Differences in larval size of Calliphora vicina reared on pig brain and liver and the effects of changing food substrate mid-development: Implications for the estimation of Post Mortem Interval using blow fly evidence.

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1. Abstract

The blow fly *Calliphora vicina* (Robineau-Desvoidy) is one of the most forensically important insects found in the UK. Its rapid and consistent time of arrival at a body following death makes it a potentially very accurate tool for the calculation of Post Mortem Interval, thus allowing investigators to accurately determine the likely time frame within which an individual died. The estimation of Post Mortem Interval using insect evidence depends largely upon the accurate estimation of age of juvenile flies with several data sets available to the forensic entomologist to aid estimation. However, recent studies have pointed out some of the flaws in these data sets, including the effects of food substrate on development. Here, two experiments were carried out to estimate the effects of substrate on development times: firstly, juvenile *C. vicina* were reared on both pig liver and pig brain to determine whether there was a developmental difference between the two substrates and secondly, food substrates were swapped at an interval of 48 hours to investigate the effect of substrate on different larval stages and its impact on development times. A significant difference was found between juveniles reared on brain and those reared on liver, with the former being larger in width, length and weight. However, there was no significant difference found when food substrate was swapped after 48 hours. Different explanations for the findings are postulated and are discussed within the context of implications for the accurate estimation of both age and Post Mortem Interval using insect evidence.
2. Introduction

Insect evidence has been useful in death investigations (Anderson, 1995), drug consumption (Introna, 1990), food contamination and cases of neglect (Gennard, 2007). Insects have been used as a forensic indicator for many hundreds of years and their uses are well documented (Gennard, 2007; Anderson, 1995; Marchenko, 2001; Benecke, 2001). Insects are known to colonise a cadaver during various stages of decomposition or even a living organism which may have been wounded or neglected. In this respect the cadaver can be thought of as an ecosystem divided into a series of micro-habitats.

Some of the most useful insects within this category are the family Calliphoridae or blow flies (also known as bottle flies or flesh flies). These are carrion feeding flies. There are over 1000 species worldwide (Turner, 2005) and they have been the subject of a great many studies relating to the field of forensic identification (Kaneshrajah and Turner, 1994; Grassberger and Reiter, 2001; Ireland and Turner, 2006; Clark, Evans and Wall, 2006; Woolridge, Scrase and Wall, 2007 and Adams and Hall, 2003). Within the UK and the rest of Europe, Calliphora vicina (Robineau-Desvoidy) is one of the most important of these flies, along with Calliphora vomitoria (L.) and Lucilia sericata (Meigen) (Gennard, 2007; Turner, 2005; Marchenko, 2001). The usefulness of these species lies in their consistent and very early time of arrival at a cadaver. They are also associated with infestation of food stuffs and fly strike or myiasis in animals and humans (Turner, 2005).

A common way of interpreting insect evidence is to use the estimated age of an individual or cohort of insects to calculate the time since death or Post Mortem Interval (PMI) (Amendt et al., 2007; Wells and LaMotte, 2001; Greenberg, 1991) This method can produce much more accurate results than purely physiological indicators such as rigor mortis or body temperature and can be determined long after these methods become useless: around 36 hours after death (Jackson and Jackson, 2004). As well as measuring PMI, insects can indicate if a body has been moved, covered, submerged or buried for any length of time, which can often be the case if an act of homicide has been committed.

The extremely predictable and well documented behaviour of arthropods, models of development, and (more generally) successions of insects on a cadaver may be reliable indicators of the time of death. As Wells and LaMotte (2001) have observed, “It has long
been observed that insects associated with vertebrate carrion display PMI-dependent processes.” This is essentially what makes insects useful as a forensic tool. The use of insect evidence to calculate a PMI estimation provides information to the investigation regarding a likely time of death, or at least the period a cadaver has been exposed to insect succession.

