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Development of immature blowflies and their application to forensic science

A thesis submitted by Donnah Marie Day for the degree of Masters of Science - Research at the University of Wollongong, NSW.

Supervisor: Dr James F. Wallman

School of Biological Sciences

January 2006

I, Donnah Marie Day, declare that the work recorded in this thesis is entirely my own effort, except where otherwise acknowledged. I also certify that this work is original and has not been previously submitted in any other course of study at this or any other institution.

Signature

Date

Student number

All nature is lovely and worthy of our reverent study.

Anonymous

ABSTRACT

Data on the development of immature blowflies and other carrion-breeding flies can assist with determination of the post-mortem interval and thus be used as a tool to help solve crime. The main focus of this research was to develop reliable data for growth at constant temperatures in larvae of *Calliphora augur*. Constant temperatures were chosen because fluctuating regimes can be location specific and may therefore have limited application. A number of other blowfly species were also studied, but only *C. augur* and *Lucilia cuprina* were used in planned experiments. Since *C. augur* is ovoviviparous, and therefore has a small clutch size, the fecund egg-laying species *Lucilia cuprina* was also cultured and used as a model for pilot experiments, in feasibility studies and to explore the broader applicability of results from trials with *C. augur*.

Some of the current ideas behind estimation of post-mortem interval using blowfly larvae have been expanded upon and prediction intervals for larvae of *C. augur* are presented. In forensic entomology, plots of this type usually present the variables differently than the strict mathematical method, where a known predictor (x) is used to estimate an unknown value of interest (y). In forensic entomology, these axes are often reversed. In my work, I have adopted a more classical mathematical method and present a way of estimating time as related to larval age (y) from the (known) somatic measurement of body length (x). Whilst this has been the main core of my work, some important practical difficulties of working in this field have also been given attention.

A way to salvage some damaged specimens has been discovered; measurement of body width at the junction of the 5th and 6th abdominal segments can be used as an alternative measurement to body length, and I present a means to convert from one measurement to the other. A paper on this topic has been published in *Forensic Science International*.

I have also explored the effect of freezing and thawing developmental media on larval growth in *Calliphora augur* and *Lucilia cuprina*, and it appears that there is no significant difference. The effect of different tissues from sheep on larval growth was also examined in the above two species, with growth on sheep's liver being slower than growth on sheep's brain or sheep's meat. A paper on this topic has been accepted by the *Journal of Forensic Sciences*.

The effect of preservative solutions on different larval stages of *C. augur*, and on the third instar larvae of *C. augur* and *L. cuprina*, was also examined. It was found that the larvae of each species reacted differently to the preservatives, as did the different instars within a

species. Ten per cent formalin and Kahle's solution effected the least change in larval body length, but when larvae were placed into preservatives alive only 10% formalin had no deleterious effect on both species. However, since 10% formalin can affect the analysis of larval DNA, it is not recommended. In fact, it appears that choosing an optimum preservative may be difficult until more work is done in this area.

While work in forensic entomology is far from straightforward, it is hoped that the decidedly practical nature of my studies will serve to equip forensic entomologists with more tools to help solve crime.

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ABBREVIATIONS

PMI	post-mortem interval	mcg	microgram
DNA	deoxyribonucleic acid	mg	milligram
sp.	species	g	gram
<i>C.</i>	<i>Calliphora</i>	mL	millilitre
<i>Ch.</i>	<i>Chrysomya</i>	h	hour
<i>L.</i>	<i>Lucilia</i>		
%	percent	R-sq	R-square
+	plus	R-sq adj	R-square adjusted
±	plus or minus	ANOVA	analysis of variance
		d.f.	degrees of freedom
		std dev.	standard deviation
pers comm.	personal communication	p=	probability
e.g.	for example	P	probability
i.e.	that is	n=	number
c.f.	compare with		
vs.	versus		
am	morning		
pm	afternoon/evening		
mm	millimetres	°C	degrees Celsius
cm	centimetres	EtOH	ethanol
km	kilometres	CO ₂	carbon dioxide
		E	east
		S	south

I sincerely hope you never have need of these findings.

CHAPTER 1

Introduction

Throughout history flies have been both venerated and detested. Maggots crawling on dead bodies are widely considered to be just another disgusting element of decay. However, maggots, flies and other insects can be used to help solve crime. The earliest account [1] is a case from thirteenth century China where a slashing murder was solved when officials observed flies congregating on the blade of a murderer's sickle, attracted there by traces of blood. The owner of the tool maintained his innocence under interrogation but confessed when he was shown that only his sickle attracted the flies. Other beneficial aspects of flies are many and include pollination, wound therapy, human food and fish bait [2]. Flies are also important decomposers [3-5]. They have been revered as the god Baalzebub and feature in the Bible as one of the plagues in the story of Exodus [in 6]. Images of flies carved into amulets have been used to ward off evil spirits and ensure prosperity. Flies have even been used as messengers of love [2].

The present day application of insects to the investigation of crime, known as forensic entomology, began with the work of several European pioneers (Bergeret, Broudel and Yovanovitch) who published in the last half of the 19th century [7]. Some observers, for example Erzinclioglu [8] have felt that the application of entomology to forensic medicine was placed on a firm footing late in the 19th century by Megnin. Megnin proposed the novel concept that a corpse exposed to air undergoes a series of eight changes, with insects colonising in successive waves in response to these changes. By identifying the insects on a corpse, Megnin was able to determine rough estimates of the time of death [7, 9]. Recognition of the species involved in their different immature stages in the succession, coupled with a knowledge of their rates of development, has continued to be used since as an indication of the age of a corpse [10]. Because of this, entomologists may be called upon to identify specimens for medico-legal purposes, particularly as an aid in establishing the time of death – a crucial factor in the investigation of murder cases [10]. The application of flies and other arthropods to forensics also includes civil matters [6, 7, 11], stored product entomology [12] and contraband trafficking [13]. In this thesis, the focus and discussion are limited to the application of flies to human death, and post-mortem interval estimation.

The term *forensic entomology* is generally applied to the study of insects and other arthropods associated with criminal events, especially murders, for the purpose of uncovering information useful to an investigation [13]. After death, the tissues of animals, including humans, are attractive to a variety of insects and other vertebrates [10]. Decomposing remains provide a temporary micro-habitat offering a progressively changing food source to a wide variety of organisms; the arthropods constitute a major element of this fauna, with insects and especially flies and their larvae, being the predominant group [14]. Blowflies are ubiquitous and are typically the first insects to visit a dead body, often well before it is discovered by humans, including police [6].

The postmortem interval, or PMI, is the period of time between the moment of death and the discovery of a body [13]. It can be an integral part of an investigation because determination of an accurate PMI can either implicate or exonerate a suspect. The PMI can also provide information related to movement of a corpse or the manner of the death [15]. Hence, the PMI can be a pivotal point in evidence in a wide range of cases including homicide or other untimely death, child neglect [16] and wildlife poaching [17]. The method used to determine the PMI is largely dependant on the state of decay of the body and how soon after death it is found. Pathology and histology are most often used to determine the PMI if a body is found in the first hours after death [18]. When a body is found from approximately two days to a month after death pathology becomes less reliable and data on the development of colonising insects are most useful [2]. The use of entomology to estimate post-mortem intervals (PMIs) in criminal investigations has been found to be more reliable than autopsy and as reliable as police eyewitness information [148]. When liver mortis, rigor mortis, body temperature or bloating can no longer be observed, for example if a body is burnt, scattered, or only partial remains are discovered, an entomological approach is an indispensable tool for estimating PMI [149]. Whilst the PMI cannot be estimated in every case, many accurate case accounts exist in the literature [16, 17, 39, 40, 118, 150, 151]. Furthermore, examination of insects at a crime scene can offer other useful information, such as linking a suspect to a crime scene, providing analysis of badly decomposed bodies, indicating movement of a body, period of neglect, and time of day and weather conditions at the time of insect colonisation [151]. Entomology can also assist in determining the manner of death [111], the victim's movements before death, the social status of the victim [92] or the presence of drugs in the body [152].

An entomologist's estimate of post-mortem interval is based on a series of generally valid assumptions, but variance in any one of these assumptions can skew the accuracy of the estimate [111]. Factors such as macro and micro habitat [112-114], the oviposition behaviour

[62, 86-88, 115] and reproductive habits [43-45, 84, 116] of different species, corpse accessibility and suitability [117-119], maggot mass heating [91], geographical variation [48, 120, 121], the influence of drugs [122-124] and food shortages [125] can all have a bearing on the estimated PMI. Indeed, the initial process of collecting and preserving maggots can also lead to error [25, 50, 109].

Given that there is a chance that erroneous PMI determinations could impede justice, and that there is a risk of this occurring when using inaccurate published data for making PMI determinations [14, 20], it is imperative that local developmental data be used where possible. Whilst work has been done on forensically important diptera in Perth [21], Brisbane [22-26], Adelaide [27], Canberra [28, 29] and Melbourne [30], not all of these data have been published. Although some of the species examined already include New South Wales in their normal distributions, there appear to be no comprehensive studies focusing specifically on populations of forensically important flies from this state. This is what I set out to address in this thesis, specifically with regard to blowfly populations from the Illawarra region.

The work presented in this thesis has been largely applied to particular practical problems. Some new ideas have been explored and alternative approaches to current practice are presented. Studies have been conducted on blowfly development and an alternative measurement to larval body length has been discovered. The effects of substrate type and the effect of freezing and thawing developmental media on larval development have been explored. The effects of different preservative solutions on different growth stages and different species have also been ascertained. I also present prediction intervals for larval growth in *Calliphora augur* – one of the commonest species associated with human remains in New South Wales [31].

CHAPTER 2

Initial Observations

When commencing this project it was important for me to determine which blowfly species were present in the local area and also to gain some familiarity with their biology and culture. To this end, initial trapping was undertaken in the local area of Wollongong and captured individuals of interest were observed in the laboratory. After identification, captured adults for observation were individually housed in plastic cages with some panels removed and replaced with fine mesh. They were maintained on tap water, raw sugar and sheep's liver. All cages were housed in a temperature-controlled room. When identification was uncertain, a captured adult was housed individually until young were produced and the adult was then pinned and identified. The finer details of the methods used in this project are the focus of Chapter 3.

2.1 Initial trapping

Preliminary trapping detected *Calliphora hilli hilli* Patton, *Calliphora fallax* Hardy, *Calliphora stygia* (Fabricius), *Calliphora augur* (Fabricius), *Lucilia cuprina* (Wiedemann), *Lucilia porphyria* (Walker), *Sarcophaga* sp., *Chrysomya rufifacies* (Macquart), *Chrysomya varipes* (Macquart) and *Musca* sp. Identification followed Wallman [32]. Practice in trapping and identifying was undertaken prior to any culturing. In the first few instances, the identities of pinned dead adults were checked by Dr James Wallman at the University of Wollongong.

2.2 Initial observations

The calliphorid and sarcophagid flies of forensic importance have holometabolous metamorphosis, i.e. the larvae pupate and emerge as a new, fully formed winged adult. There are generally three instars (two moults) prior to pupation [33, 34]. The general pattern of blowfly development is illustrated in Figure 2.1. Adult female flies will mate and produce eggs. The eggs are laid on a protein medium where they hatch into first-instar larvae. These first-instar larvae then feed and grow prior to moulting into second-instar larvae. The second-instar larvae continue to feed and grow prior to moulting into third-instar larvae.

Figure 2.1: General schema of blowfly development [Source: 35]. After mating, the adult female lays eggs (1) which hatch into first-instar larvae (2). These feed prior to moulting into second-instar larvae (3). The second instar larvae continue to feed prior to moulting into third-instar larvae (4). On completion of feeding the third-instar larvae leave the food and burrow into the soil where they pupate with puparia (5). New adult flies (6) emerge from these puparia, mature and continue the cycle.

On completion of feeding, the third-instar larvae usually migrate away from the food source and burrow into the ground where they pupate. New adult flies emerge from these pupae, mature and mate to start the cycle again.

Although a number of identification keys exist [28, 34, 36, 37], the larval forms of flies can be difficult to identify [38], especially in the younger instars. The most reliable method for identifying fly larvae found in a human corpse is to rear the larvae through to adult flies [39] but this is not always possible [39, 40]. Although newer molecular techniques are quite powerful, the technology is still expensive and DNA sequences are available for only some carrion-breeding species at the present time. Furthermore, specific gene sequences are not appropriate for all species [41, 42].

Species studied in the laboratory included *C. stygia*, *C. hilli hilli*, *C. augur*, *L. cuprina* and *Ch. rufifacies*. The initial culture of *C. stygia* was begun with adult flies from South Australia provided by Dr James Wallman. Adult *Ch. rufifacies* were kindly supplied by Dr Garry Levot. All other species were captured by trapping.

2.2.1 *Calliphora stygia*

The flies were approximately seven days old when received and housed in a cage in the temperature control room. On day seven the adult flies were provided with sheep's liver and observed to feed from liver on days seven and eight. Egg masses were observed approximately 23 hours after receipt. Two days later the chaff was observed to be teeming with second-instar larvae. The next day a mix of second- and third-instar larvae was observed, with one noticeably large larva, indicative of the phenomenon of precocious egg production [43] in this species. On day 15, the adults were given more liver and the eggs and liver were transferred to a rearing container approximately 27 hours later. These larvae began to migrate away from the food after seven days. They appeared to be light sensitive at this time. The first brown pupa was observed the next day (day 8) and the first new adults emerged at day 15. The first adult mortality was observed at day 21 (3 adults dead). At day 23 another seven adults died and by day 30 only four adults survived.

In a second observed generation of larvae light brown pupae were seen 16 days after oviposition. Light brown, brown and white pupae were seen 17 days after oviposition. Only brown pupae were observed 22 days after oviposition, with the first adult emergence observed after 25 days. Four more new adults were observed at 26 days,

with the main emergences occurring after 27 days (30+ new adults), and after 28 days (50+ new adults). The first mortalities were observed to occur after 30 days. A ventilated lid was not used with this second generation and larvae were at times observed on the sides and underside of the lid away from the food.

In a third observed generation of larvae, second instars were observed 48 hours after oviposition with third instars present after four days. Larval wandering began at day 12 and continued to day 15 and the first light brown and dark brown pupae were seen at day 16, with only dark brown pupae observed at day 19. The first emergence occurred at day 24 and the first mortality occurred at day 32.

Further studies were not conducted on *C. stygia* because the colony crashed whilst in the care of a colleague at a time when it was difficult to re-establish a colony with local stock. Furthermore, this species is reported to be facultative between ovoviviparous and multilarviparous (viviparous) [44] depending on the season [45]. The observed phenomenon of precocious egg production also detracted from its suitability for the proposed experiments. This phenomenon is detected when one larva is observed to be much larger than all the other larvae that hatch from an egg mass laid by a particular female. The final reason that this species was not pursued further was that adult activity was observed to be markedly reduced with continued captive breeding such that the animals appeared to slow down and become somewhat sluggish. This has also been observed by other workers (Dr Melanie Archer, pers comm.).

2.2.2 *Calliphora hilli hilli*

A single female *C. hilli hilli* was trapped and transferred to a cage in the temperature controlled room. Liver was provided and second instars were observed approximately 23 hours later. Third instars were observed approximately two days later. The first white pupa was seen on day 5 and wandering larvae were noticed on day seven. Brown pupae were observed at day 12 and brown dead maggots were seen at day 14. Since there were no emergences by day 20 the pupae were discarded, perhaps prematurely. The female was given more liver at day 20 but died the next day. Because this species may be confused with *C. fallax*, the dead female was then pinned and confirmed as *C. hilli hilli*.

Detailed studies were not conducted on *C. hilli hilli* because the adults failed to emerge from pupation. These were discarded to avoid contaminating the culture area with

parasitic wasps which are a main means of population control in the natural situation [29]. *Calliphora hilli hilli* has been noted as a difficult species to culture [e.g. 46] and Kamal [47] experienced difficulties with *Calliphora* sp. whereby he observed much prepupal mortality, low emergence and deformities in new adults.

2.2.3 *Calliphora augur*

Traps set out in October 2002 captured *Calliphora augur*. This species was also caught in traps in November 2002 and December 2002.

Individual females trapped at 4 pm were observed to larviposit between 5.45 and 6.00 pm inside the tied off freezer bag from the top of a trap. These larvae were placed onto liver at 6.20 pm. The next day they continued feeding and were observed to be strongly negatively phototrophic. This light sensitivity was also observed on the following two days. Third instars were observed on day three. By day six all larvae had wandered away from the liver. The individual larvae were sometimes observed to be inside a cylindrical piece of chaff making them difficult to see (Figure 2.2).

White and brown pupae were observed on day seven with brown pupae only observed at day eight. The first emergence was observed on day 16 with seven or more new adults seen. The main emergences occurred on days 17 and 18. First mortalities were observed at day 25. Eleven days after the first emergences, the adults were provided with liver, in which they showed immediate interest. The liver was changed the next day. Immediate interest was again observed and also continued the next day. The first new egg masses were observed 14 days after first emergence. These eggs did not hatch and by 28 days after first emergence all adults were dead.

A second (morning) catch of one wild-type female *C. augur* was transported to the laboratory and given liver. Eggs were transferred to a rearing container the next day. On day three third-instar larvae were observed in the rearing container. It appears that the female had laid some infertile eggs and that the larvae had already moved beneath the liver, away from the light. White pupae were first observed at day six and brown pupae were first observed at day seven. The adult female died nine days after larviposition. The first emergence (one individual) was observed at day 15 and the main emergence occurred on day 16. The new adults were provided with liver six days later and showed immediate interest, but no eggs or larvae were produced.



Figure 2.2: A dead *Calliphora augur* larva inside a cylindrical piece of chaff.
When alive they may be very difficult to see in this situation.

This cage was then supplemented with three new wild-type females and their larvae. Again, some eggs were noted and first-instars were observed beneath the liver on the second day. Third-instars were observed two days later. Pupae were noticed on day seven (but no observations were done on days four, five or six) and emergence was first observed on day 15.

It appeared that the provision of liver for oviposition was occurring too long after emergence to allow the females adequate nutrition to mature their ovaries. It was found that if liver was not available to females at or soon after emergence females did not produce viable offspring. When females of *C. augur* have mature larvae ready for laying the abdomen takes on a milky white appearance on the undersurface. It is even possible at times to see the larvae moving inside the abdomen.

Females with an abdomen with a white undersurface do not always larviposit and will at times lay eggs which do not hatch in what appears to be a kind of practice laying. The new approach desired from these initial observations was to provide liver prior to or immediately after emergence. This remedied the problem of infertile eggs and allowed for continued successful culture.

Fresh sheep's liver was provided for ovary maturation and ovi/larviposition from just prior to emergence, from emergence throughout the practice laying period, when infertile eggs are laid, until the first fertile lay of first-instar larvae. Liver was then only provided to adult flies when required for experimental or culture purposes.

2.2.4 *Lucilia cuprina*

Wild-type *Lucilia cuprina* adults were trapped in the morning and transported to the temperature controlled room. The adults were provided with sugar and water only on day one. On day three eggs had been laid on the base of the cage. These eggs became desiccated and did not hatch. On day eight the adults were provided with liver and eggs were observed the next day. These were removed to a rearing container. Third instars were observed two days later. Three days after oviposition, the third instars were observed to be dispersing into the chaff. Four days after oviposition most of the larvae had left the liver and were present in the chaff. Only a few were observed to still be feeding on the liver. Light brown pupae were observed five and six days after oviposition. Brown pupae were observed six days after oviposition and by day seven only brown pupae were observed. Thirteen days after oviposition between 40 and 80

new adults had emerged. No observations were done the previous day, so emergence may have begun then.

Four days after the observed emergence the new adults were provided with liver to which the adults were immediately attracted. Six days after emergence the first clumps of egg were observed. These hatched the following day. Five days after oviposition third-instars were observed and brown pupae were observed 11 days after oviposition. No observations were made on days seven to ten after oviposition, so pupation may have occurred earlier than 11 days. Newly emerged adults were first observed 18 days after oviposition, however no observations were done on days 13 to or 17.

One of the first-trapped adults was observed making a clicking noise 14 days after capture. This continued for four days, by which time all adults had disappeared from their cage. Adults of *L. cuprina* were found to be particularly adept at escaping from the cages, squeezing their bodies through the smallest tears in the mesh top, or through areas where the mesh top or the access sleeve had come away from the cage. Rigorous checks of all cages remedied the problem.

Similarly, *L. cuprina* larvae were also quite adept at escape, squeezing their bodies through the lip of the rearing container and the lid, even when it was properly sealed. Similarly, Mackerras [48] observed the larvae to be capable of escaping a jar with a screw top lid. He also observed the larvae to be intensely active when wandering, as was the case in my studies. On more than one occasion the wandering larvae were observed to have left the rearing container, out of the room and beneath the door, and making their way along the hallway of the building. It appears that the activity of many migrating *L. cuprina* disturbs settlement of larvae into the wheat chaff shelter and so leads to this escape activity. It does not appear to be a problem with small densities or with deeper (40 mm) chaff. This behaviour was not seen with larvae of *C. augur*.

The remedy to this was to place the rearing containers on an upturned round plastic take-away food container in a foam box containing water up to the level of the container. Any escaping larvae fell into the 'moat' of water and drowned. The larvae tended to escape the rearing containers more often when the density of larvae was greater. Culling the larvae into smaller groups in individual rearing containers also helped to stop the larvae escape from their container.

Because *L. cuprina* may be confused with *L. sericata* (indeed the two species have been observed to interbreed in captivity [48]), dead wild-type adults were pinned and confirmed as *L. cuprina*. All cohorts were separated until identity was confirmed.

While some workers report that *L. cuprina* females appear to retain protein from the larval/pupation stage and use it to mature their ovaries, thus not requiring a protein meal for ovary maturation [48], this is not fully supported in the literature [c.f. 49]. Since, on one occasion, eggs of *L. cuprina* were observed on the bottom of a mixed sex cage containing newly emerged flies which had not been given any protein, protein was provided to *L. cuprina* in the same way as for *C. augur* to minimise stress on the breeding colony.

2.2.5 *Chrysomya rufifacies*

The adult *Chrysomya rufifacies* obtained from Dr Garry Levot had been trapped in Toongabbie in Sydney. The adults were transported to the temperature controlled room and provided with fresh liver. There was some interest immediately but no eggs were laid. The next day the adults were provided with liver containing larvae of *L. cuprina*. Within 20 minutes the *L. cuprina* larvae began to leave the food and tried to escape. Approximately an hour later eggs of *Ch. rufifacies* were observed on the base of the cage. The next day the larvae of *L. cuprina* were observed on the walls and floor of the cage. These were all removed, as was the liver. Fresh liver containing first-instar *L. cuprina* was then introduced to the cage. Twenty-five minutes later, female *Ch. rufifacies* were observed on the liver with ovipositors extended. Larvae of *Ch. rufifacies* were first observed five days later. These were too large to be first-instars. These larvae did not pupate and no adult emergences were observed.

It appears that larvae of *L. cuprina* initially introduced were too large for predation. The second introduction of first instar larvae did appear to initiate oviposition but few *Ch. rufifacies* larvae hatched. Trials involving cotton wool may have been more successful but were not conducted.

Chrysomya rufifacies was studied only briefly. This species was not considered for ongoing culture and study because, while there are records of it being involved in primary strike in the north of Australia [26], it is generally regarded as a secondary coloniser, dependent upon previous infestation of carrion by other species [29]. The larvae are actively predaceous and feed both on the carrion and the healthy living

maggots of *Lucilia* and *Calliphora* spp. [29]. This might therefore have required trials with mixed species and the confounding influences of predation and competition. The limited work done in this study with this species indicated that it can be difficult to have gravid females lay under controlled conditions unless a suitable prey species is present, and the prey species needs to be at a suitable stage of development. Another reason that this species was not examined in this study was because same sex clutches have been observed [50] and the space and resources were not available to work with the numbers required to avoid this. Space restrictions also meant that cultures of predator and prey species could not be separated.

2.3 Species choice

Calliphora augur and *Lucilia cuprina* were finally selected for detailed study because:

- 1) they were relatively abundant when cultures were being set up,
- 2) they are relatively easy to identify,
- 3) both species have been recorded in crime scene samples by Dr Garry Levot [31] and Dr James Wallman (pers comm.).
- 4) problems culturing them had been easily overcome.

The initial intention was to culture and perform extensive studies on both species. Although some studies were conducted on *L. cuprina*, due to limited space and time, this species was used as a model species for preliminary trials with only some detailed studies being conducted. Larvae of *L. cuprina* were observed to feed mainly on the surface of the meat but *C. augur* larvae tended to burrow into the substrate and often needed to be carefully dissected out. Recoveries of *C. augur* were rarely 100% and some of the larvae recovered were useless because of damage inflicted when recovering them.

The main focus of this research became examination of aspects of using *C. augur* as a forensic indicator.

2.3.1 *Calliphora augur*

Adults of *Calliphora augur*, also called the lesser brown or blue bodied blowfly (Figure 2.3), have a metallic greenish shield on the abdomen [51]. This species occurs mostly in summer and females lay live young which are capable of feeding immediately [51].

It is regarded as consecutively actively viviparous [44], i.e. females control laying of larvae and always lay larvae if fertile and sperm are present. Robust and highly active first-instar hatch promptly after oviposition, giving this species a great advantage over oviparous species for exploiting small, quickly perishable carcasses [49]. Johnston and Tiegs [52] found that this species was capable of depositing eggs or maggots or both, with the eggs usually hatching within six hours. I did not observe this. Mackerras [48] noted maggots of *C. augur* breaking through the chorion when laid and found that this species takes from 10 to 14 days to become sexually mature in captivity [48]. I also observed a similar delay. Similarly to Mackerras [48] and O'Flynn [24], I observed that, whilst *C. augur* normally deposits living larvae, it will also lay soft infertile eggs for some days before normal larviposition occurs. The average number of larvae per female has previously been noted as 50 [48] or 58 maggots [49]. Callinan [53] recorded a range of between 22.5 and 98.3 larvae per female in a controlled environment and a range of between 0.3 and 45.2 larvae per female in a field environment.

Calliphora augur is a blowfly commonly encountered in forensic cases in south-eastern Australia. It is abundant, relatively easy to identify in the hand and does not lay eggs [19]. It is regarded as ovoviviparous [25] or consecutively actively viviparous, whereby females control laying of larvae and always lay larvae when fertile and sperm are present [44]. It larviposits in a scattered manner. Aside from its frequent presence in crime scene samples in Australia [31], *C. augur* has also been recorded as an agent of human myiasis [54].

Calliphora augur breeds mostly in carcasses, but will also lay in such places as wounds and weeping eyes [51]. While it has a preference for fresh carcasses [55], it has been observed to oviposit on both fresh and putrid liver [48] and maggots have been found in sour milk, grain, old bags, and dead insects [29]. It deposits its maggots under the slightest stimulus [29], even on glass if confined to small glass tubes [48]. Females larviposit in a scattered, non-communal manner.

Calliphora augur is most abundant in south-eastern Australia and does not occur in the western half [49]. *Calliphora augur* largely replaces *C. dubia (nociva)*, its Western Australian sister species, in south-eastern Australia [56]. These two species will interbreed in captivity but there is no F₂ generation from the hybrid and no evidence to suggest hybridisation in nature [55].

Figure 2.3: Adult *Calliphora augur* (source: [51]).

Aside from its importance to forensic medicine, *C. augur* has also been recorded as a primary strike fly in sheep [28, 49] and has been listed as a primary and secondary facultative agent of myiasis by Hall and Wall [57]. It is a wide ranging fly with maggots obtained from sheep in Canberra (ACT), Albury, Jerilderie, Inverell (NSW) [28], and Thallon and Moggill (Qld) [22, 24] and from human bodies indoors in Victoria [58] and New South Wales [31]. It is commonly found in houses [49, 59] and has also been recorded in human myiasis in Australia [54].

Descriptions and illustrations of the various growth stages of *C. augur* are available in Levot [51], O'Flynn [24], Fuller [28] and Wallman [37]. Studies on the development of this species have been undertaken by Johnston [23], Mackerras [48], Levot [60] and O'Flynn [25].

2.3.2 *Lucilia cuprina*

Adults of *L. cuprina*, the Australian sheep blowfly (Figure 2.4), are a metallic green/bronze colour [51]. This species is oviparous and females prefer to oviposit in communal laying sites being used by other females of the same species [61]. These communal laying sites used by *Lucilia cuprina* have high humidity and low illuminance [62]. *Lucilia cuprina* does not appear to exhibit precocious egg development [c.f. *Lucilia sericata* in 19]. It is sometimes referred to as *Phaenicia cuprina* and is cosmopolitan, being widely distributed from the Mediterranean to the Oriental Region, throughout the Afrotropical Region (including Madagascar, Réunion and Mauritius), and the Australian, Nearctic and Neotropical Regions [10]. Most adult *L. cuprina* do not migrate more than 1-2 km in their lifetime [59]. They migrate from carrion at night as post-feeding larvae, overwintering in this form and pupating the following spring [59].

Lucilia cuprina was first found in New Zealand in the early 1980s and now occurs in 62% of flystrike cases in that country [63]. In Australia, *L. cuprina* has displaced *L. sericata* in urban Brisbane and Sydney [pers comm. M J Rice, in 63].

Studies on this species have been done by Mackerras [48, 64], Levot [60, 65], Vogt and Woodburn [66], O'Flynn [25], Williams [67], Dallwitz [68] and Nishida [69].

Figure 2.4: Adult *Lucilia cuprina* (source: [51]).

2.4 Working cultures

The working cultures of *L. cuprina* and *C. augur* were established from individuals trapped in the Bellambi and Woonona areas of Wollongong, respectively. The cultures were refreshed with the addition of wild type individuals whenever possible.

CHAPTER 3

General Methods

These studies were conducted at Wollongong (34° 25' S, 150°53' E) in New South Wales, Australia.

3.1 Trapping

In order to determine which dipteran species could be important in the region, the initial stages of the study involved trapping for blowflies. The traps used to catch live flies were similar in design to the 'West Australian' blowfly trap, first described by Newman and Clarke [70]. Williams [71] and Cole [72] improved on the original design to meet their own needs, and a similar trap was described by Anderson [73].

The traps consisted of a lure, an inverted cone with a small hole at the apex and a sink from which the flies cannot escape once captured. The lure was placed inside an empty black plastic plant pot with holes in the bottom. The flies were attracted to the lure, entered the holes of the pot to feed from or lay upon the lure, and then flew up into the inverted cone. Once inside the cone they were channelled up toward the light and through the apex into a clear plastic freezer bag from which they were then collected. The freezer bag was secured to the PVC tubing with a large elastic band. One corner of the freezer bag was snipped off to allow the removal of individual flies without disturbing the trap. The snipped off corner was securely closed with a clothes peg when required. The small apex of the cone meant that, once through the apex, flies usually did not manage to get back to the bait but rather remained trapped in the upper reaches of the trap.

The inverted cone was made from fine flymesh in a similar way to that described by Newman and Clark [70] and was placed within a length of 150 mm diameter PVC tubing. The lengths of tubing varied between 200 mm and 250 mm. The end of the PVC tubing with the base of the cone was placed snugly into a standard black 150 mm diameter plant pot such that the holes at the base of the pot were not covered. The base of the black plastic plant pot was painted yellow to enhance the attractiveness of the trap [71].

Ant invasion happened rarely due to the traps being elevated by bamboo stilts in a similar way to that described by Anderson [73] or suspended in trees with coathanger wire. If traps were invaded by ants, trapping was discontinued for a few days, the area washed down to remove the ant trail and subsequent trapping ensued with the stilts placed in trays of water. A few drops of detergent was sometimes added to break the surface tension of the water.

Meat lures were placed inside a 'dixie' cup inside the trap on a small hard plastic platform raised from the base of the pot by pieces of coathanger wire bent into a squarish upside down 'U' shape. The flies were not excluded from the bait because initial trapping was for the purpose of familiarity and did not target gravid females alone. The flies could have been excluded from the bait with slight modification if needed. A photograph of the traps used is shown in Figure 3.1. A schematic of a similarly designed trap is shown in Figure 3.2.

Sheep products were most commonly chosen for bait because the species of forensic interest have a well documented history of flystrike in sheep [22, 49, 59, 63, 74, 75], but other protein sources were also used. Although sodium sulfide can enhance and prolong the attractiveness of a lure [71, 76], and is used widely in both Australia and New Zealand for control trapping and research [66, 72, 74, 77], it was not used here because sufficient numbers of flies for identification and culture establishment were caught without it.

Trapped individual adults for culture were conveyed to the university within two hours of capture. If transport was delayed for any reason, a wad of wet cotton wool was placed inside the freezer bag and the bag was tied off. Trapped individual adults for identification were killed with acetone fumes and pinned.

3.2 Materials and Equipment

3.2.1 Culture establishment

The cultures of *Calliphora augur* and *Lucilia cuprina* used for these studies were established from individuals trapped in Wollongong, New South Wales (34° 25' S, 150°55' E). Identification followed Wallman [32].

3.2.2 Culture

To begin the cultures, wild-type flies (F_0) were introduced to a cage in a temperature-controlled room. The offspring of these flies were collected on sheep's liver and placed into rearing containers with excess liver. The larvae were allowed to grow and pupate and then the pupae were manually sifted from the wheat chaff and placed inside a new cage labelled F_1 .



Figure 3.1: Trap used to capture adult flies.

Figure 3.2: Trap design (similar to the one used in this study) [source: 72].

The flies were provided with a protein source for ovary maturation and then this process was repeated for each subsequent generation. The cultures were maintained out of phase such that each generation comprised a number of emergences separated over time to provide a mix of different aged gravid females. Generations were conserved in that no intergenerational mating occurred, except when newly trapped wild-type individuals were added to the culture.

The cages containing adults and larval rearing containers were kept on shelving in a room maintained at $25 \pm 3.5^\circ\text{C}$ (range 24.0 to 28.5°C) and ambient humidity. This room was well ventilated to prevent the accumulation of excess ammonia that may affect larval development (Dr Garry Levot, pers comm.). Cultures were kept in a 12:12 light:dark light regime with a 15 minute 'dusk' transition period of low light between the shift from light to dark.

3.2.3 *Adult cages*

Adult flies for culture were maintained in square plastic cages measuring 330 mm long, 220 mm wide and 250 mm high (external dimensions) (Figure 3.3 a). The plastic of the tops of the cages was cut away and replaced with fine mesh to allow adequate ventilation (Figure 3.3 b).

A hole was also cut at the front of the cages and a sleeve of fine mesh which could be tied off was attached to allow access to the cage for the provision of food and water without flies escaping (Figure 3.3 a). Protein was also added and removed from the cages using this access port.

Petri dishes were used for the sugar and reused as needed (Figure 3.4 a). Plastic 50 g weigh boats, which were inexpensive and readily replaced, were used to contain the protein (Figure 3.4 b). Small round plastic containers with screw-on lids and a dental wick inserted snugly through a hole in the middle of the lid provided water (Figure 3.4 (c)) and a source of moisture that many flies could use concurrently without risk of drowning. The flies in each cage were provided with water and raw sugar *ad libidum*. Fresh sheep's liver was provided as a protein source for ovary maturation and ovi/larviposition.



a) front view



b) top view

Figure 3.3: Adult fly culture cages.



a) petri dish with raw sugar



a) weigh boat containing liver



b) water container with dental wick

Figure 3.4: Materials used for fly culture maintenance.

Sheep's liver and other protein products were purchased fresh in bulk, cut into suitably sized portions and frozen at -20°C. Each sheep's liver gave approximately eight to twelve 50 g pieces of liver, depending on its size and degree of bloodiness. All protein presented to females was thawed and equilibrated to room temperature prior to use.

3.2.4 *Larval growth*

Square white two litre ice cream containers, measuring 170 mm wide along each side and 90 mm deep (external measurements), were used to rear larvae (Figure 3.5). Most of the centre of each lid of the containers was cut away and replaced with very fine mesh, allowing ventilation but preventing escape of larvae. The collected offspring from any given lay were placed inside a rearing container with excess (~ 50 g) liver in a weigh boat. Wells and Kurahashi [78] have noted that larvae may fail to pupate if they have no shelter or are very wet. The weigh boats sat above a 2 cm deep base of wheat chaff (Figure 3.6), which provided dry shelter for pupation. If the layer of chaff was too deep, much chaff was wasted and it took much longer to sort for pupae. If the layer of chaff was too shallow, it was observed that some larvae failed to pupate properly or attempted to leave the rearing containers, climbing up the sides and onto the underside of the lid. When the larvae had finished feeding and migrated away from the food source, they entered the chaff and settled there. After pupation had occurred, pupae were sorted from the chaff manually and transferred to a weigh boat. The pupae were then sprinkled with a small amount of chaff, sufficient to cover them, and transferred to a cage containing sugar and water until emergence.

3.2.5 *Waste management*

After each procedure surfaces were wiped down with 75% or 80% EtOH. All items for washing-up were cleaned with very hot water and detergent, rinsed extensively to remove all detergent residues [79] and air-dried. Since no cleaners frequented the animal house, the floor was regularly swept and mopped to remove chaff, sugar, traces of blood and other dirt.

The spent chaff was discarded and never reused. All waste or surplus material was frozen and held at -20° C prior to disposal to ensure that any excess larvae or pupae were dead. Cultured adult flies needing to be euthanased were destroyed in the same way.



Figure 3.5: Larval rearing containers for flies.



Figure 3.6: Wheat chaff shelter used to line base of larval rearing containers.

3.2.6 *Temperature-controlled-cabinet*

Most of the experiments were conducted inside an Axyos (Brisbane, Australia) environmental chamber fitted with a Eurotherm 2604 temperature-control unit (Quantum Scientific). At the conclusion of each trial in the temperature cabinet, the cabinet was reset to the next temperature and allowed to equilibrate for two days prior to beginning the next temperature trial. Rearing containers inside the growth cabinet were maintained in continual darkness.

3.3 **Sample handling and preservation**

3.3.1 *Sample handling*

There are many factors that confound larval development. Reports on the effects of handling appear to vary [23, 73, 80]. In early studies, larvae of both *L. cuprina* and *C. augur* (but particularly *L. cuprina*) were observed to reanimate from the shrunk white prepupae stage when disturbed. This is in contrast to the observations of others [23, 80]. It was decided to use unhandled replicates in all trials, and unhandled controls where handling was necessary, e.g. the preservative experiments.

Larvae less than 7 mm long were handled with a fine artist's or horsehair brush. Larvae greater than 7 mm long could be handled carefully with flat-headed forceps. Larvae were killed with boiling water [81], to produce maximum elongation, which is comparable to maximum extension of a live larva [82]. The larvae were then patted dry gently and preserved in 80% EtOH [83]. When transferring larvae <7 mm long from the feeding substrate to boiling water for killing, the brush was dipped in water at room temperature. When transferring larvae <7 mm from blotting paper to 80% EtOH for preservation, the brush was dipped in 80% EtOH to avoid diluting the preservative and therefore the amount of shrinkage and thence final measurements.

Larvae which had been placed into vials head-first, and larvae which were head-down when killed, showed a characteristic curled-head shape (Figure 3.7) and could not be measured accurately. All larvae were therefore placed into vials posterior-end first to help minimise this. Swirling the larvae in the killing solution, e.g. boiling water, also helped to minimise head-curling.



Figure 3.7: Head-curling in larvae.

The top larva shows the natural camber of a dead maggot.
The bottom larva demonstrates the head-curling phenomenon.

3.3.2 *Mixing and storage of preservative solutions*

80% EtOH for preservation of immature stages of flies was prepared using a measuring cylinder to decant the desired volume of 100% EtOH. This was then mixed with the appropriate volume of deionised water when it was available. If deionised water was not available, tap water was used.

3.4 **Measurement, data handling and statistics**

3.4.1 *Larvae and pupae*

Larvae and pupae were measured with Mitutoyo (Kawasaki, Japan) Absolute digimatic digital callipers. For individuals under 12 mm, measurement was done using a dissecting microscope to ensure the larva was not being crushed in the teeth of the callipers, and that the teeth of the callipers did actually make contact with the correct parts of the larva. Measurements of body length were done as shown in Figure 3.8. Length was measured as the distance, viewed laterally, between the most distal parts of the head and last abdominal segment.

At collection, all individuals were lifted off or dissected out of the developmental medium. On occasion it was difficult to collect all larvae, particularly second- and early third-instars. Attempts to improve collection involved flooding the substrate with tap water at room temperature in order to find and collect the larvae. Initially, the influx of water appeared to make the larvae return to the surface, presumably to avoid drowning [84] but this practice was discontinued when it became apparent that some larvae were drowning inside the liver. A new approach of pulsating the liver with flat-headed tweezers was found to discourage feeding and encourage migration away from the food, thus making collection easier.

Growth stage (i.e. pre- or post- feeding) and/or instar number were recorded. Instars were differentiated based upon the illustrations of Johl and Anderson [85]. Where growth stages differed behaviourally (i.e. feeding third-instars and wandering third-instars) the different types observed were preserved in separate vials. Post-feeding or wandering larvae were defined as those that had moved away from the liver and present in the chaff.

Figure 3.8: Axes of measurement for body length (all species).
[Source: 37]. H=head, TS=thoracic segment, AS=abdominal segment.

3.4.2 *Temperature*

The ambient temperature within the temperature-controlled cabinet was monitored with small dataloggers (iButtons; Maxin Integrated Products, Sunnyvale, USA) at all stages of the cabinet trials. A manual Checkpoint 1 temperature probe was also used to gather data on temperature in some experiments.

3.4.3 *Humidity*

The temperature-controlled cabinet was set at $60 \pm 5\%$ humidity. Readings were recorded regularly and all readings were recorded manually just prior to sample removal so as to indicate the humidity samples had been growing at. Humidity was usually recorded by the cabinet's sensor, by another sensor placed inside the temperature controlled cabinet and by a wet bulb hydrometer, also placed inside the cabinet.

3.4.4 *Data handling and statistics*

Temperature data from the iButtons were downloaded with an iButton-TMEX program. These data were then imported into Excel® (Microsoft Corporation, Redmond, USA). Excel® was also used to enter raw data and plot basic graphs. JMP® (SAS Institute Inc., Cary, USA) and Genstat® (VSN International Ltd, Hemel Hempstead, UK) was used for analyses.

CHAPTER 4

Pilot Experiments

Summary:

A number of pilot experiments were conducted. These have been grouped together in this chapter to illustrate the basis for the chapters that follow. This chapter will help the reader understand the rationale for conducting the various experiments in this study.

Since the development of *Calliphora augur* larvae (Chapter 8) was the strongest focus of my work, these studies were begun very early and were done over the length of my candidature. The studies on preservatives (Chapter 7) and different protein types as larval fodder (Chapter 6) were conducted after commencement of the developmental studies on *C. augur* larvae. The discovery of body width as a useful alternative to body length (Chapter 5) also occurred after the main studies had been commenced. Improvement in techniques from the pilot experiments fortunately meant that I did not need to use the conversion from width to length. The fresh/frozen trials (Chapter 6) were done to validate the techniques being used in my main study.

4.1 Experiment A - Egg hatch and development in *Lucilia cuprina*

It was necessary to determine egg hatching times for *L. cuprina* prior to running any development studies to allow for controlled counts of early first-instar larvae. Initially, cages containing gravid females and males of *L. cuprina* were presented with a portion of liver at room temperature for one hour to permit females to oviposit. Groups of a known number of eggs were then transferred to a new piece of liver, which was placed inside the temperature-controlled cabinet at 20°C. These eggs did not hatch.

A new approach was therefore taken, whereby whole cohorts of eggs, containing groups of eggs from multiple females, were removed from the adult cage after allowing one hour for oviposition. After oviposition, the undisturbed eggs were placed inside the cabinet at 20°C immediately and then monitored for hatching. Observations were done hourly where possible. This process was repeated five times.

The times chosen to give females liver for oviposition were based upon observations from trapping, but also from consideration of the fact that flies mostly do not oviposit at night [86] [but see 87, 88], and can be inactive in cool and hot, sunny conditions [84]. The times chosen were mid-morning and mid-afternoon, i.e. 9-10 am and 4-5 pm, although oviposition was also observed around dusk in summer. Obviously, seasonal differences in peak laying times are likely to occur. The times chosen in this study may not be the peak periods and daylight savings adjustments were not made. Attempts were made to have females oviposit in the dark. However, they remained resting on the top of the cage and did not show interest in the liver.

The numbers hatched per hour are shown in Table 4.1. At 20°C, *L. cuprina* eggs began to hatch (i.e. first-instar larvae were apparent) at approximately 22 hours (the earliest was 21 hours). At 23 hours 30+ eggs had hatched in two trials but some took longer, e.g. 26 hours in trials 1 and 4. From this it was decided to use 26 hours as a guide to hatching time for eggs of *L. cuprina* at 20°C. These preliminary trials possibly should have been extended to examine broader time frames.

Hatching times for eggs of *L. cuprina* were also examined at 15°C. This temperature was examined because the temperature and development trials for *Calliphora augur* had begun at that temperature and only one temperature-controlled cabinet was available. O'Flynn [25] observed eggs of *L. cuprina* hatching at approximately 45 hours at 15°C, so the eggs were left undisturbed for 40 hours before observations began. Results are shown in Table 4.2.

Table 4.1: Number of *Lucilia cuprina* larvae hatching per hour at 20°C.

(n.d.=no data; n.c.=no change)

Hours	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
21	n.d.	n.d.	n.d.	2	n.d.
22	n.d.	4	2	2	6
23	n.d.	4	30+	10	30+
24	6+	6	30+	15	n.c.
25	12+	15	30+	15	n.d.
26	30+	8	n.c.	30+	n.d.
27	30+	10	n.d.	n.c.	n.d.
28	n.c.	n.c.	n.d.	n.d.	n.d.

Table 4.2: Number of *Lucilia cuprina* larvae hatching per hour at 15°C.

(n.c.=no change; n.d.=no data; white indicates the eggs are not yet ready to hatch; opaque indicates the egg is nearly ready to hatch)

Hours	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
39	n.d.	n.d.	n.d.	n.d.	white
40	n.d.	n.d.	n.d.	white	white
41	n.d.	n.d.	white	white	white
42	n.d.	opaque	white	white	n.c.
43	opaque	~20	opaque	n.c.	opaque
44	~10	~50	n.c.	~10	opaque
45	~20	n.c.	n.c.	n.c.	n.c.
46	n.c.	½ egg mass	~10	~50	n.c.
47	n.c.	n.c.	n.c.	~100	few
48	½ egg mass	¾ egg mass	n.c.	n.c.	~50
49	¾ egg mass	n.c.	n.c.	n.c.	~100
50	n.c.	n.c.	n.c.	n.c.	n.c.
51	n.c.	n.c.	n.c.	n.d.	n.d.
52	n.c.	n.c.	n.d.	n.d.	n.d.
53	n.c.	n.d.	n.d.	n.d.	n.d.

Hatches began at 43, 44, 46 and 47 hours – 44 hours was the most common. Most hatching occurred at 46, 47 or 48 hours. The few eggs which had been oviposited in a scattered way hatched first if they did not become desiccated. In masses of eggs, hatching occurred first around the edges.

These experiments were not continued with because the focus of the main study had shifted to development in *Calliphora augur* and space and resources were limited. When *L. cuprina* was required as a model, e.g. in pilot trials for other experiments, eggs were incubated at 20°C.

4.2 Experiment B - Average cohort size in *Calliphora augur*

The average number of larvae per female has previously been noted as 50 [48] or 58 maggots [49]. Callinan [53] recorded an average range of between 22.5 and 98.3 larvae per female in a controlled environment and between 0.3 and 45.2 larvae per female in a field environment. Because these ranges appeared to be quite large, a small study was set up to determine an average cohort size for the culture I would be working with.

A cage containing gravid females and males of mixed ages was provided an approximately 50 g piece of thawed sheep's liver which had equilibrated to room temperature. As the flies settled on the liver, individuals were collected and transferred to 50 mL specimen pots containing approximately 10 g of sheep's liver. These were then sealed. 20 adult flies were collected in this way.

Counts of larvae were done the following day. Five flies laid masses of infertile eggs, 10 flies died without laying and five flies larviposited with an average of 58 ± 14 per gravid female. The range was between 34 and 72 larvae.

4.3 Experiment C - Minimum larval density to induce elevated temperatures

There are a number of accounts of elevated temperatures due to endogenous heat from a feeding maggot mass [e.g. 27, 89, 90, 91]. In order to determine growth at constant temperatures, attempts were made to ensure that the developing larvae were in fact exposed to the temperature being examined, and that temperature elevation from maggot mass heating was not occurring. To do this several small density trials were constructed to examine the effect of known numbers of larvae, i.e. a known larval density, on the temperature of developing maggots. New larvae of *C. augur* were placed on excess liver in the following densities: 0, 25 and 50. Temperatures were monitored with a manual probe. Readings were taken daily.

