrier dressing and a four layer compressive dressing was re-applied to the site. On further inspection of the removed dressing, hundreds of small larvae between 3 and 5 mm were observed along with one newly formed pupae. A second case was presented in which three larvae were collected from a leg wound of a patient. In both cases the specimens were sent for identification. In both cases the specimen was identified as *M. scalaris*, which supports our findings of larvae working their way through the numerous layers of bandage to feed.

Hira and colleague (2004) reported a case of myiasis of a man that had a building site accident which resulted in numerous fractures to the pelvis and many lacerations to the leg. The leg was bandaged and placed in traction however after 14 days the gauze bandaged was observed to be discoloured with a foul smell coming from it. Upon removal of the dressing small white worms were seen coming from the bandage. The specimens were collected and identified as *M.scalaris.* Preventative measures are

discussed however in the case of Phorids these measures are seen as difficult due to the Phorids small size which helps them enter through most fly screening as well as bandages, however by regularly changing the dressings and regular observations of the wounds may help reduce the infestations.

# 6: Conclusion

## 6.1 Conclusion

Determing the post mortem interval (PMI) is the most important function of entomological evidence. There are many different factors that play a role in insect development with temperature playing the most important role; other factors may include weather, food type, access to food, buried, or exposed, presence of toxins along with geographical regions. The mPMI estimation requires knowledge of the development data for the specific species of insect collected from a cadaver (Byrd and Castner, 2009). Without this knowledge and understanding of the behaviour of the insects then specific species may not be used or classed as a forensically important species. It is of great importance that the size and age of the insect is correct when working with forensic cases.

Based on the results of the research carried out within in this thesis, many observations have been made about the species *Megaselia scalaris* which may help towards this fly being accepted as a forensically important species.

In Chapter 2, whilst our adult, eggs and larval imaging are in agreement with other work completed by researchers. The first observations carried out with a Nikon XT H 225 and Synchrotron at Elettra found that it may be possible to age the pupa by looking internally at the reproductive organs which seem to move from the posterior to anterior region of the abdominal cavity, the older the pupa. Whilst this was a preliminary experiment and further work needs to be run, the images clearly show the movement of the internal organs; however we learnt that the minimum energy output to successfully image *M.scalaris* requires an energy source between 8 and 35kV.

During Chapter 3, observations showed that adult *M.scalaris* flies of both sexes are more inclined to go towards pet food rather than the fresh pig liver and that they are able to move and find food both in light and dark conditions. Females have been shown to be more active in dark conditions however different foods and rearing temperatures have shown that they have an effect on developmental rates.

Collaborative worldwide research in the area of growth development using the same parameters would be beneficial and more reliable. Different geographical areas may bode different results however currently as researchers are using many different parameters, the results cannot be used as a comparative method.

Our research investigating toxicological analysis from *M.scalaris* showed that there was no effect on the size but saw an effect on developmental time. Results also showed the puparia did not show any drug present may suggest that further research into toxicological analysis is required.

The observations presented in Chapter 4, using both the maze and activity monitor conclude that *M.scalaris* were active in both dark and light periods. It was also found that *M.scalaris* were able to oviposit during the dark phase in which the adults were active. Locomotor activity concludes that *M.scalaris* is both diurnal and nocturnal in activity with a period of activity spanning over 24 hours.

Light has shown to be an important stimulus to the flies, whilst the light maze has shown that different colours have different effect on the two sexes. Our observations have also shown that *M.scalaris* are able to oviposit during the daytime hours which is expected fly behaviour, the experiment also showed that flies are able to lay eggs in a dark environment during daytime conditions which coincides with the coffin fly label. The experiment has also shown that these flies are able to oviposit in dark conditions during the night.

The pattern of emergence and flight periodicity is discussed by Lewis and Taylor (1965). They comment that the emergence rhythm may influence the time of flight in the insect. Our observations concluded that males do emerge prior to females and that different rhythms are present during full darkness conditions and light/dark conditions. Our experiments demonstrated that this species is clock regulated and that emergence in continuous dark may occur both during the dark or the light subjective phase.

The observations in Chapter 5 showed that *Megaselia scalaris* adults were able to burrow down in to soil and continued their reproduction through all developmental stages. The adult flies were observed returning to the surface after emerging from the puparia possibly to mate as after a couple of days they disappeared back down into the soil. The sand was found to be a hard material for the adults to burrow down into possibly due to the small very dry environment resulting in the death of all the adults a few days after the experiments were set up.

The study of myiasis has been used to provide useful evidence in forensic entomology cases such as abuse or neglect and nosocomial myiasis. Preventative

measures are seen as difficult in the case of Phorids due to their small size, which helps them enter through most fly screens as well as bandages; the regularly changing of the dressings may help towards the reduction of infections. Overall our experiments showed that the most successful covering was cling film which saw numerous eggs laid on the surface, neither *M.scalaris* adults nor larvae were not able to penetrate the film to reach the meat inside.

This thesis was completed with the aim of trying to resolve previously asked questions along with addressing issues which allows us a better understanding of the species *Megaselia scalaris* and the effect it has on the Forensic Entomological field. Based on the results of this research, numerous observations were made which may impact what entomologists know about *M.scalaris* behaviour and may assist in future mPMI estimations.

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