According to Ireland and Turner (2006), there are two common methods of estimating PMI: the first involves the sizing and aging of sampled maggots using standard experimental data to determine the maximum period of time they have been present at the corpse. All species of blow flies will follow a similar curvilinear pattern of growth which is ultimately dependent on temperature (Higley and Haskell, 2001). The second method, commonly adopted in modern practice, is to calculate the Accumulated Degree Hours (ADH) or Accumulated Degree Days (ADD) that are required for larvae to reach a particular stage in development and from this, derive their age. According to Higley and Haskell (2001) “Linear models are most commonly called degree-day models because development is regarded as a combination of temperature above minimum developmental threshold multiplied by time.” This second method is used in other more general areas of entomology and is a more accurate estimation since it is linear and so makes a simpler assumption that there will be an increase in development rate with an increase in temperature. The first method, being based on a complicated curvilinear model, is rather more complicated to carry out (Higley and Haskell, 2001).

In their study, Grassberger and Reiter (2001) reared *Lucilia sericata* in a laboratory on beef liver and subsequent average developmental rates were compiled for different temperature regimes. They also produced isomegalen-diagrams based on their findings. In an isomegalen diagram, “time from hatching to peak feeding is plotted against temperature, each line representing identical larval length at various temperatures” (Grassberger and Reiter, 2001: 34). Their ultimate aim was to produce a diagram from which maggot age could be drawn from, given that the temperature is constant. In practice, this has limited

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1 Alternatively there are now computer programs available such as ‘DEGDAY’ put forward by Higley, Pedigo and Ostlie (1986).
value since temperature in very infrequently constant, particularly within the UK. It can be argued that this particular set is only useful for cases where specimens have been drawn from beef liver, given that this was the food substrate used to rear the maggots in the experimental studies. These limiting factors therefore have a potential to produce inaccurate results, and by inaccurate estimation of developmental stage - such as making comparisons with data which have limited resemblance to field specimens - a PMI estimation can become very unreliable.

Laboratory controlled studies, whilst useful in many respects such as studying temperature and food regimes, are limited in their usefulness with regard to real life cases. Specimens collected from cadavers in the field will no doubt have developed under changing temperature and weather conditions and may have been subject to other external factors such as food availability and natural predators. Therefore, the major flaw in the techniques outlined above is the standard data upon which they are based and their reliance on controlled conditions which wouldn’t apply in the field. There are an increasing number of these experimental data which the forensic entomologist can consult (for example: Byrd and Butler, 1998; Grassberger and Reiter, 2001; Greenberg, 1991 and Nishida, 1984) which are compiled from the controlled-condition rearing of flies and a range of different food sources have been used however they have not been considered an important variable and are not discussed as a possible source of error (Ireland and Turner, 2006). A recent set of standards put forward in a paper by Amendt et al. (2007) has discussed how the forensic entomologist may accurately estimate the size and age of a blowfly maggot. However, there are references to several temperature-based standard data sets, which differ and none of which account for variations in food source. It has been shown that not only do development rates of Calliphora vicina vary on different tissues (Kaneshrajah and Turner, 1994) but that Lucilia sericata shows different development rates between pig tissues and beef tissues (Clark, Evans and Wall, 2006) This raises questions regarding the reliability of laboratory databases as a source of comparison when the data may not accurately compare to field data. The problem becomes compounded when other factors such as weather conditions (Williams, 1961), nocturnal oviposition behaviour (Singh and Bharti, 2001), food availability (specifically, the amount of food available) and the
presence of toxins or poisons (Strehler et al., 2008; O’Brien and Turner, 2004; Goff and Lord, 2001) are also taken into account.