Temperatures in a rearing container holding chaff and meat only were also measured concurrently. This experiment was conducted in the temperature-controlled cabinet.

To examine the number of larvae which could elevate temperature, three dataloggers (iButtons) were glued to the base of 50 g plastic weigh boats underneath a volume of sheep's liver which filled the volume of the container, away from the light, so as to record the temperature where the larvae were actively feeding (larvae were observed to be negatively phototrophic). A manual probe was used to take periodic readings to validate the iButtons. When the larvae had finished feeding and migrated away from the liver to pupate the dataloggers were collected and cleaned, and the data were downloaded. The temperatures recorded by manual probe and clusters of three iButtons for the different densities of *C. augur* larvae are summarised in Table 4.3. The full data recorded by the iButtons are provided in Appendix I.

Although the larvae were observed to move away from the probe when measuring, slight thermal heating was detected with as few as 25 larvae of *C. augur* in excess liver. Slight heating was also observed with the density of 50 larvae.

The larvae were observed to not always be in direct contact with the iButton clusters, having at times invaded the liver itself such that larvae were feeding inside the piece of liver rather than underneath. Slight temperature elevations were detected by the iButtons. Examination of the data collected by the iButtons indicates that elevations of approximately 0.5°C were prolonged for the density of 25 but not 50 larvae. The peaks in the iButton data correlate with opening the rearing containers to take manual probe temperature readings and are probably caused by warm ambient air entering the rearing container during the probing process. Without access to a constant temperature room it was not possible to examine the effects of density in more detail.

Because the difference in number between the two treatments was large (25) another trial was set up to examine smaller increments of density. Unfortunately, at that time it was difficult to obtain the large numbers of *C. augur* larvae required, so the second trial was conducted with the much more easily obtained larvae of *L. cuprina*. The densities examined were 0, 10, 20, 30 and 40 larvae. Larvae of the same age were transferred to liver when newly hatched. Temperatures in a control rearing container with chaff and meat were measured concurrently, as was a rearing container with chaff only.

Table 4.3: Temperatures in degrees Celsius recorded by manual probe and iButton for different densities of *Calliphora augur* larvae grown on excess liver. (μ =mean).

Time (h)	Density					
	0		25		50	
	Manual probe	iButton μ (n=3)	Manual probe	iButton μ (n=3)	Manual probe	iButton μ (n=3)
0	23.6	19.5	23.6	19.2	23.6	19.5
24	19.7	19.5	20.0	19.6	19.8	19.5
48	19.9	20.0	20.0	20.0	20.4	19.8
61	20.0	19.8	20.4	19.6	20.4	19.8
72	20.0	19.8	20.0	19.6	19.9	19.5
85	20.1	19.8	19.8	19.6	19.8	19.5

The dataloggers failed in the controls for the *L. cuprina* trial and so these data cannot be used. Since the dataloggers failed in the *L. cuprina* trial, trials planned to examine the effect of densities in increments of 10 individuals for *C. augur* had to also be abandoned and it was necessary to make decisions about experimental design on the available data only.

Since it was not possible to confidently conclude that densities of 20 or 50 larvae would not increase developmental temperatures above ambient, and given the small observed clutch size of *C. augur*, it was concluded that a sample size of 10 would be used in the developmental studies to avoid temperature elevation due to maggot mass agglomeration. The experimental design for the development studies on *C. augur* was therefore altered from three replicates of 20 larvae to three replicates of 10 larvae with an additional three replicates of 10 larvae for every 24-hour period.

4.4 Experiment D - Growth of *Calliphora augur* larvae on fresh substrate compared with frozen/thawed substrate

As there has been no examination of the effect of freezing and thawing larval substrate in developmental studies, and because substrates were being purchased in bulk necessitating freezing, I wanted to ensure that freezing and thawing protein given to larvae did not unduly influence their growth. These experiments sought to validate the techniques being used in the developmental studies on *C. augur* larvae.

A small experiment was set up using *C. augur* larvae to explore this idea. Three fresh lamb's livers were cut into 12 pieces each and randomised into two groups such that half the pieces from each liver were in each group. The contents from one group were kept fresh at 4°C for two days in a refrigerator. The contents of the other group were frozen to -20°C for two days. On the third day the frozen liver pieces were allowed to thaw, and these and the refrigerated pieces were then all allowed to equilibrate to room temperature. A cage containing gravid females and males was presented with a portion of liver from another source, and the females allowed one hour to oviposit. Enough larvae were obtained to conduct the experiment from the one cohort. Counts of approximately 20 new larvae were allocated to each treatment. Treatments were not replicated. This experiment was run in the temperature-controlled room.

Placement of samples on the rack inside the temperature-controlled room was randomised by lottery as follows (the numbers indicate the day on which the larvae were to be killed):

Left	8	3	5	6	1	2	7	9	4	Right
Front	FT	Fr	FT	FT	FT	Fr	FT	Fr	Fr	Front
Back	Fr	FT	Fr	Fr	Fr	FT	Fr	FT	FT	Back

Fr=fresh; FT=frozen/thawed

Day allocations were also chosen by lottery. Day 0 samples were killed and preserved immediately. Each of the fresh/frozen pairs was removed together on the day allocated. The day eight and nine samples were missed but reallocated to days nine and 11, respectively. Results for the fresh/frozen pairs are shown in Figures 4.1 and 4.2, and Table 4.4.

Plots of the raw data (Figures 4.1 and 4.2) show similar curves with peak feeding at day five and shrinkage apparent from day six. Instar moulting appeared to be at the same time but no transitional forms between instars were detected. Pupae were first detected in the fresh treatment on day six and in the frozen/thawed treatment on day seven. Statistically significant differences in mean larval/pupal body length were detected on days one, three, six and 11.

There were no statistically significant differences in the mean body/pupal lengths of the treatments on days 0, two, five, seven and nine. The day four frozen/thawed sample had a mouldy growth in it and was unusable.

Since these results indicated that freezing developmental media may have an effect on larval growth, other, replicated, experiments were designed. The details and results of these further experiments comprise Chapter 6.

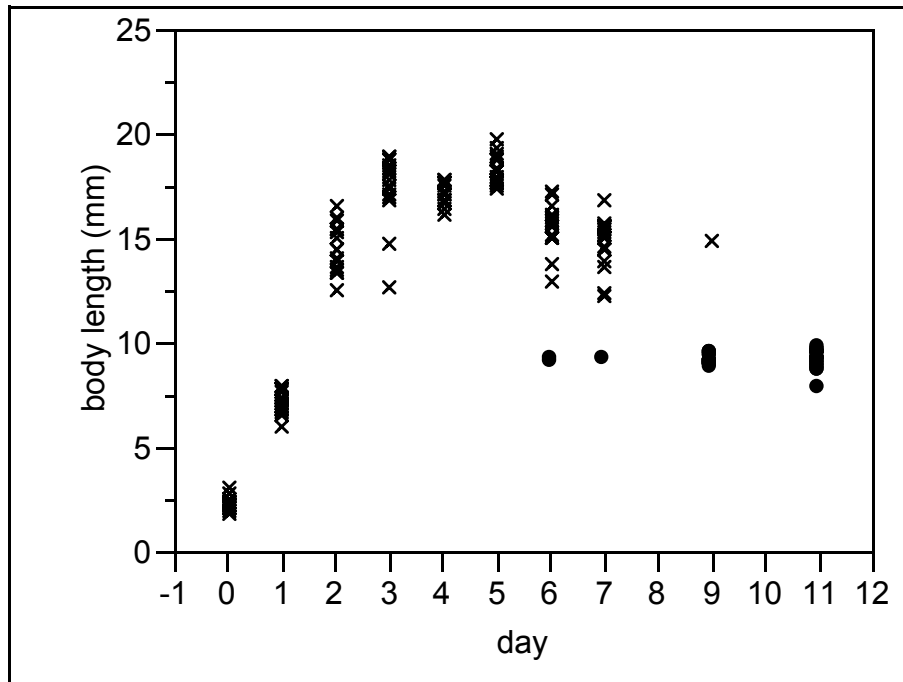


Figure 4.1: Growth of *Calliphora augur* larvae on fresh sheep's liver.

Legend: x = larvae, • = pupae

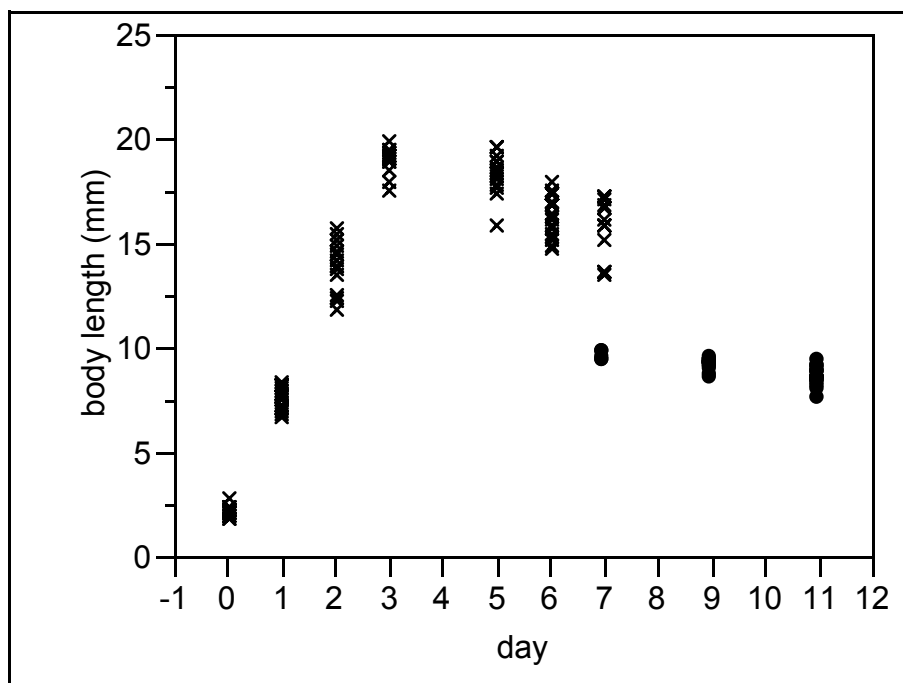


Figure 4.2: Growth of *Calliphora augur* larvae on frozen/thawed sheep's liver.

Legend: x = larvae, • = pupae

Table 4.4: Growth of *Calliphora augur* larvae on fresh vs. frozen/thawed sheep's liver. 1st=first-instar, 2nd=second-instar, 3rd =third-instar, WP=white pupae, LBP=light brown pupae, BP=brown pupae, n.d.=no data, NA=no analysis.

	Fresh sheep's liver			Frozen/thawed sheep's liver			
Day	Growth stage	Mean body length (mm)	Range and N	Growth stage	Mean body length (mm)	Range N	P
0	1 st	2.206	1.68-2.92 (18)	1 st	2.12	1.73-2.73 (24)	0.2514
1	2 nd	7.11	5.92-7.86 (20)	2 nd	7.51	6.65-8.32 (20)	0.0216
2	3 rd	14.46	12.37-16.44 (16)	3 rd	13.87	11.79-15.59 (16)	0.1661
3	3 rd	17.41	12.60-18.81 (17)	3 rd	18.91	17.48-19.73 (16)	0.0015
4	3 rd	17.13	16.09-17.72 (15)	n.d.	n.d.	n.d.	NA
5	3 rd	18.19	17.26-19.62 (19)	3 rd	18.23	15.82-19.57 (15)	0.8242
6	3 rd	15.46	12.86-17.21 (15)	3 rd	16.33	14.6-17.89 (20)	0.0197
6	BP	9.36	9.30-9.41 (2)	BP	n.d.		NA
7	3 rd	14.81	12.20-16.75 (18)	3 rd	15.64	13.37-17.17 (12)	0.0927
7	WP	n.d.		WP	9.75	9.60-9.89 (2)	NA
7	LBP	9.35	9.35 (1)	LBP	9.685	9.5-9.87 (2)	NA
7	BP	n.d.		BP	9.49	9.49 (1)	NA
9	3 rd	14.78	14.78 (1)	n.d.			NA
9	BP	9.27	8.91-9.69 (18)	BP	9.31	8.72-9.59 (18)	0.5943
11	BP	9.34	8.00-10.00 (22)	BP	8.72	7.76-9.45 (19)	<0.0001

4.5 Experiment E - Larval growth on different tissue substrates

Erzinclioglu [92] discussed the possibility of chemical differences between animal tissues and human tissues but experiments to evaluate these differences have not yet been performed. Previously, developmental studies on fly larvae have used various types of liver, including beef [46, 73, 93], pork [78], ox [60], lamb [94] and a mix of minced ox liver and jelly meat [21]. Other workers have used fish [95], pet mince (a mixture of muscle and offal) [25], mammalian muscle [33], (unspecified) meat [96], lean pork [97-100], ground beef [101, 102] and mouse carcasses [103]. Few studies have compared larval preference for different substrates prior to rearing [97-100] and only one study examined the suitability of different substrates for both oviposition and rearing [47]. None of these comparative studies have included sheep products and, since the species of interest in this study are species which are locally common in sheep myiasis, and in light of ethical problems with using human flesh for comparison, it was decided to use sheep's liver for rearing larvae for developmental studies on *Calliphora augur*. Economics, availability and a need for conservative estimations also strongly influenced this decision, notwithstanding the fact that liver is the most common fodder used in developmental studies on Calliphoridae larvae. This was not run as a design validation study because there was no *a priori* reason to suspect that previous workers had not been using optimal media.

4.5.1 Choice of substrates

Maggot invasion of a corpse often occurs through the eyes and into the skull cavity [50]. Hence, for a large part of their development, such larvae are feeding on brain. In a wound situation, the larvae may be feeding on muscle and the associated epithelial or adipose tissue. The tissues chosen for comparison were sheep's liver, sheep's meat in the form of lamb chops, and sheep's brains.

4.5.2 Observed growth of *Lucilia cuprina* larvae on different tissue substrates

The first trial was conducted in the temperature-controlled room and involved observation of larvae only. Second-instars were observed on day two in all fodder types, although the larvae in brain appeared smaller. Second-instars were observed on day three in all fodder types, with those grown on meat appearing slightly larger than those grown on brain or liver. On day four the larvae grown on liver were difficult to locate but still appeared to be second-instars. Those grown on meat and brain were third-instars. A hard crust had formed on the top of all substrate types. On day five the larvae grown on liver still appeared to be in their second-instar. Those grown on meat and liver were in their third-instar. On day six I was unable to locate the larvae growing

on liver without extensive disruption. Those growing on meat and brain were all third-instars. On day seven third-instar larvae were observed growing on liver. Those growing on meat were wandering away from the food. Those growing on brain were also wandering away from the food and some were nearly sessile. On day eight I was unable to locate any larvae without extensive disruption of their food. On day nine the larvae growing on liver were still third-instars. Those growing on meat and brain were post-feeding third-instar larvae or young pupae (white in colour).

4.5.3 *Measured growth of Lucilia cuprina larvae on different tissue substrates*

Lucilia cuprina larvae were used once again. Each protein type was given in excess at the beginning of the experiment such that the feeding maggots would feed on progressively decayed protein, as they would on normal carrion. The experiment was run in duplicate at 25°C. Eggs were collected on sheep's liver over a one-hour period of non-continuous laying. The eggs were then removed and left undisturbed for 48 hours. After 48 hours approximately 70 first-instar larvae were transferred to each rearing container. Samples of approximately ten larvae (range 2-19) were then collected at approximately 12-hour intervals.

The average pooled sample sizes were eight for brain, ten for liver and nine for meat. The total number of animals collected was 95 from brain, 129 from liver and 103 from meat. Results are shown in Figures 4.3-5 and summarised in Table 4.5.

Interestingly, it was found that the highest average larval length was achieved fastest with meat (111 h). Whilst larvae grown on brain and liver both had longer average larval lengths than larvae grown on meat at 135 hours, the average for those individuals grown on liver was smaller than those grown on brain.

No significant difference was detected between the groups at 135 hours but a significant difference was detected between the groups at 111 hours. Statistically significant differences were consistently detected between the groups up to 111 hours. The exceptions were at 0 and at 88 hours. Statistically significant differences were not detected between the groups at 135, 171 and 197 hours but the numbers collected after 111 hours are so small statistical tests are not likely to be reliable. This pattern of diminishing numbers was caused by it being progressively more difficult with time to find the larvae.

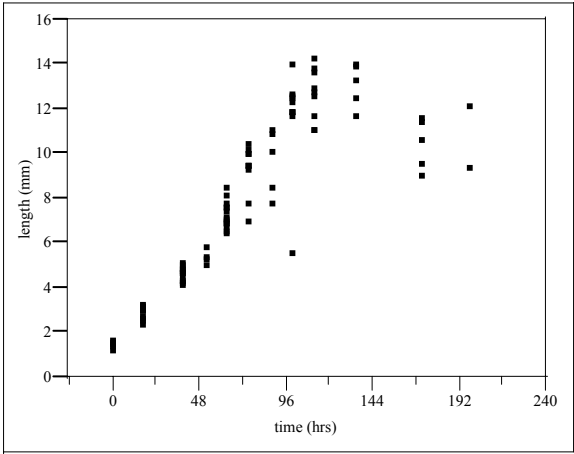


Figure 4.3: Growth of *Lucilia cuprina* larvae on sheep's brain (no controls for handling or mass heating).

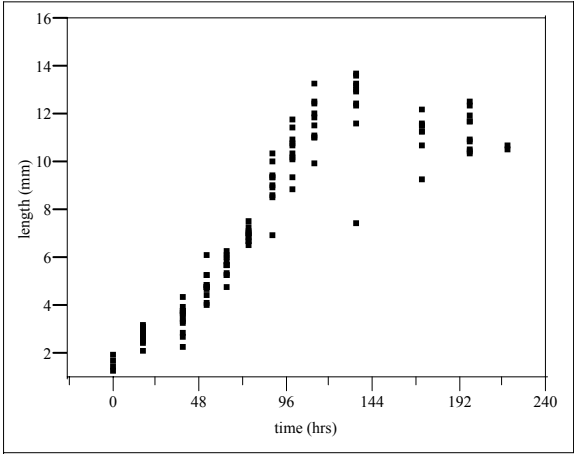


Figure 4.4: Growth of *Lucilia cuprina* larvae on sheep's liver (no controls for handling or mass heating).

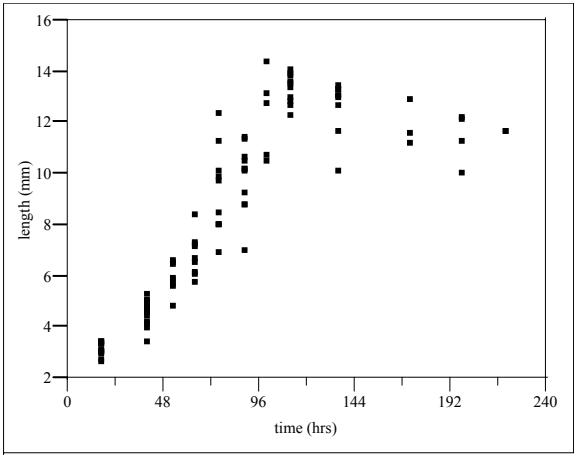


Figure 4.5: Growth of *Lucilia cuprina* larvae on sheep's meat (no controls for handling or mass heating).

Table 4.5: Mean lengths (mm) \pm standard deviation of *Lucilia cuprina* larvae grown on different sheep's tissues. *=outlier/s removed, the number removed is shown in brackets.

Time (hours)	Brain	Liver	Meat	P value
0	1.56 \pm 1.66 range = 1.33-1.77 n = 5	1.70 \pm 0.298 range = 1.41-2.06 n = 4	nd	0.397
16	3.03 \pm 0.28 range = 2.47-3.33 n = 14	2.94 \pm 0.20 range = 2.53-3.26 n = 18*(1)	3.23 \pm 0.26 range = 2.72-3.53 n = 14	0.0091
39	4.71 \pm 0.32 range = 4.18-5.18 n = 12	3.55 \pm 0.55 range = 2.34-4.46 n = 13	4.69 \pm 0.44 range = 4.08-5.40 n = 17*(1)	<0.001
52	5.44 \pm 0.33 range = 5.08-5.87 n = 4	4.92 \pm 0.56 range = 4.11-6.21 n = 12	6.01 \pm 0.60 range = 4.90-6.70 n = 8	0.0011
63	7.35 \pm 0.60 range = 6.54-8.61 n = 14	5.74 \pm 0.43 range = 4.91-6.36 n = 11	6.90 \pm 0.81 range = 5.87-8.52 n = 9	<0.001
75	9.94 \pm 0.41 range = 9.42-10.56 n = 10*(2)	7.077 \pm 0.28 range = 6.66-7.61 n = 10	9.36 \pm 0.1.70 range = 7.03-12.48 n = 10	<0.001
88	9.72 \pm 0.1.47 range = 7.80-11.15 n = 5	9.39 \pm 0.61 range = 8.61-10.48 n = 9*(1)	9.94 \pm 1.29 range = 7.12-11.54 n = 11	0.571
99	12.26 \pm 0.41 range = 11.75-12.79 n = 10*(2)	10.56 \pm 0.88 range = 8.99-11.90 n = 10	12.38 \pm 1.65 range = 10.58-14.48 n = 5	0.0017
111	12.74 \pm 1.17 range = 11.13-14.33 n = 9	11.77 \pm 0.96 range = 10.04-13.37 n = 10	13.42 \pm 0.59 range = 12.38-14.18 n = 10	0.0020
135	13.17 \pm 0.99 range = 11.74-14.10 n = 5	13.01 \pm 0.67 range = 11.68-13.79 n = 9*(1)	13.21 \pm 0.24 range = 12.81-13.53 n = 7*(2)	0.8222
171	10.52 \pm 1.15 range = 9.09-11.69 n = 5	11.54 \pm 0.16 range = 11.38-11.73 n = 5*(3)	12.01 \pm 0.88 range = 11.32-13.01 n = 3	0.0723
197	10.83 \pm 1.95 range = 9.44-12.21 n = 2	11.43 \pm 0.80 range = 10.48-12.60 n = 10	11.50 \pm 1.00 range = 10.10-12.27 n = 4	0.7082
219	nd	10.71 \pm 0.10 range = 10.63-10.78 n = 2	11.76 \pm 0.02 range = 11.74-11.77 n = 2	0.0053

On average, larvae grown on meat were the longest. Larvae grown on brain were found to be longer on average than those grown on liver. Comparison of the maximum average body lengths did not show a significant difference ($p=0.2669$) between the groups grown on the different tissues. It was difficult to see the larvae on brain due to the similarity of colour. Decaying brain was also particularly unpleasant to work with because of its pungent odour.

These initial trials with *L. cuprina* may have been confounded by mass heating and feeding disturbance. Since it did appear that the tissues on which the larvae were feeding may have an influence on the rate of larval growth, replicated trials were set up to examine measured differences between these substrates without the confounding influences of mass heating and feeding disturbance. The results of this experiment have been incorporated into Chapter 6. Also included in Chapter 6 are the results of a similar, replicated, experiment with larvae of *C. augur*. Parts of Chapter 6 have been prepared as a manuscript which has been accepted by *The Journal of Forensic Sciences*.

4.5.4 Suitability of substrate types for capture of adult calliphorids

Since larvae were being transferred to the different protein types from sheep's liver, traps were set with the different sheep protein types to establish whether they attract flies for oviposition purposes. To this end, four identical traps similar in design to those described by Newman [70] were set out during summer in an urban environment in Wollongong, New South Wales, Australia (34° 25' S, 150°55' E). Each trap was allocated to its treatment and not used for any other treatment or purpose for the duration of the experiments. One trap was baited with sheep's liver, one with sheep's brain and a third trap was baited with a lamb chop. Sodium sulfide solution was not used to enhance the aroma of the baits. A fourth, unbaited, trap was used as a control. Each trap was rotated through four positions randomly selected by lottery such that each trap was in each position once for each of two replicate experiments. The general layout of the traps is shown in Figure 4.6. Trapping was only conducted on warm, non-windy, sunny days. Traps were out from dawn to dusk. The experiment was run twice; once in December 2002 and once in January 2003. Catch results are summarised in Tables 4.6 and 4.7.

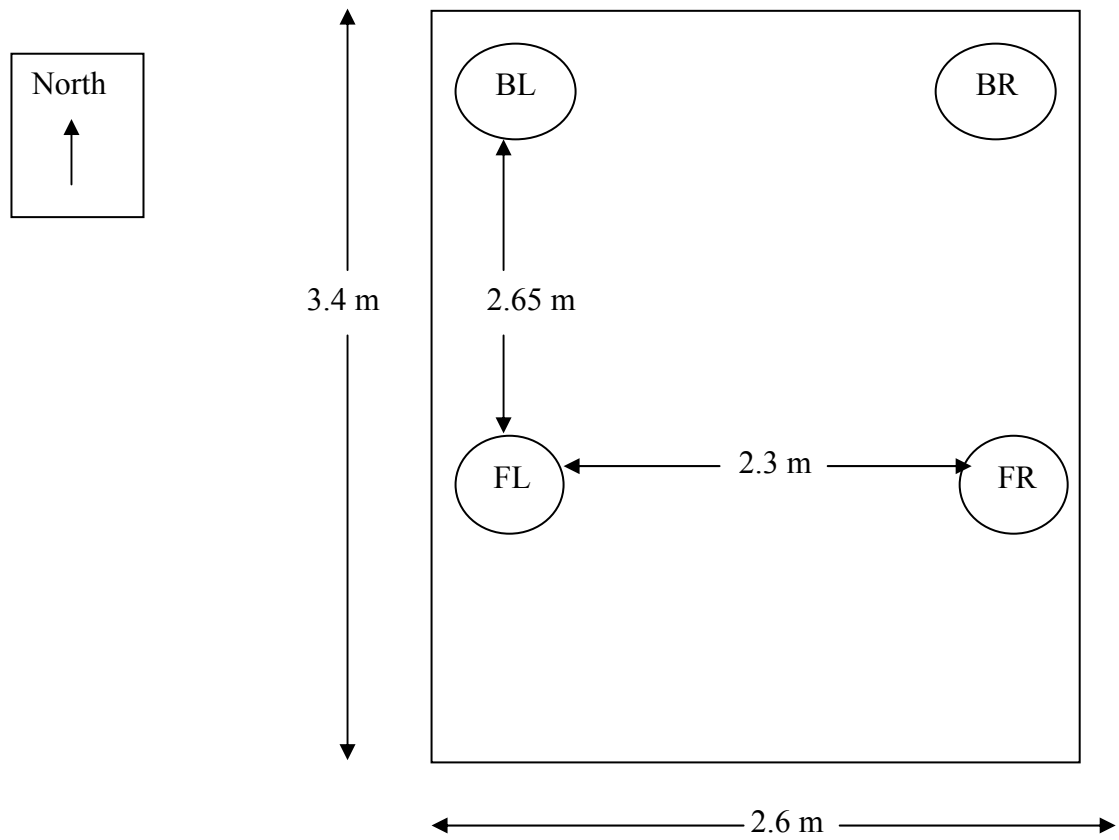


Figure 4.6: Layout for trapping

BL=back left, BR=back right, FL= front left, FR=front right

Table 4.6: Total catch numbers for fly traps baited with different sheep's tissues in an urban environment in summer.

Date	Position	Lure	Total catch	<i>Lucilia</i> sp.	<i>Calliphora augur</i>	Sarcophagidae	unknown
18/12/2002	FL	Brain	5	1	2	0	2
	FR	Meat	1	0	1	0	0
	BL	Control	0	0	0	0	0
	BR	Liver	1	0	0	0	1
19/12/2002	FL	Meat	11	1	10	0	0
	FR	Liver	1	1	0	0	0
	BL	Brain	5	2	2	1	0
	BR	Control	0	0	0	0	0
20/12/2002	FL	Liver	1	0	0	1	0
	FR	Control	0	0	0	0	0
	BL	Meat	1	0	1	0	0
	BR	Brain	0	0	0	0	0
21/12/2002	FL	Control	0	0	0	0	0
	FR	Brain	5	0	4	1	0
	BL	Liver	5	3	0	2	0
	BR	Meat	1	0	0	0	1
14/1/2003	FL	Brain	3	2	0	0	1
	FR	Liver	1	0	1	0	0
	BL	Control	0	0	0	0	0
	BR	Meat	0	0	0	0	0
15/1/2003	FL	Liver	12	5	7	0	0
	FR	Control	0	0	0	0	0
	BL	Meat	4	2	0	2	0
	BR	Brain	4	4	0	0	0
16/1/2003	FL	Control	0	0	0	0	0
	FR	Meat	1	0	0	0	1
	BL	Brain	9	8	1	0	0
	BR	Liver	5	2	3	0	0
17/1/2003	FL	Meat	1	1	0	0	0
	FR	Brain	0	0	0	0	0
	BL	Liver	5	4	1	0	0
	BR	Control	0	0	0	0	0
Totals			82	35	33	7	5

Table 4.7: Summarised catch for fly traps baited with different sheep's tissues in an urban environment in summer.

BL=back left, BR=back right, FL= front left, FR=front right

	Trial 1	Trial 2	Totals
Position			
FL	16	17	33
FR	2	7	9
BL	18	11	29
BR	9	2	11
Lure			
Liver	23	8	31
Meat	6	14	20
Brain	16	15	31
Control	0	0	0

The control trap caught nothing. All other traps caught flies of forensic interest. Brain caught the most flies on three occasions, liver caught the most flies on two occasions and meat caught the most flies on one occasion. The front left position caught the most flies on four occasions. The back left trap caught the most flies on two occasions.

The individual species caught did not appear to show a preference for any particular protein type. Traps baited with liver and brain caught the most flies overall. All protein types lured flies of forensic interest to the traps. Application of a G-test [104] to an $r \times c$ contingency table of trap location and bait type detected no significant difference between the treatments ($G=8.55$, $df=5$).

The results from trapping show that all protein types are suitable oviposition media and that no particular preference was exhibited. The trapping experiments were to serve only as an indication of oviposition suitability and no other inference was intended.

4.6 Experiment F - Body width as an alternative to body length

As mentioned in the General Methods, larvae which had been placed into vials head-first, and larvae which were head-down when killed, showed a characteristic curled-head shape and could not be measured accurately. Although I took steps to remedy the problem in my studies by swirling larvae in the killing agent, e.g. hot water, and then placing them into the preservative solution posterior-end first, the phenomenon is likely to continue to occur if care is not taken by collectors. Head-curling can be defined as the bending of the head of a maggot towards the ventral surface of the body upon killing and preservation. This phenomenon can make accurate measurement of body length very difficult; whilst a larva can sometimes be straightened, this is not always possible. Furthermore, there is a certain natural camber in the shape of a maggot that should be preserved. Artificial straightening can over-compensate and result in a measurement that is larger than it should be. It occurred to me that this might actually be quite widespread, and something of a challenge to consulting forensic entomologists.

It was speculated that the utility of body width could be explored as an alternative measurement to body length for predicting larval age, and therefore post-mortem interval. It was also speculated that larval width might perhaps be able to be converted to larval length, such that existing reference length data can be utilised even when accurate larval lengths are difficult to obtain.

The *L. cuprina* maggots grown in Experiment E (Section 4.5) were measured for both body length and body width. Larvae were measured with digimatic digital callipers. Measurement was done using a dissecting microscope to ensure that the animals were not being crushed in the teeth of the callipers and that the teeth of the callipers were actually making contact with the correct parts of each larva. The length of a larva, viewed laterally, was measured between the most distal parts of the head and the eighth abdominal segment. The width of a larva, viewed laterally, was measured between the ventral and dorsal surfaces at the junction of the fifth and sixth abdominal segments (Figure 4.7).

Plots of the raw data are shown in Figure 4.8. The shapes of the plots of the raw data are very similar for length and width. A linear line of best fit for width gave an R-sq of 0.76. A quadratic model line of best fit for width gave an R-sq of 0.83. A cubic model line of best fit for width gave an R-sq of 0.87. Following on from these results, a pilot trial was run with *C. augur* larvae grown at $20.0 \pm 0.5^\circ\text{C}$ (range $19.5\text{--}20.0^\circ\text{C}$) with two replicates of 10 larvae.

Results from this pilot trial are included in Chapter 5, which also includes results for *C. augur* larvae grown at 25°C . These results were included in the paper published by *Forensic Science International*.

4.7 Experiment G - Initial studies with preservatives

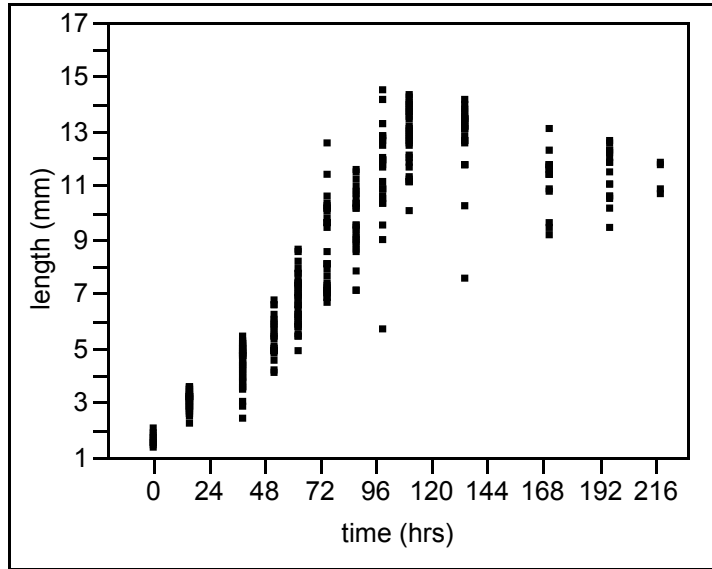
4.7.1 Preservatives in current use

Personal communication with crime scene examiners has indicated that they do not always preserve larvae from crime scenes in the recommended way, i.e. killing in boiling water and then preserving in acetic alcohol [8], because glacial acetic acid is difficult to obtain, ethanol is provided to them as 100% only and boiling water is not available at most crime scenes. Instead, crime scene entomology specimens have often been placed directly into 100% EtOH (pers comm. Constable Paul Maloney). This does not kill the larvae immediately and larvae have been observed alive and writhing up to 10 minutes after immersion.

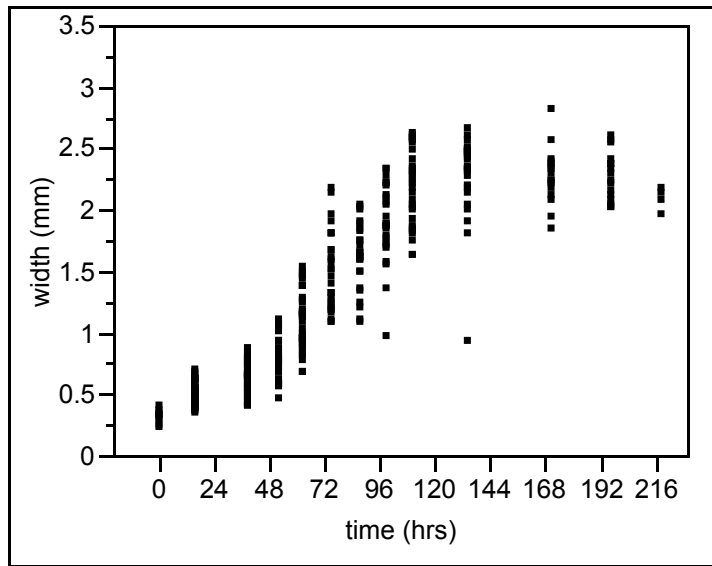
It is not clear why use of 80% EtOH is recommended as best practice [83] compared with other preservatives in current practice locally, e.g. 10% formalin at Glebe Morgue (pers comm. Professor John Hilton) and Royal North Shore Hospital Morgue (pers comm. Mr Aldo Severino), and Kahle's solution at Westmead Morgue (pers comm. various staff members). The effect of different preservative solutions on *Protophormia terraenovae* (Robineau-Desvoidy) has been explored by Tantawi and Greenberg [82].

Figure 4.7: Axes of measurement for length and width [After 37].

H=head, TS=thoracic segment, AS=abdominal segment.



(a) body length



(b) body width

Figure 4.8: Body length and body width in *Lucilia cuprina* larvae.

They found that shrinkage varied from 3.2 to 30.8% depending on the preservative used. They also found that different concentrations of the same preservative led to quite different amounts of shrinkage, for example 17.3% for 70% EtOH, compared with 24.4% for 90% EtOH. To date there has only been one other, very recent study of this kind [105] and no work of this type on the species of particular interest in this study. Further, there are currently no data on shrinkage of larvae preserved in 100% EtOH.

4.7.2 *Time in preservative*

A review of the literature did not indicate how long samples could remain in preservatives prior to measurement without their size being affected. A series of trials was set up to examine time to maximum change in length for the different instars. Since these trials were also begun after commencement of the *C. augur* development trials, no larvae from the temperature and development trials, food substrate trials or preservative type trials were measured until having been in any given preservative for more than 10 days. Ten days was chosen as the time interval because the different preservative types may take different times in which to have their full effect. Also, a ten-day preservation period allows for sample collection, exhibit handling, transport, autopsy and time management by the entomological consultant.

In the first trials, *C. augur* larvae were examined. Three groups of approximately 20 larvae were killed in boiling water, dried and transferred to 80% EtOH. These were then measured immediately and left unhandled until the relevant day allocation when they were measured again. Day allocations were chosen randomly by lottery. Unfortunately, it was extremely difficult to collect enough larvae to set the experiment up all at once (3 replicates x 20 individual larvae x 11 days [one group of replicates for day 0 allocations]=660 larvae required). This was achieved once with feeding third-instar larvae but problems with the recording device led to some early data being lost and the experiment therefore being abandoned. A recording device was being used to record dictated measurements so as to have the samples out of preservative for the shortest possible time and therefore reduce evaporation of the preservatives from both the vials and the larvae themselves. This experiment was not attempted again. Another possible approach, involving staggered feeding groups, was not taken because the larvae would not have been exactly the same age nor grown in the same conditions, requiring additional controls and therefore even larger numbers of larvae.

Since the recommended preservative is 80% EtOH [83], and since preservation of samples in the *C. augur* development trial was needed immediately, 80% EtOH was used. A short experiment was set up to examine when the best time to measure would be because there are no data on this in the current literature. More detail on this issue is included in Chapter 7.

4.8 Experiment H - Culture control

The genetic integrity of the flies used and how the results are applied is also of concern. Adult flies in the culture of *C. stygia* showed signs of becoming sluggish the longer that this species had been under culture. Literature review revealed that little attention has been given to this.

Whilst some studies have been conducted on purpose-caught wild-type flies and their eggs or larvae, which have either been trapped or collected from carrion [21, 23, 33, 47, 73, 93, 95, 97-101, 103, 106, 107], other studies have been conducted using flies from established cultures [66, 68, 78, 94, 108]. In some cases the age of the culture is known [68, 78] but in others it is either not known or not stated [66, 94, 108]. Some workers supplement their cultures with wild-caught adults from time to time [73, 93], presumably to avoid problems which may be associated with inbreeding. For example, Norris [49] observed that after 12 months in culture some species became independent of a protein meal for ovary maturation. Inbreeding in cultures of flies may lead to behavioural changes, due to a high level of wing damage from space constraints in small cages (personal observation). Inbreeding may also have other affects that are not fully understood.

The use of inbred cultures may be necessary if adults of a certain species are very difficult to identify. In such cases, only cultures established from a gravid single female can be confidently regarded as monospecific. This single gravid female approach was taken by Wells and Kurahashi [78] for *Ch. megacephala*. Another complicating factor is that some species can be difficult to maintain under laboratory conditions, e.g. *C. hilli hilli* [46, personal observation], necessitating the dissection of larvae or eggs from anaesthetised gravid females in order to work with them [46]. Concerns about applying results from flies collected from one area to other more geographically distant areas have been raised because the physiology and rate of development of species may vary from place to place [8]. Furthermore, some forensic entomologists have indicated that they frequently have to estimate the developmental time of a lesser-known fly species based on that of a close relative that has been better studied [109]. This approach is not ideal, but may be shown to have some validity in the future.

I decided to examine my *C. augur* culture for differences between early and late generation larvae. The results are incorporated into Chapter 8.

CHAPTER 5

Body width as an alternative to body length in *Calliphora augur* and *Lucilia cuprina*

Summary:

The length of fly larvae collected from corpses is often used to help provide an entomological estimate of time since death. However, 'head-curling' by larvae can affect the accuracy of length measurements. To investigate a possible resolution to this problem, larvae of *Calliphora augur* and *Lucilia cuprina* were grown on sheep's liver at constant temperatures. Replicate samples were collected at set time intervals until pupation. Body length and width were measured for individual larvae and examined as predictors of age. It was found that body width, as measured at the junction of the fifth and sixth abdominal segments, is comparable with body length for age prediction of maggots of *C. augur* and *L. cuprina* grown at these temperatures. Furthermore, conversion of width to length can be done with 95% accuracy from a simple linear model.

5.1 Introduction

Where flies are the primary colonisers of a corpse, the PMI is best estimated by rearing larvae under temperature conditions consistent with those at the death scene, as well as consulting growth data on the relevant fly species published in the scientific literature [13]. Various methods for calculating maggot age have been suggested. Examples include: examination of the internal mouthparts and posterior spiracles of each maggot instar [111], biochemical estimates such as increases in the DOPA decarboxylase titer [6, 50] and uptake of plasma protein by the fat body [126], daily growth layers on skeletal apodemes (ridge-like ingrowths of the exoskeleton of an arthropod that support internal organs and provide attachment points for muscles) [92], examination of gut contents [127], and measurement of crop length [50, 85, 127] and crop colour changes [6]. Some workers use larval weight [20, 60, 67, 101, 103], but dry weights involve destructive sampling. Furthermore, since preservative solutions may affect the mass and density of a subject being preserved, wet weight cannot be used if there are no published standards for conversion back to live weight.

Measurement of body length is by far the most common parameter from which to estimate the age of a maggot [73, 93, 97-100, 107]. There are a few studies which have included both length and weight of larvae [69, 128, 129], and it appears that these parameters correlate well [128]. Greenberg [6] provided a brief review of the various methods of aging larval, pupal and adult blowflies.

Whilst the phenomenon of maggot 'head-curling' does not appear to have been reported in the literature, it may be a problem for forensic entomologists using body length for PMI estimations. Head-curling can be defined as the bending of the head of a maggot towards the ventral surface of the body upon killing and preservation. This phenomenon can make accurate measurement of body length very difficult; whilst a larva can sometimes be straightened, this is not always possible. Furthermore, there is a certain natural camber in the shape of a maggot that should be preserved. Artificial straightening can over-compensate and result in a measurement that is larger than it should be.

While head-curling can be easily reduced by swirling larvae in the killing agent, e.g. hot water, and then placing them into the preservative solution posterior-end first, the phenomenon is likely to continue to occur if care is not taken by collectors.

In light of these difficulties, it was speculated that the utility of body width could be explored as an alternative measurement to body length for predicting larval age, and therefore post-mortem

interval. It was also speculated that larval width might perhaps be able to be converted to larval length, such that existing reference length data can be utilised even when accurate larval lengths are difficult to obtain.

5.2 Materials and methods

The general methods described in Chapter 3 were followed. For the experiments in this chapter however, specific detail is outlined below.

Initial studies conducted with *L. cuprina* (see Section 4.6) led to encouraging results. These experiments were therefore expanded to include *C. augur* at two constant temperatures.

5.2.1 Sample generation

Cages containing males and gravid females of *C. augur* were presented with a portion of liver for one hour at room temperature to permit oviposition. New larvae (up to one hour old) were then transferred undisturbed to approximately 50 g of sheep liver in individual rearing containers. All rearing containers were then transferred to an Axyos environmental cabinet and left undisturbed until an allocated time had elapsed.

The first (pilot) trial with *C. augur* was run at $20.0 \pm 0.5^\circ\text{C}$ (range 19.5 - 20.0°C) with two replicates of 10 larvae. The larvae were grown for time allocations of 0, 6, 12, 18, 24, 36, 48, 60, 72, 84, 96, 108, 120, 144, 156, 168, 180 or 192 hours and were then collected. Encouraging results from this pilot trial led to examination of a greater number of individuals (three replicates of 10 individuals) at another temperature ($25.0 \pm 0.5^\circ\text{C}$, range 24.5 - 25.0°C). The time allocations at 25°C were 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 48, 52, 56, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180 and 192 hours.

5.2.2 Sample collection

At collection, all individuals were lifted off or dissected from the substrate. They were then transferred to just-boiled water for one to two minutes to fix the larvae and stop enzyme activity [130], and to produce elongation [81] comparable to maximum extension of a live larva [82]. The larvae were then towel-dried and preserved in 80% EtOH [130-132].

All larvae were placed into vials posterior-end first. Where growth stages differed either physically or behaviourally (i.e. feeding third instars and wandering third instars) the

different types observed were preserved in separate vials. Post-feeding or wandering larvae were defined as having moved away from the liver and being present in the chaff.

5.3 Results

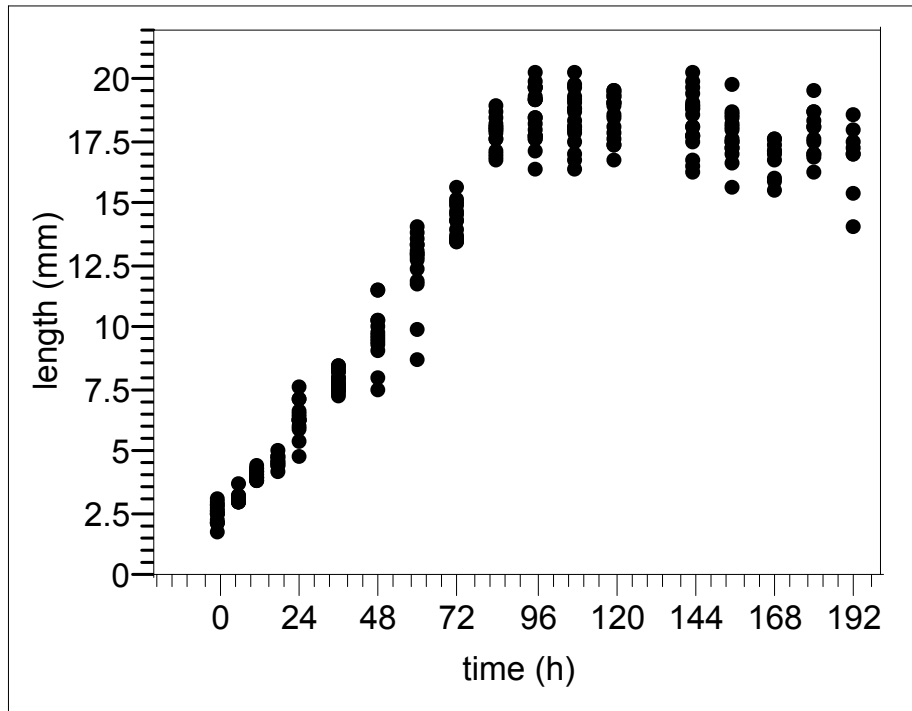
5.3.1 *Larvae grown at 20°C*

At 20°C larvae began to migrate away from the meat at 120 h. A total number of 10 outlying measurements (i.e. those more than two standard deviations from the mean) were detected in the complete data set of all times examined (four in the length data and six in the width data). No individual maggot was observed to have outlying measurements for both length and width. Removal of these outliers did not influence when maximum mean length or maximum mean width were observed (96 and 156 h respectively). Similarly, removal of these outliers had no influence on the value observed for maximum mean length (no outliers at 96 h) and they did not change the value of the maximum mean width (no significant difference). The outlying measurements have therefore been included in all subsequent analyses.

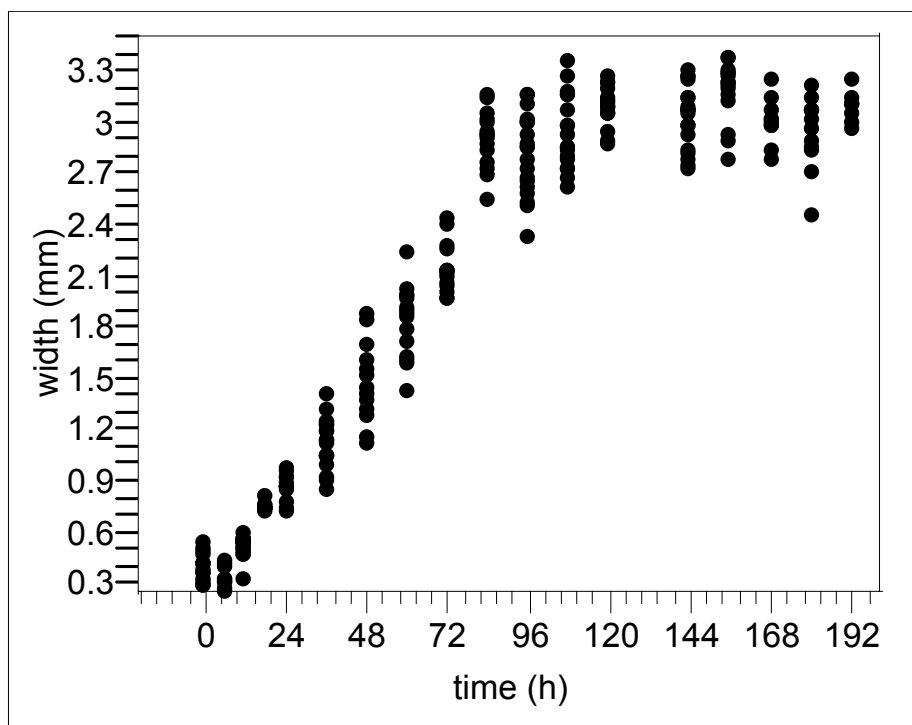
Plots of lengths against time and widths against time for larvae grown at 20°C are shown in Figure 5.1 (a and b). Details of measurements are collated in Table 5.1. The shapes of the plots of the raw data are very similar for length and width.

Mean length increased steadily from a minimum of 2.53 mm at 0 h to a maximum of 18.62 mm at 96 h. Shrinkage in length began soon afterward. Mean width showed an initial decrease from 0.41 mm at 0 h to 0.34 mm at 6 h, increased quite steadily to 2.95 mm at 108 h and then increased to a maximum of 3.18 mm at 156 h (Table 5.1).

The calculated r-squared values for lines of fit for these plots are shown in Table 5.2. The R-square (adjusted) value for a linear line of best fit is slightly higher for width compared with length (0.820 c.f. 0.743), indicating that width may in fact be a better predictor of time for these larvae when grown at 20°C.



a) body length



(b) body width

Figure 5.1: Change over time in body length and body width of *Calliphora augur* larvae grown at 20°C - untrimmed raw data.

Table 5.1: Summary of body length and width data over time for *Calliphora augur* larvae grown at 20°C.

Time (h)	Length (mm)				Width (mm)			
	mean	std dev.	range	n	mean	std dev.	range	n
0	2.53	0.36	1.79-3.06	15	0.41	0.08	0.29-0.55	15
6	3.13	0.25	2.94-3.75	9	0.34	0.07	0.26-0.44	9
12	4.13	0.21	3.82-4.51	15	0.52	0.06	0.34-0.60	15
18	4.64	0.24	4.21-5.06	9	0.75	0.03	0.72-0.82	9
24	6.35	0.72	4.86-7.66	14	0.87	0.07	0.72-0.98	14
36	7.85	0.38	7.25-8.48	18	1.13	0.15	0.85-1.42	18
48	9.72	1.14	7.49-11.51	13	1.48	0.24	1.13-1.89	13
60	12.52	1.46	8.72-14.07	15	1.84	0.20	1.43-2.24	15
72	14.36	0.69	13.46-15.66	14	2.15	0.15	1.97-2.44	14
84	17.86	0.66	16.76-18.95	15	2.91	0.16	2.55-3.16	15
96	18.62	1.07	16.42-20.39	20	2.82	0.23	2.33-3.17	20
108	18.48	1.09	16.38-20.37	18	2.95	0.22	2.63-3.36	18
120	18.61	0.89	16.76-19.62	17	3.10	0.11	2.88-3.28	17
Beginning of post-feeding migration								
144	18.43	1.14	16.37-20.38	19	3.03	0.18	2.73-3.31	19
156	17.79	1.00	15.72-19.91	15	3.18	0.18	2.78-3.39	15
168	16.84	0.74	15.58-17.67	10	3.01	0.13	2.78-3.26	10
180	17.86	0.94	16.34-19.62	12	2.95	0.20	2.46-3.22	12
192	16.89	1.44	14.09-18.58	8	3.10	0.09	2.98-3.25	8

Table 5.2: Summary of best fit of body length and width data over time for larvae of *Calliphora augur* grown at 20°C (n=256).

	Length		Width	
	R-square adjusted	Probability	R-square adjusted	Probability
Linear	0.743808	<.0001	0.82036	<.0001
2nd deg poly	0.953669	<.0001	0.951692	<.0001
3rd deg poly	0.955465	<.0001	0.956021	<.0001

A second-degree polynomial model improved prediction for both length and width (length up to an R-sq adj of 0.953; width up to an R-sq adj of 0.951). Neither measurement appears much better than the other using this second-degree model. A third-degree polynomial model hardly improved prediction of length and width at all (R-sq adj of 0.955 for length cf an R-sq adj of 0.956 for width) for either measurement.

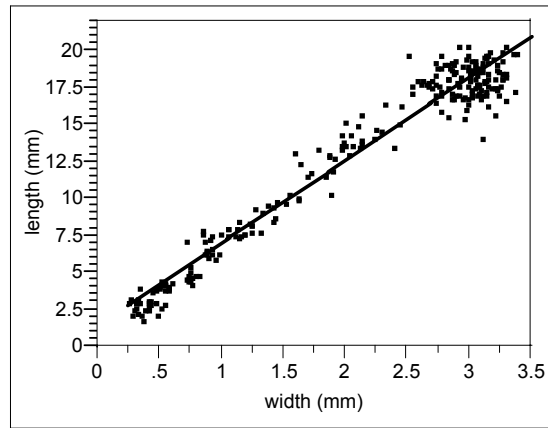
A plot of length vs. width, with width as the fixed variable or predictor, shows that a linear line of best fit [$l = 1.3384611 + 5.6048817 w$] gives an R-sq adj of 0.957 (Figure 5.2). A second-degree polynomial model [$l = 3.1604818 + 5.1983248 w - 0.8776608 (w - 2.08844)^2$] gives an R-sq adj of 0.967. A third-degree polynomial model [$l = 1.3586539 + 6.1962641 w - 1.4700879 (w - 2.08844)^2 - 0.7291668 (w - 2.08844)^3$] gives an R-sq adj of 0.971. The linear model shows good congruence and the improvements in predictability that come from increasing the complexity of the model are slight.

5.3.2 Larvae grown at 25°C

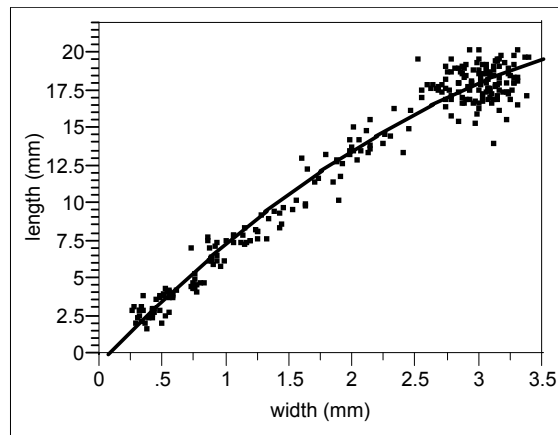
Larvae grown at 25°C began to migrate away from the meat at 96 h and pupae were observed from 144 h. Unfortunately, some of the samples had dried out due to inadequate seals on the vials in which they were preserved.

Between-sample variation was examined with ANOVA where three replicates for a time allocation were available and *t*-tests where only two replicates were available. Some replicate groups were significantly different from other replicates for length only (12b, 28b and 72a), while one replicate group was significantly different for width only (20c) and one replicate group was significantly different for both length and width (108b). Replicate samples which were not significantly different at the 0.01 level were pooled. Since pupae were observed from 144 h, all subsequent analyses contain only data for up to 132 h because the different life stages confound results from 144 h onwards.

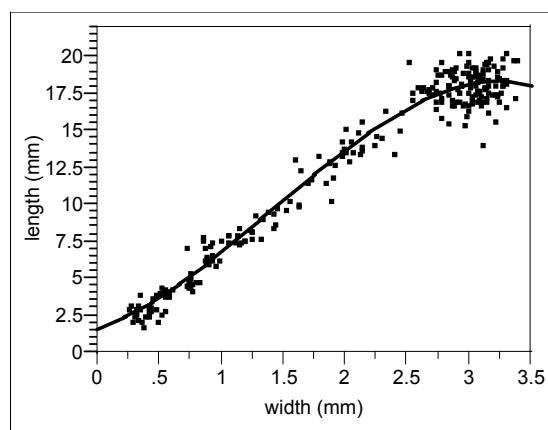
Examination of distribution data also revealed some within-sample variation. Initial examination of the distribution of data revealed 23 outliers (values more than two standard deviations from the mean) - 13 in the length data and eight in the width data. Two individual maggots were outliers for both length and width. The individuals from which these outlying values were derived were removed and the distributions were re-examined. A further six outliers were then detected - four in the length data and two in the width data - and these were also removed.



a) Simple linear model: $R\text{-sq adj}=0.957$. $l = 1.3384611 + 5.6048817 w$



b) A second-degree polynomial model: $R\text{-sq adj}=0.967$. $l = 3.1604818 + 5.1983248 w - 0.8776608 (w - 2.08844)^2$



c) A third-degree polynomial model: $R\text{-sq adj}=0.971$.
 $l = 1.3586539 + 6.1962641 w - 1.4700879 (w - 2.08844)^2 - 0.7291668 (w - 2.08844)^3$

Figure 5.2: Three models of estimated predictability of larval body length from larval body width for *Calliphora augur* grown at 20°C.

A final examination of the distribution of the data revealed one final outlying measurement in the length data, which was removed to produce the final data set.

Plots of lengths against time and widths against time for larvae grown at 25°C are shown in Figure 5.3 for 0-132 h. Details of measurements are collated in Table 5.3. The shapes of the plots of the raw data are similar for length and width, and not dissimilar to those generated for the larvae grown at 20°C. A notable difference is the expanded range of values observed for the larvae grown at 25°C, particularly above 52 h.

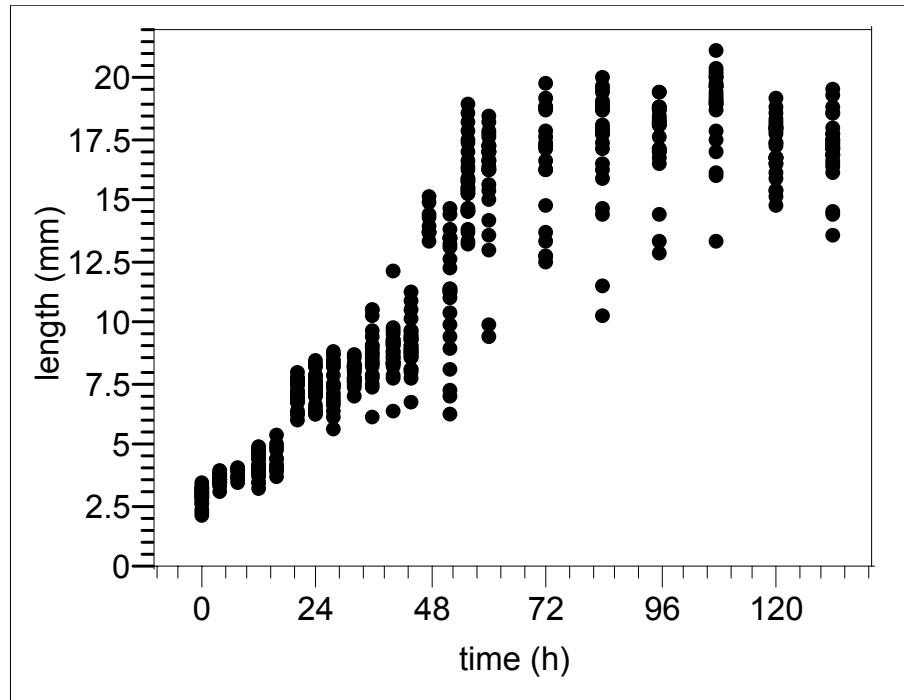
Mean length increases more or less steadily from a mean of 2.92 mm at 0 h to 18.07 mm at 72 h (Table 5.3). However, a maximum mean of 19.57 mm occurs at 108 h, which is 12 hours after migrating third instars were first detected.

The longest individual recorded was from the 108 h sample. Some oscillation is seen around maxima of between 18 and 20 mm from 72 to 108 h.

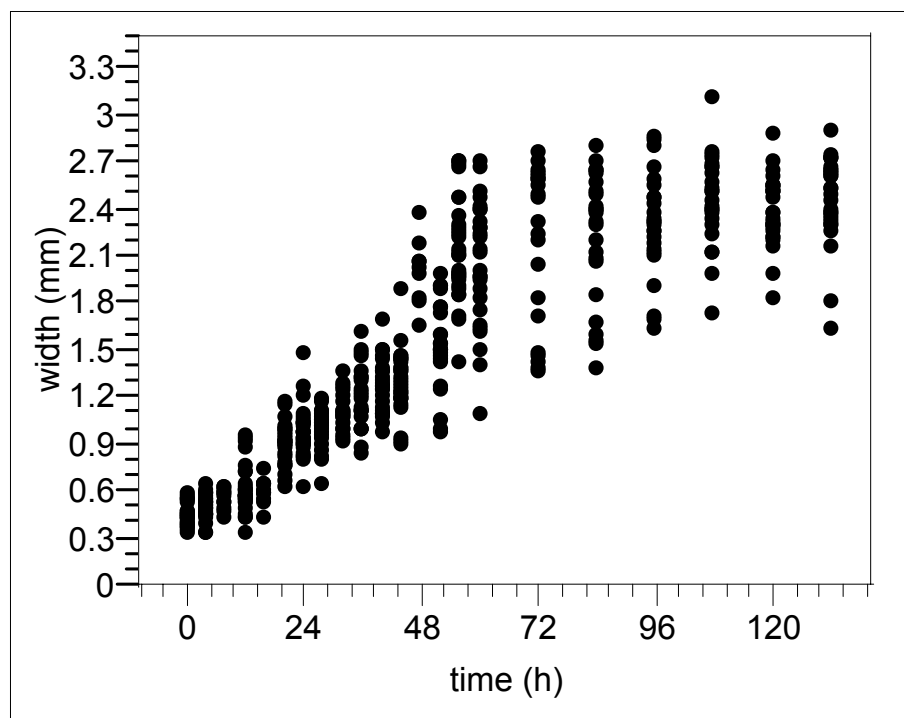
Mean width does not exhibit the initial decrease seen in the larvae grown at 20°C (Table 5.1). Mean width increases quite steadily from 0.45 mm at 0 h to a maximum of 2.53 at the experimental end point (Table 5.3).

The calculated r-squared values for lines of fit for these plots are shown in Table 5.4. The R-square (adjusted) values for linear lines of best fit (Figure 5.4) are comparable (0.794 c.f. 0.795). A second-degree polynomial model improved prediction for both length and width (length up to an R-sq adj of 0.896; width up to an R-sq adj of 0.872). Length appears to be the better predictor using this model. A third degree polynomial model hardly improved prediction at all for either measurement (R-sq adj of 0.919 for length cf an R-sq adj of 0.882 for width).

A plot of length vs. width, with width being the fixed variable or predictor, shows that a linear line of best fit [$l = 0.3189932 + 7.1514971 w$] gives an R-sq (adj) of 0.950 (Figure 5.4). The second-degree polynomial model [$l = 0.675289 + 7.2167512 w - 0.7689426 (w - 1.53621)^2$] gives an R-sq (adj) of 0.954. The third-degree polynomial model [$l = -1.379374 + 8.5722903 w - 0.6939537 (w - 1.53621)^2 - 1.4339317 (w - 1.53621)^3$] gives an R-sq (adj) of 0.960.



a) body length



b) body width

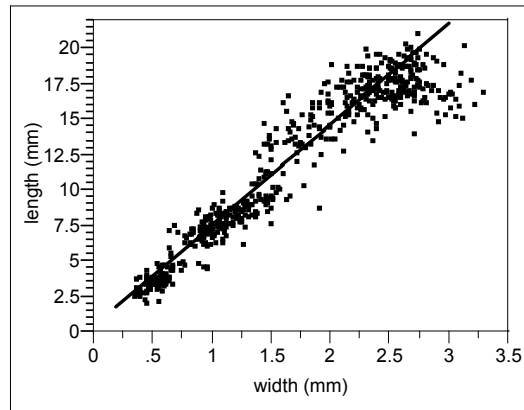
Figure 5.3: Change over time in body length and body width of *Calliphora augur* larvae grown at 25°C - untrimmed raw data.

Table 5.3: Summary of body length and width data over time for *Calliphora augur* larvae grown at 25°C - outliers removed.

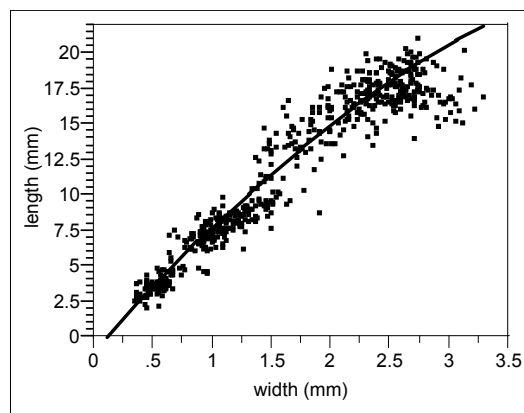
Time (h)	Length (mm)				Width (mm)			
	mean	std dev.	range	n	mean	std dev.	range	n
0	2.92	0.35	2.18-3.48	20	0.45	0.08	0.34-0.59	20
4	3.60	0.19	3.10-3.94	27	0.50	0.08	0.35-0.66	27
8	3.81	0.21	3.44-4.11	11	0.56	0.07	0.44-0.63	11
12	3.97	0.34	3.29-4.46	17	0.54	0.09	0.35-0.73	17
16	4.45	0.51	3.77-5.41	12	0.61	0.08	0.44-0.75	12
1st to 2nd instar transitional forms detected								
20	6.94	0.50	6.10-7.81	16	0.85	0.11	0.64-1.02	16
24	7.32	0.64	6.30-8.48	26	0.97	0.10	0.81-1.22	26
28	8.14	0.64	7.01-8.89	12	1.02	0.10	0.83-1.20	12
32	7.97	0.45	7.01-8.69	18	1.11	0.12	0.92-1.37	18
36	8.67	1.08	6.18-10.62	22	1.21	0.20	0.85-1.62	22
2nd to 3rd instar transitional forms detected								
40	8.84	0.56	7.80-9.84	23	1.26	0.15	0.98-1.51	23
44	9.07	0.68	7.760-10.62	24	1.29	0.16	0.92-1.57	24
48	14.18	0.60	13.43-15.22	9	2.00	0.21	1.67-2.38	9
52	11.28	2.48	6.25-14.76	23	1.53	0.29	0.98-1.99	23
56	15.81	1.53	13.28-19.02	29	2.15	0.32	1.43-2.72	29
60	16.89	0.86	15.04-18.49	22	2.19	0.29	1.63-2.71	22
72	18.07	1.07	16.30-19.84	13	2.49	0.22	2.05-2.78	13
84	17.89	1.52	14.54-20.16	22	2.29	0.36	1.54-2.81	22
96	18.18	0.80	16.62-19.52	21	2.41	0.25	1.92-2.86	21
Migrating post-feeding 3rd instars detected								
108	19.57	0.75	17.91-21.19	18	2.52	0.20	2.00-2.78	18
120	17.21	1.58	14.82-19.24	27	2.39	0.22	1.83-2.89	27
132	17.38	0.69	16.25-18.87	23	2.53	0.20	2.16-2.91	23
144	Pupae detected							

Table 5.4: Summary of best fit for body length and width data over time for larvae of *Calliphora augur* grown at 25°C (outliers removed; n=435).

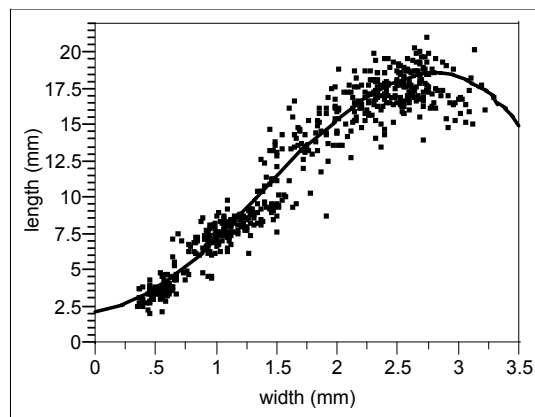
	Length		Width	
	R-square adjusted	Probability	R-square adjusted	Probability
Linear	0.79463	<.0001	0.795113	<.0001
2nd deg poly	0.896093	<.0001	0.872158	<.0001
3rd deg poly	0.919197	<.0001	0.882638	<.0001



a) Simple linear model: $R\text{-sq adj}=0.950$. $l = 0.3189932 + 7.1514971 w$



b) A second-degree polynomial model: $R\text{-sq adj}=0.954$.
 $l = 0.675289 + 7.2167512 w - 0.7689426 (w - 1.53621)^2$



c) A third-degree polynomial model: $R\text{-sq adj}=0.960$
 $l = -1.379374 + 8.5722903 w - 0.6939537 (w - 1.53621)^2 - 1.4339317 (w - 1.53621)^3$

Figure 5.4: Three models of estimated predictability of larval body length from larval body width for *Calliphora augur* grown at 25°C.

As was found for the larvae grown at 20°C, the linear model shows good congruence and the improvements in predictability coming from increasing the complexity of the model are slight.

5.4 Discussion

Growth of *C. augur* in these experiments was as expected and correlated well with the findings of other workers [25]. This species was found to be easy to rear in the laboratory provided protein was available prior to or very soon after emergence. The clutch size of *C. augur* is smaller than other species of *Calliphora* because it is ovoviparous. Although smaller clutch sizes can make working with ovoviparous species more labour intensive than working with egg-laying species, ovoviparous species still require detailed study and deserve equal attention. Problems encountered in the rearing of other species of *Calliphora*, such as *C. hilli hilli*, were not seen in *C. augur*. For example, it was not difficult to maintain in the laboratory, there was no need to dissect larvae or eggs out of anaesthetised gravid females in order to work with them [46] and this species does not produce precocious eggs [19, 43, 116]. It does not switch between reproductive strategies in different seasons as *C. stygia* may do in New Zealand [see 45], nor does it not produce mixed clutches of fertile eggs and larvae like *C. hilli hilli*.

The high predictability of a measurement as simple as width is perhaps surprising. Furthermore, the level of congruence of width to length is much better than expected. The initial reduction in width seen from 0 h to 6 h at 20°C possibly relates to expansive or elastic properties of the cuticle of a young larva. Newly laid individuals (0 h specimens) may not yet be fully extended. The possibility of segments being telescoped within each other also exists [50]. The fact that a similar decrease was not seen in individuals grown at 25°C probably relates simply to accelerated growth at the higher temperature.

An initial decrease may still occur at that temperature but was not detected in this study, in spite of the more frequent sampling regime, because it fell between the times examined. Similarly, the reduction in predictability seen with an increase in temperature, as evidenced by a reduction in R-sq adj values for both length and width over time, is likely to be due to an acceleration of growth due to the increase in temperature.

It is interesting to observe that the robustness of prediction of length from width was quite well conserved in spite of the increase in temperature. It is also interesting to note that such good predictability is possible with such a small sample of the wild population, especially when between-sample and within-sample variation were both observed.

The inclusion of the outliers in the 20°C data was dictated partly by a consideration of the influence of the individual data points on the full data set, but also by awareness that small data sets are often inadequate. Even though the data sets in these experiments were small, they were found to be sufficient for their purpose. It is worth noting that many of the individuals deemed to be significantly different were detected just prior to a change in life stage, usually a moult. It is possible then that the greatest variation in these parameters is observed at a change in life stage. Examination of calculated standard deviations in samples prior to moults in the 25°C data set, particularly length at 16 h, and length and width at 36 h, partially support this idea.

The main advantage of using larval width as an adjunct to larval length for estimating a PMI is that no extra equipment or chemicals are needed. The additional measurements take moments to perform and allow for easy conversion to length. They therefore have the potential to salvage certain crime scene specimens whose length cannot be reliably determined, and thus still use data sets from the literature to estimate their age. The use of damaged specimens by measuring body width will not only allow possible age estimation, but also perhaps contribute to overall insect community and successional data. In any case, the availability of body width as an alternative measurement of maggot samples adds another valuable tool to the entomologist's arsenal.

5.5 Conclusions

This study shows that larval width, measured at the juncture of the fifth and sixth abdominal segments, is comparable with larval length for prediction of larval age for *C. augur* grown at multiple temperatures. This study also shows that, for *C. augur*, a simple linear model of the form $y = x + b$ can be used to easily transform measurements of larval width to estimates of larval length with 95% accuracy. So, for *C. augur* at least, head-curling may no longer present an obstacle to determination of size and therefore the estimation of age of individual maggots, and permit salvage of some damaged specimens.

A preliminary study on *L. cuprina* larvae has shown that width is also a good substitute for length in this species (Section 4.6) irrespective of the substrate type. Investigation of still more fly species will reveal the true applicability of this approach.

CHAPTER 6

Studies on developmental media

Summary:

While research has examined the effect of freezing and subsequent thawing on the decomposition of carcasses, no studies have investigated the effect of the freezing and thawing of tissues used as a developmental substrate by fly larvae. This chapter presents results of such studies using larvae of *Calliphora augur* (Fabricius) on sheep's liver. Approximately 20 first-instar larvae were collected on sheep's liver and subsequently transferred to paired treatments of fresh and frozen/thawed liver equilibrated to room temperature. They were then left undisturbed for one to ten days. When the allocated time had elapsed the body length of the larvae in each pair of groups was compared. No significant differences were detected between any pairs at a 1% level and only one pair was significantly different at a 5% level. It is concluded that freezing and thawing of a developmental medium of sheep's liver has no significant effect on growth of *C. augur* larvae.

The size of fly larvae is an important variable in the use of these insects to estimate post-mortem interval. A study was therefore conducted to investigate the effect of type of food substrate on larval growth in two species of forensically important Australian blowflies. After collection on sheep's liver in the laboratory, larvae of *Lucilia cuprina* and *Calliphora augur* were grown on sheep's liver, lamb chops and sheep's brains, and their body lengths compared. Results indicated that the development of larvae fed sheep's liver was adversely affected compared with the development of larvae fed meat and brain; they moulted later, reached maximum length more slowly and sometimes produced significantly smaller pupae. These findings, similar to those of another recent study, have obvious implications for post-mortem interval determinations. Estimates may be considerably skewed if the site of collection of larvae at a death scene contains tissue types different to those used in reference experiments. It is therefore recommended that caution be exercised in forensic analyses that interpret crime scene data using developmental studies done with a single type of larval food substrate.

6.1 Introduction

6.1.1 *Fresh vs. frozen developmental media*

While Catts [111] has noted that freezing and subsequent thawing of animal carcasses greatly accelerates their decomposition, the use of defrosted carcasses is quite common in forensic entomological studies [87, 89, 114, 122, 123, 133-138]. To date, the only study examining the effect of freezing and thawing on the growth of larvae feeding on carrion has been that of Micozzi [90], who found internal differences between the fresh and frozen animals. He also noted changes in terms of internal organ degeneration and the sites and timing of bacterial and larval activity. In general, his findings were 1) that the rate of disarticulation is slower in fresh-killed animals than in frozen/thawed ones but the sequence is the same, and that freeze/thawing appears to accelerate the rate of disarticulation; and 2) that mechanical disruption of the tissues caused by freezing weakens the skin, connective tissue, and joints, thus facilitating aerobic decay and skeletal disarticulation, and making internal organs more susceptible to invasion by foreign organisms and insects.

Use of frozen and thawed tissues, rather than whole carcasses, for studies on oviposition and development in carrion flies is also quite common [88, 139], but there have been no published studies comparing larval growth on fresh vs. frozen/thawed tissues. An experiment was conducted to examine the effect of freezing and thawing the larval developmental medium on development in the blowfly, *Calliphora augur*.

6.1.2 *Larval growth on different tissues*

It is not uncommon to use non-human vertebrate models when studying development and succession in carrion-breeding fly species, especially Calliphoridae (blowflies), with the aim of applying the data to post-mortem interval determinations. Previous developmental studies on fly larvae have used various types of liver as the developmental medium, including beef [46, 73, 93], pork [78], ox [60], lamb [94], an unspecified type [40], and a mix of minced ox liver and jelly meat [21]. Liver is a commonly used substrate because it is readily available, relatively inexpensive and of uniform consistency. Other substrates used include fish [95], pet mince (a mixture of muscle and offal) [25], mammalian muscle [33], (unspecified) meat [96], lean pork [97-100], ground beef [101, 102] and mouse carcasses [103]. A few studies have compared larval preference for different substrates prior to rearing [97-100] and only one study appears to have examined the suitability of different substrates for both oviposition and rearing [47].

Uvarov [140] has reviewed insect nutrition and metabolism. Hobson [141-144] conducted various studies on nutrition in blowfly larvae specifically, and Mackerras and Freney [64] observed nutrition and development in maggots of Australian blowflies. Studies on *Calliphora vomitoria* [Wollman in 140] found that larvae developed much better on brains sterilised at 130°C than on meat sterilised at 115°C. Kozantsikov [in 40] observed a variation between 29.1 and 75.6 days in the development of *Calliphora erythrocephala* (*vicina*) larvae grown on various meats at a temperature of 15.1°C.

Maggot invasion of a corpse often occurs through the eyes and into the skull cavity [50]. Hence, for a large part of their development, such larvae are feeding on brain. A recent study by Kaneshrajah and Turner on *C. vicina* [139] found that an error of up to two days might be expected in a post-mortem interval estimate using larvae grown on pig's liver compared with brain, heart, kidney and lung. Further, these workers observed puparia of reduced weight and size when larvae were grown on brain or heart. The present study, begun independently of that of Kanaeshrajah and Turner [139] reports the results of comparative larval development in *Lucilia cuprina* and *Calliphora augur*. Larvae in my study were grown on sheep's liver (representative of the commonest fodder type in developmental studies), superficial sheep's muscle with associated adipose and epithelial layers (i.e. lamb chops) (possibly similar in structure to a wound environment), and sheep's brains (to examine growth of larvae infesting the brain cavity). There are established nutritional differences between these particular tissues [145]. *Calliphora augur* and *L. cuprina* were selected for detailed study because they are relatively abundant and have been recorded in local crime scene samples in New South Wales, Australia [31]. *Calliphora augur* is regarded as consecutively actively viviparous [44], i.e. females control laying of larvae and always lay larvae if fertile and sperm are present. In contrast, *L. cuprina* is oviparous and females prefer to oviposit in communal laying sites being used by other females of the same species [61]. These communal laying sites have high humidity and low illuminance [62]. *Lucilia cuprina* does not appear to exhibit precocious egg development [c.f. *Lucilia sericata* in 19].

6.2 Materials and Methods

The general methods described in Chapter 3 were followed. For the experiments in this chapter however, specific detail is outlined below.

6.2.1 *Fresh vs. frozen developmental media*

First-instar larvae were collected on sheep's liver over a one-hour period. Approximately 20 new larvae were allocated to paired samples of approximately 50 g pieces of fresh and frozen/thawed sheep's liver. During the preparation of each whole liver, prior to freezing, half of each was allocated to the frozen treatment and half to the fresh treatment. Pieces from a number of livers were randomly pooled in each category. Liver pieces in the fresh category were refrigerated overnight at 4°C. Those in the frozen/thawed category were frozen to -20°C overnight and allowed to thaw the next day. Partially frozen samples were not used. All meat pieces were equilibrated to room temperature prior to use.

Paired samples and their positions in the laboratory were chosen by lottery. After sample allocation, the larvae were allowed to develop undisturbed for each of one to ten days until their allocated time had elapsed. Developing larvae were kept on shelving in an Axyos temperature-controlled cabinet and maintained at 25±1°C. Collected larvae were immediately killed by immersion in boiling water, dried with paper towel and preserved in 80% EtOH [83, 130]. Larvae were placed into vials posteriad to prevent head-curling [146]. Where larval growth stages differed behaviourally (i.e. feeding third instars and wandering third instars) the different types observed were preserved in separate vials. Pupae were killed by direct immersion in 80% EtOH.

After a minimum of ten days in preservative, the length of the larvae and pupae was measured with the aid of a dissecting microscope and Mitutoyo Absolute digimatic digital callipers. Larval body length was measured as the distance, viewed laterally, between the most distal parts of the head and last abdominal segment. The instar of each larva was determined by examination of posterior spiracular slits under a dissecting microscope. The ambient temperature within the temperature-controlled cabinet was monitored with small dataloggers (iButtons). Data entry and analysis were done using JMP[®] and Excel[®].

6.2.2 *Larval growth on different tissues*

Lamb's fry (liver) and lamb chops were readily obtained from butcher's shops and supermarkets. Brains were ordered from a local abattoir. All tissues were purchased fresh, in bulk, and of a standard fit for human consumption. For any planned experiment, the three tissue types were all purchased together. Each liver was then cut into suitable sized portions (approximately 50 g lots). Half the portions were frozen at -

20° C for storage. All tissues presented to females and used in the experiments were thawed and equilibrated to room temperature prior to use.

Cages containing gravid females and males of each species were presented with a portion of sheep's liver at room temperature for one hour to permit females to oviposit or larviposit. First-instar *C. augur* larvae were transferred to the different protein types immediately following their initial collection on liver. Eggs of *L. cuprina* were allowed to hatch undisturbed on the liver on which they were collected and first-instar larvae were transferred to the treatment tissue type approximately 25 hrs after oviposition.

Approximately 20 of the new larvae were transferred to each rearing container with a moistened fine artist's brush. For separate periods of between 0 and 10 days there was one rearing container allocated to each fodder type for *C. augur*. For *L. cuprina* there was one rearing container allocated to each fodder type for separate periods between 1 and 11 days. A total of 33 rearing containers was used for each species. The day and substrate type to which a set of larvae was allocated was determined by lottery to randomise microclimatic effects in the laboratory.

After sample allocation, all rearing containers were placed on a rack in a temperature-controlled room and maintained at $25 \pm 3.5^{\circ}\text{C}$ (range 24.0 to 28.5°C) and ambient humidity. All rearing containers were left undisturbed until the allocated time had elapsed.

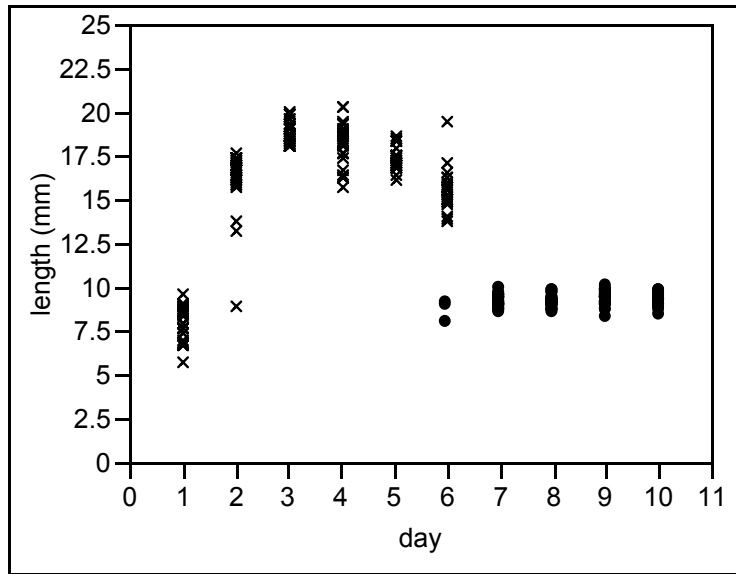
6.3 Results

6.3.1 Fresh vs. frozen developmental media

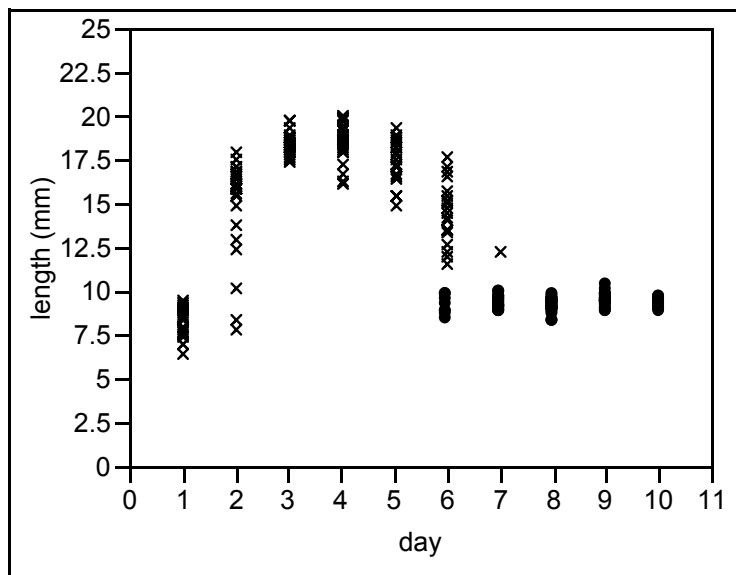
The ambient temperature was found to oscillate between 24.0 and 25.0°C for the duration of the experiment. The results of growth of *C. augur* larvae on fresh and frozen/thawed sheep's liver at this temperature are shown graphically in Figure 6.1. The curves are very similar and the spread of the data points is almost mirrored between the two treatments.

A significant difference in body length was detected at a 5% level (but not a 1% level) between larvae grown on fresh liver and larvae grown on frozen/thawed liver on day 6, but not between pupae on the same day. Comparisons between larvae grown on fresh liver compared with frozen/thawed liver on all other days showed no significant differences in body length.

Both groups of larvae moulted from first instar to second instar within 24 hours and from second instar to third instar within 48 hours. No transitional forms were seen. The group grown on frozen/thawed liver achieved maximum mean larval length at day 3. The group grown on fresh liver achieved maximum mean length on day 4. Two larvae had migrated from the frozen/thawed liver by day 4, but none from the fresh liver.



a) fresh liver



b) frozen/thawed liver

Figure 6.1: Growth of *Calliphora augur* larvae (length in mm) over time on a) fresh sheep's liver and b) frozen/thawed sheep's liver. All tissues were equilibrated to room temperature prior to use. Legend: x = larvae, • = pupae

By day 5 all but one larva had migrated from the frozen/thawed liver and approximately half the larvae had migrated from the fresh liver. The process of pupation did not appear to be as highly synchronised as instar moult. On day 6 all larvae had migrated and pupae were observed from those grown in both treatments, with more pupae observed from those grown on the fresh liver. By day 7 the larvae had almost all pupated, but one third-instar larva was observed on the fresh liver. The presence of this larva in the fresh group is explained by the occasional persistence of individual immatures as prepupae after others have pupated [48]. Pupae only were observed on days 8, 9 and 10. Descriptive data for each group are shown in Table 6.1.

6.3.2 *Larval growth on different tissues*

6.3.2.1 Growth of *Lucilia cuprina* larvae on different sheep's tissues

The average sample sizes of larvae recovered from the different tissues were 13 for brain, 11 for liver and 17 for meat. The total numbers of larvae collected were 142 from brain, 124 from liver and 187 from meat. Growth curves of the raw data are shown in Figure 6.2. Percent recoveries are shown in Table 6.2. Recoveries range from 5% to 130%. The 130% recovery is unlikely to be contamination, but rather a counting error. The habit of *L. cuprina* larvae to curl around themselves and each other may also have contributed to the counting error. The final data set with outliers removed is summarised in Table 6.3.

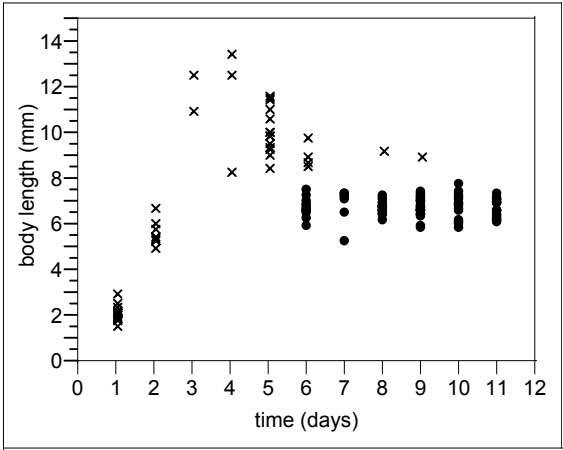
Transitional forms, first to second and second to third instars, were observed on liver and meat but not on brain. Mean maximum length was reached by day 3 for the groups grown on brain and meat, and on day 5 for the group grown on liver. Migration was first observed on day 4 from both brain and meat, and on day 5 from liver. After day 8 all individuals (larvae and pupae) were found in the chaff.

Pupae were first observed on day 6 for all tissue types (all dark brown pupae). Younger, white and light brown pupae were not observed. Migration from developmental medium to chaff was not well synchronised in any treatment for this species. Migration behaviour and proportions on meat and in chaff are shown in Figure 6.3.

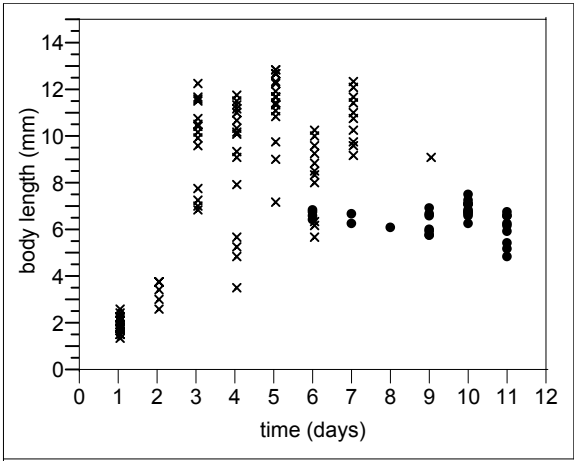
Significant differences were not detected between the groups on day 1, the day of hatching. The non-significant difference between the larvae on day 2 was between meat and brain only. The larvae grown on liver could not be compared because multiple larval stages were collected from the liver sample (Table 6.1).

Table 6.1: Summary growth data of *Calliphora augur* larvae on fresh vs. frozen/thawed sheep's liver. P= mature [dark brown] pupae, C=number migrated to chaff [in superscript], M=number still on meat [in superscript], †=excluded from analysis.

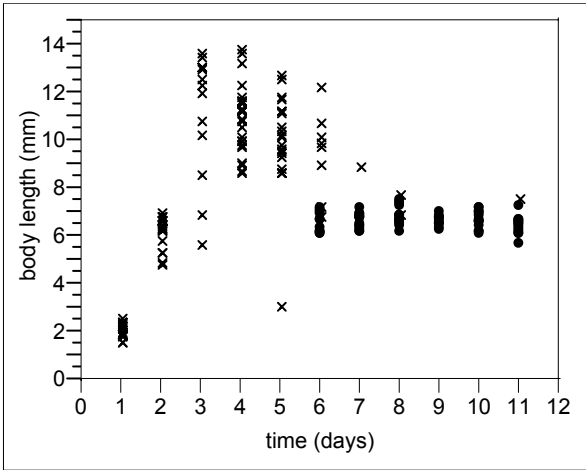
Time (days)	0	1	2	3	4	5	6	7	8	9	10
FRESH											
Life stage	1st	2nd	3rd	3rd	3rd	3 rd M ^{9†}	3 rd /P ^{8†}	3 ^{1†} /P	P	P	P
Mean (mm)		8.12	15.04	18.38	18.42	16.53	14.39	9.42	9.29	9.60	9.36
Range (mm)		6.35- 9.36	7.75- 17.80	17.35- 19.68	16.08- 19.88	14.79- 18.09	11.39- 17.62	8.93- 10.05	8.41- 10.00	9.02- 10.48	9.01- 9.86
Std dev		0.81	2.63	0.60	1.00	1.03	1.71	9.42	0.37	0.34	0.25
n		23	27	23	27	11	21	22	26	28	17
FROZEN/THAWED											
Life stage	1st	2nd	3rd	3rd	3 rd C ^{2†}	3 rd M ^{1†}	3 rd /P ^{3†}	P	P	P	P
Mean (mm)		7.80	16.10	18.69	18.23	17.27	15.46	9.29	9.27	9.47	9.38
Range (mm)		5.63- 9.55	8.87- 17.56	17.92- 19.96	15.68- 20.25	16.08- 18.51	13.75- 19.32	8.66- 10.01	8.72- 9.99	8.36- 10.22	8.56- 9.93
Std dev		0.96	1.72	0.62	1.14	0.65	1.23	0.34	0.33	0.41	0.34
n		27	28	28	26	22	21	28	26	29	27



a) sheep's brain



b) sheep's liver



c) lamb chops

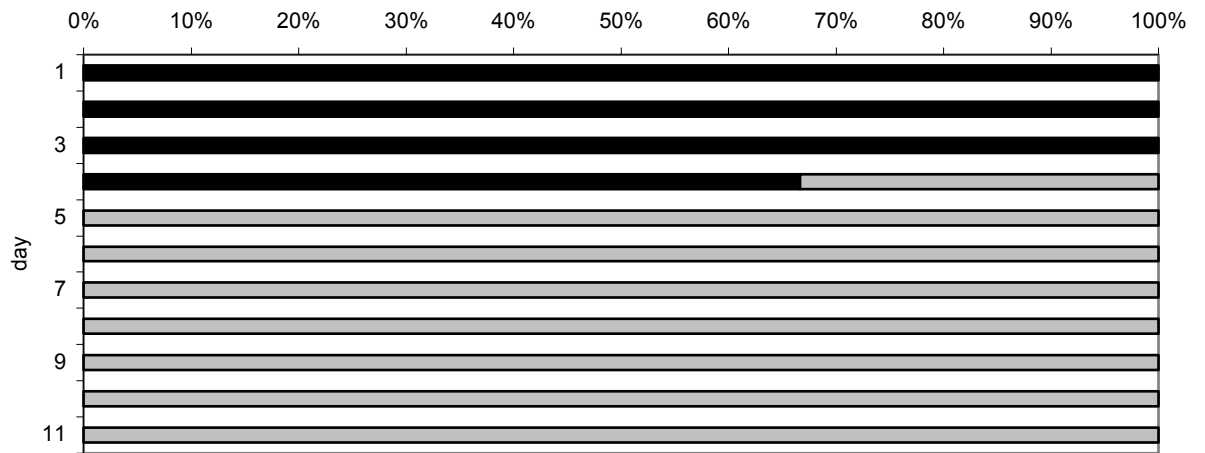
Figure 6.2: Growth of *Lucilia cuprina* larvae on different sheep's tissues - raw data shown.
Legend: x = larvae, ● = pupae

Table 6.2: Recoveries of *Lucilia cuprina* larvae grown on different sheep's tissues - brain, liver and meat.

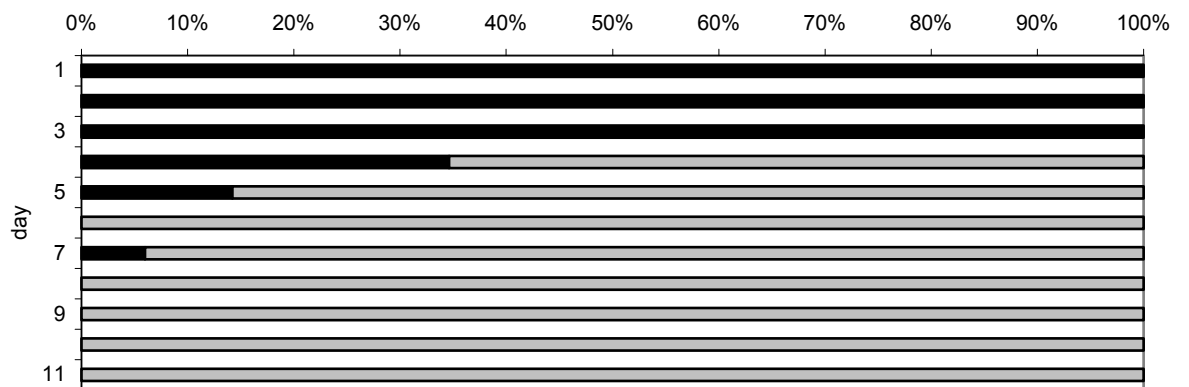
Time (days)	Recovery (%)		
	Brain	Liver	Meat
1	80	85	90
2	35	25	80
3	10	70	65
4	15	80	130
5	70	80	105
6	85	75	95
7	35	60	80
8	95	5	85
9	130	40	60
10	70	55	70
11	85	45	75
Average	64.5	56.3	85

Table 6.3: Mean body length (mm) \pm std dev. of larval instars and growth stages by day of *Lucilia cuprina* grown on different sheep's tissues. WP=white pupae, LBP=light brown pupae, BP=brown pupae. Where std dev.= 0, n=1. nd=no data. *[#]=outliers removed, [#]=number removed. [§]=excluded from analysis. ^f=pupae compared.