The empirical data obtained from these studies clearly shows a developmental difference between food substrates, but do not attempt to explain such differences. By understanding the physiological development and biological processes which occur in growing maggots, we might better understand this difference and its implication for accurate PMI estimation. Hobson (1931) carried out some of the first in depth investigations into the physiology of blow fly larvae. He made a number of interesting observations, including those relating to the digestion of certain substrates such as lipids and proteins in a normal developing larva. He actually stated: “The presence of fat in larvae reared on a protein diet proves the synthesis of fat from protein; it is not possible to decide whether the fat found in the mid-gut has been synthesised in situ or deposited in the gut after synthesis elsewhere.” (Hobson, 1931) Brain has a higher lipid content than brain, with approximately 8.58g/100g compared with 5.02g/100g, respectively² (Day and Wallman, 2006). Liver is composed of denser connective tissue and a number of ligaments, whilst the brain consists of the much softer material, myelin, designed to protect the delicate neurons (Gray, 1918). These differences are also observable during the decomposition of these two tissues under the same conditions; brain will quickly liquefy whereas liver will dehydrate and become a hardened lump. Day and Wallman suggest (2006); “…larvae consuming more fat will be expending less energy on metabolism…these larvae can thus, in principle, direct more energy into growth.”

It is important that forensic scientists have the most accurate and well researched resources available to them to maximise the usefulness of any evidence. Estimation of the age of a maggot can be extremely difficult since there are no clear visual or physiological markers at any given stage of development, but with the use of systematic and rigorous rearing strategies this could be achieved. Following on from the work of Kaneshrajah and Turner (1994) Calliphora vicina reared on pig liver might be expected to develop more

² This information was obtained from the website of the United States National Agricultural Library suggesting that the figures listed are for pig brain – however this is not mentioned in the text and the original document is no longer available.
slowly than those reared on pig brain and the resulting adults might be larger and more robust. Previous research in this laboratory suggests that a pivotal change could occur in development at around 48 hours, with the feeding taking place during this period being the most important to overall development. Therefore it may also be hypothesised that if larvae feed on liver for the first 48 hours proceeding hatching, they will expend more energy freeing up nutrients from that liver resulting in an overall energy deficit which cannot be regained even if the larvae then feed on a more fat-rich substrate such as brain. As a result there will be a longer period of development and an overall smaller size and weight compared to larvae which have fed upon brain for the first 48 hours. The main experiment of the current study aims to establish whether there is a significant difference between brain tissue and liver as the main development indicator of C. vicina larvae of different ages and whether or not the first 48 hours of feeding has a significant effect on duration to larval stage and also to pupation.
3. Method and materials

The larvae in this study were reared on both pig’s liver and pig’s brain. Liver was chosen as a baseline control since this is food most commonly used in published studies. Brain was used as a comparison as previous findings in this laboratory have found brain to be a particularly good food source for blow flies and also because flies will typically begin to infest a corpse at open orifices, including the head. There are also nutritional and structural differences between the two tissues. C. vicina were chosen as they are known to be one of the first visitors to carrion within the UK and therefore one of the most likely insect species to be sampled in a forensic case.

3.1 Experiment A (preliminary testing)

Preliminary test runs were carried out for two purposes. Firstly, to increase the number of flies in the population thus ensuring a strong group to draw eggs from, and secondly to determine that there was a significant difference between maggots reared on liver tissue and maggots reared on brain tissue.

Preparation of eggs

The eggs used in this experiment were taken from a culture reared from individuals collected on the roof of South Bank University using a pig liver bait (see Figure 1). They were then fed on sugar water ad libitum and pig liver to encourage ovary development. Eggs were placed in small plastic containers covered with a nylon stocking to allow for ventilation but preventing the larvae from escaping. Portions of food substrate were placed in each container and the bottoms of the containers were covered in sawdust to provide a migrating ground for pupating larvae.

Substrate preparation

Fresh pig brain was sourced from a local butcher, with the brains being taken fresh from the heads of the pigs on the day of delivery. Pig’s liver was purchased either from the same butcher or from the local supermarket. All the tissue was refrigerated until it was needed. It was possible to purchase liver for use on the day, however, due to delivery
constraints, the brain frequently needed to be refrigerated for a few days before use. The freshest possible tissue was used for experimental purposes.