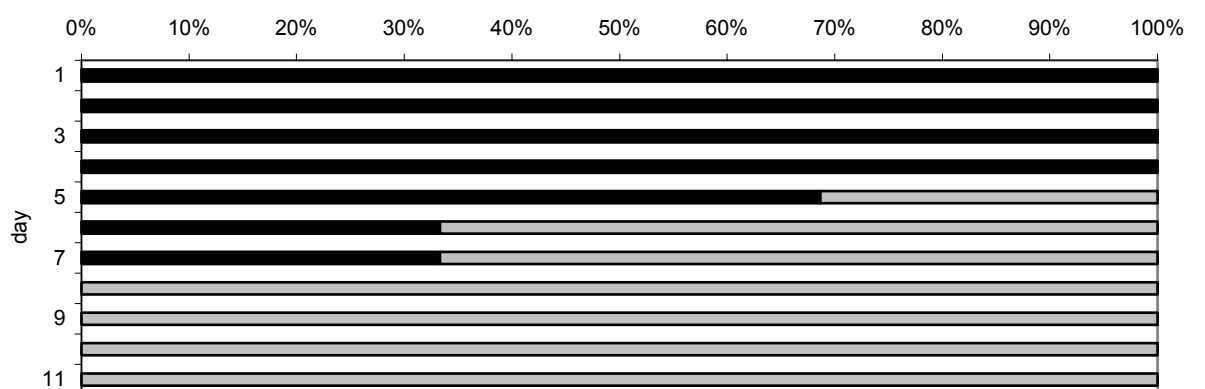
Day		Life stage								
		1st	1st-2nd	2nd	2nd-3rd	3rd	WP	LBP	BP	p
1	Meat	1.95 \pm 0.30	-	-	-	-	-	-	-	0.6944
	Brain	1.97 \pm 0.23 ^{*1}	-	-	-	-	-	-	-	
	Liver	1.88 \pm 0.33	-	-	-	-	-	-	-	
2	Meat	-	-	6.02 \pm 0.69	-	-	-	-	-	0.1620
	Brain	-	-	5.59 \pm 0.57	-	-	-	-	-	
	Liver	2.75 \pm 0.28 [§]	3.35 \pm 0 [§]	3.71 \pm 0.02 [§]	-	-	-	-	-	
3	Meat	-	-	-	5.53 \pm 0 [§]	11.53 \pm 2.11	-	-	-	0.0310
	Brain	-	-	-	-	11.69 \pm 1.09 [§]	-	-	-	
	Liver	-	-	-	-	9.75 \pm 1.84	-	-	-	
4	Meat	-	-	8.62 \pm 0 [§]	-	10.93 \pm 1.35	-	-	-	0.0204
	Brain	-	-	-	-	11.36 \pm 2.73 [§]	-	-	-	
	Liver	-	-	5.60 \pm 0 [§]	-	8.92 \pm 2.70	-	-	-	
5	Meat	-	-	-	-	10.22 \pm 1.27 ^{*1}	-	-	-	0.0009
	Brain	-	-	-	-	10.04 \pm 1.00	-	-	-	
	Liver	-	-	-	-	11.58 \pm 1.11 ^{*1}	-	-	-	
6	Meat	-	-	-	-	9.36 \pm 1.77	-	-	6.68 \pm 0.40	0.2944
	Brain	-	-	-	-	8.94 \pm 0.55	-	-	6.81 \pm 0.34 ^{*1}	
	Liver	-	-	-	-	8.22 \pm 1.58	-	-	6.7 \pm 0.21	
7	Meat	-	-	-	-	8.83 \pm 0 [§]	-	-	6.65 \pm 0.30	0.4470 ^f
	Brain	-	-	-	-	-	-	-	6.90 \pm 0.75	
	Liver	-	-	-	-	10.76 \pm 1.09 [§]	-	-	6.53 \pm 0.26	
8	Meat	-	-	-	-	7.21 \pm 0.63 [§]	-	-	6.86 \pm 0.39	0.4096 ^f
	Brain	-	-	-	-	9.14 \pm 0 [§]	-	-	6.76 \pm 0.29	
	Liver	-	-	-	-	-	-	-	6.09 \pm 0 [§]	
9	Meat	-	-	-	-	-	-	-	6.65 \pm 0.22	0.0097 ^f
	Brain	-	-	-	-	8.85 \pm 0 [§]	-	-	6.78 \pm 0.41	
	Liver	-	-	-	-	9.08 \pm 0 [§]	-	-	6.26 \pm 0.48	
10	Meat	-	-	-	-	-	-	-	6.72 \pm 0.37	0.5150 ^f
	Brain	-	-	-	-	-	-	-	6.80 \pm 0.57	
	Liver	-	-	-	-	-	-	-	6.92 \pm 0.40	
11	Meat	-	-	-	-	-	-	-	6.45 \pm 0.36	0.0023 ^f
	Brain	-	-	-	-	-	-	-	6.73 \pm 0.40	
	Liver	-	-	-	-	-	-	-	5.99 \pm 0.68	



a) sheep's brain



b) lamb chops



c) sheep's liver

Figure 6.3: Proportions of immature *Lucilia cuprina* individuals observed on meat and in chaff over time when grown on different sheep's tissues.

Legend: = located on meat , = located in chaff .

The significant differences detected on days 3 and 4 were between larvae grown on liver and meat only. The recovery of larvae from brain on these days was low and thus not compared. Because larvae were a similar colour to both the brain and fat in the lamb chops, it was difficult to see them on these tissues, especially when they were very small.

The significant difference between the groups on day 5 further illustrates a lag in larval growth on liver. The group grown on liver achieved maximum body length on day 5 but larvae grown on brain and meat achieved this on day 3 and were already beginning to reduce their body length in preparation for pupariation.

The larvae grown on liver began this process between days 5 and 6. Comparison of the groups exhibiting the highest mean body length, i.e. day 3 for brain and meat and day 5 for liver, showed no significant difference in length ($p=0.7625$, $n=30$), indicating that larvae can potentially reach the same maximum size regardless of the tissue type that forms the larval developmental medium.

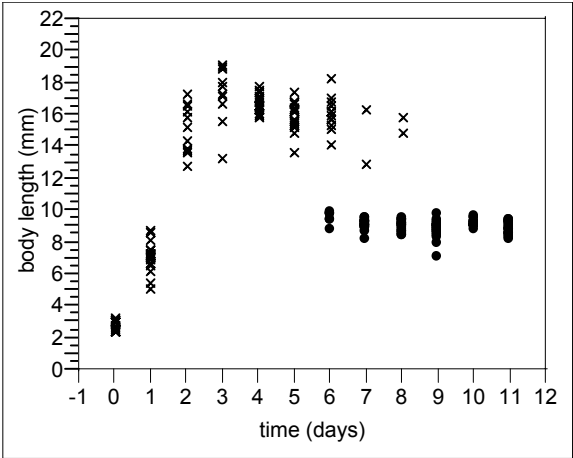
Across days 6-11 larvae grown on liver mostly produced smaller pupae than the other substrate groups. This difference was significant for days 9 and 11. It is interesting that day 10 showed no significant difference; this may indicate that although larvae grown on liver seem to lag developmentally, they can achieve a similar pupal size.

6.3.2.2 Growth of *Calliphora augur* larvae on different sheep's tissues

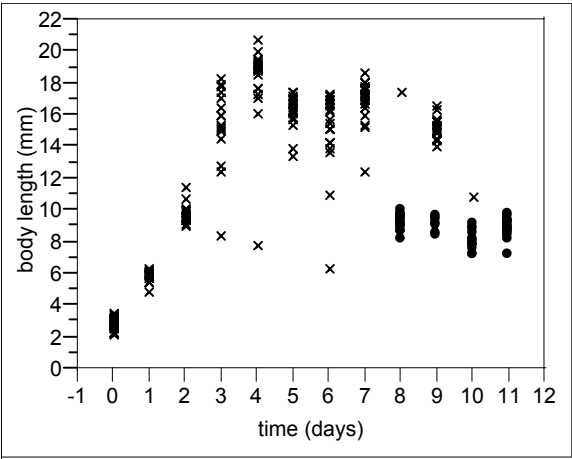
The average sample sizes of larvae recovered from the different tissues were 17 for brain, 17 for liver and 18 for meat. The total numbers of larvae collected were 201 from brain, 203 from liver and 195 from meat. Growth curves of the raw data are shown in Figure 6.4. Percent recoveries are shown in Table 6.4. The final data set, with outliers removed, is summarised in Table 6.5.

Transitional forms, first to second and second to third instars, were observed on liver but not on the groups grown on brain or meat. Mean maximum length was reached by day 3 for the groups grown on brain and meat, and on day 4 for the

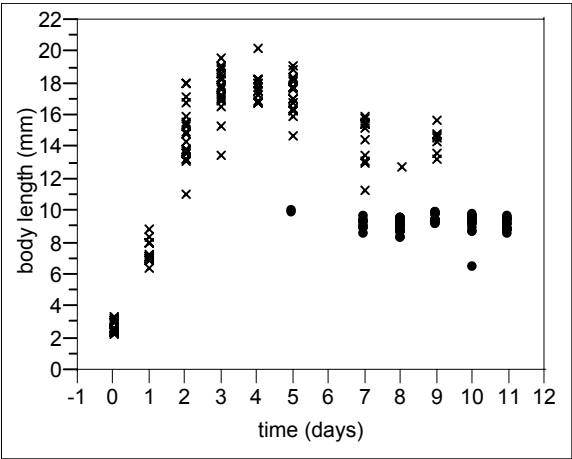
group grown on liver. Migration was first observed on day 4 from both brain and meat, and on day 6 from liver.



a) sheep's brain



b) sheep's liver



c) lamb chops

Figure 6.4: Growth of *Calliphora augur* larvae on different sheep's tissues - raw data shown.
Legend: x = larvae, ● = pupae

Table 6.4: Recoveries of *Calliphora augur* larvae grown on different sheep's tissues - brain, liver and meat. n.d.=no data.

Time (days)	Recovery (%)		
	Brain	Liver	Meat
1	95	60	70
2	55	70	100
3	50	80	90
4	85	90	75
5	85	85	90
6	95	95	n.d.
7	110	95	95
8	100	90	95
9	95	100	100
10	85	70	95
11	85	90	75
Average	85.5	84	89

Table 6.5: Mean body length (mm) \pm std dev. of larval instars and growth stages by day of *Calliphora augur* grown on different sheep's tissues. WP=white pupae, LBP=light brown pupae, BP=brown pupae. Where std dev.= 0, n=1. nd=no data. *#=outliers removed, #=number removed. §=excluded from analysis. ^f=pupae compared.

Day		Life stage								
		1st	1st-2nd	2nd	2nd-3rd	3rd	WP	LBP	BP	p
0	Meat	2.58 \pm 0.34	-	-	-	-	-	-	-	0.48
	Brain	2.67 \pm 0.31	-	-	-	-	-	-	-	
	Liver	2.72 \pm 0.36	-	-	-	-	-	-	-	
1	Meat	-	-	7.32 \pm 0.70	-	-	-	-	-	<0.0001
	Brain	-	-	6.99 \pm 0.28* ⁶	-	-	-	-	-	
	Liver	-	4.73 \pm 0 [§]	5.74 \pm 0.29	-	-	-	-	-	
2	Meat	-	-	-	-	14.66 \pm 1.78	-	-	-	0.6183
	Brain	-	-	-	-	14.97 \pm 1.51	-	-	-	
	Liver	-	-	9.06 \pm 0.214 [§]	9.67 \pm 0.42 [§]	11.3 \pm 0 [§]	-	-	-	
3	Meat	-	-	-	-	17.77 \pm 1.09* ¹	-	-	-	0.0015
	Brain	-	-	-	-	17.17 \pm 1.79	-	-	-	
	Liver	-	-	-	-	15.67 \pm 1.75* ¹	-	-	-	
4	Meat	-	-	-	-	17.69 \pm 0.89* ²	-	-	-	<0.0001
	Brain	-	-	-	-	16.59 \pm 0.54	-	-	-	
	Liver	-	-	-	-	18.50 \pm 1.16* ¹	-	-	-	
5	Meat	-	-	-	-	17.16 \pm 1.17	9.91 \pm 0 [§]	10.03 \pm 0 [§]	-	0.0011
	Brain	-	-	-	-	15.94 \pm 0.71* ¹	-	-	-	
	Liver	-	-	-	-	16.38 \pm 0.62* ²	-	-	-	
6	Meat	nd	nd	nd	nd	nd	nd	nd	nd	0.2856
	Brain	-	-	-	-	15.97 \pm 1.04	-	9.86 \pm 0.09 [§]	9.5 \pm 0.37 [§]	
	Liver	-	-	-	-	15.38 \pm 1.66* ¹	-	-	-	
7	Meat	-	-	-	-	14.32 \pm 1.52	8.62 \pm 0 [§]	-	9.29 \pm 0.28 [§]	<0.0001
	Brain	-	-	-	-	14.50 \pm 2.38	-	9.31 \pm 0 [§]	9.20 \pm 0.23* ^{1§}	
	Liver	-	-	-	-	16.7 \pm 1.40	-	-	-	
8	Meat	-	-	-	-	12.64 \pm 0 [§]	-	-	9.06 \pm 0.38	0.1089 ^f
	Brain	-	-	-	-	15.24 \pm 0.67 [§]	-	-	9.07 \pm 0.34	
	Liver	-	-	-	-	17.33 \pm 0 [§]	-	-	9.31 \pm 0.45	
9	Meat	-	-	-	-	14.33 \pm 0.80 [§]	-	-	9.58 \pm 0.28	<0.0001 ^f
	Brain	-	-	-	-	-	-	-	8.95 \pm 0.31* ³	
	Liver	-	-	-	-	15.10 \pm 0.73 [§]	-	-	9.19 \pm 0.48	
10	Meat	-	-	-	-	-	-	-	9.39 \pm 0.33* ¹	<0.0001 ^f
	Brain	-	-	-	-	-	-	-	9.25 \pm 0.21	
	Liver	-	-	-	-	10.7 \pm 0 [§]	-	-	8.17 \pm 0.59	
11	Meat	-	-	-	-	-	-	-	9.20 \pm 0.33	0.1913 ^f
	Brain	-	-	-	-	-	-	-	8.94 \pm 0.38	
	Liver	-	-	-	-	-	-	-	9.05 \pm 0.42* ¹	

In the group grown on brain not all individuals migrated on any one day; pupae were sometimes observed in the drying meat as well as in the chaff. Three days were recorded on which all larvae had migrated from meat, and two days were recorded on which all larvae had migrated from liver. Migration behaviour and proportions on meat and in chaff are shown in Figure 6.5. Migration from the larval developmental medium to the chaff was most synchronised in those larvae grown in brains. Larvae grown on liver and meat migrated in a much more fragmented way.

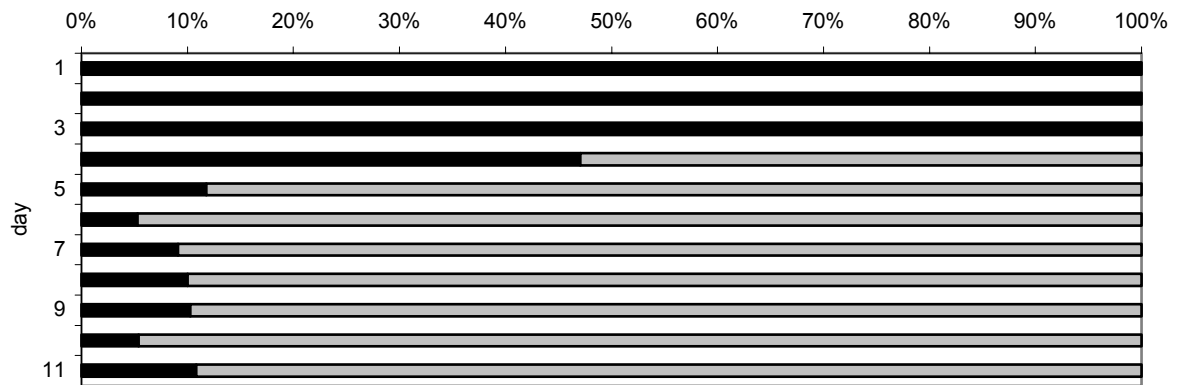
Pupae were first observed on day 5 from meat (white pupae) and day 6 from brain (dark brown pupae). Light brown pupae were observed on day 6 from meat. Dark brown pupae were not observed from meat until day 7. Dark brown pupae were first observed from liver at day 8.

Significant differences were detected between the substrate groups on many days (days 1, 3, 4, 5, 7, 9 and 10). No significant difference between the groups on day 0 indicates that the batches of new first-instar larvae were similar enough for comparisons of the treatments to ensue.

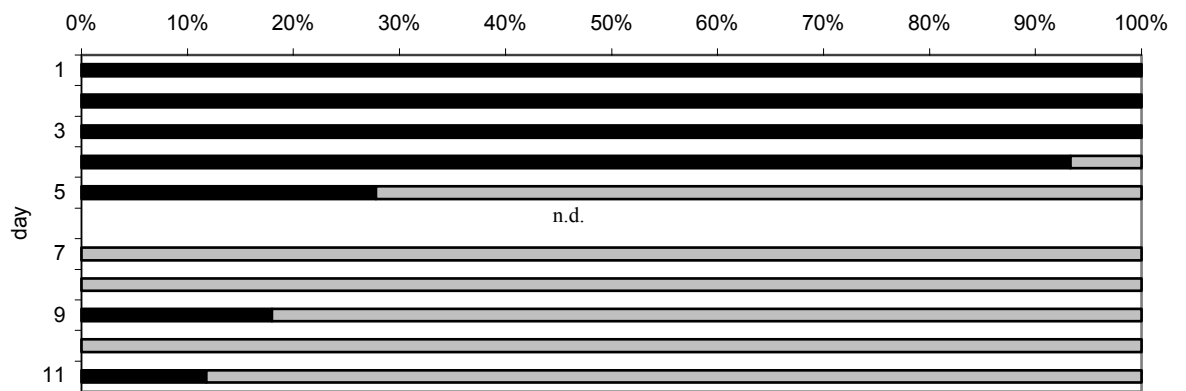
At day 2, most larvae grown on liver were second instars. There was one third-instar larva and it was not possible to compare this one larva with the third instars for brain and meat. There were no second instars observed in either the brain or meat treatments at that time. The non-significant difference for day 2 indicates that, at that time, the group grown on meat was not significantly different to the group grown on brain, and it was not possible to examine a statistical relationship with those grown on liver. On day 6 it was only possible to compare brain and liver, and although no significant difference was detected between the larval lengths, the presence of pupae and most of the larvae from brain in the chaff indicates that there were developmental differences that were significant.

The pupae from larvae grown on brain were smaller on two occasions, significantly so on one occasion. The pupae from larvae grown on liver were significantly smaller once and actually larger on one occasion. The non-significant difference for the measurements of pupae at day 8 indicates that larvae of *Calliphora augur* grown on liver seem to lag developmentally, but can

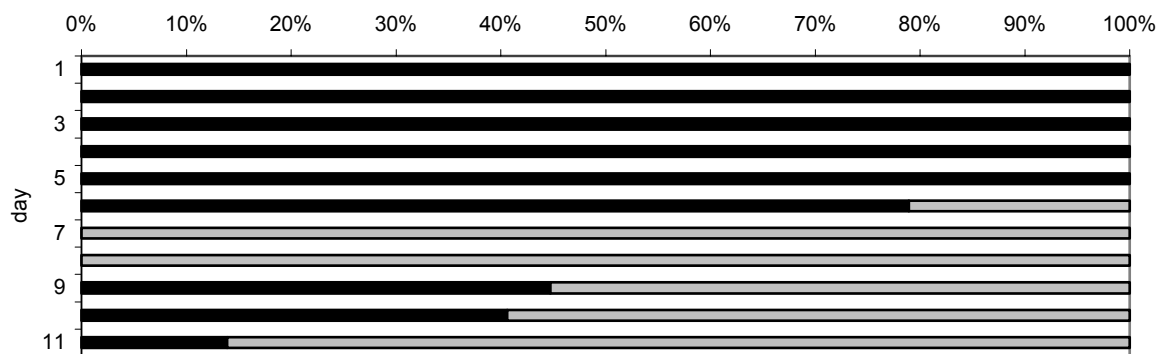
potentially eventually achieve a similar pupal size to those grown on other tissues.



a) sheep's brain



b) lamb chops



c) sheep's liver

Figure 6.5: Proportions of immature *Calliphora augur* individuals observed on meat and in chaff over time when grown on different sheep's tissues. n.d.=no data.

Legend: ■ = located on meat , ■ = located in chaff .

6.4 Discussion

6.4.1 *Fresh vs. frozen developmental media*

Subtle differences between larvae grown on fresh and frozen/thawed liver, such as that frozen and thawed treatments achieved maximum larval length and began migration a day earlier than those grown on fresh liver, may have disappeared with greater replication.

Calliphora augur is regarded as consecutively, facultatively viviparous [44]. The experiment was not repeated due to the low expected cohort size of 50 maggots [48, Section 4.2] and a desire to contain the study to one generation to avoid any intergenerational differences. The subtle differences detected appear to be offset by full migration and pupation occurring at the same time for both treatments and there being no significant differences between the length of larvae in any treatment pair at a 1% level.

When examining the effect of freezing on female rat carcasses, Micozzi [90] observed that frozen/thawed animals predominantly showed decay (aerobic decomposition) and that fresh animals predominantly showed putrefaction (anaerobic decomposition). This appears to be largely due to a reduced gut flora after freezing and does not apply to my study, which used a single type of tissue butchered and tested for human consumption, i.e. with most of the surrounding connective tissue removed.

6.4.2 *Larval growth on different tissues*

The developmental lag seen in larvae grown on liver was seen in both species examined and largely reflects the results of Kaneshrajah and Turner [139]. Significantly smaller pupae from larvae grown on brain were only observed once for each species, whereas Kaneshrajah and Turner [139] observed that larvae grown on brain and heart showed a marked wet weight loss during the post-feeding stage leading to puparia of reduced weight and size. Significantly smaller pupae from larvae grown on liver were observed twice with *L. cuprina* but only once with *C. augur*. The pupae of *C. augur* from liver were actually the largest on one occasion.

Investigations of *Lucilia* larvae feeding on meat have suggested that the main factors involved in the breakdown of the meat are mechanical maceration and an alkaline reaction resulting from bacterial action [143]. Hobson [142] found that trypsin is present in *Lucilia* larvae reared aseptically on sterilised brain and that growth proceeds

at almost the same rate as in the presence of bacteria. Other proteolytic enzymes are present in larval excreta (e.g. collagenase) and these may serve to digest the structural parts of muscle tissue [143]. Mackerras and Freney [64] expanded on Hobson's work and found that both peptic and tryptic enzymes are present in all instars and developmental stages of *L. cuprina*, as well as in *L. sericata* and *Ch. rufifacies*, sometimes strongly so. A study by Constable [147], which included both *C. augur* and *L. cuprina*, found evidence to suggest that proteolytic enzymes have an important role in larval survival, and studies on *L. cuprina* and *Ch. rufifacies* with various media found that an increased content of soluble sheep protein was necessary for complete development [64].

It therefore seems that the main factors contributing to the observed differences in growth rate are tissue structure, activity of proteolytic enzymes and the amount of soluble protein, but other nutrients may also have a play a role. Using United States Department of Agriculture (USDA) data on food composition [145], a comparison of nutrients in the tissues examined indicated that liver has less total lipid than meat or brain (5.02 g/100 g cf 16.97 and 8.58 respectively). Studies on fat metabolism in *Calliphora* [Weinland in 140] showed that a larva is able to build fat from proteins and accumulate it in the fat bodies, to be freely spent during metamorphosis, while a pupa builds no further fat. Generally, fatty substances are used and stored for energy. It follows then that larvae consuming more fat will be expending less energy on metabolism than those feeding on substances which are purely proteinaceous. These larvae can then, in principle, direct more energy into growth.

Again, using the USDA data [145], comparison of other nutrients between the tissues examined indicated that liver has much more iron than meat or brain (7.37 mg/100g for liver cf 1.43 for meat and 1.75 for brain), a much higher copper load (6.979 mg/100g cf 0.145 in meat and 0.24 in brain), a much higher selenium load (82.4 mcg/100 g cf 7.5 for meat and 9.0 for brain), higher levels of thiamine, riboflavin, niacin, pantothenic acid, total folate and vitamin B12. Retinol is present in liver but not in brain. The role of these nutrients in larval growth and development is, however, unclear. The accumulation of these substances, particularly retinol, in liver reflects the role of this organ in the body. The liver acts as a filter and accumulates and metabolises toxic and other foreign substances. It also serves as a store for vitamin A. Liver differs structurally from the other tissues in that it is denser, and contains less fat and more connective tissue. It is likely that a larger output of energy is necessary to liberate the

nutrients in liver to feeding larvae, and that upon consumption of nutrients some toxins may also be ingested.

6.5 Conclusions

6.5.1 Fresh vs. frozen developmental media

It is concluded that larval growth in *C. augur* on frozen/thawed sheep's liver is not significantly different to growth on fresh sheep's liver. This short study deserves to be extended using other fly species to confirm whether my conclusions have wider applicability. For now, workers in forensic entomology may have improved confidence that a frozen food substrate probably does not significantly affect development of fly larvae.

6.5.2 Larval growth on different tissues

Larvae of both blowfly species grown on liver were often smaller than those grown on sheep's brains and sheep meat, moulted later, reached maximum length more slowly and sometimes produced significantly smaller pupae. It is common in forensic entomology for workers to consult data on the development of flies in arriving at estimates of post-mortem interval. These results suggest that there may be limitations in the forensic application of such data if they derive from a type of animal tissue different from that on which larvae at a death scene have been feeding. The advice of Kaneshrajah and Turner [139] is therefore repeated, i.e. that forensic practitioners should note the part of a corpse from which larvae are collected and exercise caution in their application of developmental data based on only one type of larval food substrate.

CHAPTER 7

Effect of preservative solutions on preservation of *Calliphora augur* and *Lucilia cuprina* larvae

Summary:

A major role of forensic entomology is to estimate the post-mortem interval. An entomologist's estimate of post-mortem interval is based on a series of generally valid assumptions, error in any of which can alter the accuracy of an estimate. The initial process of collecting and preserving maggots can itself lead to error, as can the method of killing and preservation. Since circumstances exist where it is not possible to rear maggots, methods of killing and preservation can be vital to preserving the integrity of entomological evidence. In this study, a number of preservation techniques used at crime scenes and in mortuaries were examined, and their effect on feeding third-instar larvae of *Calliphora augur* and *Lucilia cuprina* evaluated. The preservatives used were 70, 75, 80, 90 and 100% EtOH, Kahle's solution and 10% formalin. Each treatment was replicated three times. The effect of handling on first- and second-instar, feeding and post-feeding third-instar larvae of *C. augur* was also examined and compared to unhandled controls. Finally, the effects of preservatives were noted when larvae of *C. augur* and *L. cuprina* were placed into preservatives alive. It was found that continued handling is detrimental to specimens because preservative evaporates from both the vial and the specimens. No single preservative type was found to be entirely suitable for both species if DNA retrieval is desired. Specimens placed into most preservatives alive exhibited adverse colour changes, desiccation, sunkeness and agglomeration. It is concluded that the reaction to preservative type might be species specific and that different instars of the same species might also react differently.

7.1 Introduction

The rigorous collection of appropriate evidence is an integral part of using insects in forensic investigations. O'Flynn [25] stressed the importance of presenting entomological specimens in a viable state, not preserved or moribund from anoxia or refrigeration, and Erzinçliouglu [92] emphasised that collection by entomologists at a crime scene can potentially yield much more information than is possible from samples collected and submitted by a non-entomologist. Adequate preservation of larval stages can be vital, particularly in cases where chemical contamination may have occurred and the larvae do not survive the rearing process [39, 40].

The crime scene environment is very different to that of a laboratory and, when an entomologist is unable to attend a crime scene, crime scene personnel must instead collect the entomological evidence. However, such personnel may not preserve larvae as recommended in the literature, either because the required chemicals or dilutions are not available to them, or because suggested techniques, such as killing larvae in hot water, may be impractical or impossible in the field.

In practice, the approach to preserving larval specimens is rarely uniform. Review of various case accounts reveals that collection appears to occur mostly at autopsy [11, 40, 150, 153, 154], but may also happen before autopsy [151] and at the crime scene [118, 153]. Ethanol appears to be the preservative of choice, [16, 39, 151, 153-156] but the concentrations vary and it appears that hot water, despite being recommended, is not always used as a killing agent [86, 101, 153, 155, 157]. In some cases the concentration of ethanol is not revealed [39, 117, 133, 149], and while some workers suggest that preservation has occurred, they do not identify the preservative solution [17, 148, 150]. In controlled experiments, workers have killed larvae with hot water and preserved them with ethanol [97, 99, 100]. Wells and Kurahashi [78] removed all larvae and killed them in boiling 70% ethanol. Other workers have killed eggs and larvae immediately and mounted them on glass slides [158], and Rodriguez and Bass, in their landmark study on human cadavers in East Tennessee [159], did not appear to immediately kill the insects collected, rather placing and preserving them in a solution containing 85 cm³ of 90% ethanol, 10 cm³ of 40% formalin and 5 cm³ of glycerin (a recipe for Pampel's solution). Similarly, Wolff *et al.* [160] appear to have simply preserved their samples in 70% ethanol. Rodriguez and Bass were not the only workers to use relatively complex preservation solutions. For example, Shean *et al.* [161] first fixed immatures in KAA solution (85 mL 95% ethanol, 10 mL kerosene, 20 mL glacial acetic acid and 10 mL dioxane) and then transferred them to 75% ethanol and 3% glycerin as soon as possible. Turchetto *et al.* [149] employed Pampel's solution for some of

their work and Wells and LaMotte [101] preserved larvae in Kahle's solution. The use of preservatives has actually been discouraged by some workers [67] because it may affect dry weights.

The most recent text for students of forensic entomology [83] recommends fixing the larvae by either placing them in Kahle's solution or KAA, or by boiling the larvae in hot water. Specimens should then be removed from the fixative and placed into 80% ethanol [83]. Fixing in hot water accommodates the limited resources of law enforcement agencies and avoids specimens becoming brittle from being in chemical fixation for extended periods (more than 12 hours) [83]. Other handbooks on the subject recommend proper fixation and preservation in ethyl alcohol (ethanol) [130], and treating them with near-boiling water before transfer to 70-80% alcohol or Pampel's fluid (6 parts 35% formalin, 15 parts 95% ethyl alcohol, 2 parts glacial acetic acid and 30 parts distilled water), which is apparently particularly suitable for dipteran larvae requiring dissection [10]. Lord and Burger [81] and Wallman [162] have published protocols for collection of forensic entomological specimens, including similar recommendations for immature and soft-bodied insects.

Few studies compare different preservative types on dipteran species of forensic interest. Tantawi and Greenberg [82] examined the effects of a range of preservative types on *Protophormia terraenovae* and *Calliphora vicina*. They compared 70, 80 and 90% ethanol, benzene, kerosene, formalin, 99.7% isopropanol, 70% ethanol/AAG; 90% ethanol/AAG, KAAD, KAA, XAAD, XA, Pampel's fluid and San Veino. They found shrinkage in body length with all solutions in both species, but that *C. vicina* underwent greater shrinkage than *P. terraenovae*. They also found more shrinkage with younger third-instars than with older ones. A recently published paper by Adams and Hall [105] examined various killing techniques and preservatives on post-feeding third-instar larvae of *C. vomitoria* and *Lucilia sericata*. The preservatives examined were 10% formalin, 80% ethanol and 90% ethanol. Adams and Hall [105] also examined the effects of placing live larvae into these solutions and the effects of water temperature when killing. They found extension of larvae in both species with some treatments and poor preservation from placing live specimens into all preservative types.

To date there have been no other studies in this area, and no work of this type on Australian species. Independently of Adams and Hall's work, studies were commenced on the effects of preservative types on larvae of *Calliphora augur* and *Lucilia cuprina*.

7.2 Materials and Methods

7.2.1 *Preservative choice*

Discussion with crime scene investigators has indicated that larvae are often placed straight into ethanol as ordered from stores (often 95% EtOH) without being killed in hot water. Furthermore, different morgues use different preservative solutions for those insects collected during autopsy. The preservation treatments in this study were chosen to reflect current practice, help determine best practice and allow for comparison of data in the current literature. Discussion with crime scene and mortuary staff indicated that ethanol, formalin and Kahle's solution are in current use in this region. 10% formalin was kindly supplied by Aldo Severino of North Shore Hospital Mortuary. It is also the preferred method of Glebe Mortuary attached to the Institute for Forensic Medicine (Prof. John Hilton, pers. comm.). Kahle's solution was kindly supplied by staff of Westmead Hospital Coronial Mortuary. The various concentrations of ethanol were prepared by me in the laboratory.

7.2.2 *Sample generation*

Separate cages containing gravid females and males of each species were presented with a portion of sheep's liver which had been frozen and thawed, then equilibrated to room temperature (see Section 6.3.1). The females were given one hour to oviposit or larviposit. Eggs and larvae were then left undisturbed until the appropriate instar had been reached. When the larvae were at the appropriate growth stage, counts of approximately 20 of the new larvae were transferred with a fine artist's brush [132] to each preservative treatment.

7.2.3 *Sample collection, handling and preservation*

Except for the samples placed into preservative alive, collected larvae were immediately killed by immersion in boiled water, dried with paper towel and preserved. All larvae were placed into vials posteriad [146]. Growth stage (i.e. pre- or post- feeding) and/or instar number were recorded. Instars were differentiated based upon the illustrations of Johl and Anderson [85].

7.2.4 *Measurement, data handling and statistics*

The body length of larvae was measured with the aid of a dissecting microscope and Mitutoyo Absolute digimatic digital callipers. Body length was measured as the distance, viewed laterally, between the most distal parts of the head and the last abdominal segment. The ambient temperature within the temperature-controlled cabinet was monitored with small dataloggers (iButtons). Data entry and analysis were done using JMP[®] and Excel[®].

7.3 Experiment A – effects of 80% EtOH on larval body length over time in *Calliphora augur*

Different growth stages of *C. augur* larvae were killed, dried, measured (body length) and allocated to vials of 80% EtOH. They were then removed from the vials, measured and returned to the same vial each day for ten consecutive days. Unhandled controls were also prepared. These were killed, dried, measured and preserved in the same way but left undisturbed for ten days, when they were removed from the vials and remeasured. Three replicates of ten individuals were examined. The controls were one replicate of ten individuals. The growth stages examined were first-instar, second-instar, feeding third-instars and late third-instars. A ten-day period was chosen because the different preservative types may take different times to show a maximum effect. Also, a ten-day preservation period may be similar to that needed for sample collection, exhibit handling, transport, autopsy and time management by the entomological consultant.

7.4 Experiment B – effects of different preservative types on feeding third-instar larvae of *Calliphora augur* and *Lulia cuprina*

In order to determine how different preservative solutions influence the body length and general integrity of larvae of *C. augur* and *L. cuprina*, a number of preservation trials were conducted. In the first part of this experiment, feeding third-instar larvae of both species were allocated to the following treatments: 70% EtOH, 75% EtOH, 80% EtOH, 90% EtOH, 100% EtOH, 10% formalin and Kahle's solution. Three replicates of 20 feeding third-instar larvae were run for each measured treatment. So that comparisons could be made between measurements from the preserved larvae and the just-killed larvae, all larvae were measured after killing in boiling water and drying, prior to allocation to the preservative treatment. Preservative treatments for individual samples were selected by lottery. Feeding third-instars were chosen because these are the growth stage most commonly collected.

In the second part of this experiment, I placed three lots of 10 alive, feeding third-instar larvae directly into each preservative type. Preservative allocations were determined by lottery. These samples were observed and examined only. They were neither handled nor measured.

7.5 Results

7.5.1 Effects of 80% EtOH on larval body length over time in *Calliphora augur*

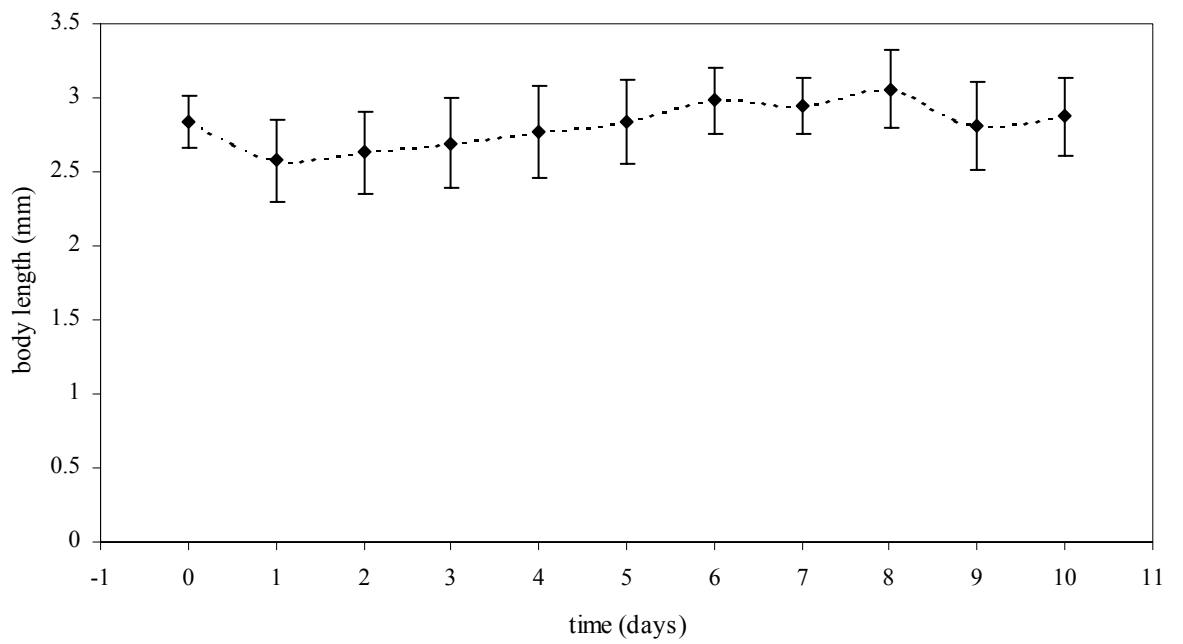
The results of studies on the various larval growth stages of *C. augur* are shown in Figures 7.1-4. In the first-instars, one of the replicates was significantly different to the other two at day 0 and also to the control sample at day 0. This sample was omitted from further

analyses. The two remaining samples were pooled. There was an initial decrease in body length and then a steady increase to approximately the same mean length at day 10 (Figure 7.1a). In the controls for this group, a slight mean increase was observed between day 0 and day 10 (Figure 7.1b). The increase in the controls was from a mean of 2.58 mm at day 0 to 2.90 mm at day 10. Interestingly, this increase of 0.32 mm (12.4%) was not found to be statistically significant (d.f.=1, $F=2.2366$, $P>0.05$). Comparisons of the means of measurements of the two handled replicates on each day showed no significant difference on most days, an exception being day 7 (d.f.=1, $F=8.7603$, $P<0.05$).

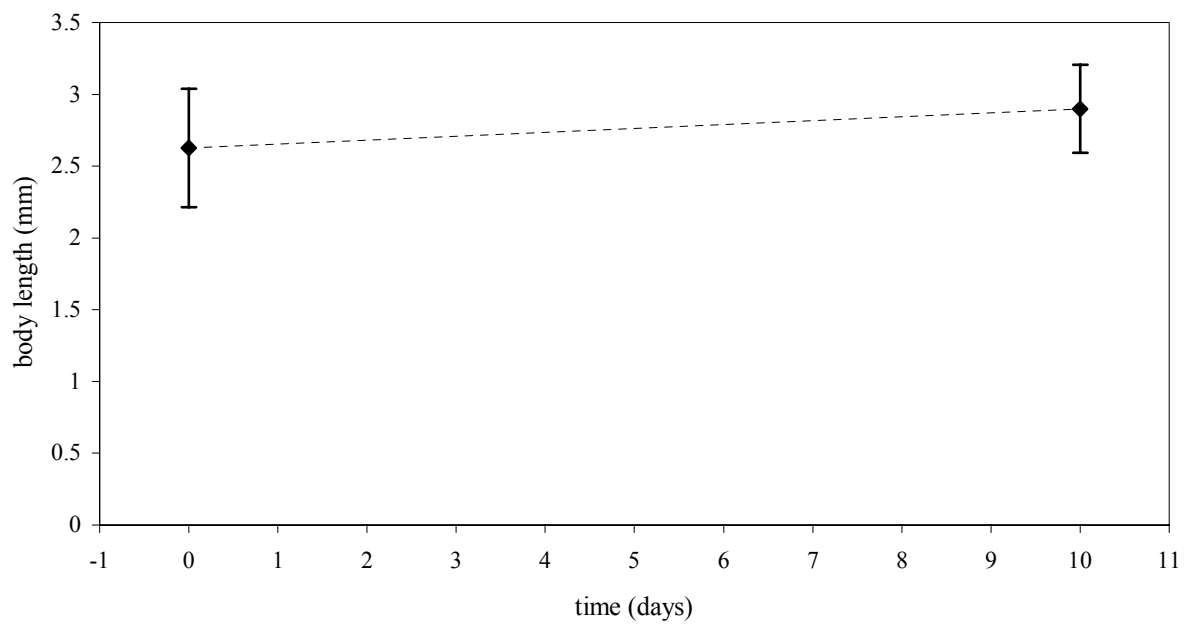
Among the second-instars, since no significant differences were detected between replicates at day 0, and the replicates were not significantly different to the control sample at day 0, the three replicates were pooled. There was a steady increase in body length and then a slight tapering off to a slight mean increase in length at day 10 (Figure 7.2a). The increase in the controls was from a mean of 4.91mm at day 0 to 5.68 mm at day 10 (Figure 7.2b). This increase of 0.78 mm (16%) was found to be statistically significant (d.f.=1, $F=7.7392$, $P<0.05$). Comparisons of the means of the three replicates on each measured day showed no significant difference on most days, but exceptions were days 5 (d.f.=2, $F=3.4928$, $P<0.05$) and 9 (d.f.=2, $F=4.722$, $P<0.05$).

In the feeding third-instars, since no significant differences were detected between replicates at day 0, and the replicates were not significantly different to the control sample at day 0, the replicates were pooled. There was a steady increase in body length and then a slight tapering off to a slight mean increase in length at day 10 (Figure 7.3a). The increase in the controls (Figure 7.3b) was from a mean of 9.60 mm at day 0 to 10.50 mm at day 10. This increase of 0.89 mm (9.3%) was not found to be statistically significant (d.f.=1, $F=1.6653$, $P>0.05$). Comparisons of the means of the three replicates on each measured day showed no significant differences.

In the late (post-feeding) third-instars, since no significant differences were detected between replicates at day 0, and the replicates were not significantly different to the control sample at day 0, the replicates were pooled. There was a steady increase in body length and then a slight tapering off to a slight mean increase in length at day 10 (Figure 7.4a). The change in the controls was actually a decrease (Figure 7.4b) from a mean of 14.04 mm at day 0 to 13.92 mm at day 10. This decrease of 0.12 mm (0.86%) was not statistically significant (d.f.=1, $F=0.0841$, $P>0.05$). Comparisons of the means of the three replicates on each measured day showed no significant differences on days 1 and 10 only, with significant differences detected on all other days.

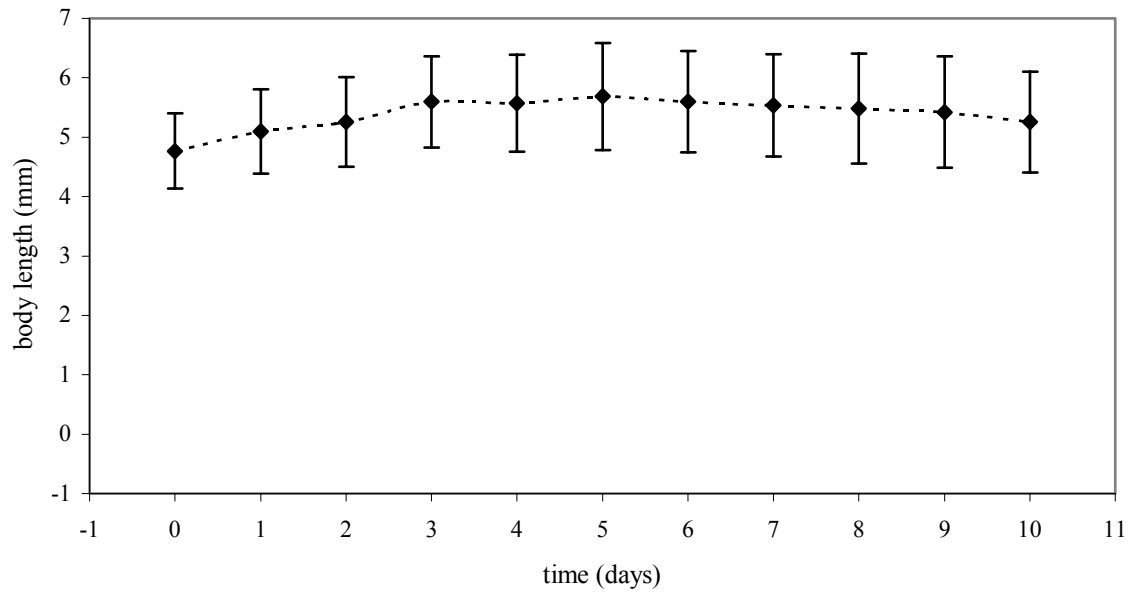


a. with constant handling

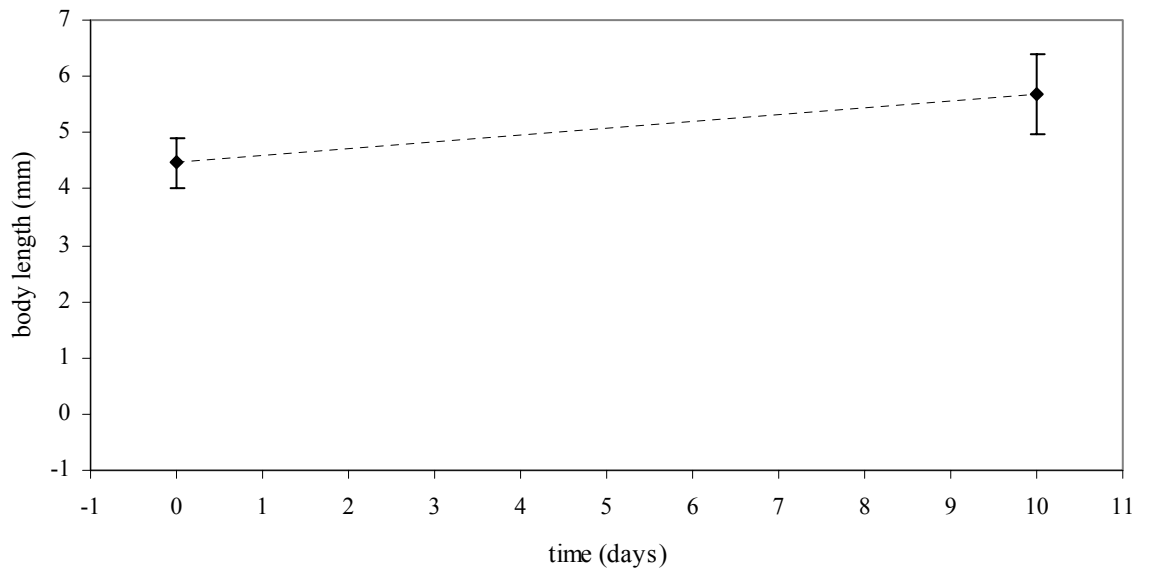


b. controls left undisturbed for 10 days

Figure 7.1: Effect of 80% EtOH on body length measurements of first-instar *Calliphora augur*.

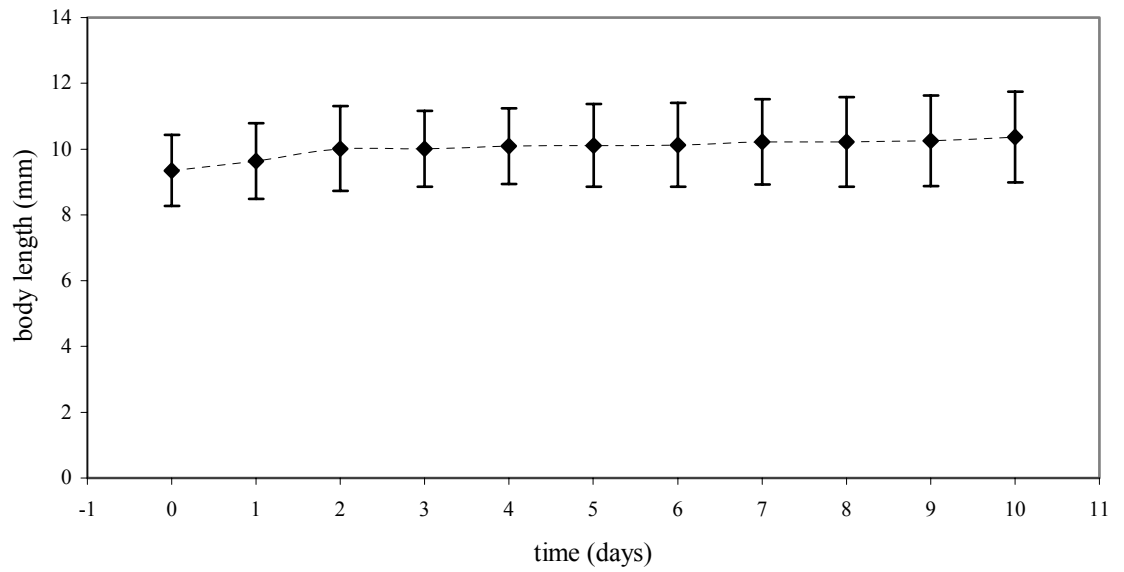


a. with constant handling

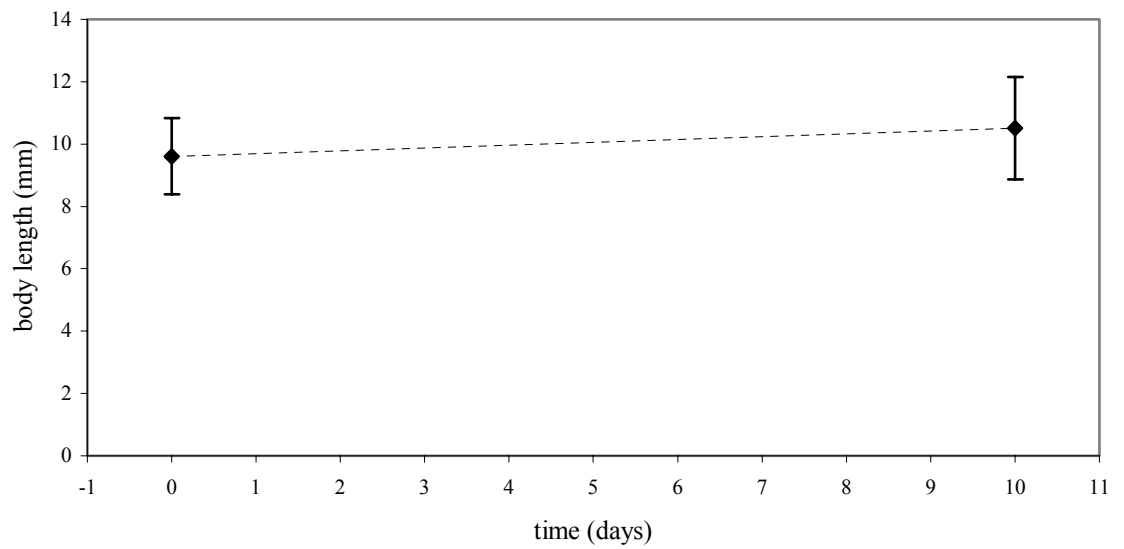


b. controls left undisturbed for 10 days

Figure 7.2: Effect of 80% EtOH on body length measurements of second-instar *Calliphora augur*.

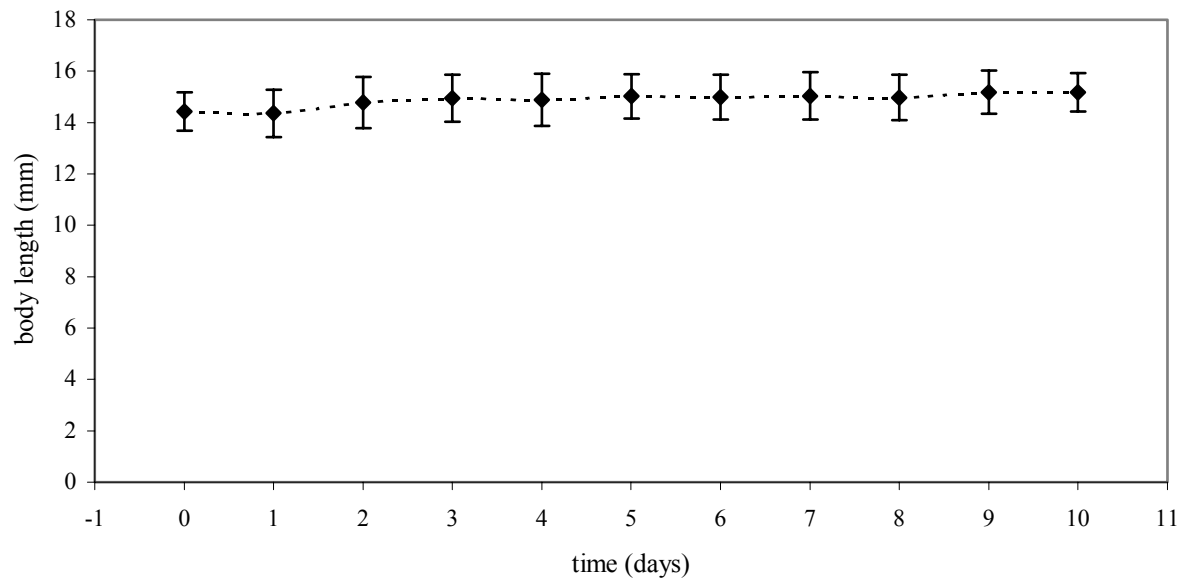


a. with constant handling

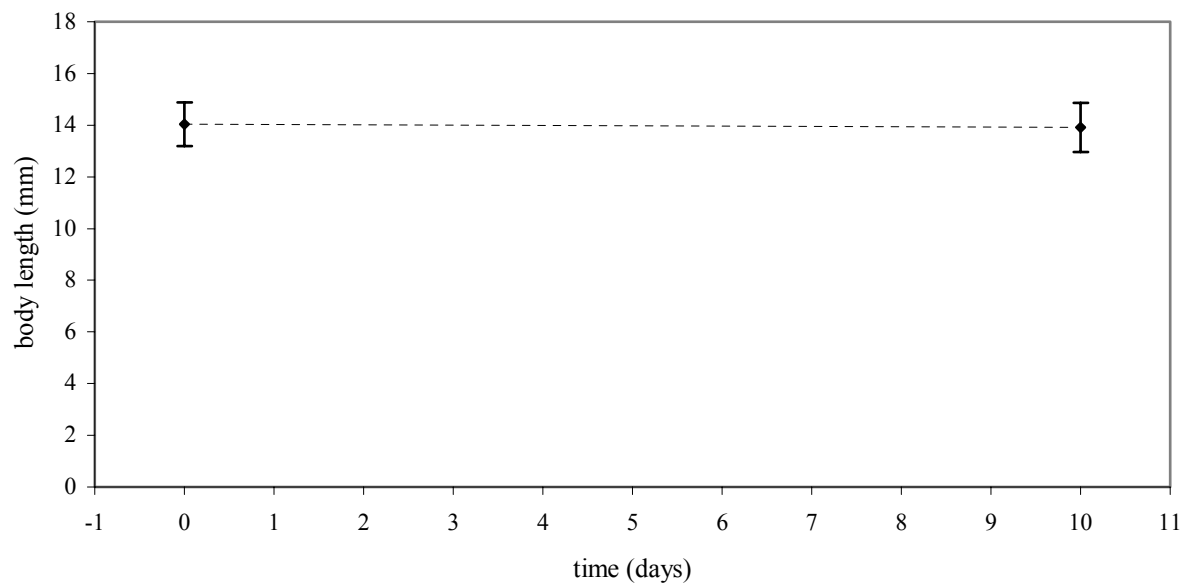


b. controls left undisturbed for 10 days

Figure 7.3: Effect of 80% EtOH on body length measurements of third-instar *Calliphora augur*.



a. with constant handling



b. controls left undisturbed for 10 days

Figure 7.4: Effect of 80% EtOH on body length measurements of late third-instar *Calliphora augur*.

7.5.2 *Effects of different preservative types on feeding third-instar larvae of Calliphora augur and Lucilia cuprina*

In some cases, individual replicates tested as statistically significantly different to the other replicates for particular treatments. These are indicated in the text below. Outliers (values more than two standard deviations from the mean) were observed but were not removed because larvae were placed into vials of preservative in groups of approximately 20 rather than individually. In some cases such outliers were obvious from a single very small or very large individual. However, examination of the distributions of the data for the same groups on days 0 and 10 revealed different numbers of outlying measurements recorded for the same group on the different days. It was not possible to confidently identify the particular individuals from which these outlying measurements came.

7.5.2.1 *Calliphora augur*

All treatments were found to induce an elongation in measured body length. Plots showing the effects of the preservative solutions on body length in feeding third-instar *C. augur* are shown in Figures 7.5-11.

The replicates treated with 70% EtOH (Figure 7.5) were pooled because none was significantly different from any other (d.f.=2, $F=0.1056$, $P>0.05$). There were no outlying measurements for this treatment. The day 0 mean body length of 14.73 ± 1.34 (std dev.) (n=62) was significantly different to the day 10 mean of 15.41 ± 1.42 (std dev.) (d.f.=1, $F=7.4745$, $P<0.01$). The percentage difference in mean body length was +4.6%.

One of the replicates treated with 75% EtOH (Figure 7.6) was significantly different to the other two at day 0 (d.f.=2, $F=5.6319$, $P<0.01$). This sample was omitted from further analyses. The two remaining samples were pooled. One outlying measurement was observed on day 0 and one outlying measurement was observed at day 10. The day 0 mean body length of 16.65 ± 1.21 (std dev.) (n=30) was significantly different to the day 10 mean of 17.61 ± 1.09 (std dev.) (d.f.=1, $F=10.4004$, $P<0.01$). The percentage difference in mean body length was +5.8%.

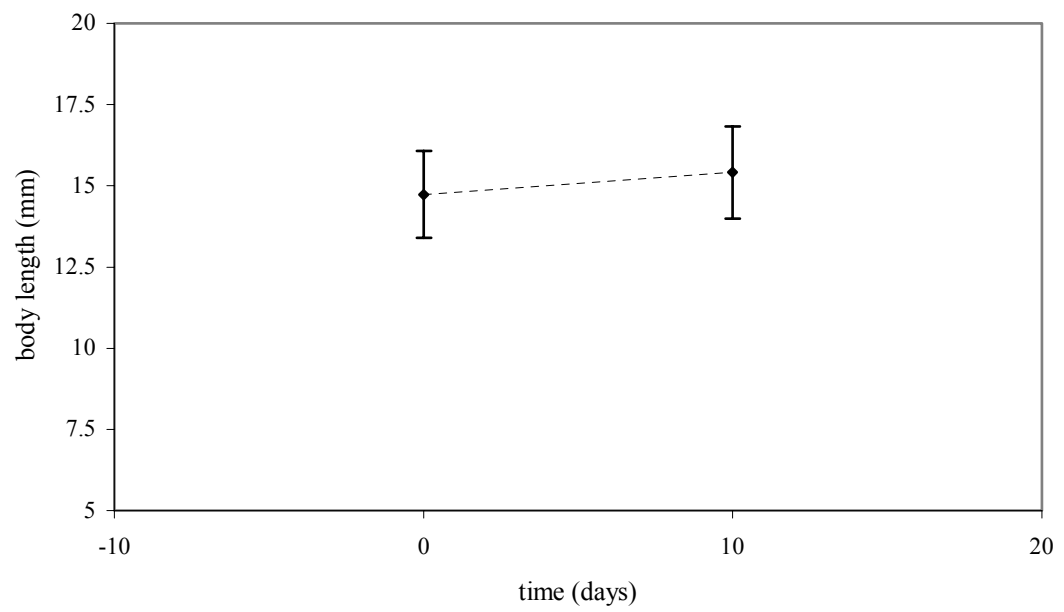


Figure 7.5: Change in body length of feeding third-instar *Calliphora augur* larvae hot-water killed and left undisturbed in 70% EtOH for 10 days.

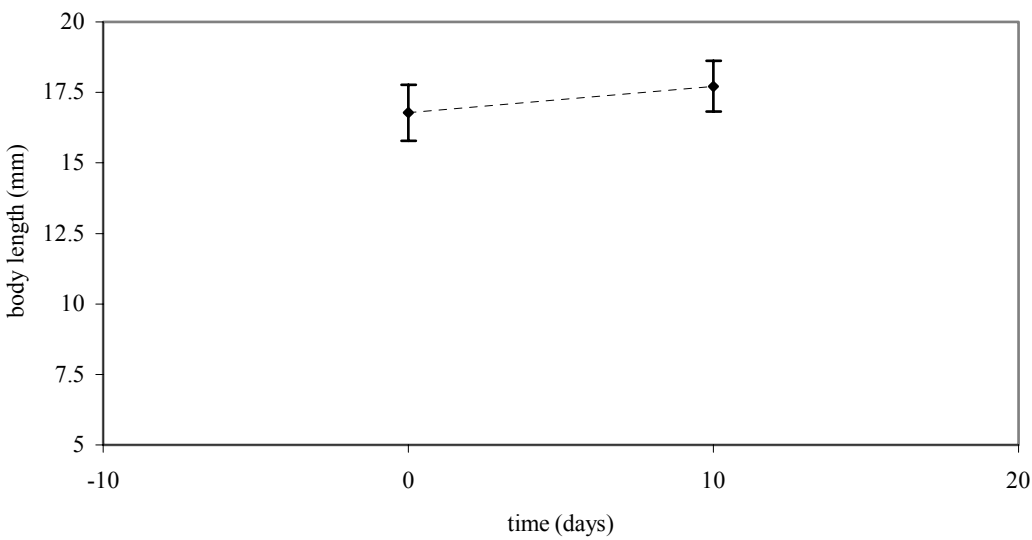


Figure 7.6: Change in body length of feeding third-instar *Calliphora augur* larvae hot-water killed and left undisturbed in 75% EtOH for 10 days.

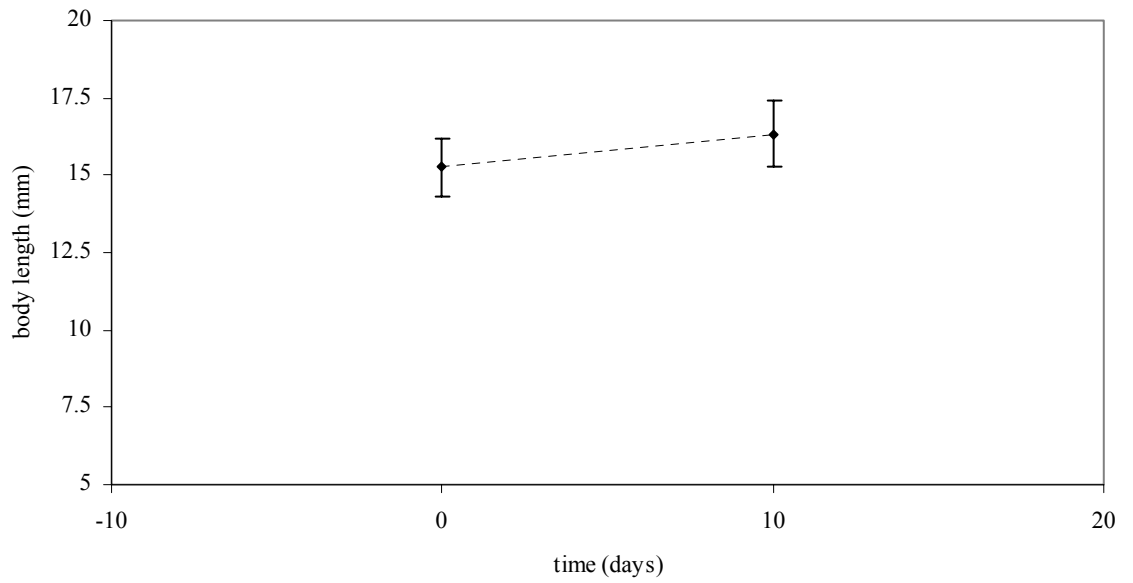


Figure 7.7: Change in body length of feeding third-instar *Calliphora augur* larvae hot-water killed and left undisturbed in 80% EtOH for 10 days.

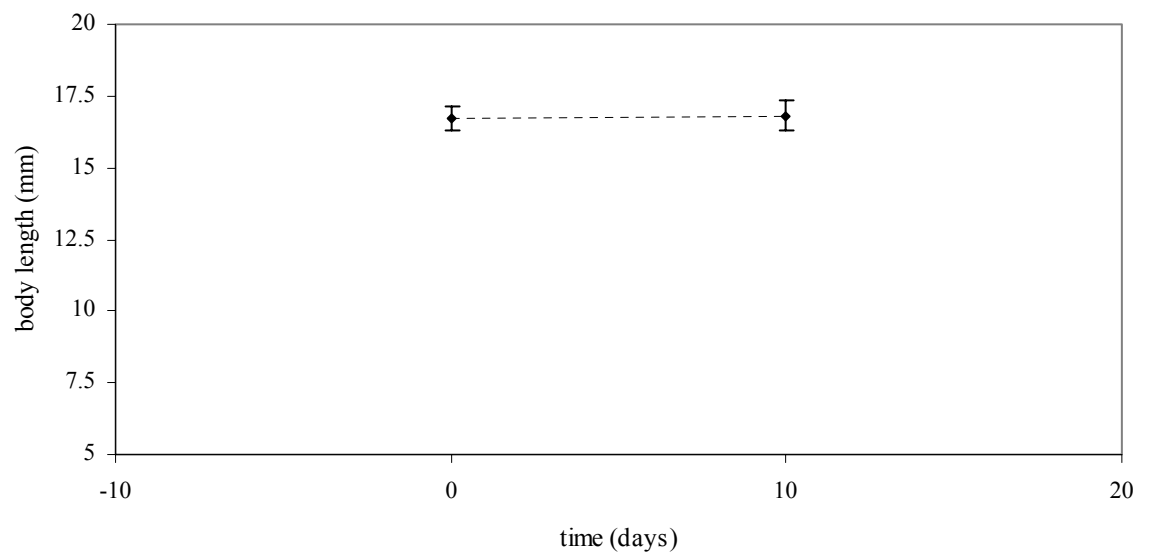


Figure 7.8: Change in body length of feeding third-instar *Calliphora augur* larvae hot-water killed and left undisturbed in 90% EtOH for 10 days.

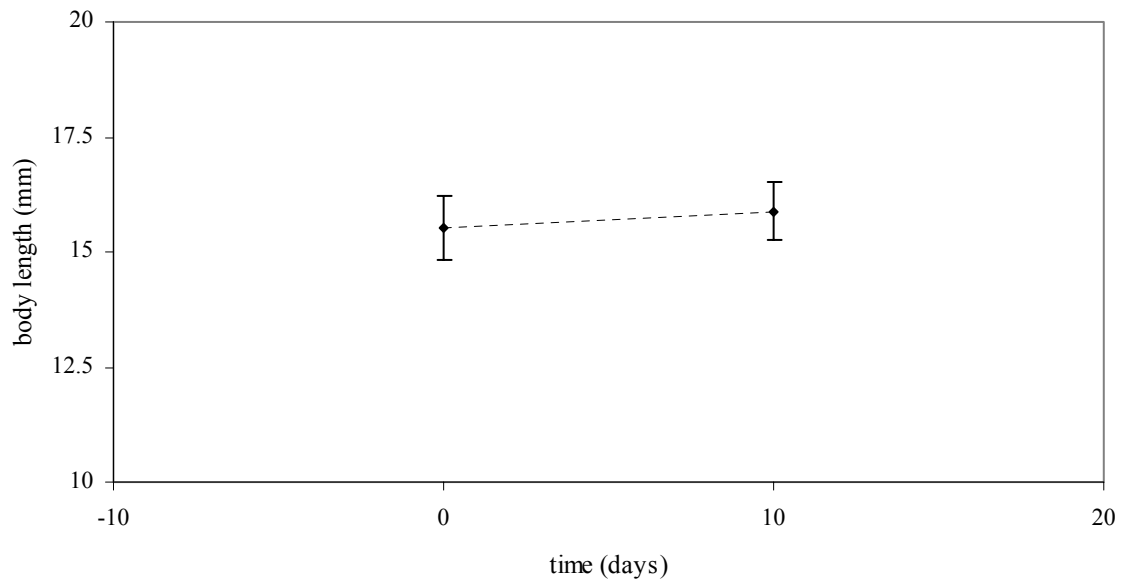


Figure 7.9: Change in body length of feeding third-instar *Calliphora augur* larvae hot-water killed and left undisturbed in 100% EtOH for 10 days.

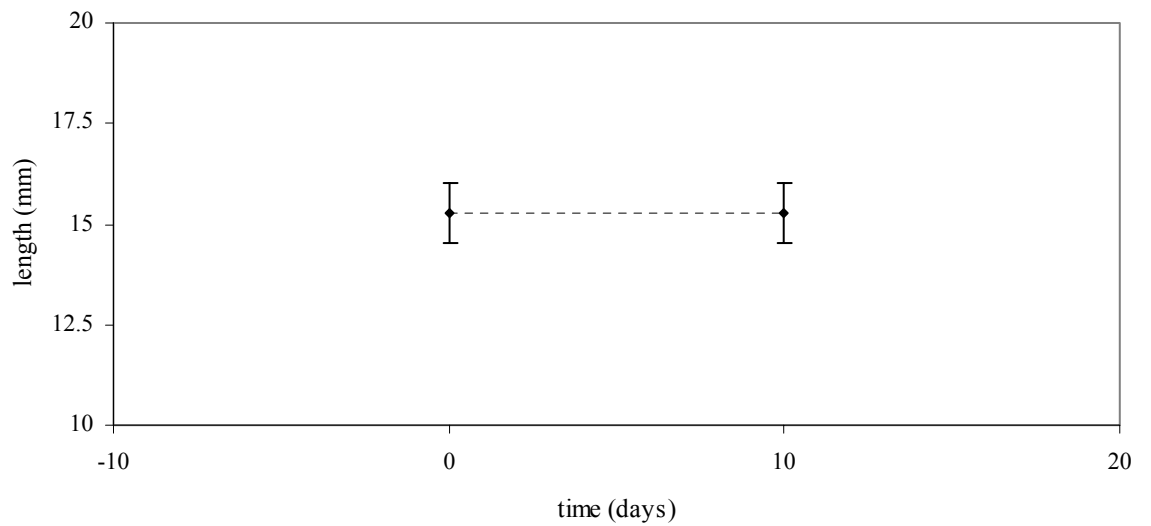


Figure 7.10: Change in body length of feeding third-instar *Calliphora augur* larvae hot-water killed and left undisturbed in 10% formalin for 10 days.

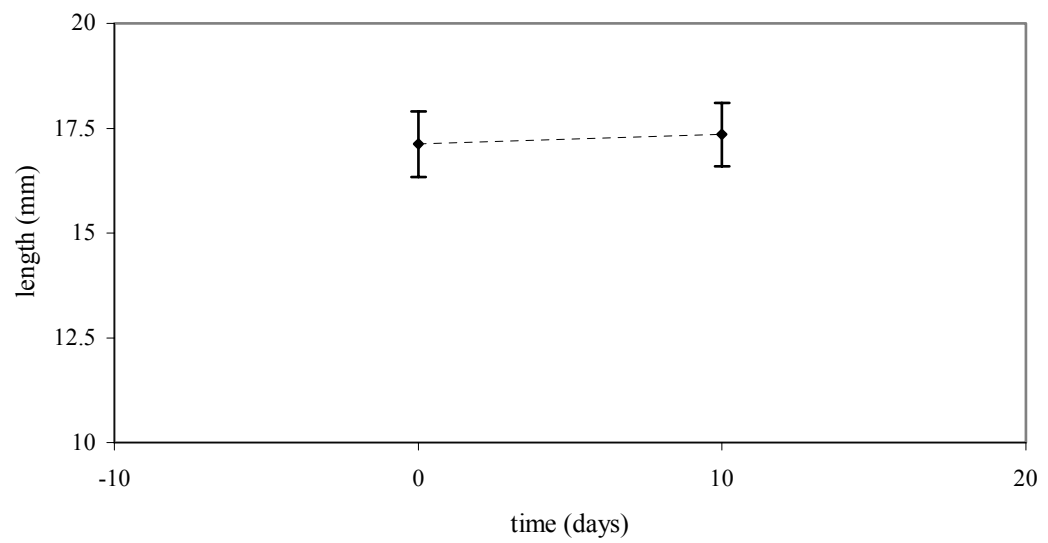


Figure 7.11: Change in body length of feeding third-instar *Calliphora augur* larvae hot-water killed and left undisturbed in Kahle's solution for 10 days.

The replicates treated with 80% EtOH (Figure 7.7) were pooled because none was significantly different from any other (d.f.=2, $F=1.4352$, $P>0.05$). Two outlying measurements were observed on day 0 and no outlying measurements were observed at day 10. The day 0 mean body length of 15.14 ± 1.09 (std dev.) ($n=58$) was significantly different to the day 10 mean of 16.34 ± 1.09 (std dev.) (d.f.=1, $F=34.7322$, $P<0.001$). The percentage difference in mean body length was +7.9%.

One of the replicates treated with 90% EtOH (Figure 7.8) was significantly different to the other two at day 0 (d.f.=2, $F=6.6065$, $P<0.01$). This sample was omitted from further analyses. The two remaining samples were pooled. No outlying measurements were observed for this treatment. The day 0 mean body length of 16.32 ± 0.72 (std dev.) ($n=39$) was significantly different to the day 10 mean of 16.69 ± 0.69 (std dev.) (d.f.=1, $F=5.2930$, $P<0.05$). The percentage difference in mean body length was +2.3%.

The replicates treated with 100% EtOH (Figure 7.9) were pooled because none was significantly different from any other (d.f.=2, $F=2.6846$, $P>0.05$). Two outlying measurements were observed on day 0 and four outlying measurements were observed at day 10. The day 0 mean body length of 15.42 ± 0.84 (std dev.) ($n=60$) was not significantly different to the day 10 mean of 15.72 ± 0.89 (std dev.) (d.f.=1, $F=3.6526$, $P=0.0584$). The percentage difference in mean body length was +1.9%.

One of the replicates treated with 10% formalin (Figure 7.10) was significantly different to the other two at day 0 (d.f.=2, $F=8.1900$, $P<0.01$). This sample was omitted from further analyses. The two remaining samples were pooled. Three outlying measurements were observed on day 0 and one outlying measurement was observed at day 10. The day 0 mean body length of 15.51 ± 0.73 (std dev.) ($n=39$) was not significantly different to the day 10 mean of 15.57 ± 0.71 (std dev.) (d.f.=1, $F=0.1171$, $P>0.05$). The percentage difference in mean body length was +0.3%.

The replicates treated with Kahle's solution (Figure 7.11) were pooled because none was significantly different from any other (d.f.=2, $F=0.7149$, $P>0.05$). Three outlying measurements were observed on day 0 and three outlying measurements were observed at day 10. The day 0 mean body length of 16.95 ± 1.07 (std dev.) ($n=55$) was not significantly different to the day 10 mean of 17.12 ± 1.12 (std dev.) (d.f.=1, $F=0.6963$, $P>0.05$). The percentage difference in mean body length was +1.0%.

The effects of the preservative solutions on body length in feeding third-instar *C. augur* are summarised in Table 7.1 and illustrated graphically in Figure 7.12. All preservatives induced elongation, which appears to increase with increasing ethanol concentration up to 80% EtOH, where the greatest elongation occurs, and then decrease again in 100% EtOH. Kahle's solution and 10% formalin effected the least elongation.

7.5.2.2 *Lucilia cuprina*

Larvae of *L. cuprina* were found to curl around each other, making accurate counting for sample allocation more difficult than for *C. augur*. I have also observed this behaviour in first- and second-instar larvae of *L. cuprina*.

All treatments were found to induce a reduction or shrinkage in body length. Plots showing the effects of the preservative solutions on body length in feeding third-instar *L. cuprina* larvae are shown in Figures 7.13-19.

One of the replicates treated with 70% EtOH (Figure 7.13) was significantly different from the other two (d.f.=2, $F=7.6704$, $P<0.01$). This sample was omitted from further analyses. The two remaining samples were pooled. There were no outlying measurements for this treatment. The day 0 mean body length of 9.33 ± 0.91 (std dev.) ($n=54$) was not significantly different to the day 10 mean of 9.15 ± 1.10 (std dev.) (d.f.=1, $F=0.9077$, $P>0.05$). The percentage difference in mean body length was -1.9% .

The replicates treated with 75% EtOH (Figure 7.14) were pooled because none was significantly different from any other at day 0 (d.f.=2, $F=0.9060$, $P>0.05$). One outlying measurement was observed on day 0 and two outlying measurements were observed at day 10. The day 0 mean body length of 10.01 ± 0.66 (std dev.) ($n=67$) was significantly different to the day 10 mean of 9.63 ± 0.88 (std dev.) (d.f.=1, $F=8.2446$, $P>0.01$). The percentage difference in mean body length was -3.8% .

The replicates treated with 80% EtOH (Figure 7.15) were pooled because none was significantly different from any other at day 0 (d.f.=2, $F=3.0239$, $P>0.05$). Two outlying measurements were observed on day 0 and two outlying measurements were observed at day 10. In this case, at day 0 the two outliers were low range, but at day 10 one of the outliers was low range and one was high range.

Table 7.1: Change in measured body length of feeding third-instar larvae of *Calliphora augur* and *Lucilia cuprina* in various preservative treatments. All larvae were hot-water killed and towel dried prior to placement in treatments.

n=number of observations

Preservative type	Mean length (mm) \pm std dev. when just hot-water killed	Mean length (mm) \pm std dev. after 10 days in preservative	Change in measured length (%)	p value	n
<i>Calliphora augur</i>					
70% EtOH	14.73 \pm 1.34	15.41 \pm 1.42	+4.6	0.0072	62
75% EtOH	16.65 \pm 1.21	17.61 \pm 1.08	+5.8	0.0021	30
80% EtOH	15.14 \pm 1.09	16.34 \pm 1.09	+7.9	<0.0001	58
90% EtOH	16.32 \pm 0.71	16.69 \pm 0.69	+2.3	0.0242	39
100% EtOH	15.42 \pm 0.84	15.72 \pm 0.89	+1.9	0.0584	60
10% formalin	15.51 \pm 0.73	15.57 \pm 0.71	+0.3	0.7331	39
Kahle's soln	16.95 \pm 1.07	17.12 \pm 1.12	+1.0	0.4059	55
<i>Lucilia cuprina</i>					
70% EtOH	9.33 \pm 0.91	9.15 \pm 1.10	-1.9	0.3429	54
75% EtOH	10.01 \pm 0.66	9.63 \pm 0.88	-3.8	0.0048	67
80% EtOH	9.49 \pm 0.87	9.42 \pm 1.01	-0.8	0.6007	91
90% EtOH	11.11 \pm 0.78	10.84 \pm 0.92	-2.4	0.0805	60
100% EtOH	10.98 \pm 0.82	10.39 \pm 1.02	-5.4	0.0007	60
10% formalin	9.66 \pm 0.79	9.58 \pm 0.83	-0.8	0.5731	71
Kahle's soln	11.28 \pm 0.74	11.00 \pm 0.78	-2.4	0.0958	39

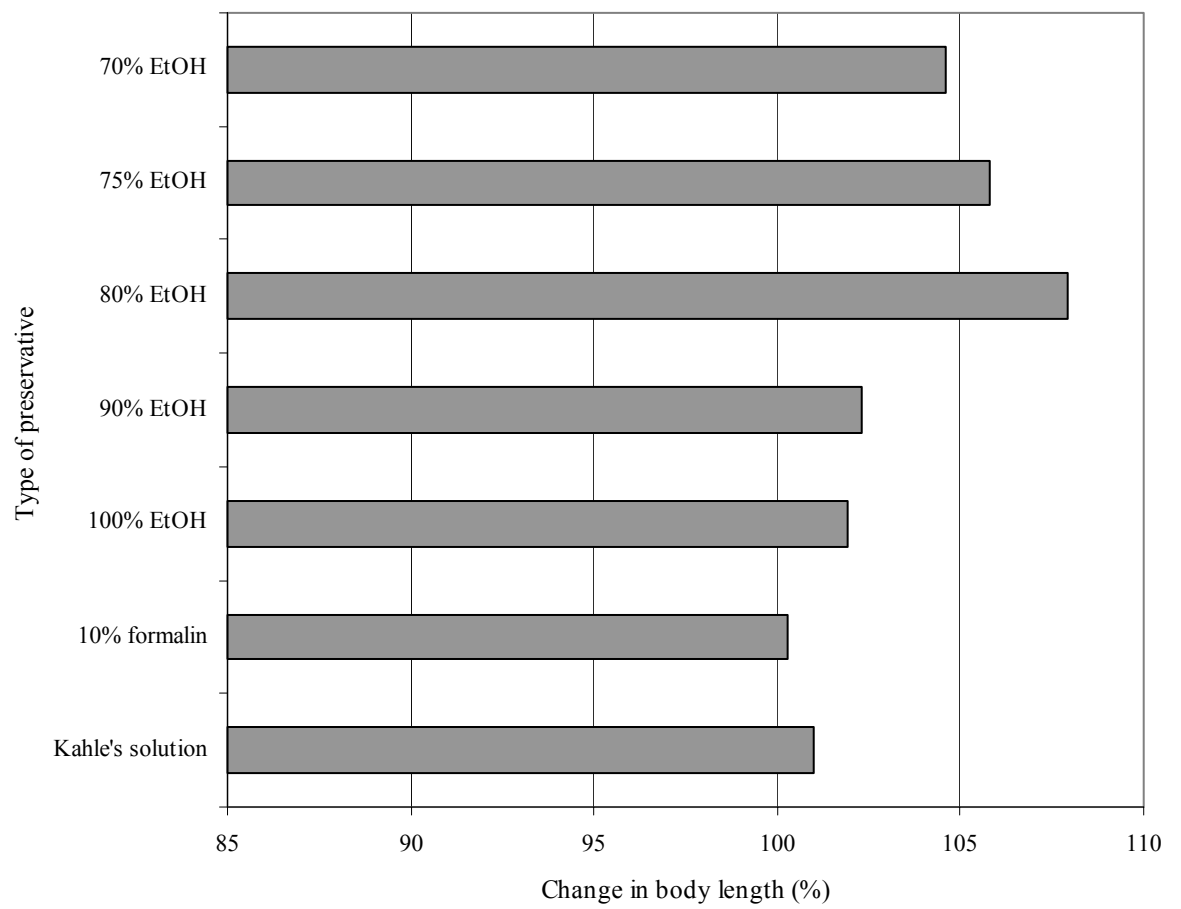


Figure 7.12: Comparative effects of various preservative solutions on body length of feeding third-instar *Calliphora augur* larvae.

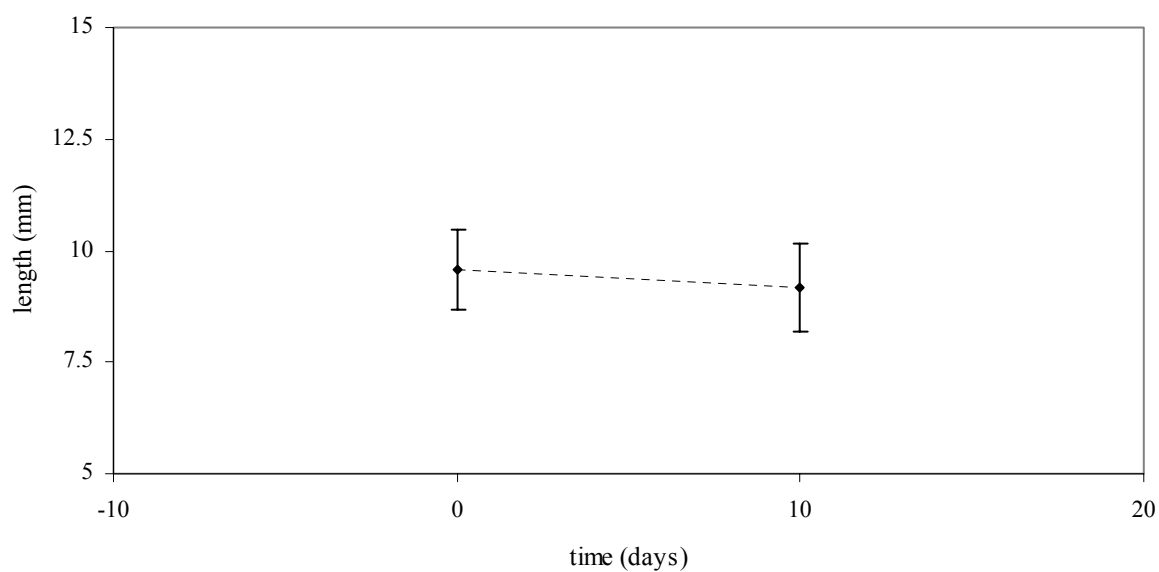


Figure 7.13: Change in body length of feeding third-instar *Lucilia cuprina* larvae hot-water killed and left undisturbed in 70% EtOH for 10 days.

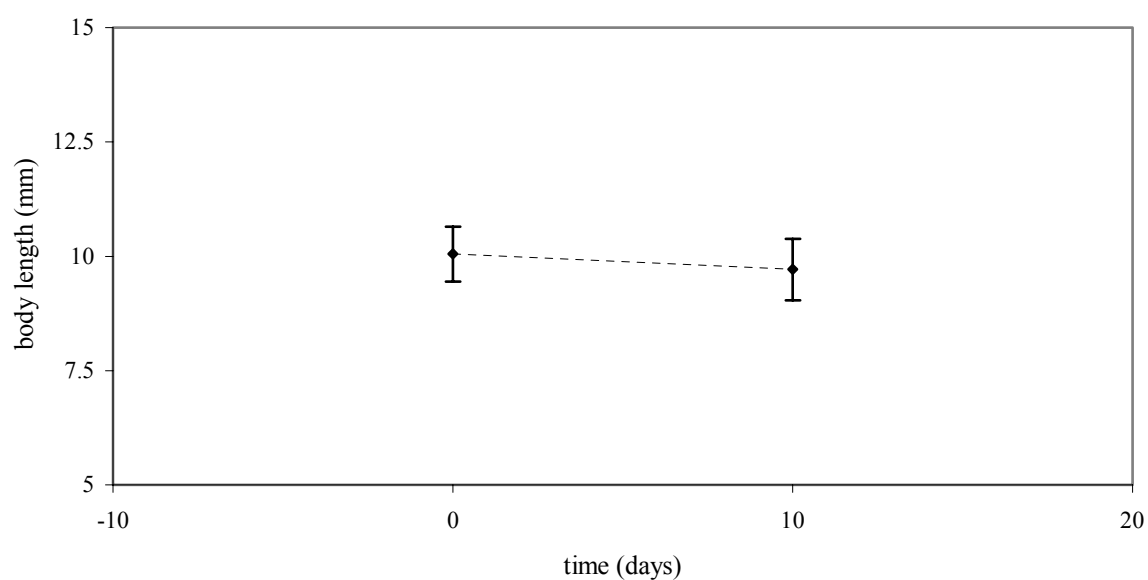


Figure 7.14: Change in body length of feeding third-instar *Lucilia cuprina* larvae hot-water killed and left undisturbed in 75% EtOH for 10 days.

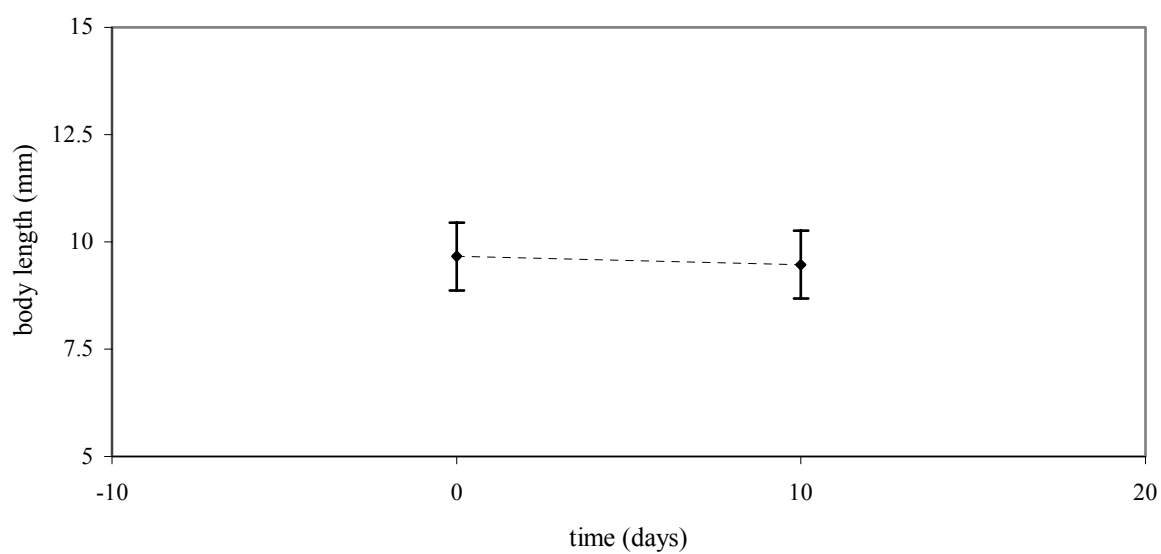


Figure 7.15: Change in body length of feeding third-instar *Lucilia cuprina* larvae hot-water killed and left undisturbed in 80% EtOH for 10 days.

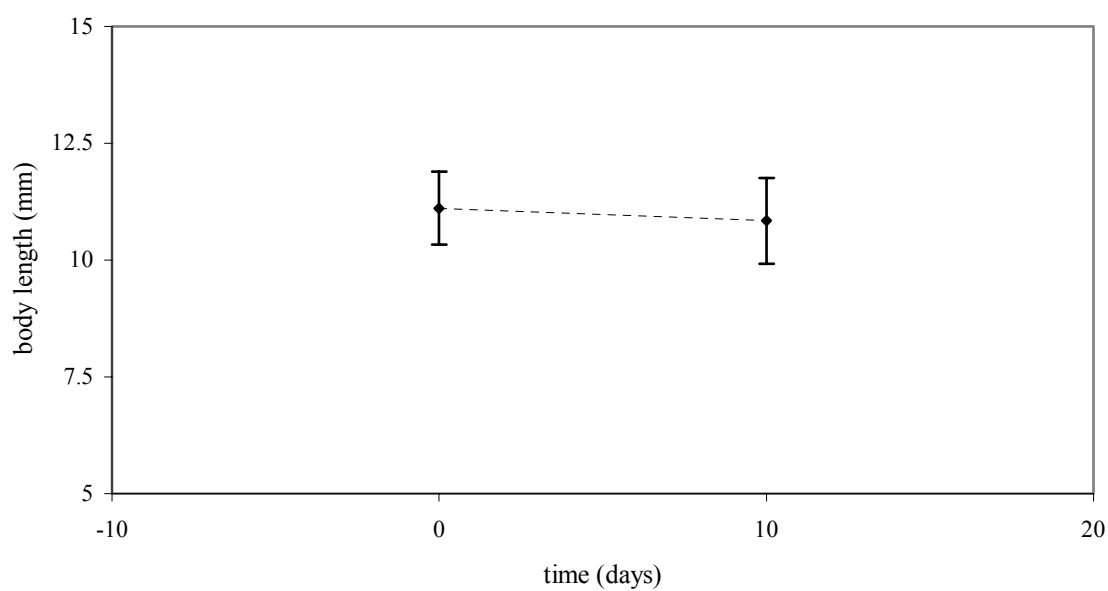


Figure 7.16: Change in body length of feeding third-instar *Lucilia cuprina* larvae hot-water killed and left undisturbed in 90% EtOH for 10 days.

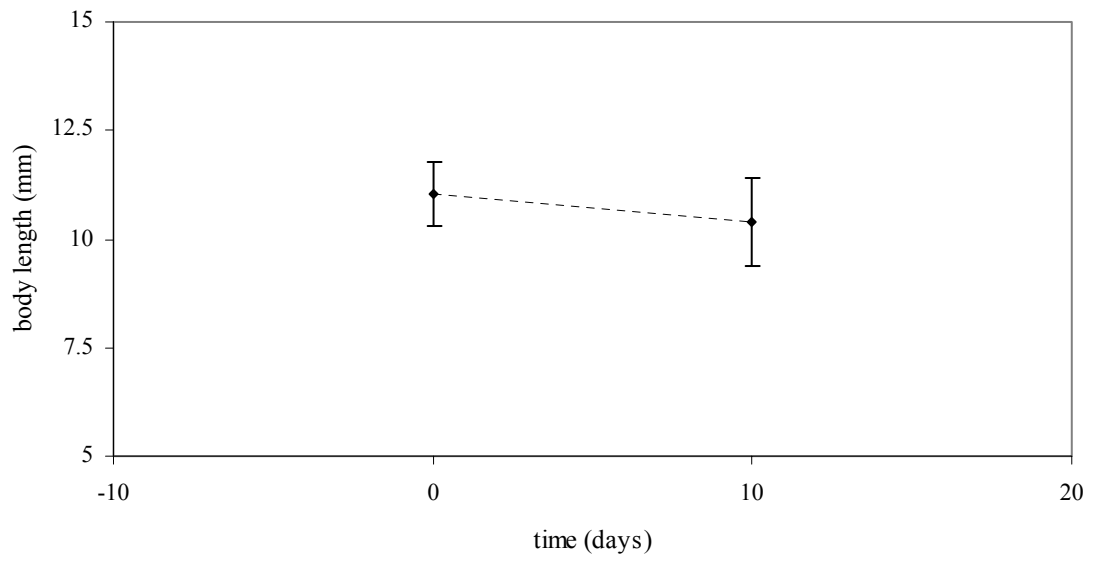


Figure 7.17: Change in body length of feeding third-instar *Lucilia cuprina* larvae hot-water killed and left undisturbed in 100% EtOH for 10 days.

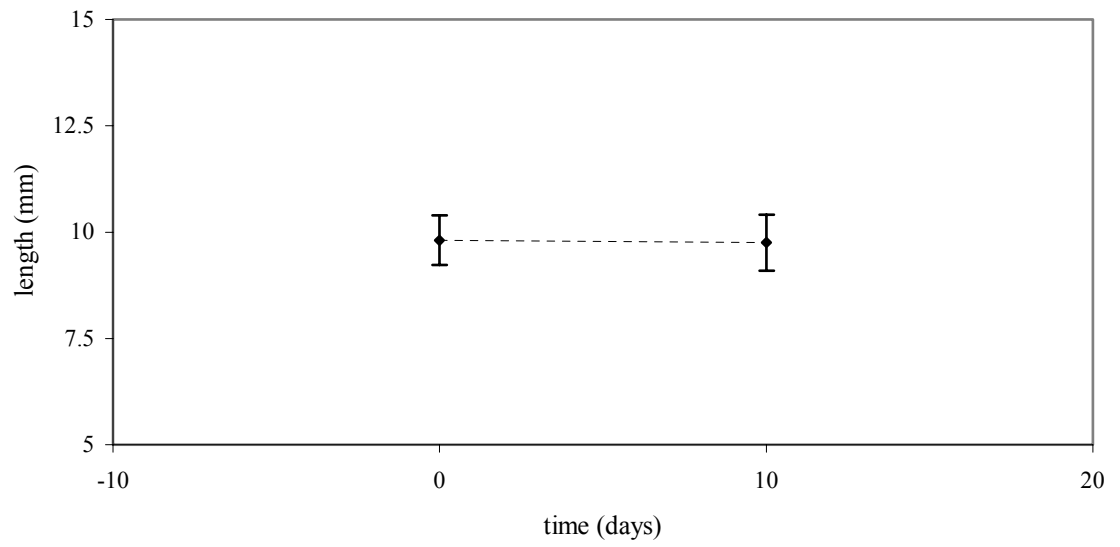


Figure 7.18: Change in body length of feeding third-instar *Lucilia cuprina* larvae hot-water killed and left undisturbed in 10% formalin for 10 days.