**Sampling generation and method**

Gravid female and male flies were kept in ventilated nets in a greenhouse for warmth. They were presented with large portions of pig’s liver to encourage oviposition. The subsequent eggs were transferred directly onto the sampling containers – 100 eggs on 100g of either pig’s brain or pig’s liver – using a moistened paint brush and incubated at 20°C and 60-65% humidity.

Eggs were typically collected within 24 hours of oviposition and were either used immediately or refrigerated for a maximum of five days. Fresh pig liver and pig brain were cut into 25g portions and 25 eggs were transferred to each of the portions inside small plastic containers, into which sawdust was also added. The purpose of the sawdust was to act as a dry area for pupation. The containers were then covered with nylons and incubated at 20°C (see Figure 2). Following incubation, the containers were monitored daily (except weekends) and once the maggots had reached the post-feeding stage they were collected, killed and measured.

Maggots were killed by submersion in very hot water and fixed in 70% alcohol (see Adams and Hall, 2003 and Amendt et al. 2006). Specimens were then weighed and measured in length and width according to suggestions made by Day and Wallman (2006).

Specimens were measured by being placed onto a clean plastic board and against a standard 15cm ruler. Both length and width were measured in this manner and all measurements were rounded up to the nearest 0.5mm, according to Amendt et al. (2007). Specimens were then weighed using Oxford A1204 balance scales (see Figure 3). All maggots were cleaned of any tissue which became stuck to them during feeding, a more common problem with those on brain tissue as it became liquidised very quickly. The same ruler and balance scales were used during both experiments to ensure consistency.
3.2 Experiment B

The second round of experiments was designed to test the hypothesis that the first 48 hours of development is pivotal, with regard to the changing of food substrate. Pig brain and liver were used again, as with experiment A.

Preparation of larvae eggs and substrate preparation

The eggs for this experiment were taken from a culture reared from maggots purchased at a local fishing tackle supplier. Flies were fed sugar water *ad libitum* and small pieces of pig liver to encourage ovary development in the females. Once oviposition had taken place, eggs were placed onto containers in the same manner as in experiment A. Eggs were used within 48 hours of hatching, being refrigerated in-between to avoid early hatching. Pig liver and brain were obtained by the same means as experiment A.

Sampling generation and method

Eggs collected from gravid females using fresh pig liver were transferred to prepared containers with 100g of either pig liver or brain and sawdust as a pupating medium. 300 eggs were transferred to each container initially. The resulting maggots were left to feed for 48 hours when 200 eggs were transferred across 2 prepared containers containing the opposite food substrate. The experimental variables are shown in Table 1 below.

*Table 1: The four experimental variables used in experiment B. The maggots feeding on containers 3 and 4 were transferred to fresh meat as indicated after 48 hours.*

<table>
<thead>
<tr>
<th>Container</th>
<th>Food source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Brain throughout</td>
</tr>
<tr>
<td>2</td>
<td>Liver throughout</td>
</tr>
<tr>
<td>3</td>
<td>Brain for first 48 hours, followed by liver for the remaining duration</td>
</tr>
<tr>
<td>4</td>
<td>Liver for first 48 hours, followed by brain for the remaining duration</td>
</tr>
</tbody>
</table>

All the containers were incubated at 20°C and 60-65% humidity. Each container was sampled every 24 hours, except in the first instance that maggots had been added to it. Each
specimen was killed by immersion in hot water for 30 seconds and then fixed in 70% ethanol to preserve. Maggots were weighed and measured in length and width.
4. Results

4.1 Experiment A

The purpose of this experiment was to confirm that there was a significant difference in developmental rates between larvae reared on brain and larvae reared on liver. The brain tissue is considered to have a higher nutrient value to the larvae, maximising developmental potential.

Table 2: The mean length, width and weight of the C. vicina larvae which were collected are shown -those reared on liver and those reared on brain. The standard deviations are also shown in brackets alongside the means.