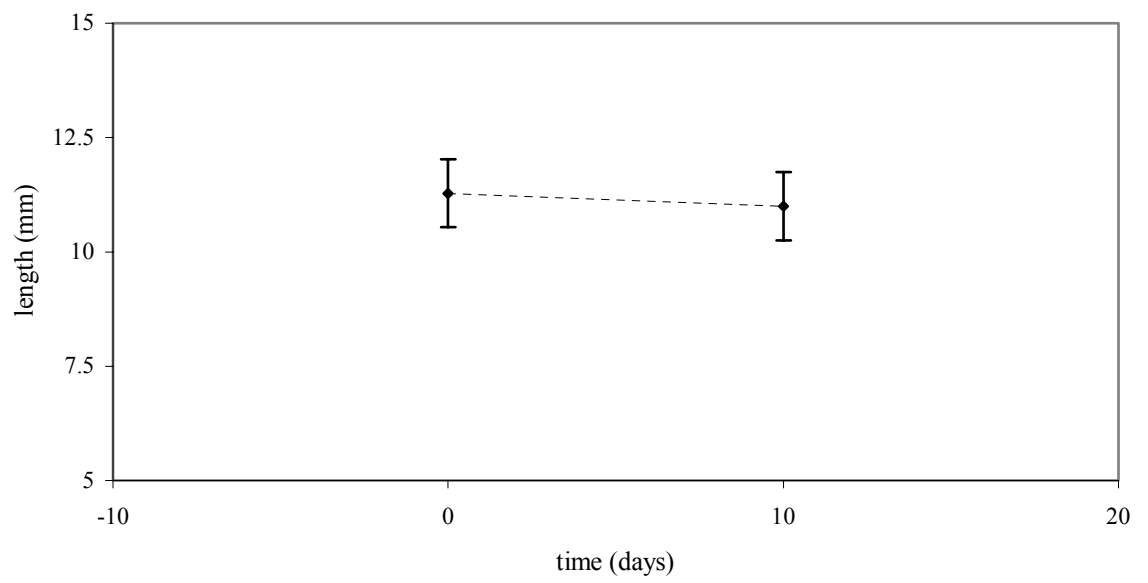


Figure 7.19: Change in body length of feeding third-instar *Lucilia cuprina* larvae hot-water killed and left undisturbed in Kahle's solution for 10 days.

The day 0 mean body length of 9.49 ± 0.87 (std dev.) ($n=91$) was not significantly different to the day 10 mean of 9.42 ± 1.01 (std dev.) ($d.f.=1$, $F=0.2749$, $P>0.05$). The percentage difference in mean body length was -0.8% .

The replicates treated with 90% EtOH (Figure 7.16) were pooled because none was significantly different from any other at day 0 ($d.f.=2$, $F=0.2346$, $P>0.05$). No outlying measurements were observed for this treatment. The day 0 mean body length of 11.11 ± 0.78 (std dev.) ($n=60$) was not significantly different to the day 10 mean of 10.84 ± 0.92 (std dev.) ($d.f.=1$, $F=3.1079$, $P<0.05$). The percentage difference in mean body length was -2.4% .

The replicates treated with 100% EtOH (Figure 7.17) were pooled because none was significantly different from any other ($d.f.=2$, $F=0.0036$, $P>0.05$). Three outlying measurements were observed on day 0 and one outlying measurement was observed at day 10. The day 0 mean body length of 10.98 ± 0.82 (std dev.) ($n=60$) was significantly different to the day 10 mean of 10.39 ± 1.02 (std dev.) ($d.f.=1$, $F=12.207$, $P<0.001$). The percentage difference in mean body length was -5.4% .

The replicates treated with 10% formalin (Figure 7.18) were pooled because none of the three was significantly different at day 0 ($d.f.=2$, $F=1.7680$, $P>0.05$). Two outlying measurements were observed on day 0 and two outlying measurements were observed at day 10. The day 0 mean body length of 9.66 ± 0.79 (std dev.) ($n=71$) was not significantly different to the day 10 mean of 9.58 ± 0.83 (std dev.) ($d.f.=1$, $F=0.3190$, $P>0.05$). The percentage difference in mean body length was 0.8% .

One of the replicates treated with Kahle's solution (Figure 7.19) was found to be significantly different from the other two ($d.f.=2$, $F=7.7638$, $P<0.01$). This sample was omitted from further analyses. The two remaining samples were pooled. No outlying measurements were observed for this treatment. The day 0 mean body length of 11.28 ± 0.74 (std dev.) ($n=39$) was not significantly different to the day 10 mean of 11.00 ± 0.76 (std dev.) ($d.f.=1$, $F=2.8439$, $P>0.05$). The percentage difference in mean body length was -2.4% .

The effects of the preservative solutions on body length in feeding third-instar *L. cuprina* are summarised in Table 7.1 and illustrated graphically in Figure 7.20.

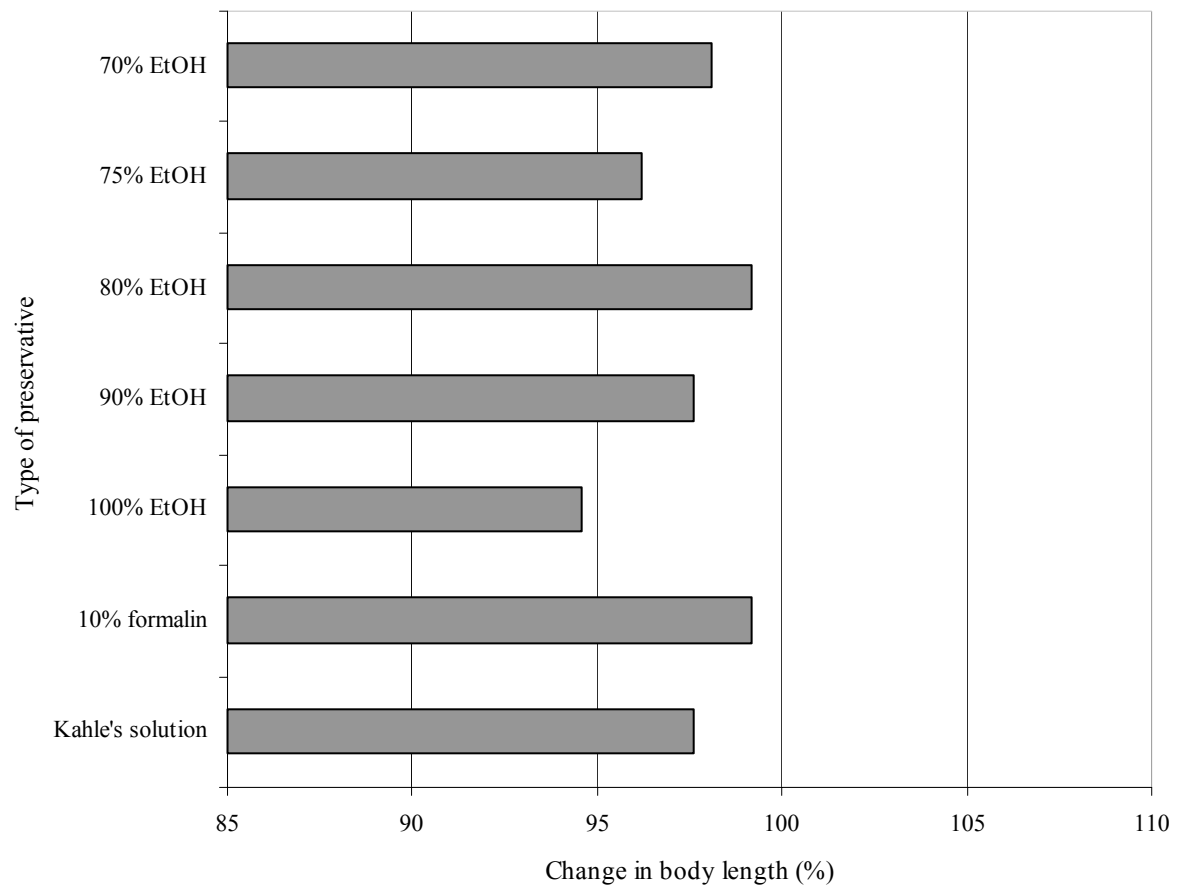


Figure 7.20: Comparative effects of various preservative solutions on body length of feeding third-instar *Lucilia cuprina* larvae.

Shrinkage does not appear to correlate with increasing EtOH concentration in the same way as for *C. augur* larvae. The greatest shrinkage occurred with 100% EtOH. The least shrinkage occurred with 10% formalin and 80% EtOH.

7.5.2.3 Larvae killed by immersion in preservative

The effects on the physical appearance of larvae not hot-water killed prior to preservation in the different treatments are shown in Table 7.2. The colours of these larvae ranged from cream (regarded as normal) to orange, brown and black. Browning and blackening was observed in both species for all of the EtOH treatments conducted. No browning or blackening was observed with either 10% formalin or Kahle's solution in either species.

While this browning/blackening does not prevent the instar of a larva from being determined from the appearance of the spiracles, it may make it difficult to identify transitional instar forms. Also, the darkened larvae were quite rigid and their spiracles harder to see. A sunken, desiccated appearance was observed for all EtOH-treated samples of *L. cuprina*, but this was only seen in *C. augur* larvae treated with undiluted EtOH. This sunkenness and desiccation was not observed in samples of either species treated with 10% formalin or Kahle's solution. Maggot agglomerations were observed in the 90% EtOH, 100% EtOH and Kahle's *L. cuprina* samples, but not in any of the *C. augur* samples. Agglomerations took the form of two to eight individual maggots joined together mouth to mouth or mouth to anus. Agglomerations also made it difficult to examine the animals properly, particularly their spiracles. Separating the agglomerations caused damage to the individual larvae.

7.6 Discussion

7.6.1 Different growth stages of Calliphora augur preserved in 80% EtOH

The different growth stages of feeding *C. augur* larvae reacted similarly to preservation in 80% EtOH by elongating, but the post-feeding larvae did not. The measured body length of the post-feeding third instars was mostly conserved. The only group with statistically significant day 0 and day 10 controls was the second-instar group. This could be due to second-instar larvae having thinner, more pliable cuticles [82]. The reason that the first-instars were not also significantly different is unknown but could relate to a metabolic delay between being deposited and assimilation of feeding products into the epidermis and cuticle. I have observed first-instars to have a reduced width shortly after larviposition [164], which appears to be related to such a time lag.

Table 7.2: Table showing effects different preservatives have when live feeding third-instar larvae of *Calliphora augur* and *Lucilia cuprina* are preserved without boiling-water fixation.

C=cream, O=orange, Br=brown, Bl=black, Y=yes, N=no.

Preservative type	Colours observed				General Appearance			
	C	O	Br	Bl	Linear	Sunken/plump	Dessication	Agglomeration
<i>Calliphora augur</i>								
70% EtOH	Y	N	Y	N	Y	Plump	No	No
75% EtOH	Y	N	Y	Y	Y	Plump	No	No
80% EtOH	Y	N	Y	Y	Y	Plump	No	No
90% EtOH	Y	N	Y	N	Y	Plump	No	No
100% EtOH	Y	N	Y	N	N	Sunken	Yes	No
10% formalin	Y	N	N	N	Y	Plump	No	No
Kahle's solution	Y	N	N	N	Y	Plump	No	No
<i>Lucilia cuprina</i>								
70% EtOH	N	N	Y	Y	N	Sunken	Yes	No
75% EtOH	N	N	Y	N	N	Sunken	Yes	No
80% EtOH	N	Y	Y	N	N	Sunken	Yes	No
90% EtOH	N	N	Y	N	N	Sunken	Yes	Yes, up to 6
100% EtOH	Y	N	Y	Y	N	Both	Yes	Yes, up to 8
10% formalin	Y	N	N	N	Y	Plump	No	No
Kahle's solution	Y	N	N	N	Y	Plump	No	Yes, pairs

The controls included in this experiment clearly show that continued handling of larvae can have a detrimental effect on the length they attain. It appears that the opening and closing of vials to examine larvae probably changes the concentration of the preservative. Each time the vials were opened for removal and replacement of measured larvae, some of the preservative probably evaporated, not only through the mouth of the vial but also from the larvae themselves. Minimal handling should reduce this problem. Examination of the effect of placing measured larvae into fresh solutions after each measurement may further clarify the matter. Since Adams and Hall [105] did not appear to place measured larval groups into fresh preservative each time they measured them, the concentrations of their preservatives may have changed by the end of their study. Thus, the lack of unhandled controls in Adams and Hall's study [105] limits the possible interpretation of their results. While my data show similar trends to those of Adams and Hall, it is worth noting that I handled the larvae more frequently.

7.6.2 *Effect of different preservative types on feeding third-instar larvae of Calliphora augur and Lucilia cuprina*

Tantawi and Greenberg [82] noted that the crop size of *C. vicina* larvae expanded when larvae were preserved in 70% EtOH, but that other treatments all caused shrinkage. They found that the amount of crop shrinkage did not exceed what normally occurs in the first three hours post-feeding and concluded that the effect of different preservative solutions on crop length is not useful for forensic purposes. I did not examine crop size but nonetheless found interesting and unexpected results. The different preservative treatments affected the two species differently. Four of the seven preservative treatments caused a significant difference in measured body length in larvae of *C. augur* (70% EtOH, 75% EtOH, 80% EtOH and 90% EtOH) and two of the seven treatments caused a significant measured difference in measured body length in larvae of *L. cuprina* (75% EtOH and 100% EtOH).

It appears that larvae of most species tend to shrink when treated with preservatives [82, 105], although Adams and Hall [105] observed elongation in both *C. vomitoria* and *L. sericata* larvae when placed into preservatives alive. Tantawi and Greenberg [82] noted that maggots that are first killed in solutions such as 70% ethanol retain some elasticity and extensibility of the cuticle. This can lead to further error in measuring body length, whereas maggots killed in boiling water are fully extended and rigid. I did not measure body lengths of live larvae but found that hot-water killed and preserved larvae of *L. cuprina* shrunk as anticipated. However, larvae of *C. augur* elongated in every preservative treatment examined. The amount of change

in body length caused by each preservative type was not comparable between the two species in my study. Species of *Calliphora* other than *C. augur* responded differently in the studies of Tantawi and Greenberg [82] (*C. vicina*) and Adams and Hall [105] (*C. vomitoria*). However, the species examined by these workers were both oviparous. As mentioned earlier, *C. augur* is ovoviviparous [44]. Examination of the preservation of larvae of other ovoviviparous species of forensic importance would clarify whether my findings are characteristic of such species.

The difference between my results for *L. cuprina* and those of Adams and Hall [105] for hot-water killed and preserved larvae of *L. sericata* are surprising given their close evolutionary relationship [165]. *Lucilia cuprina* larvae shrunk in both 80% ethanol and 10% formalin. Adams and Hall [105] observed elongation in hot-water killed *L. sericata* larvae preserved in 80% ethanol and shrinkage in 10% formalin. Adams and Hall did not replicate among individual species they examined. Replication and unhandled controls might have altered their results.

Tantawi and Greenberg [82] found that shrinkage varied between 3.2 and 30.8% depending on the preservative used. They also found that different concentrations of the same preservative led to quite different amounts of shrinkage. For example, 70% EtOH led to 17.3% shrinkage, while 90% EtOH caused 24.4% shrinkage. Tantawi and Greenberg [82] accurately measured live larvae without anaesthetising them. Live larvae are very mobile and maximum elongation is only achieved by killing with hot water. This study may have been more informative if hot water killing had been assessed as another treatment, with and without preservation. Furthermore, the sample numbers were small and there appears to have been no replication.

My findings also suggest more subtle changes in body length than found by Tantawi and Greenberg [82] when larvae were treated with different preservative types. The range for *C. augur* was from +0.3% with 10% formalin to +7.9% with 80% EtOH. The range for *L. cuprina* was -0.8% with 80% EtOH to -5.4% with 100% EtOH. The observed pattern in shrinkage with increasing concentration of EtOH treatments in hot water-killed third-instar larvae of *C. augur* was not observed with hot water killed third-instar larvae of *L. cuprina*, indicating that the effect of a particular preservative type may be quite variable in larvae of different species.

I do, however, strongly agree with Tantawi and Greenberg [82] that boiling water should be used by criminal investigators, medical examiners, forensic pathologists, coroners and forensic entomologists as a standard killing solution for maggots of forensic importance, because boiling appears to greatly lessen autolysis by destroying digestive enzymes and the gut flora. Although

there is great merit in reducing the gut flora and therefore decomposition in the dead maggot, some workers have discouraged its use [166] because it may affect internal structures, particularly the alimentary canal. Adams and Hall [105] found that immersion in water >80°C for at least 30 sec was the best method of killing [105] and this has been recommended in recent texts [83, 162]. Since it can often be difficult to have boiling water at a crime scene, it has been recommended [39] that larvae be placed into alcohol for on-scene preservation and then removed from the alcohol and boiled as soon as possible (or within 24 h after initial preservation), once back in the laboratory. Larvae should then be removed from the initial alcohol solution and placed into boiling water and allowed to remain for about 30 sec. After this time, they should be promptly removed and placed into a fresh solution of 80% alcohol in which they will be properly fixed and ready for long-term storage or shipment [83]. Immersion and swirling of larvae in hot water may also lessen the occurrence of head-curling [164]. In an emergency, a cup of hot water or tea can be obtained from fast food outlets (Dr M. Lee Goff, pers. comm.)

Adams and Hall [105] suggest that the heating process acts directly on the larval cuticle by altering its permeability. Boiling water may act on chitin and waxy substances in the cuticle, dissolving these components; this as a possible explanation for second-instar larvae significantly changing their length during preservation in 80% EtOH. Interestingly, Tantawi and Greenberg [82] found that heating the larvae in boiling water prior to placing them into preservative prevented shrinkage. My replicated findings do not concur; change in body length was observed with all preservative types in both species that I examined.

7.6.3 *Effect of different preservative types on larvae placed into preservatives alive*

Adams and Hall [105] found that placing larvae into preservatives alive resulted in poor preservation in all cases. By contrast, I found that larvae of both *C. augur* and *L. cuprina* were well preserved when placed alive into both Kahle's solution and 10% formalin. They were cream in colour, plump, linear and there was no evidence of dessication or agglomeration. However, only *L. cuprina* larvae agglomerated when placed alive into Kahle's solution. Unlike Adams and Hall, I did not observe larvae so swollen with fluid that they appeared stretched [105]. Use of formalin as a preservative should be a last resort because formalin makes DNA difficult to amplify [167].

Adams and Hall [105] suggested that the larval cuticle acts as a semipermeable membrane. In support of this, I observed dessication and sunkeness in all ethanol treatments of non-killed *L. cuprina* larvae and also with the 100% EtOH treatment of *C. augur* larvae. These authors also

noted that the best-preserved larvae were also the longest. My study used the same age cohort groups and samples were examined in groups. Because of this, I was unable to match particular measurements to individual larvae and cannot comment on the effects of preservatives on particular size classes.

Whilst PMI determinations using larvae alone are not ideal, the need for this in criminal investigations does occur. Nuorteva [39, 40] has reported cases where larval rearing has not been possible due to insecticide contamination, and Leclercq and Vaillant [119] presented a case where lead arsenate was sprinkled or injected into two bodies to repel police dogs and/or insects. It is therefore imperative that the integrity of samples be maintained through good collection techniques and effective killing and preservation practices. Work in this regard is needed to determine what is a) practical and possible to actually do at a crime scene, b) the effects of different preservatives on other fly species of forensic interest, particularly ovoviviparous ones, and c) the effect of these solutions on DNA retrieval of fly species of forensic interest. This paper confirms the findings of Tantawi and Greenberg [82] and Adams and Hall [105] that larvae of different species react differently to different preservative types. It also highlights the fact that not all forensically interesting fly species, and indeed not all species of the same genus, react predictably to different methods of preservation. With much current research on phylogeny and species determinations from DNA of forensically important blowflies [e.g. 165], maintaining the integrity of DNA is a new and very real concern to consulting entomologists. The need for proper controls is imperative in studies in this discipline. Forensic entomology continues to enjoy increasing credibility, but is unlikely to be immune to tests of its rigour and validity.

7.7 Conclusions

I conclude that different instars of *C. augur* react differently to preservation, with second-instar larvae appearing particularly vulnerable to preservative-induced change in body length.

Different species react differently to the same preservative treatment when killed with hot water, and I suspect that optimal preservation practice could be species-specific, perhaps even instar-specific. 10% formalin and Kahle's solution were the only preservatives tested which did not induce significant change in body length. Formalin is damaging to DNA recovery and is not recommended. It would be useful to pursue studies on DNA recovery from larvae preserved in Kahle's solution.

Finally, placement of live larvae into preservatives should be discouraged, despite the well-preserved appearance of larvae placed in formalin and Kahle's solution; EtOH and Kahle's caused agglomeration of *L. cuprina* larvae, which can make it difficult to identify instar and increase the risk of damaging specimens during their examination. These and other preservatives may have a similar effect on other species.

CHAPTER 8

Development of *Calliphora augur* larvae at constant temperatures

Summary:

Calliphora augur is a carrion-breeding blowfly of forensic, medical and agricultural importance in south-eastern Australia. Studies on this species have usually been part of broader investigations and there are currently little detailed data on the development of this fly. Developmental studies were conducted at the constant temperatures of 15, 20, 25, 30 and 35°C on sheep's liver and found decreasing variation in the length reached by larvae at certain times up to 30°C, although this variation increased again when larvae were grown at 35°C. It was found that particular instars grow best at certain temperatures and that transitional forms (between moults) can have a large overlap with fully moulted instars and be present for longer than expected. These results are in agreement with some aspects of earlier studies but differences were noted. 95% prediction intervals are provided to enable the age of a feeding maggot of *C. augur* to be derived from its length.

8.1 Introduction

Information on the successional patterns of insects on remains, either alone or coupled with data on development of the species present, can help provide an estimate of the post-mortem interval. Many excellent succession studies have been done on carrion insects [25, 29, 89, 110, 157, 161, 168, 169] and knowledge of the development of individual necrophagous species is constantly increasing. The general approach previously adopted in developmental studies has been to generate growth curves and then use plots [59, 98-100], tables [47, 69] or inverse prediction [101] to estimate the age of a maggot and therefore the time of oviposition, to give at least a minimum estimate of post-mortem interval. Graphs presented from these studies often display time or age on the *x*-axis and maggot length or weight on the *y*-axis. However, since time is the unknown variable, it should, from a strict statistical perspective, be given on the *y*-axis. This chapter presents investigations into the effect of inverting a traditional larval size vs. time plot, to display time as the unknown variable. This was done using a detailed analysis of the growth, at constant temperatures, of the important ovoviviparous Australian blowfly, *Calliphora augur* (Fabricius). Investigations were based on measurements of larval length and included observations on size overlap between larval instars.

8.2 Materials and Methods

The general methods described in Chapter 3 were followed. For the experiments in this chapter however, specific detail is outlined below.

8.2.1 Development of *Calliphora augur* larvae at constant temperatures

The constant temperatures chosen for the developmental studies reflect the regimes used in earlier studies [25] to allow some comparison of results. The temperatures chosen were 15, 20, 25, 30 and 35°C. A separation of 5°C between the values is deliberate, as growth at intermediate temperatures can be inferred from the models obtained from these temperatures (Section 2.4.2).

An initial experiment using *Lucilia cuprina* as a model revealed that hourly measurements of larvae showed such gradual change that larger time frames needed to be chosen. Since it is well known that growth rate increases with temperature, it was decided to alter the sample period in line with the temperature being examined. Larvae were sampled every 24 hours at 15°C and every 12 hours at 20, 25, 30 and 35°C. These intervals were largely arbitrary, but were based on the temperature vs. growth curves of O'Flynn [25] and a desire to have an adequate number of intervals for analysis. It was therefore expected that direct comparisons could be possible for many points in time for which data were collected.

Preliminary studies on larvae density indicated that some endogenous heat generation occurred with as few as 25 larvae growing on 50 g of sheep's liver (see Section 4.3). To avoid endogenous heat generation, and to ensure that the larvae were actually growing at the ambient temperature being examined, I chose a sample size of 10.

For each time interval to be examined, 10 individual newly laid larvae were placed on an excess amount of sheep's liver that had been thawed and equilibrated to room temperature. Sheep's liver was purchased in bulk from an abattoir, cut into suitable sized portions and frozen at -20°C until needed. Early manipulations indicated that the volume of a 50 g weigh boat filled with liver was adequate to avoid starvation or feeding stress. Also, since the livers were observed to vary in their bloodiness, weight was not regarded as the best indicator of nutritive value. The weigh boats containing the liver were then placed on a bed of chaff inside rearing containers. The chaff provided the larvae with a place to pupate.

Females of *C. augur* were observed to first engage in 'practice laying'. They would then lay small numbers of larvae before a large oviposition, after which they would again lay small numbers of larvae. Since, this laying behaviour and the small cohort size of *C. augur* made it difficult to run the desired six replicates for every time frame being examined, three replicates of 10 were run for every time interval. To get an idea of variation in an average cohort size [78], without endogenous heat generation, an additional three replicates were run for each 24-hour period examined (i.e. six replicates for each 24 h). The sample time treatments were chosen by lottery with time allocations, and replicates, each recorded separately on small pieces of paper which were pooled in a large envelope and shaken. Sample numbers were then drawn from the pool individually. If a sample was missed it was added to the lottery again, and redrawn and rerun. Upon completion and examination of the sample set for each temperature, six-hourly sampling of groups of 10 larvae was conducted in triplicate for the first three to six days at 15°C to determine moult times more precisely. Similarly, four-hourly sampling was conducted in triplicate for the first three to six days at 20, 25, 30 and 35°C to determine moult times more precisely. The sample lottery technique was employed for sample allocation.

All treatments were placed into an Axyos temperature-controlled cabinet and left undisturbed until the time for collection, because disturbance can delay pupation [29, 48]. At collection, all individuals were lifted off or dissected out of the substrate and transferred to just-boiled water for 1-2 minutes, dried and transferred to 80% EtOH [83]. On occasion the substrate was flooded with tap water at room temperature in order to help find and collect the larvae, particularly with second- and early third-instars. Initially, it appeared that the influx of water

made the larvae return to the surface, presumably to avoid drowning [84]. However, this method was only partly successful because on some occasions it caused drowning of individuals inside the liver which affected recovery, especially with the smaller instars. Pulsating the liver gently with tweezers proved to be a more effective way of discouraging feeding and encouraging the maggots to leave the security of the liver.

The additional replicates for the 15°C trial were run at the conclusion of experiments at all other temperatures. This was done as a colony control to test for differences in growth between early and late culture generations and thus as a measure of confidence in the biological uniformity of the colony over time.

8.2.2 *Estimating time (age) from maggot length*

Most of the studies on insect development have looked at age prediction from larval length or larval weight, with length on the y axis and time on the x axis. Since time is the unknown and desired variable, I have endeavoured to look at predicting time from the lengths of feeding larvae with the graph inverted to show length on the x axis and time on the y axis. The raw time data were regressed against length and the estimates and standard errors of the regression coefficient were recorded. 95% prediction intervals for age were then calculated. Three sets of intervals were found for each temperature. These were worked out by fitting straight line, quadratic and cubic models to the data sets. Intervals were worked out for the age at lengths of 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 mm except where there were no data at these intervals. Occasionally negative values were encountered from the quadratic and cubic models. These were regarded as anomalies and ignored.

8.3 Results

8.3.1 *Number of lay events required from females of Calliphora augur*

Because the cohort size of *C. augur* is small, a large number of separate oviposition episodes (referred hereafter as ‘lay events’) were required. The number of lay events for each temperature examined is shown in Table 8.1. As the temperature increased, the number of lay events decreased, partly because management of the culture was improving all the time, and partly because the time frames were becoming shorter. Trials took up to 25 lay events to establish and comprised between 60 and 80 samples.

8.3.2 Development of *Calliphora augur* larvae at constant temperatures

The ambient temperatures were found to oscillate between 14.5 and 15.0°C at 15°C, between 20.0 and 20.5°C at 20°C, between 24.5 and 25.0°C at 25°C, between 29.5 and 30.0°C at 30°C and between 34.0 and 35.0°C at 35°C.

Recoveries of *C. augur* were rarely 100% and some of the larvae recovered were useless because of damage inflicted while recovering them. Numbers of larvae recovered are shown in Tables 8.2-6 (note that ‘moult refinement’ refers to the extra sampling that was carried out to determine the timing of moults more precisely). Recoveries may also have been affected by the larval being similar in colour to the chaff and on a few occasions larvae were nearly missed due to being hidden inside pieces or ‘rounds’ of chaff (also mentioned in Section 2.2.3).

At times the edges of pieces of liver dried and became stuck to a weigh boat creating a physical barrier which made it difficult or sometimes impossible for larvae to migrate and complete their development. At such times pupae were often observed beneath liver in the weigh boats. Pupae were observed in the weigh boats in some of the samples grown at 15°C, 30°C and 35°C.

Table 8.1: Number of lay events required from *Calliphora augur* females to run these experiments. ¹ First lot of replicates, ² second lot of replicates for culture control.

Temperature (°C)	Number of lay events
15	15 ¹ +10 ²
20	25
25	16
30	15
35	12

Table 8.2: Recoveries of immature *Calliphora augur* grown at 15°C.

(n_i=number of individuals in each replicate; n_r=number of replicates, N= total measurable number recovered, *=number of additional replicates is 6, not 3).

Time (h)	Mean recoveries		Overall recovery (%)
	Initial replicates (n _i =10; n _r =3)	Additional replicates (n _i =10; n _r =3)	
0	n.d.	9.8 (N=59)*	98.3
24	8.3 (N=25)	-	83.3
48	9.3 (N=28)	13.0 (N=39)	111.6
72	8.6 (N=26)	-	86.6
96	6.0 (N=18)	10.0 (N=30)	80.0
120	6.3 (N=19)	-	63.3
144	8.3 (N=25)	8.6 (N=26)	85.0
168	8.3 (N=25)	-	83.3
192	7.0 (N=21)	7.0 (N=21)	70.0
216	6.3 (N=19)	-	63.3
240	5.0 (N=15)	9.3 (N=28)	71.6
264	6.6 (N=20)	-	66.6
288	3.0 (N=9)	7.3 (N=22)	51.6
312	10.6 (N=32)	-	106.6
336	2.0 (N=6)	8.6 (N=26)	53.3
360	2.6 (N=8)	-	26.6
384	7.0 (N=21)	16.6 (N=50)	118.3
408	8.6 (N=26)	-	86.6
432	9.0 (N=27)	9.0 (N=27)	90.0
456	3.0 (N=9)	-	30.0
480	2.3 (N=7)	7 (N=21)	93.3
504	6.6 (N=20)	-	66.6
528	4.6 (N=14)	9 (N=27)	68.3
576	8.6 (N=26)	-	86.6
600	8.6 (N=26)	9.3 (N=28)	90.0

Table 8.3: Recoveries of immature *Calliphora augur* grown at 20°C.

(n_i=number of individuals in each replicate; n_r=number of replicates, N=total measurable number recovered)

Time (h)	Mean recoveries			Overall recovery (%)
	Initial replicates (n _i =10; n _r =3)	Additional replicates (n _i =10; n _r =3)	Moult refinement (n _i =10; n _r =3)	
0	5.6 (N=17)	9.3 (N=26)	-	75
6	-	-	3.6 (N=11)	36
12	4.3 (N=13)	-	-	43
18	-	-	5.3 (N=16)	53
24	7.3 (N=22)	-	-	73
30	-	-	5.0 (N=15)	50
36	6.6 (N=20)	-	-	66
42	-	-	6.6 (N=20)	66
48	8 (N=24)	3.3 (N=10)	-	56
54	-	-	8.6 (N=26)	86
60	5.3 (N=16)	-	-	53
66	-	-	8.3 (N=25)	83
72	9 (N=27)	-	-	90
84	13 (N=39)	-	-	130
96	9.6 (N=29)	8.6 (N=26)	-	92
108	8 (N=24)	-	-	80
120	6 (N=18)	-	-	60
132	9 (N=27)	-	-	73
144	6.3 (N=19)	8.3 (N=25)	-	73
156	6.3 (N=19)	-	-	63
168	6 (N=18)	-	-	60
180	5.3 (N=16)	-	-	53
192	8.3 (N=25)	9.6 (N=29)	-	90
204	6 (N=24)	-	-	80
216	8.6 (N=26)	-	-	86

Table 8.4: Recoveries of immature *Calliphora augur* grown at 25°C.

(n_i=number of individuals in each replicate; n_r=number of replicates, N= total measurable number recovered)

Time (h)	Mean recoveries			Overall recovery (%)
	Initial replicates (n _i =10; n _r =3)	Additional replicates (n _i =10; n _r =3)	Moult refinement (n _i =10; n _r =3)	
0	6.6 (N=20)	8.3 (N=28)	-	80
4	-	-	9 (n=27)	90
8	-	-	3.6 (N=11)	36
12	10 (N=30)	-	-	100
16	-	-	4 (N=12)	40
20	-	-	7.6 (N=23)	76
24	9.6 (N=29)	-	-	96
28	-	-	7 (N=21)	70
32	-	-	6 (N=18)	56
36	7.3 (N=22)	-	-	73
40	-	-	8.3 (N=25)	83
44	-	-	9.3 (N=28)	93
48	3 (N=9)	9.3 (N=28)	-	61
52	-	-	7.6 (N=23)	76
56	-	-	9.6 (N=29)	96
60	9 (N=27)	-	-	90
64	-	-	9 (N=27)	90
72	7 (N=21)	-	-	70
84	8 (N=24)	-	-	80
96	8.3 (N=25)	8 (N=24)	-	81
108	8 (N=24)	-	-	80
120	9 (N=27)	-	-	90
132	9.3 (N=28)	-	-	93
144	8.6 (N=26)	7 (N=21)	-	78
156	11.3 (N=34)	-	-	113
168	9 (N=27)	2.3 (N=7)	-	56
180	7.6 (N=23)	-	-	76
192	9 (N=27)	7 (N=21)	-	80
204	8 (N=24)	-	-	80
216	10 (N=30)	-	-	100

Table 8.5: Recoveries of immature *Calliphora augur* grown at 30°C.

(n_i=number of individuals in each replicate; n_r=number of replicates, N= total measurable number recovered)

Time (h)	Mean recoveries			Overall recovery (%)
	Initial replicates (n _i =10; n _r =3)	Additional replicates (n _i =10; n _r =3)	Moult refinement (n _i =10; n _r =3)	
0	10 (N=30)	10 (N=30)	-	100
4	-	-	9.3 (N=28)	93
8			9.6 (N=29)	96
12	7.3 (N=22)	-	-	73
16	-	-	9.6 (N=29)	96
20	-	-	7 (N=21)	70
24	5.6 (N=17)	-	-	56
28	-	-	8.6 (N=26)	86
32	-	-	8 (N=24)	80
36	8.3 (N=25)	-	-	83
40	-	-	8.3 (N=25)	83
44	-	-	10 (N=30)	100
48	7 (N=21)	5.6 (N=17)	-	63
52	-	-	9 (N=27)	90
56	-	-	8.3 (N=25)	83
60	9.3 (N=28)	-	-	93
64	-	-	11.3 (N=34)	113
68	-	-	9.3 (N=28)	93
72	7.6 (N=23)	-	-	76
84	9.3 (N=28)	-	-	93
96	9.6 (N=29)	8 (N=24)	-	88
108	0	-	-	0
120	7.6 (N=23)	-	-	76
132	4.6 (N=14)	-	-	46
144	8.3 (N=25)	7.3 (N=22)	-	78
156	7.3 (N=22)	-	-	73
168	6.3 (N=19)	-	-	63
180	9.6 (N=29)	-	-	96
192	9 (N=27)	8 (N=24)	-	85
204	8 (N=24)	-	-	80
216	7.3 (N=22)	-	-	73

Table 8.6: Recoveries of immature *Calliphora augur* grown at 35°C.

(n_i=number of individuals in each replicate; n_r=number of replicates, N= total measurable number recovered)

Time (h)	Mean recoveries			Overall recovery (%)
	Initial replicates (n _i =10; n _r =3)	Additional replicates (n _i =10; n _r =3)	Moult refinement (n _i =10; n _r =3)	
0	11 (N=33)	10 (N=30)	-	105
4	-	-	5.3 (N=16)	53
8	-	-	6.3 (N=19)	63
12	6 (N=18)	-	-	60
16	-	-	9 (N=27)	90
20	-	-	7 (N=21)	70
24	6.3 (N=19)	-	-	63
28	-	-	2 (N=6)	20
32	-	-	8.3 (N=25)	83
36	7 (N=21)	-	-	70
40	-	-	0	0
44	-	-	6.3 (N=19)	63
48	4.3 (N=13)	4.6 (N=14)	-	45
52	-	-	5 (N=15)	50
56	-	-	6.6 (N=20)	66
60	5.6 (N=17)	-	-	56
64	-	-	0	0
68	-	-	2 (N=6)	20
72	7.3 (N=22)	-	-	73
84	4.6 (N=14)	-	-	46
96	5 (N=15)	4.3 (N=13)	-	46
108	4.6 (N=14)	-	-	46
120	3.3 (N=10)	-	-	16
132	4.6 (N=14)	-	-	46
144	4.6 (N=14)	2.6 (N=8)	-	36
156	4.6 (N=14)	-	-	46
168	2 (N=6)	-	-	20
180	5.6 (N=17)	-	-	56
192	4.3 (N=13)	6.6 (N=20)	-	55
204	1.6 (N=5)	-	-	16
216	1.6 (N=5)	-	-	16

Since the purpose of this study was to obtain an indication of variation in wild-type larvae but to avoid elevated heating from maggot mass agglomeration, the replicates have been pooled. Data from an unusual prepupal form which was only observed at 30°C and 35°C have been omitted. These are assumed to be individuals unable to complete development. This form appears as elongated, sessile and predominantly white with one very tapered end. In one case it was observed to have changed to the dark brown pupal texture along the dorsal surface only. These have also been observed in a culture of *Calliphora ochracea* and also other species (Dr James Wallman, pers. comm). Very small individuals were also observed. Since these were also reported by Wells and Kurahashi [78], they have been included in my data.

Results for growth at 15°C, 20°C, 25°C, 30°C and 35°C are shown in Figures 8.1-5. The growth curves are the anticipated classical inverted 'J' shape. At 15°C pupation was first observed at 336 hours, at 20°C pupation was first observed at 216 hours, at 25°C pupation was first observed at 144 hours, at 30°C pupation was first observed at 120 hours and at 35°C pupation was first observed at 132 hours. This decrease in time to pupation with increase in temperature was expected.

Mean comparative body length data by growth stage are collated in Table 8.7. Transitional forms were observed; first to second-instars were seen at 15, 20 and 25°C but not at 30 or 35°C. Second- to third-instars were seen at all temperatures. White (young) pupae were not observed. Light brown (intermediate age) pupae were noted, but only at 20 and 25°C. Dark brown (final pupal form) pupae were observed at all temperatures. Median, range and 95% quantile range data (to reduce the influence of very small and very large larvae [101]) are shown in Table 8.8. The earliest detections of the various growth stages at the temperatures examined can be easily derived from Table 8.9.

To examine how the body lengths of the different instars overlap, plots of the full ranges measured for the different temperatures were generated and are shown in Figures 8.6-8.10. Some overlap in larval length occurred between first, first to second and second-instars, between second, second to third and third-instars, and also between second to third, and feeding and post-feeding third-instars at 15°C. At 20°C overlap occurred between first, first to second and second-instars, between second, second to third and third-instars, and between second to third feeding and post-feeding third-instars. At 25°C overlap occurred between first, first to second and second-instars, between first to second, second, second to third and third-instars, and between second, second to third feeding and post-feeding third-instars.

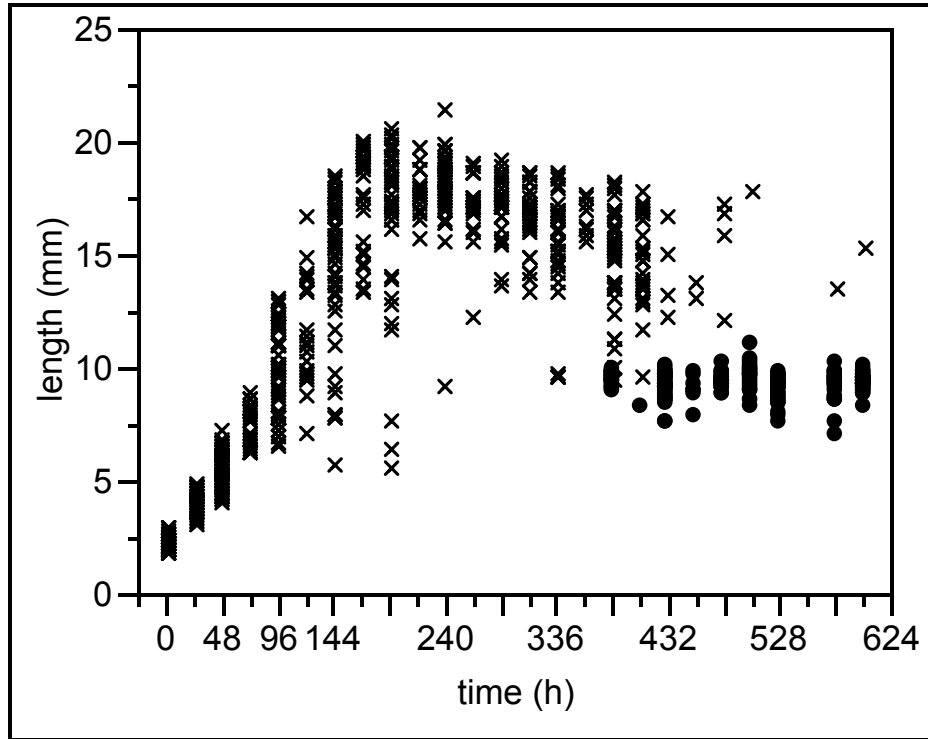


Figure 8.1: Development of *Calliphora augur* as measured by body length in mm at 15°C.

Legend: x = larvae, • = pupae

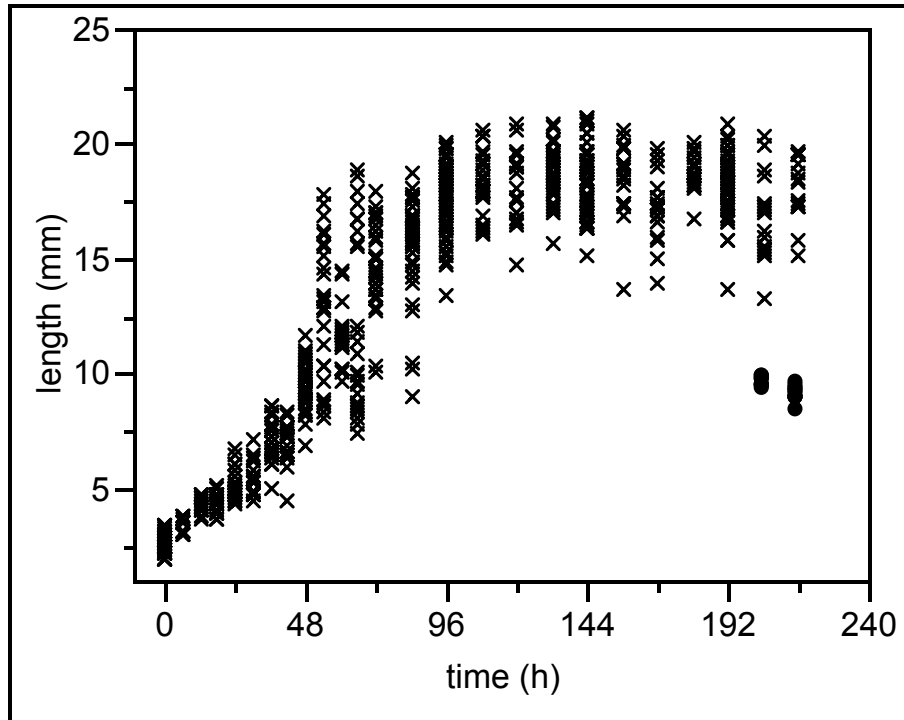


Figure 8.2: Development of *Calliphora augur* as measured by body length in mm at 20°C.

Legend: x = larvae, • = pupae

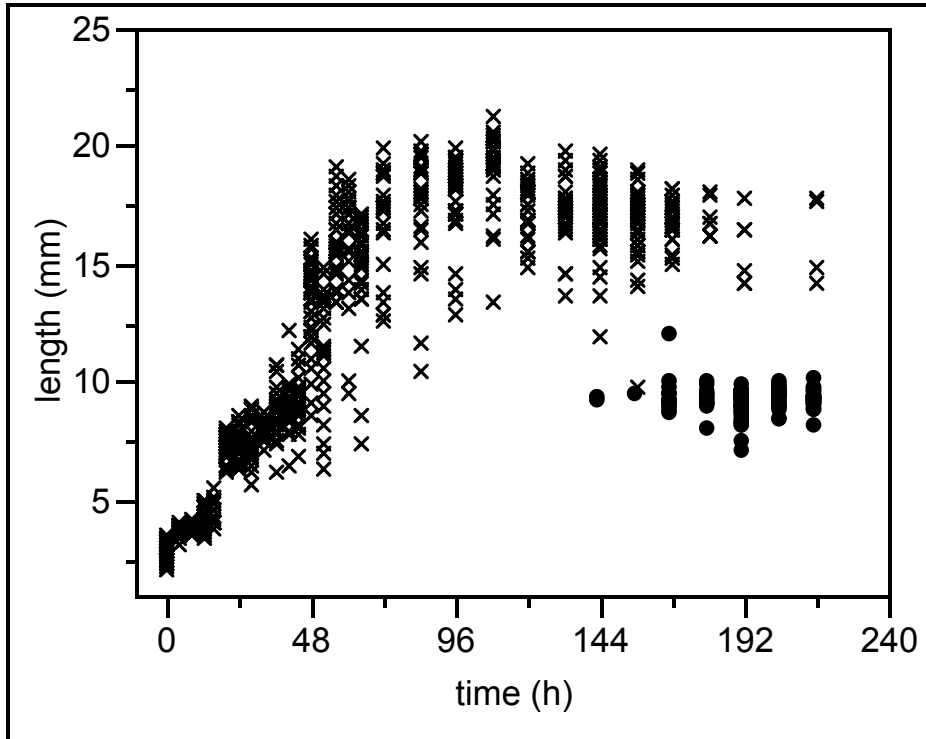


Figure 8.3: Development of *Calliphora augur* as measured by body length in mm at 25°C.