<table>
<thead>
<tr>
<th></th>
<th>Liver tissue</th>
<th>Brain tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean length (mm)</td>
<td>16.20 (2.99)</td>
<td>17.27 (3.00)</td>
</tr>
<tr>
<td>Mean width (mm)</td>
<td>2.86 (0.63)</td>
<td>3.27 (0.71)</td>
</tr>
<tr>
<td>Mean weight (g)</td>
<td>0.095 (0.074)</td>
<td>0.11 (0.045)</td>
</tr>
</tbody>
</table>

Table 2 shows that larvae reared on brain were larger in all three tested aspects, length, width and weight. The standard deviations in all three aspects across both treatments are all very similar, confirming that the degree of spread around each mean was very close.

Figure 4: Graph to show the length (mm) and frequency density over time of C. vicina larvae reared on liver. The darker red colour indicates that there was a higher frequency of larvae with the same or very similar weights at any specific sampling point.
Figure 5: Graph to show the length (mm) and frequency density over time of C. vicina larvae reared on brain. Again, higher frequency is indicated by a darker red colour.

Figure 6: Graph to show weight (g) and frequency density over time of C. vicina larvae reared on liver. Again, higher frequency is indicated by a darker red colour.
Figure 7: Graph to show the weight (g) and frequency density over time of C. vicina larvae reared on brain over time. Again, higher frequency is indicated by a darker red colour.

Table 3: Summary of results of the t-test of the effects of brain and liver as a developmental factor in overall development of C. vicina larvae
<table>
<thead>
<tr>
<th></th>
<th>T-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length</strong></td>
<td>3.45</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td><strong>Width</strong></td>
<td>6.5</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td><strong>Weight</strong></td>
<td>2.31</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

A very significant difference was found for both length (t=3.45, p<0.0001) and width (t=6.5, p<0.0001) between the two experimental groups. There was also a significant difference for weight (t=2.31, p<0.05) although this was not quite as strong as the length and width. These results support the hypothesis that flies reared on brain tissue are larger than those reared on liver tissue.
4.2 *Experiment B*

The purpose of this experiment was to determine whether the first 48 hours of feeding were pivotal to the overall development of *C. vicina* larvae.

For the purposes of more refined data analysis it was decided to omit the pupal measurements as they were unevenly represented across the treatments and caused a significant skew to the data. They were deemed a non-significant part of the overall data set and did not contribute to the testing of the hypothesis.

*Table 4: The mean length, width and weight of the *C.vicina* larvae are shown - those reared on only liver, those reared on only brain, those reared on liver then brain and those reared on brain then liver. The standard deviations are also shown in brackets alongside the means.*

<table>
<thead>
<tr>
<th></th>
<th>Brain all</th>
<th>Liver all</th>
<th>Liver, then brain</th>
<th>Brain, then liver</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length (mm)</strong></td>
<td>17.48 (1.75)</td>
<td>14.12 (4.11)</td>
<td>16.42 (4.43)</td>
<td>15.33 (3.79)</td>
</tr>
<tr>
<td><strong>Width (mm)</strong></td>
<td>3.28 (0.40)</td>
<td>1.90 (0.58)</td>
<td>2.43 (0.78)</td>
<td>2.22 (0.72)</td>
</tr>
<tr>
<td><strong>Weight (mm)</strong></td>
<td>0.11 (0.03)</td>
<td>0.05 (0.028)</td>
<td>0.079 (0.047)</td>
<td>0.063 (0.036)</td>
</tr>
</tbody>
</table>

Table 4 shows that there is a fairly large difference across all three aspects, between larvae reared on only brain and those reared on only liver, in line with the findings of experiment A. The means of each aspect for the two experimental treatments, however, appear to be very similar. Further analyses were carried out to determine if there were any significant differences.
Figure 8: Graph to show weight (g) and frequency density over time of *C. vicina* larvae reared on liver for the first 48 hours and then swapped to brain. Again, frequency density is indicated by a darker red colour.

Figure 9: Graph to show weight (g) and frequency density over time of *C. vicina* larvae reared on brain for the first 48 hours and then swapped to brain. Again, higher frequency is indicated by a darker red colour.
Table 5: Summary of results of the t-test of the effects of food substrate (brain or liver) during the first 48 hours of development, on overall development of C. vicina larvae.