Legend: x = larvae, • = pupae

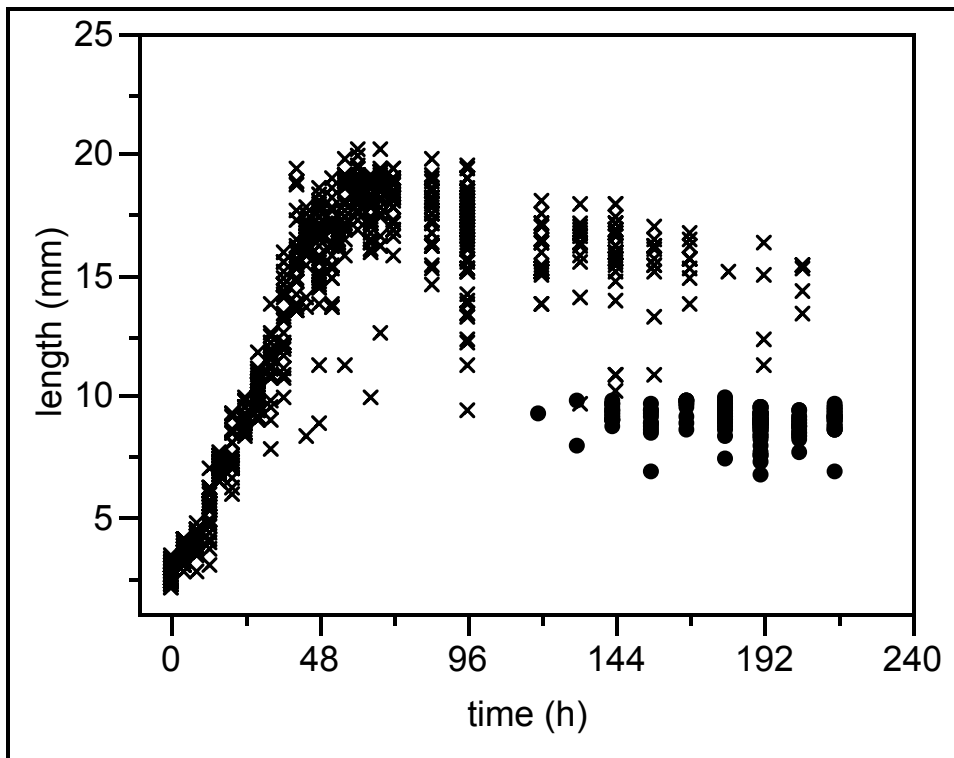


Figure 8.4: Development of *Calliphora augur* as measured by body length in mm at 30°C.

Legend: x = larvae, • = pupae

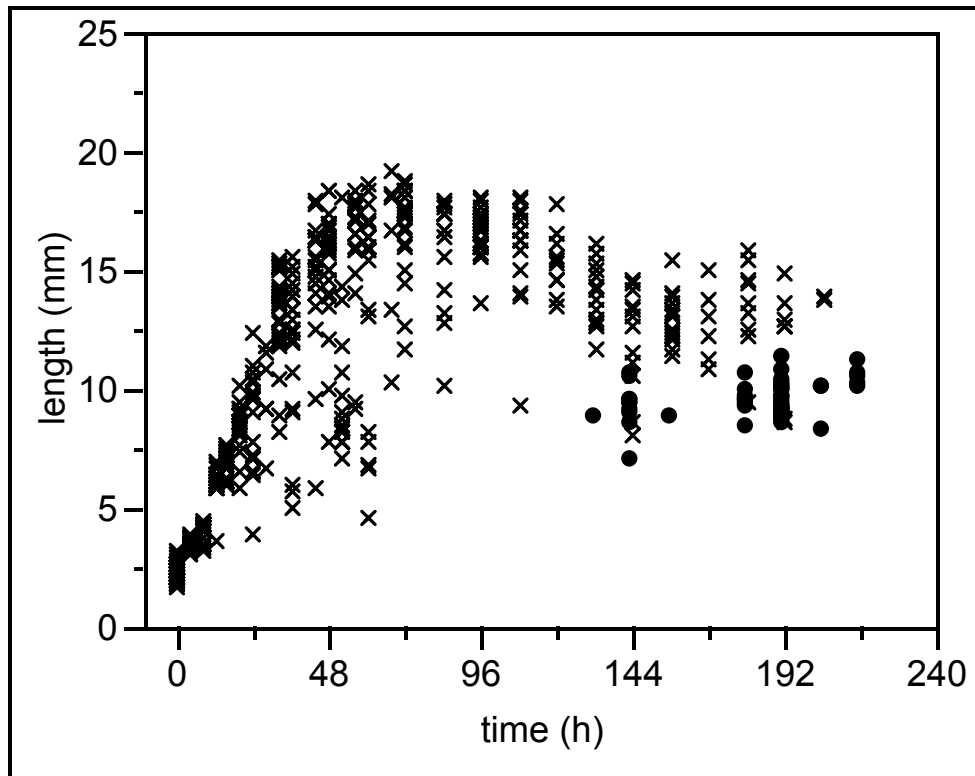


Figure 8.5: Development of *Calliphora augur* as measured by body length in mm at 35°C.

Legend: x = larvae, • = pupae

Table 8.7: Mean body length (mm) \pm std dev. of larval instars and growth stages by day of *Calliphora augur* grown at different constant temperatures. PF3rd - post-feeding third-instar, WP=white pupae, LBP=light brown pupae, BP=brown pupae. Where std dev.= 0, n=1.

Time (h)		Life stage								
		1st	1st-2nd	2nd	2nd-3rd	3rd	PF3rd	WP	LBP	BP
0	15°C	2.17 \pm 0.27	-	-	-	-	-	-	-	-
	20°C	2.55 \pm 0.40	-	-	-	-	-	-	-	-
	25°C	2.90 \pm 0.35	-	-	-	-	-	-	-	-
	30°C	2.55 \pm 0.32	-	-	-	-	-	-	-	-
	35°C	2.27 \pm 0.29	-	-	-	-	-	-	-	-
24	15°C	4.01 \pm 0.49	-	-	-	-	-	-	-	-
	20°C	-	4.79 \pm 0.23	5.15 \pm 0.72	-	-	-	-	-	-
	25°C	-	8.3 \pm 0	7.31 \pm 0.59	-	-	-	-	-	-
	30°C	-	-	9.01 \pm 0.53	9.34 \pm 0.44	-	-	-	-	-
	35°C	-	-	6.8 \pm 1.62	9.24 \pm 1.01	11.02 \pm 0.85	-	-	-	-
48	15°C	-	4.99 \pm 0	5.47 \pm 0.72	-	-	-	-	-	-
	20°C	-	-	9.08 \pm 0.87	10.06 \pm 0.88	11.57 \pm 0	-	-	-	-
	25°C	-	-	-	9.35 \pm 0.99	13.67 \pm 1.46	-	-	-	-
	30°C	-	-	-	-	16.08 \pm 1.93	-	-	-	-
	35°C	-	-	-	-	15.27 \pm 2.36	-	-	-	-
72	15°C	-	-	7.34 \pm 0.88	-	-	-	-	-	-
	20°C	-	-	-	-	14.66 \pm 1.95	-	-	-	-
	25°C	-	-	-	-	16.58 \pm 2.34	-	-	-	-
	30°C	-	-	-	-	18.08 \pm 0.93	16.84 \pm 0	-	-	-
	35°C	-	-	-	-	17.20 \pm 0.97	16.17 \pm 2.48	-	-	-
96	15°C	-	-	8.27 \pm 1.13	9.79 \pm 0.18	11.70 \pm 0.80	-	-	-	-
	20°C	-	-	-	-	17.78 \pm 1.47	-	-	-	-
	25°C	-	-	-	-	17.99 \pm 1.63	16.81 \pm 1.51	-	8.79 \pm 0	9.08 \pm 0.46
	30°C	-	-	-	-	16.03 \pm 1.74	16.44 \pm 2.35	-	-	-
	35°C	-	-	-	-	16.50 \pm 0.92	16.54 \pm 0.93	-	-	-
120	15°C	-	-	7.02 \pm 0	9.37 \pm 0	11.97 \pm 2.22	-	-	-	-
	20°C	-	-	-	-	18.06 \pm 1.60	19.55 \pm 0	-	-	-
	25°C	-	-	-	-	17.43 \pm 1.31	16.94 \pm 0.91	-	-	-
	30°C	-	-	-	-	-	15.74 \pm 1.11	-	-	9.27 \pm 0
	35°C	-	-	-	-	-	15.22 \pm 1.29	-	-	-
144	15°C	-	-	-	-	14.385 \pm 3.28	-	-	-	-
	20°C	-	-	-	-	19.01 \pm 1.53	17.50 \pm 1.07	-	-	-
	25°C	-	-	-	-	17.54 \pm 0.04	17.02 \pm 1.53	-	-	9.39 \pm 0.04
	30°C	-	-	-	-	15.01 \pm 3.09	15.94 \pm 0.92	-	-	9.34 \pm 0.31
	35°C	-	-	-	-	12.01 \pm 1.33	12.26 \pm 2.47	-	-	9.38 \pm 1.03
168	15°C	-	-	-	-	17.33 \pm 2.26	-	-	-	-
	20°C	-	-	-	-	-	17.21 \pm 1.54	-	-	-
	25°C	-	-	-	-	16.85 \pm 0.54	16.68 \pm 1.00	-	9.01 \pm 0.34	9.67 \pm 0.88
	30°C	-	-	-	-	15.78 \pm 0.66	14.65 \pm 1.31	-	-	9.52 \pm 0.44
	35°C	-	-	-	-	14.95 \pm 0	12.13 \pm 1.19	-	-	-
192	15°C	-	-	-	-	16.71 \pm 3.65	-	-	-	-
	20°C	-	-	-	-	15.69 \pm 2.97	18.20 \pm 1.15	-	-	-
	25°C	-	-	-	-	-	15.60 \pm 1.50	-	-	9.13 \pm 0.60
	30°C	-	-	-	-	13.22 \pm 2.72	14.97 \pm 0	-	-	8.88 \pm 0.52
	35°C	-	-	-	-	13.25 \pm 0.95	8.59 \pm 0.05	-	-	9.62 \pm 0.70
216	15°C	-	-	-	-	17.76 \pm 1.05	-	-	-	-
	20°C	-	-	-	-	-	18.03 \pm 1.44	-	9.09 \pm 0	9.25 \pm 0.31
	25°C	-	-	-	-	17.66 \pm 0.16	14.45 \pm 0.47	-	-	9.38 \pm 0.38
	30°C	-	-	-	-	-	-	-	-	9.06 \pm 0.56
	35°C	-	-	-	-	-	-	-	-	10.66 \pm 0.44

Table 8.8: Instar and growth stage summaries for *Calliphora augur* larvae at various constant temperatures.

Temperature and growth stage	Mean±std dev.	Median	Range and number	2.5 to 97.5% quantile range
15°C				
First instar	2.73±0.91	2.27	1.69-4.86 (85)	1.71-4.64
Second instar	6.44±1.44	6.25	4.14-10.13 (113)	4.26-9.94
Third instar	15.81±2.80	16.66	5.53-21.26 (417)	8.95-19.65
Pupae	9.41±0.50	9.45	7.15-11.17 (251)	7.94-10.26
20°C				
First instar	3.06±0.80	2.91	1.82-4.68 (72)	1.86-4.67
Second instar	7.16±1.63	7.22	4.23-10.68 (105)	4.38-10.08
Third instar	17.12±2.34	17.52	8.94-21.08 (427)	11.12-20.49
Pupae	9.44±0.36	9.48	8.57-10.01 (20)	8.57-10.01
25°C				
First instar	3.56±0.71	3.51	1.98-6.25 (123)	2.30-4.94
Second instar	7.79±0.98	7.76	4.78-9.74 (145)	5.57-9.60
Third instar	16.23±2.41	16.74	7.33-21.19 (423)	9.99-19.81
Pupae	9.31±0.54	9.36	7.16-12.14 (134)	8.21-10.12
30°C				
First instar	3.13±0.70	3.00	1.94-4.70 (118)	2.07-4.38
Second instar	7.30±1.21	7.15	4.86-9.76 (78)	5.17-9.75
Third instar	16.07±2.44	16.70	7.69-20.10 (496)	10.03-19.30
Pupae	9.03±0.57	9.11	6.78-9.96 (158)	7.35-9.88
35°C				
First instar	2.75±0.73	2.46	1.64-4.35 (98)	1.71-4.28
Second instar	7.06±1.20	6.95	3.59-10.13 (75)	3.83-9.71
Third instar	14.20±3.02	14.77	4.47-19.16 (307)	6.95-18.25
Pupae	9.65±0.80	9.56	7.12-11.48 (54)	7.60-11.42

Table 8.9: Shortest recorded times (in hours) to reach immature life stages in *Calliphora augur*. The term ‘wandering larvae’ refers to post-feeding/migrating third-instar larvae. n.o. = not observed

Temperature	15°C	20°C	25°C	30°C	35°C
First detection (hours)					
Growth stage					
First instar	0	0	0	0	0
Second instar	48	24	24	12	12
Third instar	96	60	36	28	24
Wandering larvae	264	132	96	72	72
White pupae	n.o.	n.o.	n.o.	n.o.	n.o.
Light Brown pupae	336	216	168	n.o.	n.o.
Dark Brown pupae	384	204	144	120	132
Transitional forms first detected (hours)					
First to second	48	24	16	12	n.o.
Second to third	96	48	36	24	24

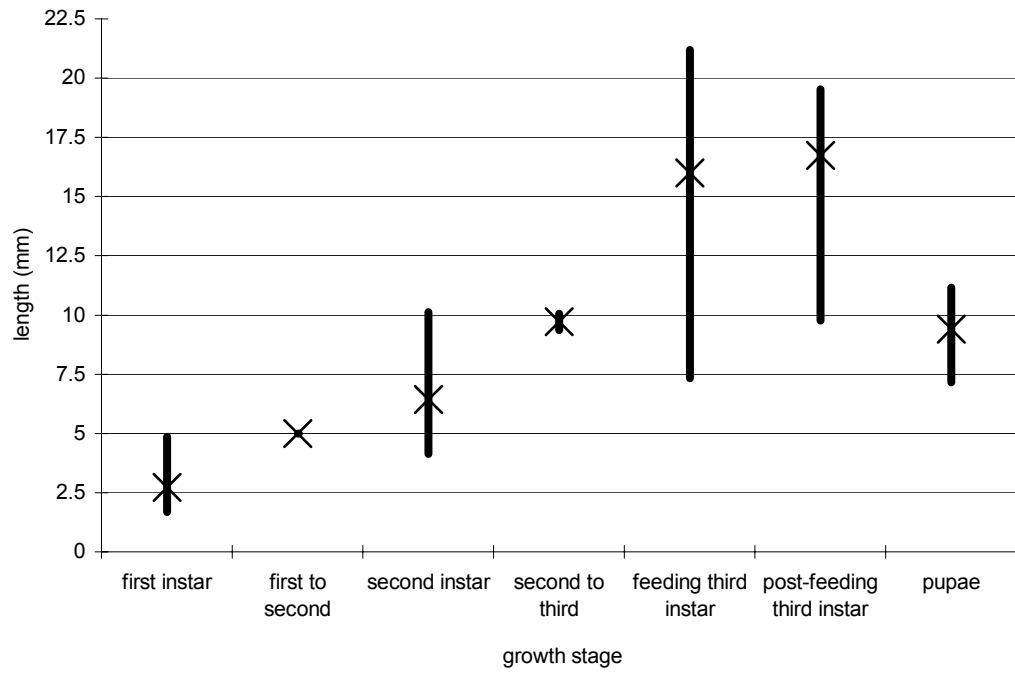


Figure 8.6: Variation in size of *Calliphora augur* larvae grown at 15°C. X=mean.

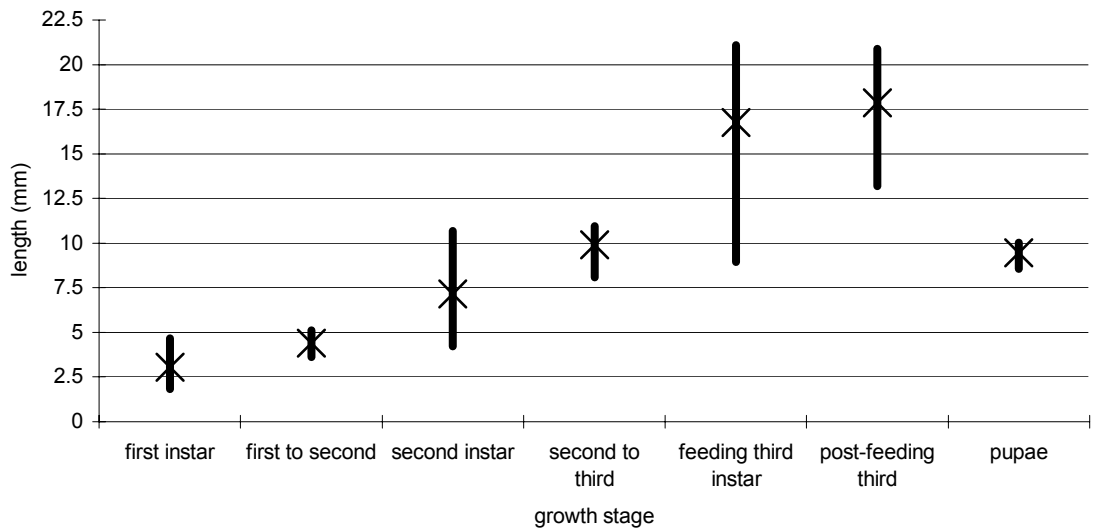


Figure 8.7: Variation in size of *Calliphora augur* larvae grown at 20°C. X=mean.

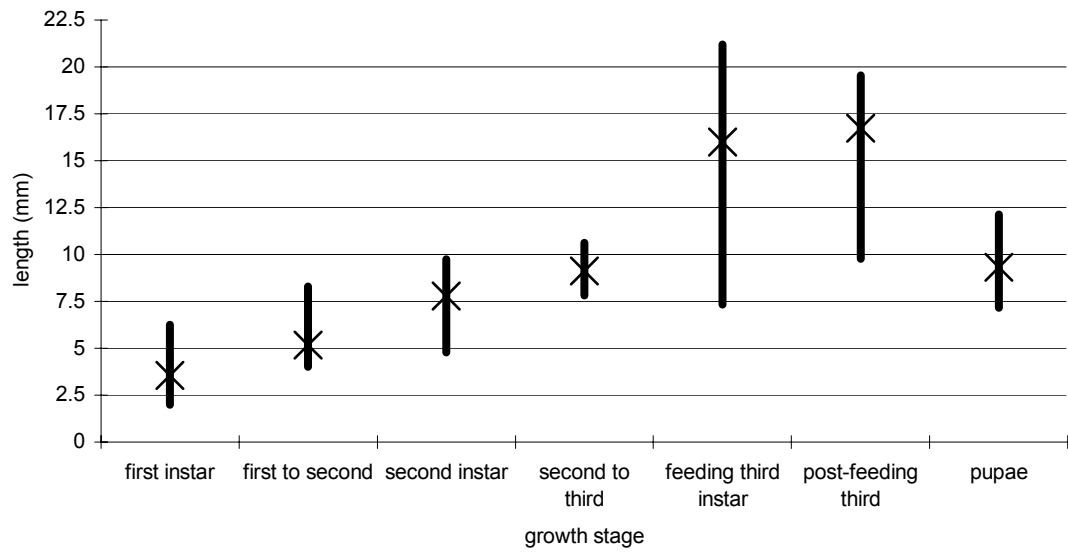


Figure 8.8: Variation in size of *Calliphora augur* larvae grown at 25°C. X=mean.

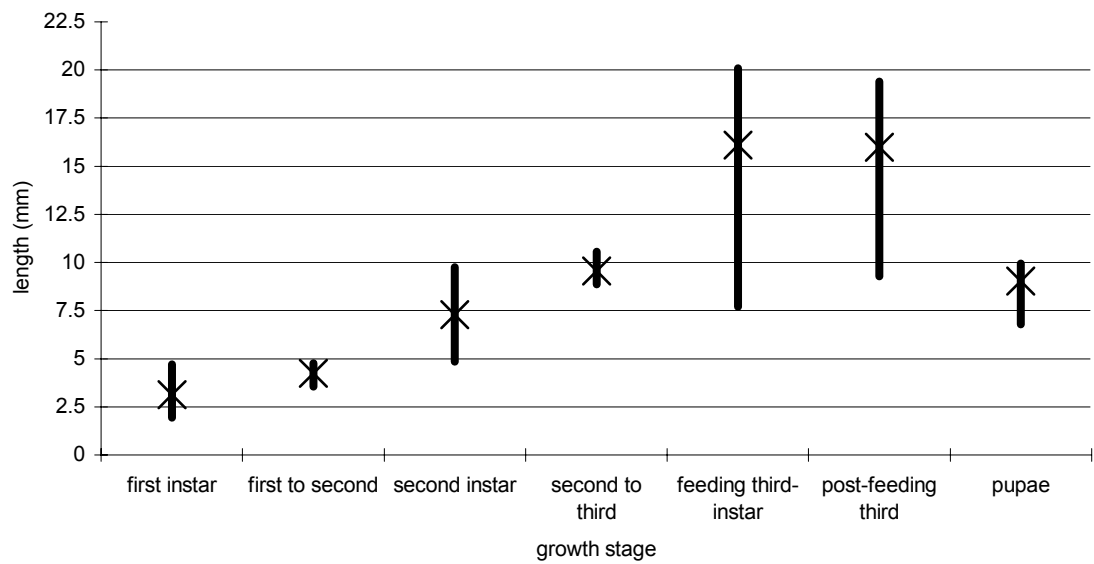


Figure 8.9: Variation in size of *Calliphora augur* larvae grown at 30°C. X=mean.

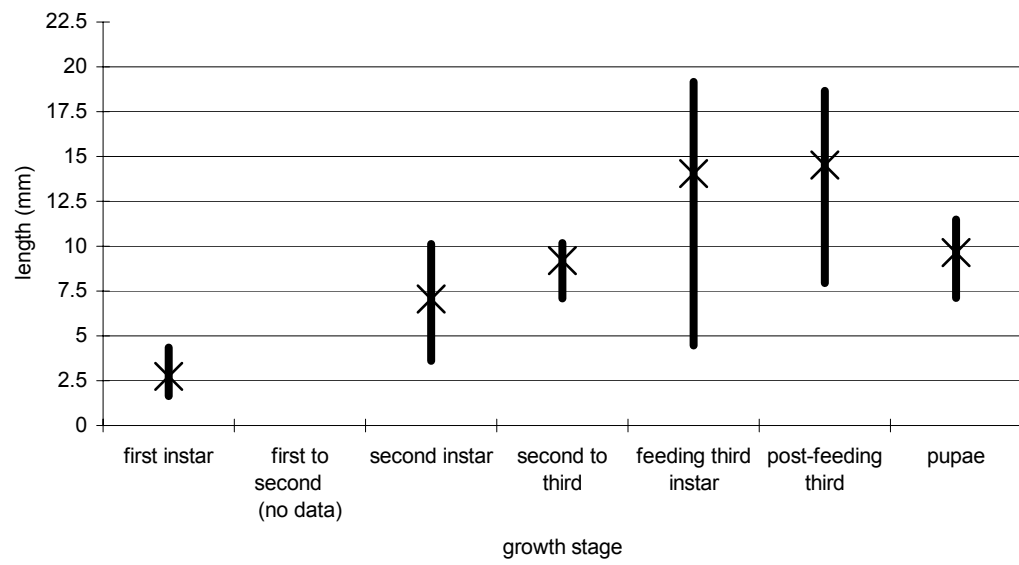


Figure 8.10: Variation in size of larvae of *Calliphora augur* larvae grown at 35°C. X=mean.

At 30°C overlap occurred between first and first to second-instars, between first to second and second-instars and between second, second to third and both feeding and post-feeding third-instars. Finally, 35°C, overlap occurred between first and second, and between second, second to third-instars, feeding third-instars and post-feeding third-instars. No first to second transitional forms were seen at this highest temperature.

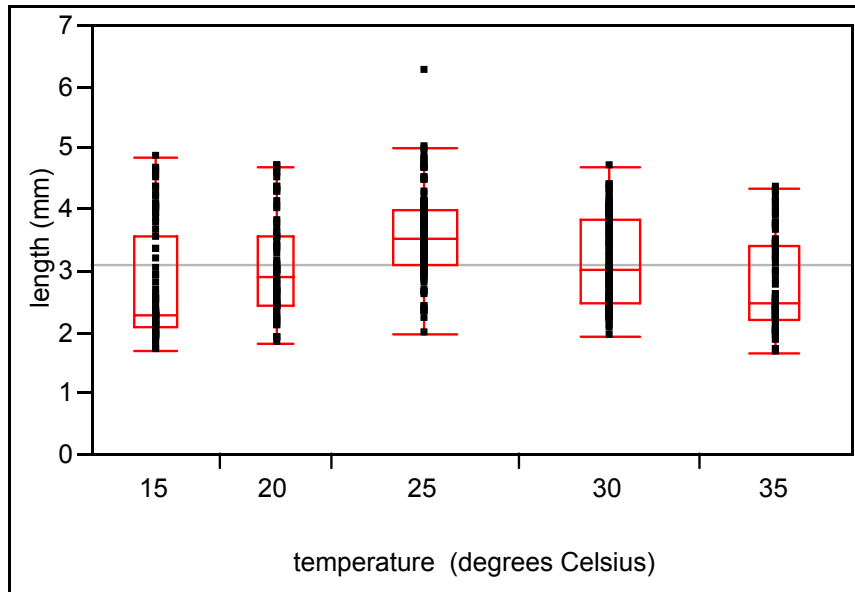
The overall influence of temperature on the various growth stages at the different temperatures is shown in Figures 8.11-8.15. First to second-instar transitions are not represented due to the numbers being small. The grand mean is represented by a horizontal line in each plot. Box plots describe the data sets. First-instar larvae were larger (longer in body length) on average when grown at 25°C. Second-instar larvae were also larger on average when grown at 25°C. Second to third transitional forms, feeding third-instars, and post-feeding third-instars were all larger on average when grown at 20°C. Feeding third-instars also performed well at 25°C and 30°C but larger variation was observed in those grown at 35°C.

Patterns of migration are shown in Figures 8.16-8.20. No clear trends are evident. There were some larvae which failed to pupate, a phenomenon observed by other workers involved in culturing blowflies (pers comm. Dr Garry Levot).

8.3.3 *Estimating time (age) from maggot length*

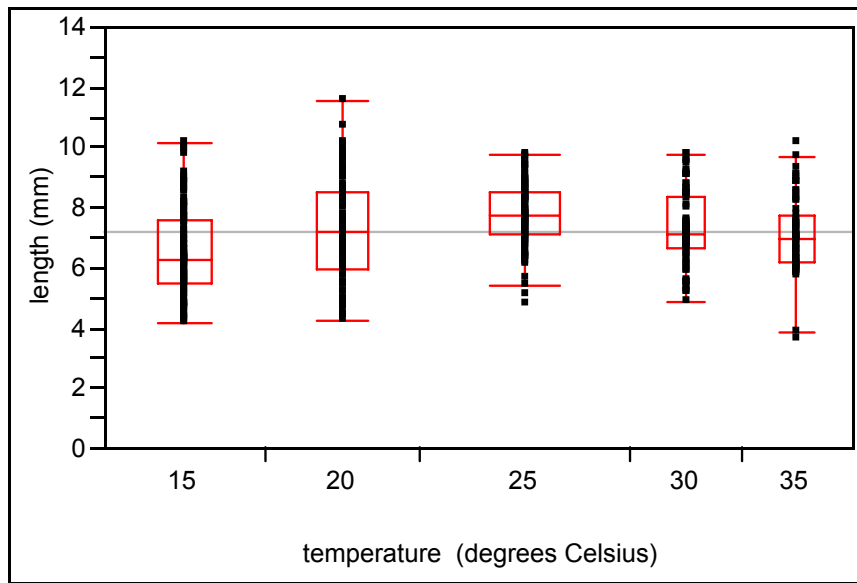
Regressions from the raw time data against length and the calculated 95% prediction intervals are shown in Figures 8.21-25. Figures show the linear, quadratic and cubic time estimates generated from larval body length data of 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 mm for each temperature examined.

At all temperatures, no great differences were observed between the estimates from the different models, with less than a day's difference predicted. Since larval growth curves are characteristically sigmoidal in shape, the quadratic model is likely to be the best indicator. The linear model is likely to be oversimplistic and the cubic model is likely to miss more at the extremes of the curve(s). The estimates generated from these curves fit the raw data reasonably well and so these prediction intervals may be used to give a quick estimate of time since oviposition. Furthermore, the time frames for which estimates have been calculated are shorter than the existing published data are able to provide [25].



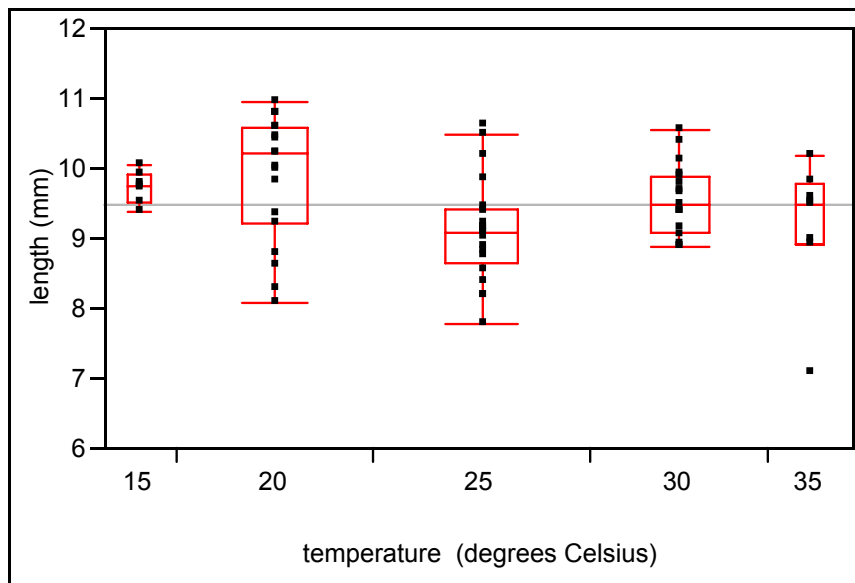
$p < 0.0001$ (n=496)

Figure 8.11: The influence of temperature on development in first-instar *Calliphora augur* larvae. The median is represented by a horizontal line within the box, while the horizontal boundaries of the box are the first and third quartiles. The range of the data is represented by the vertical lines above and below each box. 95% confidence intervals for the mean are represented by the short horizontal lines near the ends of the vertical lines. Outlying data points are represented by individual dots above or below the median confidence intervals. The large central horizontal line is the grand mean of a pool of all samples in that growth stage. The p values indicate whether there are significant differences between the mean lengths of maggots.



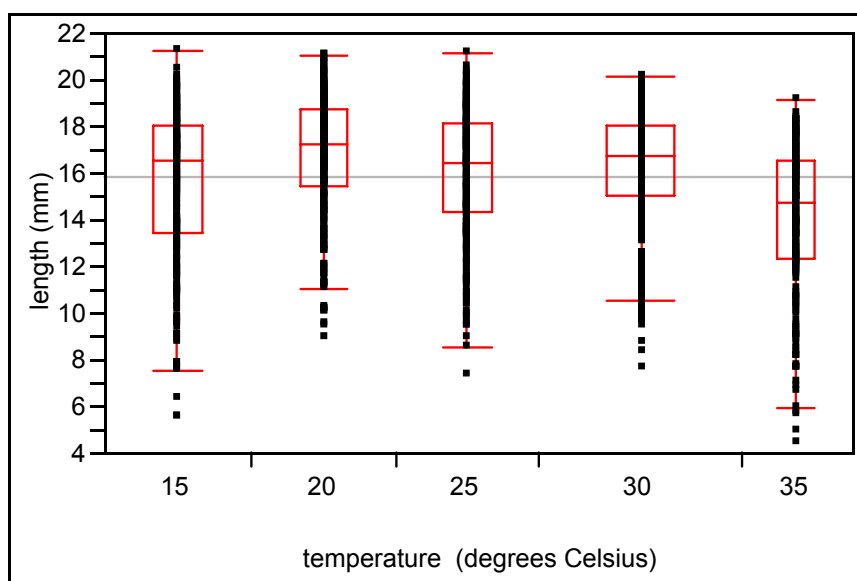
$p < 0.0001$ (n=517)

Figure 8.12: The influence of temperature on development in second-instar *Calliphora augur* larvae. The median is represented by a horizontal line within the box, while the horizontal boundaries of the box are the first and third quartiles. The range of the data is represented by the vertical lines above and below each box. 95% confidence intervals for the mean are represented by the short horizontal lines near the ends of the vertical lines. Outlying data points are represented by individual dots above or below the median confidence intervals. The large central horizontal line is the grand mean of a pool of all samples in that growth stage. The p values indicate whether there are significant differences between the mean lengths of maggots.



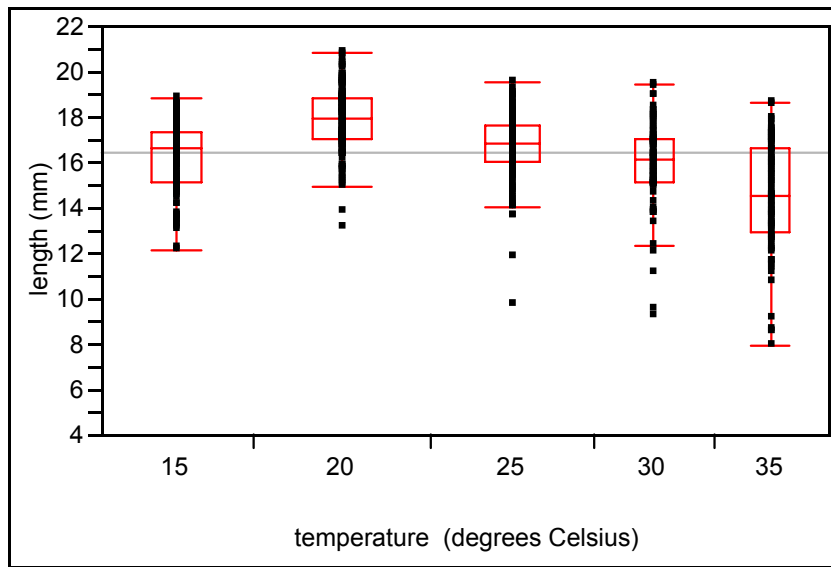
$p=0.0138$ ($n=72$)

Figure 8.13: The influence of temperature on development in second to third transition *Calliphora augur* larvae. The median is represented by a horizontal line within the box, while the horizontal boundaries of the box are the first and third quartiles. The range of the data is represented by the vertical lines above and below each box. 95% confidence intervals for the mean are represented by the short horizontal lines near the ends of the vertical lines. Outlying data points are represented by individual dots above or below the median confidence intervals. The large central horizontal line is the grand mean of a pool of all samples in that growth stage. The p values indicate whether there are significant differences between the mean lengths of maggots.



$p < 0.0001$ ($n = 1474$)

Figure 8.14: The influence of temperature on development in feeding third-instar *Calliphora augur* larvae. The median is represented by a horizontal line within the box, while the horizontal boundaries of the box are the first and third quartiles. The range of the data is represented by the vertical lines above and below each box. 95% confidence intervals for the mean are represented by the short horizontal lines near the ends of the vertical lines. Outlying data points are represented by individual dots above or below the median confidence intervals. The large central horizontal line is the grand mean of a pool of all samples in that growth stage. The p values indicate whether there are significant differences between the mean lengths of maggots.



$p < 0.0001$ (n=589)

Figure 8.15: The influence of temperature on development in post-feeding third-instar *Calliphora augur* larvae. The median is represented by a horizontal line within the box, while the horizontal boundaries of the box are the first and third quartiles. The range of the data is represented by the vertical lines above and below each box. 95% confidence intervals for the mean are represented by the short horizontal lines near the ends of the vertical lines. Outlying data points are represented by individual dots above or below the median confidence intervals. The large central horizontal line is the grand mean of a pool of all samples in that growth stage. The p values indicate whether there are significant differences between the mean lengths of maggots.

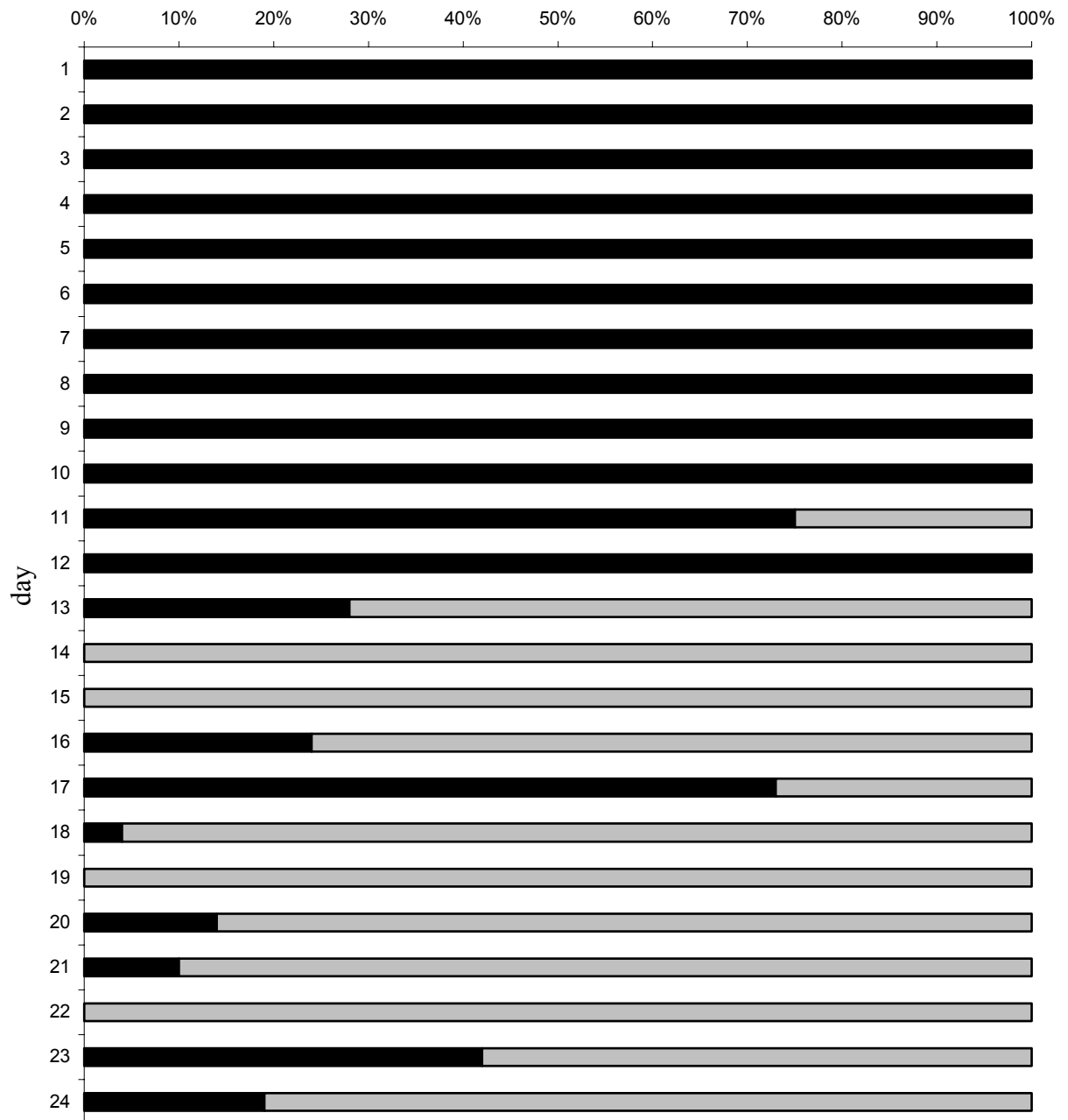


Figure 8.16: Proportions of immature *Calliphora augur* individuals observed on meat and in chaff over time when grown at 15°C.

Legend: ■ = located on meat, ■ = located in chaff .

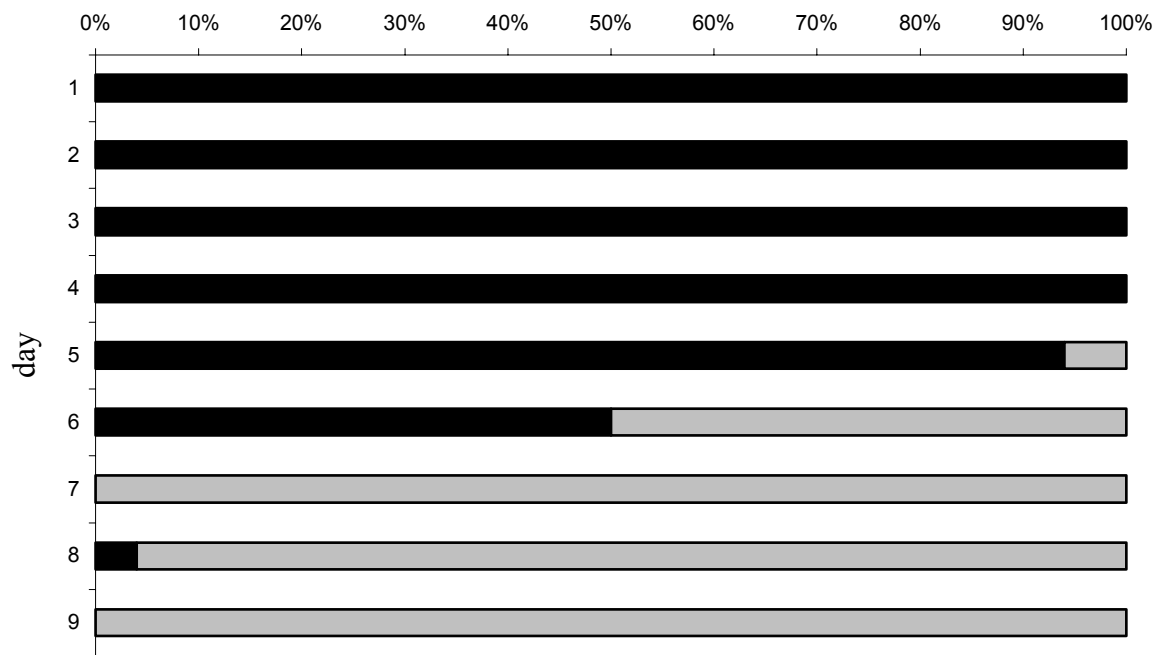


Figure 8.17: Proportions of immature *Calliphora augur* individuals observed on meat and in chaff over time when grown at 20°C.

Legend: ■ = located on meat, ■ = located in chaff .

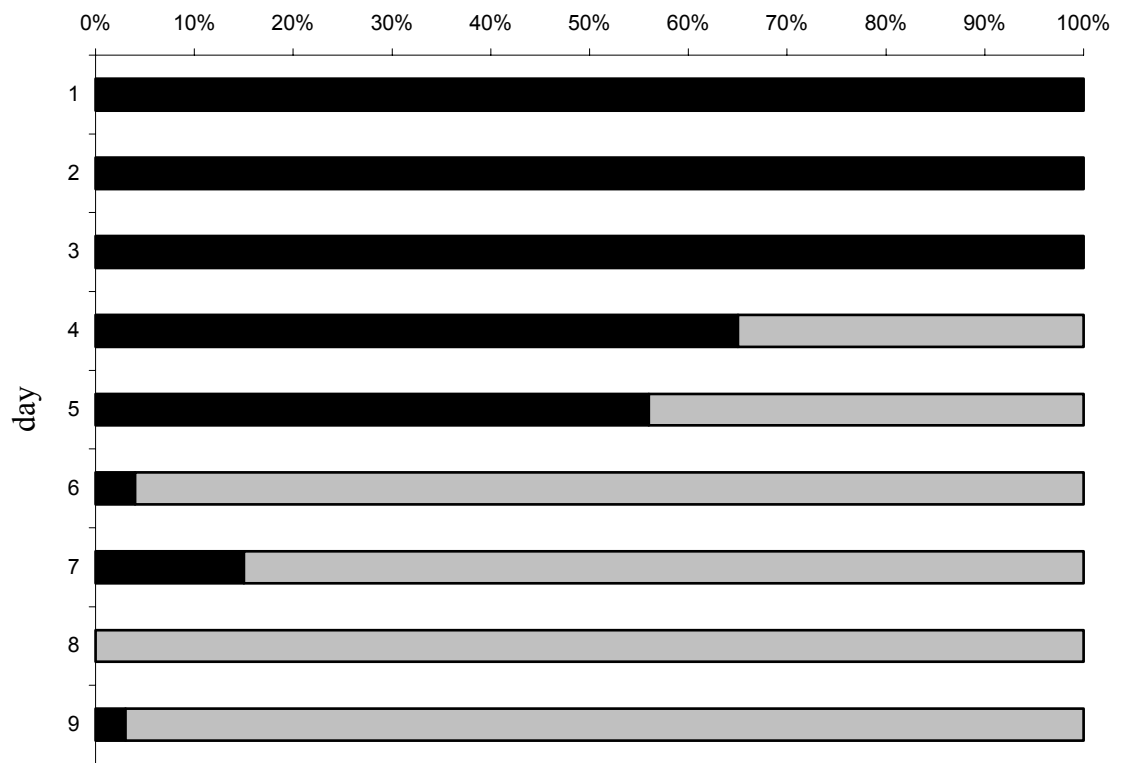


Figure 8.18: Proportions of immature *Calliphora augur* individuals observed on meat and in chaff over time when grown at 25°C.

Legend: ■ = located on meat, □ = located in chaff.

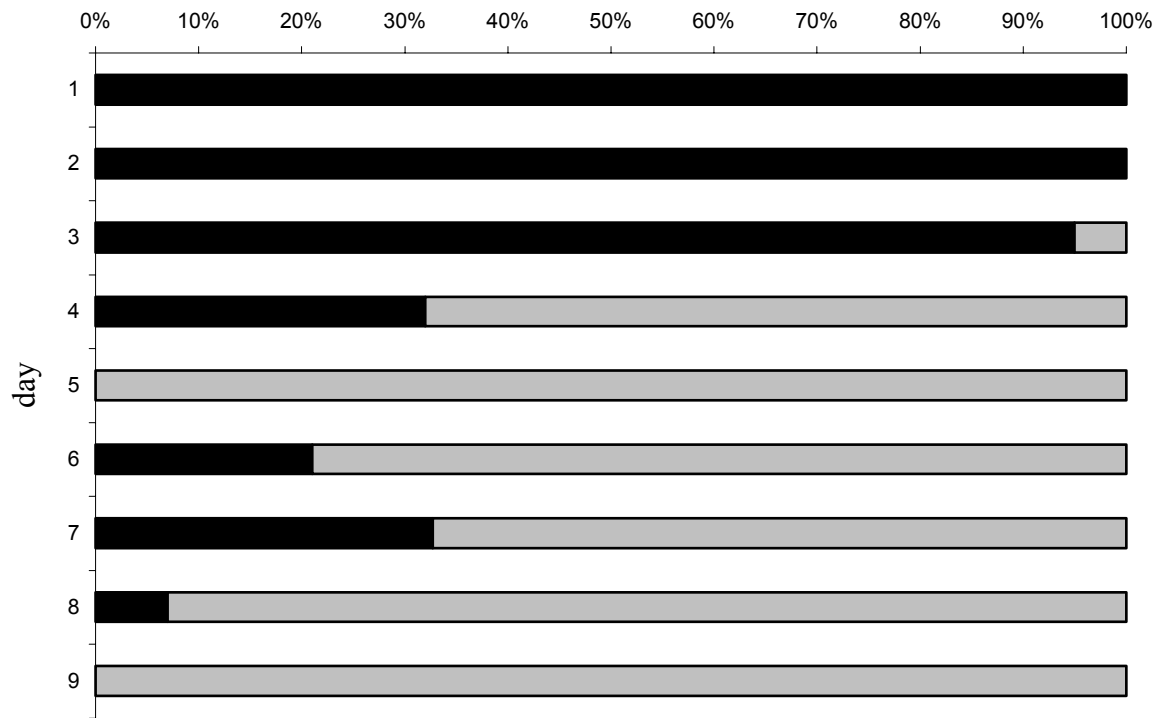


Figure 8.19: Proportions of immature *Calliphora augur* individuals observed on meat and in chaff over time when grown at 30°C.

Legend: ■ = located on meat, ■ = located in chaff .