<table>
<thead>
<tr>
<th></th>
<th>T-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>1.692</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Width</td>
<td>1.805</td>
<td>NS</td>
</tr>
<tr>
<td>Weight</td>
<td>363.636</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

No significant difference was found for width (t=1.805, p<0.5). However, a significant difference was found between length (t=1.692, p<0.05) and weight (t=363.636, p<0.0001), when food substrate was changed after 48 hours. Larvae reared on liver for 48 hours and brain for the remainder of development were significantly longer and heavier, contrary to the hypothesis that they would be smaller overall when compared to the larvae reared on brain for 48 hours and liver for the remainder.

Table 6: Summary of results for the one-way ANOVA of the effects of food substrate (brain or liver) during the first 48 hours of development, on overall development of C. vicina larvae.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>F-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>67.162</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Width</td>
<td>27.473</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Weight</td>
<td>21.739</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

A significant difference was found across all three aspects across all four treatments, length (67.162, P<0.0001), width (27.473, p<0.0001) and weight (F=21.739, p<0.0001), showing that the variability was due to the food substrate used on rearing. A further ANOVA was carried out by splitting the control treatments and the experimental treatments into two groups, ‘swapped’ (including the brain only and liver only treatments) and ‘not swapped’ (containing the two experimental treatments). A summary of the findings are shown in Table 7:
Table 7: Summary of results for the one-way ANOVAs of the effects of food substrate on C. vicina larvae being either ‘swapped’ or ‘not swapped’.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Swapped</th>
<th>Not swapped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>2.809 (NS)</td>
<td>19.170 (p&lt;0.0001)</td>
</tr>
<tr>
<td>Width</td>
<td>3.263 (NS)</td>
<td>134.944 (p&lt;0.0001)</td>
</tr>
<tr>
<td>Weight</td>
<td>5.985 (p&lt;0.05)</td>
<td>105.68 (p&lt;0.0001)</td>
</tr>
</tbody>
</table>

Table 7 shows that there was virtually no significant difference across the three aspects in the ‘swapped’ group, but a highly significant difference across all three aspects in the ‘not swapped’ group. This suggests that the overall difference in variance was due to the sizeable differences between the two control groups rather than any differences between the two experimental groups.
5. Discussion

The results from experiment A and experiment B confirm that there is a significant developmental lag observed in the larvae reared on liver tissue compared with those reared on brain tissue. Larvae took longer to develop to pupation when feeding on liver and they were also significantly smaller in both size and weight, supporting the hypothesis that the food substrate type has a significant effect on the overall development of *C. vicina* larvae. This was supported by both sets of experimental data obtained and supports the previous findings by Kaneshrajah and Turner (1994), Clark, Evans and Wall (2006), Ireland and Turner (2006) and Day and Wallman (2006). This developmental difference remained even when food substrate was swapped after 48 hours. Whilst not supporting the hypothesis that the first 48 hours of development are pivotal, this gives some insight into the substantial nutritional differences to larvae, between pig brain and pig liver.

*C. vicina* larvae are known to feed mainly on carbohydrates, proteins and lipids as part of their diet. The main purpose of feeding during the larval stage of development is to create and store fat bodies that will act as a reserve of energy, expendable during metamorphosis into adult. A review of some of the relevant literature suggests that larvae are able to synthesise fat from the protein, which they digest as part of their diet (Hobson, 1931; Day and Wallman, 2006) and that they are able to store lipids directly. The synthesis of proteins into fat would require energy to be expended, which would not occur if lipids were stored directly. This suggests that food substrates high in lipid content, such as brain, would be preferable during larval feeding as they would ensure maximum storage of energy. This possible justification of the findings of the current study is controversial as the synthesis of fat from proteins, as suggested by Hobson (1931), is highly unlikely to occur. However, the higher lipid content of brain would appear to be an important factor in its high nutritional value for larvae, given that a larvae’s development depends upon lipid storage. It is, therefore, important to understand the composition of the tissues being used as a food source and also to be aware of the possible differences between the tissues of the animal being utilised and the same tissues in a human as this may pose a source of significant error when using the standard data sets.
Another possible explanation for the developmental lag was observed during both experiments. As the liver decomposed during feeding, it became dehydrated and hard, possibly making feeding for developing maggots more difficult. The same did not apply to the brain tissue which very quickly became liquidised, enabling the maggots to move through the tissue with great ease and likely expend less energy in feeding. Maggots feeding on liver were frequently observed moving across the surface of the liver as they fed, occasionally tunnelling some way into the tissue, however, larvae feeding on brain were submerged in the tissue as it liquidised (see Figures 10 and 11 below). Findings by Clark, Evans and Wall (2006), compared larval development on liquidised tissue with larval development on whole tissue and found no significant difference between the two, however they did not compare the differences between the natural decomposing states of their chosen food substrates, a consideration that would be more useful for standard data. In the light of the observations made regarding expenditure of energy in the maggot during feeding, the physical state of the tissue could certainly have an effect on the amount of energy available for overall development. Brain tissue therefore not only has a higher lipid availability, but its liquid state during decomposition means that maggots feeding on it expend less energy breaking down and digesting tough tissue.

(from left to right) Figure 10: C. vicina larvae feeding on liver. The liver is very solid forcing the maggots to roam along the surface during feeding and possibly posing a feeding difficulty. Figure 11: C. vicina larvae feeding on brain. The maggots are completely submerged in the tissue for most of the feeding stages of development, making ingestion of food much simpler.
Experiment B found that there was no significant difference in overall size and weight of larvae when food substrate was swapped after 48 hours. Pupation time (288 hours) for these larvae fell in between the times for larvae reared only on brain (240 hours) and only on liver (360 hours). This suggests that swapping food substrate does have some affect on the development of the larvae, but that this would be difficult to measure from field specimens as the usual methods (length, width and/or weight measurements) would not show any significant difference between those feeding on one substrate and those feeding on more than one.

A possible justification for difference in time to pupation could be that the larvae go through a period of digestive adjustment when swapping from one tissue to another; perhaps the gut would need to produce different enzyme groups in order to digest the new tissue. It is important to recognise that the differences observed in the current study can only be generalised to brain and liver and that they are contrary to previous findings in this laboratory when larvae were feeding on heart muscle and skeletal muscle. This is an area of potential importance with regard to developmental effects and would likely require further investigation.

The results from the analyses of variance in experiment B showed that the overall differences in variation occurred almost completely as a result of the differences between the larvae reared either only on brain, or liver. There was a difference between time to pupation of 120 hours between the two substrates, which might suggest that larvae feeding on liver for the first 48 hours would fall behind in time to pupation, however the larvae feeding on both substrates pupated at roughly the same time. The pupae observed in the ‘brain then liver’ treatment were much darker and more abundant than those observed in the ‘liver then brain’ treatment at the same sampling time (288 hours). There were still a number of post-feeding larvae in the ‘liver then brain’ treatment, suggesting that these larvae did pupate slightly later than those in the opposite treatment, but only by a matter of hours. This observation would suggest that those larvae feeding on brain for the longest periods will develop more quickly even when moving from another food source. This is further evidence for the observation that brain is more nutritious to larvae than liver.
As discussed, many different food sources have been utilised for the purposes of producing standard laboratory data. These range from beef liver, muscle, heart or lung (Greenberg and Tantawi, 1993; Byrd and Butler, 1996, 1997, 1998; Grassberger and Reiter, 2001; Grassberger et al., 2003, Clark, Evans and Wall, 2006), lamb liver, muscle, brain or meat (Wall et al., 1992; Davies, 1998; Day and Wallman, 2006) and pork liver, brain, cheek muscle, heart, lung or kidney (Byrd and Butler, 1996, 1997, 1998; Byrd and Castner, 2001; Ames and Turner, 2003; Ireland and Turner, 2006; Clark Evans and Wall, 2006). And as Kaneshrajah and Tuner (2004) argue, there are many studies which do not list the diets or even consider them as a developmental factor. The results of this study support the argument that food substrate plays a hugely important role in the overall development rate of blow fly larvae and if this factor is ignored, it will limit the usefulness of insect evidence and PMI estimation. Both methods for calculating PMI (simple age estimation and ADH/ADD calculations) depend wholly on accurate estimation of the age of the larval specimen. This in turn is reliant upon accurate data regarding maggot length (or width/weight) compared to age. For example, from the data obtained from experiment A in the current study, larvae feeding on brain will develop approximately 24 hours faster than those reared on liver at the same temperature. These larvae will also be, on average, 1-2mm larger during most of development. Therefore, if a specimen is taken from a real case which has been recovered from the head cavity and which is 17 mm long (and therefore could be as young as 72 hours old) and is compared to standards based on liver rearing, which suggests that by this length the larvae are 120 hours old, the result is a large margin of error when using this data to calculate PMI. This error margin could be much larger if the results from experiment B were used. In a study of blind validation of post mortem intervals using the developmental data available for blow flies, VanLaerhoven (2008) found that although there was no significant difference between mathematical methods for estimating PMI, there was a significant difference between the standard data sets used. Of the 5 data sets used, each one was only usually very accurate at particular temperatures. He found that, “as lower development threshold increased, the PMI interval estimates increased.” (VanLaerhoven, 2008). This study shows that entomological evidence can be a very powerful tool, but more emphasis needs to be placed on the development of accurate standard data.
There were some methodological problems with the current study. Time and access constraints meant that sampling times were not always accurate, although this was reduced during experiment B. There were also issues with choosing the largest specimens at the time of sampling. Those maggots feeding on liver could be easily identified in masses or crawling along the tissue surface, however, those on brain were fully submerged in the liquid brain tissue during feeding, making it impossible to identify the largest ones. This may have hindered the best sampling of those maggots on brain. There were many external factors affecting the running of experiment B, leading ultimately to the loss of the original culture of flies reared from experiment A and preventing the full execution of the design for experiment B. Unfortunately, this meant that the resulting data set was smaller than anticipated, however useful conclusions were still drawn from it.
6. Conclusions

Food substrate does have a significant difference on development rate and time to pupation of *C. vicina* larvae; however that difference is reduced if the food source is swapped after 48 hours of development. Possible justifications of these findings are that the brain tissue has a higher lipid content than liver and also that brain tissue liquidises more readily during decomposition, making it easier to eat and digest.

Substrate constituent differences are just one of the many factors which need to be considered when evaluating the usefulness of laboratory data in the estimation of real case PMI. Substrate type (Kaneshrajah and Turner, 1994), larval crowding (Ireland and Turner, 2006), presence of drugs (Introna et al. 1990; 2001), temperature (Grassberger and Reiter, 2001) and process of decomposition (Campobasso et al., 2001) are some examples of factors which should be meticulously noted by the field entomologist in order to make the most accurate estimations. Studies and experiments considering these factors are extremely limited and typically only refer to one species of blow-fly. Consideration of all the different factors and indeed the effects of several factors affecting a specimen simultaneously, require further investigation.

From recent studies, including the current study, it is clear that there is a need for more accurate standard data which can be made available to forensic entomologists. This data would need to encompass many factors affecting the development of larvae of all forensically important flies, with temperature and food substrate the most important. The current standard data, although useful at certain times and temperatures (see VanLaerhoven, 2008) are relatively limited for a number of factors. An overhaul of the way blow fly larvae are reared in laboratory settings for the purposes of forensic entomology is needed. These kinds of studies would also benefit from a better understanding of the nutritional value of the food substrate being used, as this appears to have a significant effect on the development rate and may differ greatly from the same tissue in human, limiting the data even further. Investigation into the constituents of rearing tissue and their effects on larval development are also required.
7. References


8. Acknowledgements

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