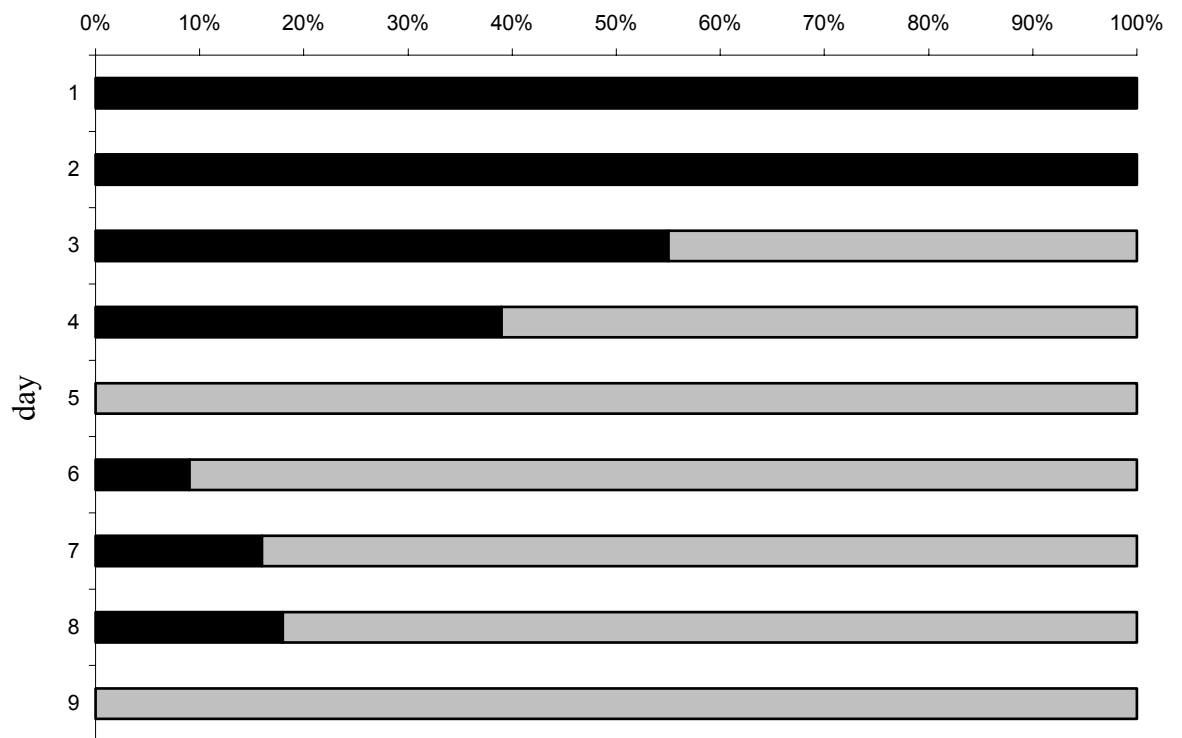
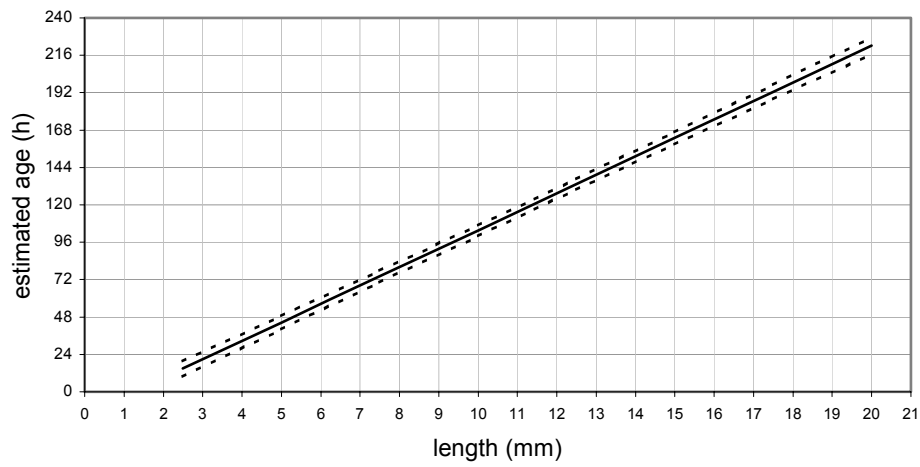


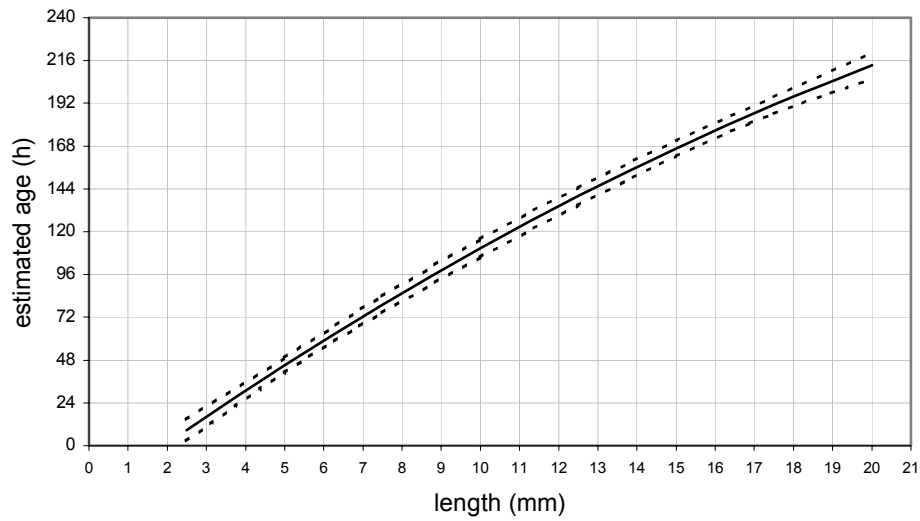
Figure 8.20: Proportions of immature *Calliphora augur* individuals observed on meat and in chaff over time when grown at 35°C.

Legend: ■ = located on meat, ■ = located in chaff .

a) linear model



b) quadratic model



c) cubic model

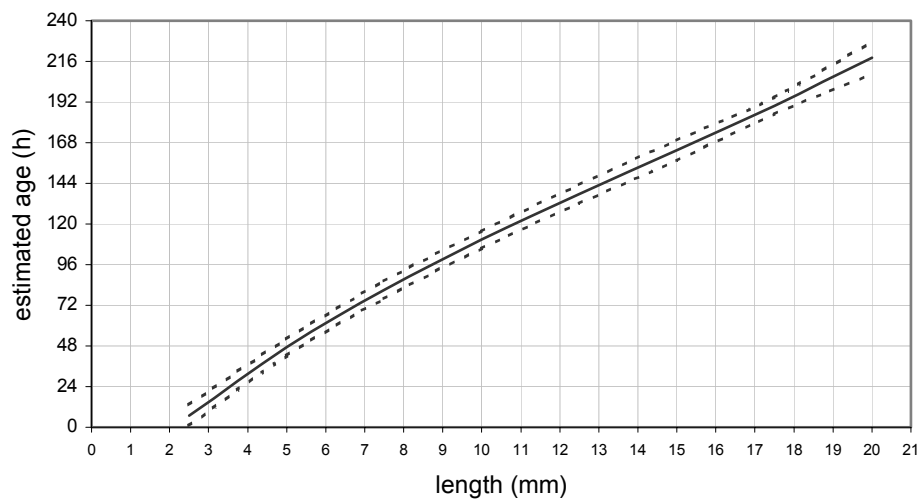
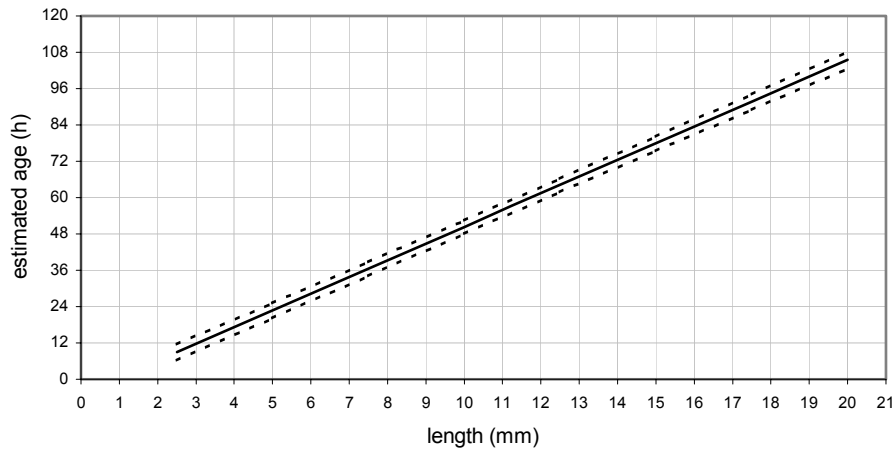
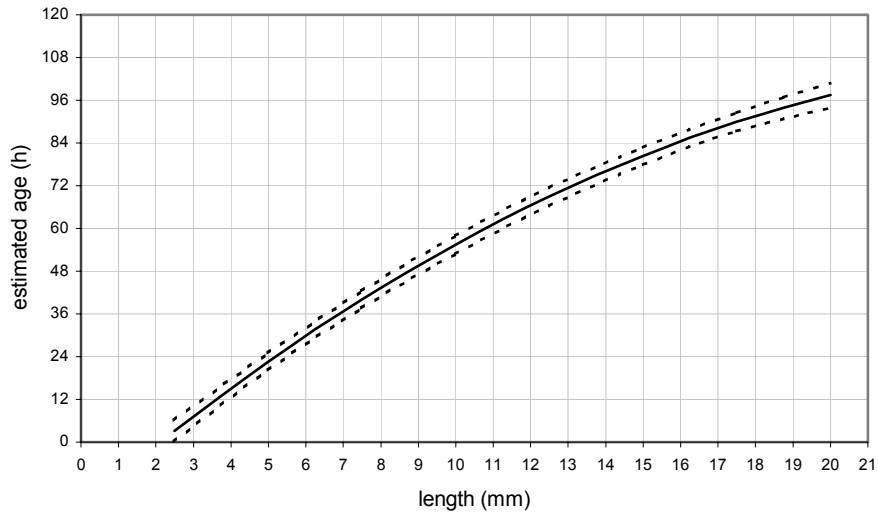


Figure 8.21: Estimation of maggot age from body length, and 95% prediction intervals for the age of feeding larvae grown at 15°C.

a) linear model



b) quadratic model



c) cubic model

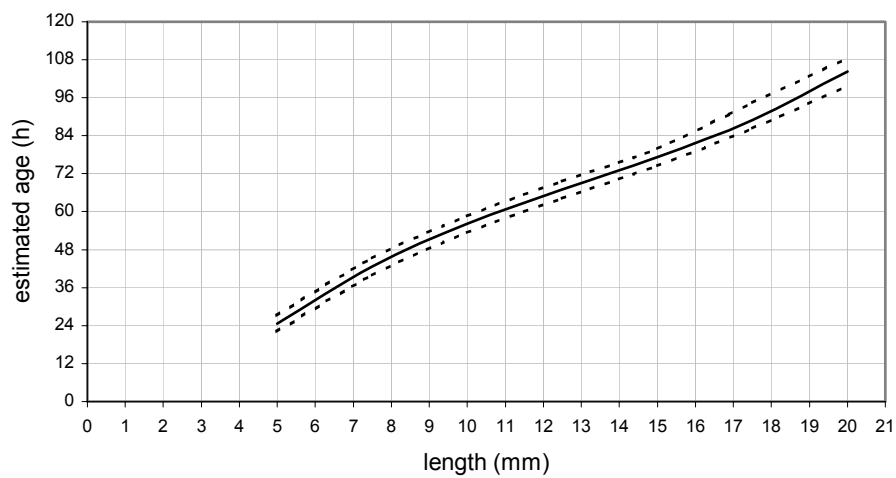
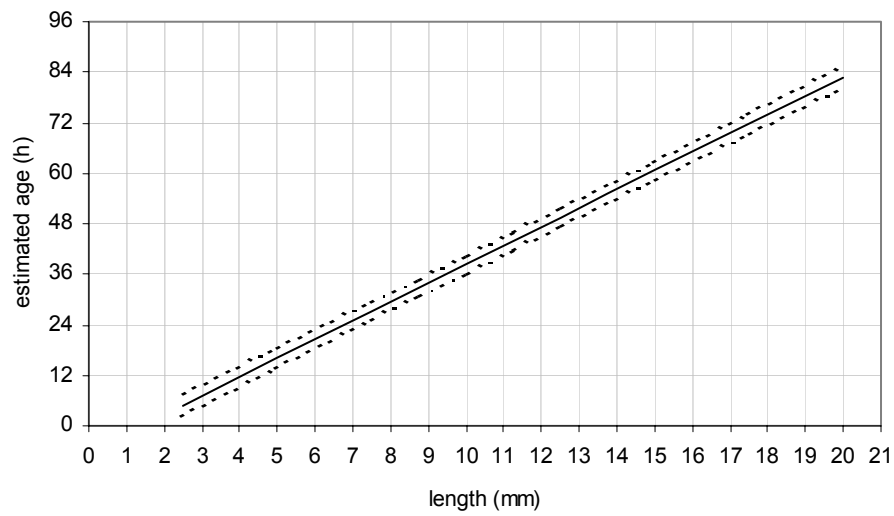
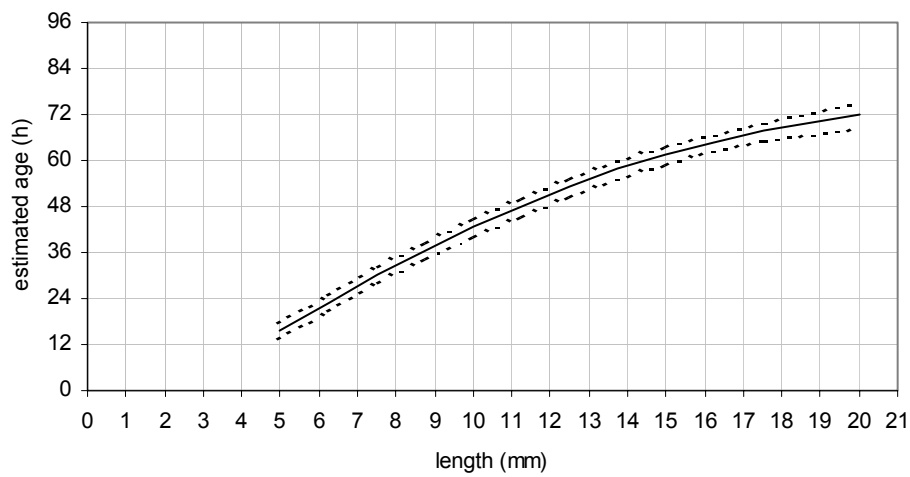


Figure 8.22: Estimation of maggot age from body length, and 95% prediction intervals for the age of feeding larvae grown at 20°C.

a) linear model



b) quadratic model



c) cubic model

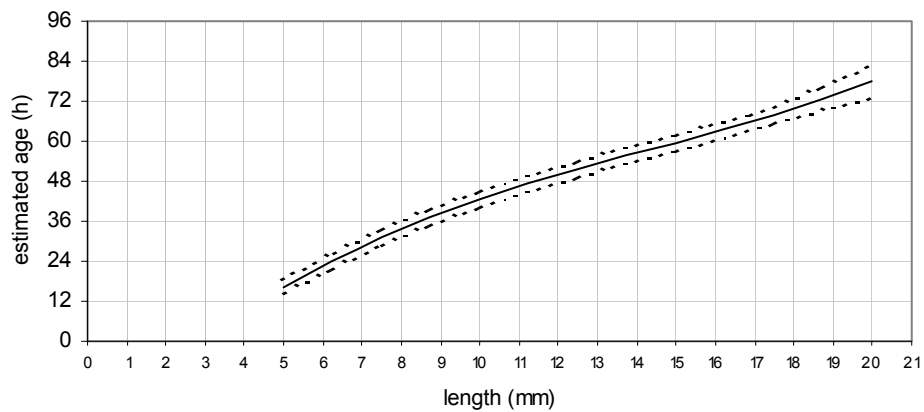
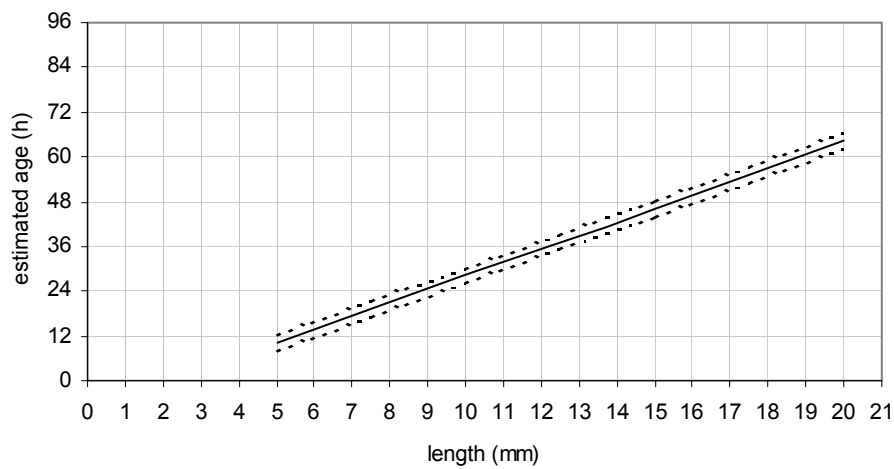
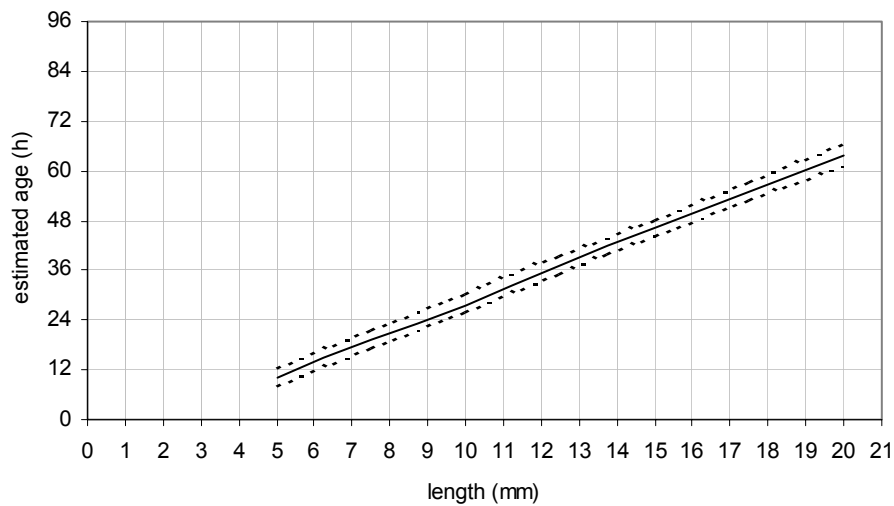


Figure 8.23: Estimation of maggot age from body length, and 95% prediction intervals for the age of feeding larvae grown at 25°C.

a) linear model



b) quadratic model



c) cubic model

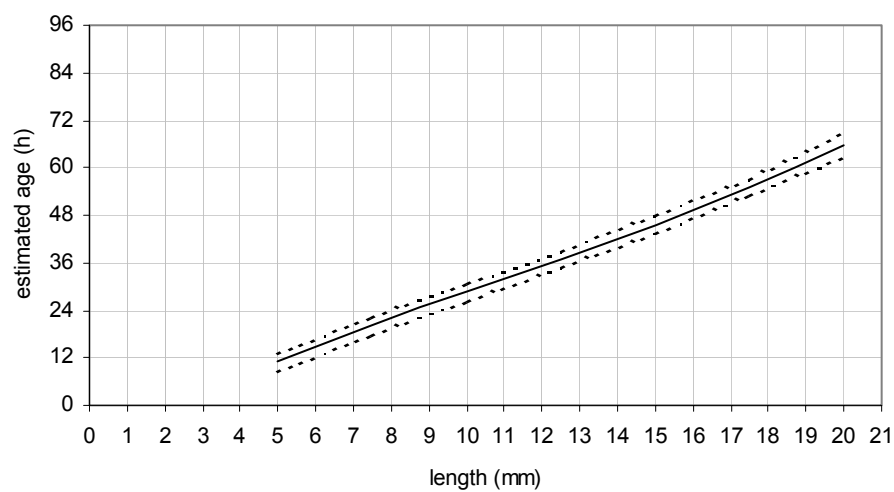
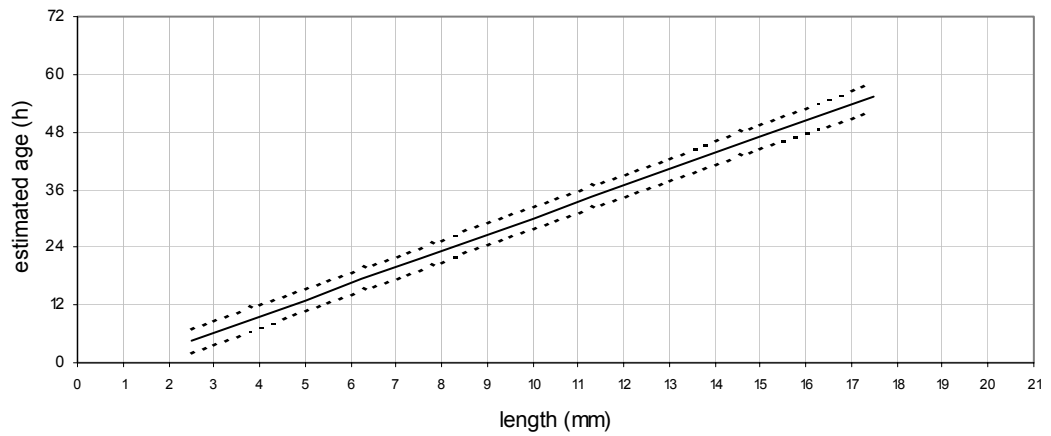
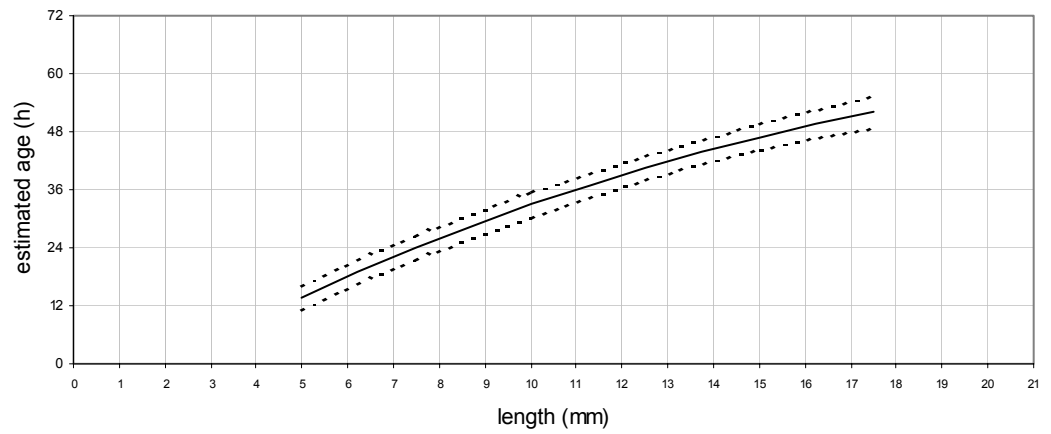


Figure 8.24: Estimation of maggot age from body length, and 95% prediction intervals for the age of feeding larvae grown at 30°C.

a) linear model



b) quadratic model



c) cubic model

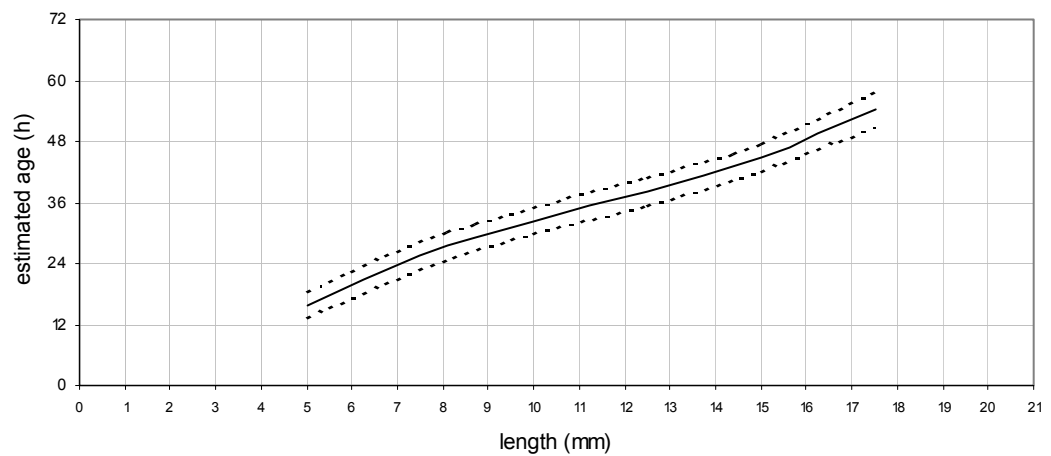


Figure 8.25: Estimation of maggot age from body length, and 95% prediction intervals for the age of feeding larvae grown at 35°C.

8.3.4 *Check of culture integrity*

Comparisons between the early (F_3) and late ($F_{10/11}$) generations (Table 8.10) showed that the first-instars were significantly different in mean length ($p < 0.0001$), as were the early second-instars (48 h, $p = 0.0259$) but not late second-instars ($p = 0.4706$). Most of the third-instars were not significantly different in mean length except those at 192 h ($p < 0.0001$). There were significant differences detected between post-feeding third-instars of the early and late generations. The pupae showed some variation, with two lots (480 and 600 h) showing no significant differences, and two other lots (432 and 528 h) showing significant differences in mean length.

8.4 Discussion

8.4.1 *Growth of Calliphora augur larvae at constant temperatures*

The decision to examine growth at constant temperatures in this study was based upon a feeling that findings from fluctuating regime studies can be largely specific to a particular location. Furthermore, I sought to explore the biology of this species more deeply than had been done previously.

Variation in larval body length appeared to decrease steadily from 15 to 30°C and then increase again dramatically at 35°C (Figures 8.1-5). The overlap observed for the transitional forms shows a steady increase with increasing temperature up to 25°C and then appears to stabilise with no great increases in overlap observed at 30 or 35°C (Figures 8.6-10). The decrease in variation probably relates to a more even growth rate at higher temperatures. The decrease at 35°C may reflect the fact that the animals are reaching their thermal limit. The variation in overlap of the growth stages, particularly the transitional forms, is puzzling and has implications for interpretation of crime scene data. These findings emphasise the importance of examining crime scene samples for instar and/or transitional forms. Whilst there has been comment on the value of transitional forms [111], few published papers include this information and some workers disregard their influence on size ranges [101]. I tend to disagree because, from my findings, a size range of up to 4 mm is possible for the one transitional type.

Shrunk forms have been included in these results but have been deliberately excluded in other studies. For example, Byrd and Butler [97] noted that their sampling method selected for the fastest growing maggots and may indicate a shorter period than could otherwise be deduced from normal collection techniques at a crime scene.

Table 8.10: Comparison of early and late generation larvae of *Calliphora augur* grown on sheep's liver at 15°C.

		Generation		
Time (h)	Growth stage	Early (F ₃)	Late (F ₁₀ /F ₁₁)	P value
		mean±standard error number		
0	First-instars	2.03±0.042 30	2.31±0.042 29	<0.0001
48	Second-instars	5.25±0.133 28	5.65±0.12 37	0.0259
96	Second-instars	8.15±0.29 15	8.56±0.46 6	0.4706
144	Feeding third-instars	13.85±0.65 25	14.89±0.64 26	0.2639
192	Feeding third-instars	14.59±0.65 21	18.83±0.65 21	<0.0001
240	Feeding third-instars	17.24±0.45 15	18.37±0.33 26	0.0515
288	Feeding third-instars	16.56±0.55 9	16.77±0.74 5	0.8180
336	Post-feeding third-instars	15.57±0.58 6	16.14±0.29 23	0.3961
384	Post-feeding third-instars	15.69±0.53 8	15.57±0.36 17	0.8604
432	Pupae	9.04±0.08 24	9.60±0.83 25	<0.0001
480	Pupae	9.49±0.15 4	9.53±0.07 20	0.7816
528	Pupae	8.84±0.12 14	9.39±0.08 27	0.0008
600	Pupae	9.47±0.07 21	9.46±0.06 28	0.9347

Wells and LaMotte [101] discussed the influence of very small and very large individuals upon variance. My inclusion of them was to demonstrate the full range that may be encountered in crime scene samples. The unusual form I observed at 30 and 35°C appears to be as yet unreported. Its presence is interpreted as an indication that the larvae are growing at or near their thermal limit. The presence of pupae in the meat trays at 15, 30 and 35°C shows a failure of larvae to migrate. It is thought that the lack of migration could relate to reduced metabolism at 15°C and to a reduced metabolism coupled with physical obstruction by the liver at the higher temperatures.

A number of other studies on the development of *C. augur* have been done [23, 25, 28, 48, 60, 64], but they were usually conducted as part of a larger project and little specific detail was given. Fuller [28] reported the average length of the maggot when full grown as 18 mm [28], and my findings concur with this. It is difficult to compare my results with those of Johnston and Tiegs [52], because they used ambient temperatures in Brisbane (which has a warmer climate than Wollongong) and reported on monthly differences. Generally though, they found larval feeding to be between 4 and 5 days, larval resting to be between 4 and 8 days, and the total duration of the larval period between 8 and 14 days. Additional, similar, studies were done by Johnston *et al.* [23] but for a longer time frame. They found the larval growth periods to be identical with Johnston and Tiegs [52], but that the prepupal stage ranged from 2 to 13 days. My results are similar to Johnston *et al.* [23] for 20 and 25°C, with larval feeding lasting between 4 days (96 h) and 5.5 days (132 h) at these temperatures, respectively. The term ‘larval resting’ used by Johnston *et al.* [23] is interpreted as the period between wandering and observation of (dark brown) pupae. Larval resting was to be between 2 and 3 days at these temperatures, which is a very much shorter period than found by them. The total larval periods in my studies at 20 and 25°C were 9 days (216 h) and 7 days (168 h). At 15°C observed a total larval period of 16 days (384 h) was observed. The extended range of the prepupal stage in the study of Johnston *et al.* [23] possibly reflects something of the seasonal variation in their locality (Brisbane). The flies in their study were grown in ambient temperatures in an insectary.

It is also difficult to compare my results with those of Mackerras [48] because he reports only on complete development, with 21-22 days reported for growth in summer conditions in an insectary and 18-20 days for development in a warm room at 20°C. Developing flies have been estimated to spend up to 40% of their preadult life as pupae [6]. If this is accepted to be applicable to all species, including *C. augur*, 40% of 18-20 days is 7.2-8

days or 172.8-192 hours. My result of 8.5 days (204 h) to dark brown pupae at 20°C therefore seems similar to the results of Mackerras [48].

Levot [60] examined weights of *C. augur* larvae at 27-28°C and found the time to maximum larval weight to be 65.5 hours. Whilst I cannot do any direct comparisons, I observed that the time to maximum larval length at 30°C was 72 h. Levot's techniques were very different to mine, with a large mass of larvae feeding on excess liver and each sampling event removing some larvae. It is possible that the larvae in his experiment were actually exposed to higher temperatures than the ambient of 27-28°C, but also that the feeding mass was better able to liquefy the substrate, and therefore grow more quickly, than those groups of larvae in my study.

Comprehensive studies by O'Flynn [24, 25] examined larval growth at temperatures between 5 and 45°C and found that at 5°C the first-instars lasted less than 3 d and the second from 14-18 d, the total of these two instars occupying between 17 and 21 d. The minimum period from larviposition to commencement of wandering was 54 d; some larvae survived for 110 d but none pupated. I did not conduct any studies at 5 or 10°C. In warm hot weather it may take only seconds for the first flies to land on a dead body placed in a wooded area [170] but this is not the case in cooler conditions, with each species having climatic preferences for activity and larvi/oviposition [171]. My results at 15°C showed second-instars still present at 4 days (96 h), which is 1.5 days longer than O'Flynn observed [25]. The duration of total larval feeding at this temperature was 11 days (264 h) which is also longer. The total larval stage I observed was approximately 14 days, 3 days more than O'Flynn. At 20°C second-instars were first observed at 1 day (24 h) and third-instars at 2.5 days (60 h) which is similar to the results of O'Flynn [25] although she observed the second-instar period to be shorter (<24 h). She observed the total duration of feeding to be between 4 and 5 days; in my study it was 5.5 days (132 h). My results for the total larval stage are similar, being 8.5 days (204 h), compared with the 7-10 days found by O'Flynn [25]. At 25°C second-instars were first observed at 24 hours and third-instars at 36 hours. These results are difficult to compare with those of O'Flynn [25], because there are data missing from her report at that temperature. She reported the total duration of feeding to be between 3 and 4 days and the total larval stage as 5-9 days. My study conforms with these results, with 4 days (96 h) observed for total feeding and 6 days (144 h) to pupation. Unlike O'Flynn [25], I did not examine 28°C, choosing instead 30°C, and she has no data for the duration of first and second-instars at this temperature. My observed 3 days (72 h) for total larval feeding at 30°C agrees with her 2.5-4 day range. My

total larval stage was shorter - 5 d (120 h) compared with 6-9 d but this could be explained by the slight difference in temperature. Although I examined 35°C rather than 34°C and, like her, found third-instars present at 24 hours, the total duration of larval feeding was 3 d (c.f. 2.5 d) and the total larval stage was 5.5 days (c.f. 4.5 days). In O'Flynn's study, the first and second-instars together occupied 24 hours at 34°C, and the total larval period lasted 4.5 days, of which the larvae fed for 2.5 days. Some larvae died in the food but most pupated, although no adults emerged. My study did not include duration of pupae so I cannot comment on this point. O'Flynn's studies [24] also included 40 and 45°C and fluctuating regimes. At 40°C she observed that larvae commenced to leave the food after 30 h, but all died without pupating. She found, however, that if larvae reared at 40°C were transferred to 25°C after ceasing to feed, they pupated and produced flies [24].

8.4.2 *Larval temperature preference*

Archer and Elgar [58] examined blowfly larvae feeding strategies in carrion and found that site preference changes with the length of time the carcass is exposed to the elements. Examination of instar size and temperature in my study (Figure 3) indicates that larvae also appear to have temperature preferences which relate to their growth stage. First-instar maggots were largest on average when grown at 25°C. Similarly, second-instars were also largest when grown at 25°C but they also displayed a much smaller variation at this temperature compared with the other temperatures examined. Due to the small number of first to second-instars observed, no comparison between temperatures is possible for this group. Second to third-instar larvae were largest on average when grown at 20°C, as were feeding third-instars. Feeding third-instars were however above the grand mean when growing at 15, 20, 25 and 30°C. The only temperature at which the average length of third-instars dropped below the grand mean was at 35°C. This drop appears to be further evidence that this is around the upper thermal limit for this species. Post-feeding third-instars were largest on average when grown at 20°C. These results are surprising and indicate that the influence of temperature on larval growth is possibly much more complex than it appears. Studies to monitor movement and temperature for large numbers of individual larvae growing in a mass would be invaluable, but could be extremely difficult to conduct without expensive equipment. *In vivo* studies on immunoglobulins have been achieved with use of fluorescent dyes [172]. Perhaps similar techniques could be utilised with maggots.

8.4.3 Comparing early and late generation larval growth

The statistically significant difference between the body lengths of new first-instar larvae of the early and late generations is thought to be due to larger females in the late generations. In culture, there is an absence of interspecific competition and the food source is not ephemeral. This is a likely explanation because Norris [49] noted the ability of cultured flies to develop eggs without a protein meal after a year or so, and I observed eggs of *Lucilia cuprina* to be laid on the bottom of a cage of mixed sex adults in the absence of oviposition media after some time in culture. These eggs, however, became dessicated and did not hatch. When the initial cultures of *C. augur* were being set up, much of New South Wales was in drought and so there were possibly some seasonal effects influencing growth and development in the flies that were caught.

Significant differences in length between generations were also detected in second-instar larvae, third-instar larvae and pupae, but not in post-feeding third-instars. This represents five comparisons of 13, or nearly 40% of the treatments. This is an area which has not received much attention to date; my results indicate that it warrants further investigation. This might ensure that data used for post-mortem interval determinations accurately reflect wild-type fly populations, and bolster the integrity of forensic entomology as a reliable science in criminal investigation.

8.4.4 Prediction intervals

All three prediction interval models generated from the 15°C data (Figure 8.21-3) are similar in their predictions. There is little predictive gain from increasing the complexity of the model. A similar pattern is seen at all other temperatures examined (Figures 8.24-35), with variation between the predicted times being small (within 4 hours or so). The quadratic and cubic models are less reliable in the lower and upper regions – there are missing data in the lower regions and a broadening range of prediction intervals in the higher regions. This was not unexpected because the extremes of the more complex models give broader estimates and involve greater measures of error.

Other workers have also explored mathematical models to describe larval development in the context of forensic entomology [20, 101]. von Zuben *et al.* [20] presented a model using larval weight as an indicator of larval age but did not indicate whether they used wet weight, or dry weight. If they used wet weight the application of their model is limited to well-collected, quickly transported, live larvae. Wells and LaMotte [101] used dry weights after preservation in Kahle's solution. They did not appear to measure wet weight first, nor

examine or control for effects on weight from their chosen preservative. They presumably chose Kahle's solution to allow examination of spiracular slits. I have found that this is easily done with a number of preservatives, including 80% EtOH. To date there appear to have been no studies at all examining the effects of different preservative types on larval weight; such studies would be very useful.

I elected to employ prediction intervals so as to be able to give a confidence of prediction. The method of calculating the intervals is such that 95% of calculated intervals will contain the true age of a maggot of a given measured body length. With forensic entomology gaining favour as an appropriate and effective tool in the investigation of time of death, some measure of confidence is needed to outline the usefulness and accuracy of any post-mortem interval determination done from crime scene samples of maggots. Forensic entomology may soon be open to the same scrutiny as other investigative tools, e.g. DNA, and forensic entomologists will need to employ the same statistical rigour that other disciplines are required to provide.

8.5 Conclusions

It is concluded that, as expected, larval growth in *C. augur* increases with increased temperature and that variation in body length decreases steadily from 15 to 30°C and then increases again dramatically at 35°C, which appears to be at or near this species' thermal limit. Transitional forms are extremely important when interpreting entomological evidence for PMI estimations, since they may cover a range of up to 4 mm in size and can be present for many hours. Unusual forms and behaviours can be indicative of the temperature regime to which maggots have been exposed and should be especially noted. I also conclude that larval temperature preference is possibly much more complex than previously thought, with certain growth stages performing better at different constant temperatures. There may be important differences between the growth of cultured individuals, subject to the age of the culture, and I suspect that this may be related to seasonal and nutritive factors. Finally, I conclude that simple linear models can estimate larval age from larval body length for the feeding stage and thus may help provide quick and convenient estimates of PMI from entomological evidence based on 95% prediction intervals.

CHAPTER 9

General Discussion

From my review of the literature and early experimental work, it became apparent that some areas of forensic entomology in Australia, and also more generally, required further investigation. It appeared that some practical problems had not been addressed but also that important validation studies had either not been done or not been published. The importance of these matters is broader than scientific validation. Consideration must be given to the general admissibility of forensic entomological evidence in court, as well as the potential for miscarriages of justice when evidence is based on poor science. Whilst detailed studies on *Calliphora augur* were the main focus of my research, more practical issues were also addressed in order to clarify matters which have been outstanding for some time, and which may have an important bearing on the validity of entomological evidence in criminal investigations. Hypotheses explored in this research were:

Hypothesis 1:

A simple alternative to body length may exist for damaged specimens, for example body width.

Hypothesis 2:

Freezing and thawing of larval substrate does not significantly affect larval growth.

Hypothesis 3:

The type of larval developmental substrate may influence larval growth.

Hypothesis 4:

Different preservative types may not have comparable effects on larval body length, either between instars of a species, or between larvae of different species.

Hypothesis 5:

Placement of live larvae into different preservative types may be detrimental to interpretation of larval evidence.

Hypothesis 6:

Prediction intervals may be possible from larval growth data of *Calliphora augur*.

Hypothesis 7:

Larvae of cultured *Calliphora augur* may be significantly different in size to those derived directly from wild-caught females.

The culmination of findings for each area of investigation is discussed briefly below.

9.1 Alternative larval measurement

The novel idea that a simple alternative to larval body length could be used on damaged specimens was explored in response to the phenomenon of head-curling. Chapter 5 outlined the main findings. Body width at the junction of the 5th and 6th abdominal segments was found to be an excellent alternative measurement. This approach required no extra equipment, applies to all larval growth stages and is extremely quick. The alternative measurement discovered was found to be appropriate for two species of local blowfly and may have broader applicability. In terms of practical application, this discovery allows consulting entomologists an alternative means of estimating post-mortem intervals based on entomological evidence, especially when damaged specimens are received. A manuscript on this finding was submitted to *Forensic Science International* and is currently in press.

9.2 Studies on developmental substrates

Chapter 6 outlined the outcomes of experiments on developmental media of larvae for two local species of blowfly.

The findings for fresh vs. frozen/thawed media not only validate my choice of technique but also validate many other published papers and serve to remove legal argument and criticism from one area of forensic entomology. The finding that growth of larvae is not significantly different on fresh media (sheep's liver) compared with frozen/thawed media authenticates the practice of using thawed media in these types of studies. In developmental studies it is often not practical to control all the variables associated with developmental media. It is therefore necessary to ensure that a suitable number of replicates are incorporated into developmental studies to randomise these effects. It is also logistically very difficult to work with fresh media on the scale required. The reassurance that my

results provide in terms of the use of frozen/thawed media not only validate many other studies, but also provide a confidence for workers approaching this type of study. My results allow workers to align their experimental setup with laying behaviour of the flies and prevent wasting time, effort and resources.

The findings for different substrate type confirm suspicions which have existed for some time and substantiate the importance of appropriate note-taking and expert, thorough collection at a crime scene or at the autopsy. A manuscript on this finding was submitted to *The Journal of Forensic Sciences* and is currently in press.

9.3 Studies on preservatives

The work presented on preservatives in Chapter 7 indicates that one preservative type may not be suitable for all blowfly species. These results were unexpected and create a need for greater investigation. That different instars of the same species showed different amounts of change in body length also raises questions on the continued use of preservatives when their specific effects are untested on the species of particular interest.

The results on the practice of placing larvae into preservatives alive clearly indicate that this practice should be strongly discouraged. The resultant effects of placement of larvae into preservatives alive, e.g. discolouration, sunkeness and head-curling, all make analysis of larvae much more difficult.

9.4 Development in *Calliphora augur*

The work on larval development in *Calliphora augur* compiled in Chapter 8 not only provides practitioners with detailed information, it also led to construction of 95% prediction intervals for larval age, something which was previously unavailable for this species. Detailed analyses indicated that larval growth is likely to be much more complex than initially understood, and that each larval instar may have a preferred temperature for maximum growth. An additional interesting comparison was between first-instar larvae from wild-caught females and those from an extended culture. The first-instar larvae from the culture were significantly larger than wild-type larvae, meaning that use of larvae cultured for several generations may not be appropriate for developmental studies where the

data will be applied to crime scene samples in which the larvae are wild-type progeny.

9.5 Conclusions

The research outcomes from these studies clearly indicate that forensic entomology is not a straightforward science. The sources of error are many and some of the published accounts could be unreliable or locale specific. I have done my best to provide good reference data and prediction guides for larvae of *Calliphora augur*, as well as to make allowance for variables such as preservative type, collection site from a body and salvage of damaged specimens. Notwithstanding this, larval development in blowflies is a complex biological process with many variables. My work has, I hope, gone some way to resolving some of the practical issues facing consulting entomologists, as well as highlight areas in need of further attention should forensic entomology become the powerful investigative tool it has the potential to be. To maintain its usefulness, and enhance its integrity and validity, forensic entomology as a discipline must become increasingly resilient to legal argument. The validation of experimental techniques used in developmental studies is certainly a vital part of developing this resilience.

BIBLIOGRAPHY

1. Tzu S (1981) *The Washing Away of Wrongs* (Original title: *Hsi yüan chi lu*). University of Michigan, (Chapter 5) (B. von McKnight, Trans.), Ann Arbor.
2. Heath ACG (1982) Beneficial aspects of blowflies (Diptera: Calliphoridae). *New Zealand Entomologist* 7:343-348.
3. Putman RJ (1978) The role of carrion-frequenting arthropods in the decay process. *Ecological entomology* 3:133-139.
4. Seastedt TR, Mameli L, Gridley K (1981) Arthropod use of invertebrate carrion. *American Midland Naturalist* 105:124-129.
5. Braack LEO (1987) Community dynamics of carrion-attendant arthropods in tropical African woodland. *Oecologia* 72:402-409.
6. Greenberg B (1991) Flies as forensic indicators. *Journal of Medical Entomology* 28:565-577.
7. Keh B (1985) Scope and applications of forensic entomology. *Annual Review of Entomology* 30:137-154.
8. Erzinclioglu YZ (1983) The application of entomology to forensic medicine. *Medicine, Science and the Law* 23:57-63.
9. Bequaert JC (1942) Some observations on the fauna of putrefaction and its potential value in establishing the time of death. *New England Journal of Medicine* 227:856.
10. Smith KGV (1986) *A manual of forensic entomology*. The Trustees of the British Museum (Natural History), London.
11. Benecke M (2001) A brief history of forensic entomology. *Forensic Science International* 120:2-14.
12. Hall RD (2001) Introduction: Perceptions and status of forensic entomology; In: J. H. B. a. J. L. Castner *Forensic Entomology. The Utility of Arthropods in Legal Investigations*. CRC Press, Boca Raton, pp 1-15.
13. Catts EP, Goff ML (1992) Forensic entomology in criminal investigations. *Annual Review of Entomology* 37:253-272.
14. Goff ML (1993) Estimation of the postmortem interval using arthropod development and succession patterns. *Forensic Science Review* 5:81-94.
15. Catts EP (1992) Problems in estimating the postmortem interval in death investigations. *Journal of Agricultural Entomology* 9:245-255.
16. Benecke M, Lessig R (2001) Child neglect and forensic entomology. *Forensic Science International* 120:155-159.
17. Anderson GS (1999) Wildlife forensic entomology: determining time of death in two illegally killed black bear cubs. *Journal of Forensic Sciences* 44:856-859.
18. Henssge C, Knight B, Krompecher T, Madea B, Nokes L (1995) *The Estimation of the Time Since Death in the Early Postmortem Period*. Edward Arnold, London.
19. Vanlaerhoven SL, Anderson G (2001) Implications of using development rates of blow fly (Diptera: Calliphoridae) eggs to determine postmortem interval. *Journal of the Entomological Society of British Columbia* 98:189-194.
20. von Zuben CJ, Bassanezi RC, von Zuben FJ (1998) Theoretical approaches to forensic entomology. II. Mathematical model of larval development. *Journal of Applied Entomology* 122:275-278.
21. Dadour IR, Cook DF, Wirth N (2001) Rate of development of *Hydrotaea rostrata* under summer and winter (cyclic and constant) temperature regimes. *Medical and Veterinary Entomology* 15:177-182.
22. O'Flynn MA (1976) *A study of blowflies breeding in sheep and in carrion*. University of Queensland, Brisbane, Queensland M. Ag. Sc.
23. Johnston TH, Walter GHH, Hall E (1923) Observations regarding the life-cycle of certain Australian blowflies. *Proceedings of the Royal Society of Queensland* 21-42.

24. O'Flynn MA (1980) *Studies on blowflies and related flies from Queensland*. University of Queensland, Brisbane, Queensland Ph. D.
25. O'Flynn MA (1983) The succession and rate of development of blowflies in carrion in southern Queensland and the application of these data to forensic entomology. *Journal of the Australian Entomological Society* 22:137-148.
26. O'Flynn MA, Moorhouse DE (1979) Species of *Chrysomya* as primary flies in carrion. *Journal of the Australian Entomological Society* 18:31-32.
27. Wallman JF (1999) *Systematics and thermobiology of carrion-breeding blowflies (Diptera: Calliphoridae)*. The University of Adelaide, Adelaide
28. Fuller ME (1932) The larvae of the Australian sheep blowflies. *Proceedings of the Linnean Society, NSW* 57:77-91.
29. Fuller ME (1934) *The Insect Inhabitants of Carrion: A Study in Animal Ecology*. Council for Scientific and Industrial Research, Melbourne
30. Archer MS (2002) *The Ecology of Invertebrate Associations with Vertebrate Carrion in Victoria, with Reference to Forensic Entomology*. University of Melbourne, Melbourne Ph D.
31. Levot G (2003) Insect fauna used to estimate the post-mortem interval of deceased persons. *General and Applied Entomology* 32:31-39.
32. Wallman JF (2001) A key to the adults of species of blowflies in southern Australia known or suspected to breed in carrion. *Medical and Veterinary Entomology* 15:433-437.
33. Kinnear JF, Martin MD, Thomson JA, Neufeld GJ (1968) Developmental changes in the late larva of *Calliphora stygia*. I. Hemolymph. *Australian Journal of Biological Sciences* 21:1033-1045.
34. Holloway BA (1985) Immature stages of New Zealand Calliphoridae; In: J. P. Dear *Fauna of New Zealand. Calliphoridae (Insecta: Diptera)*. Science Information Publishing Centre, DSIR, Wellington, pp 12-14.
35. Stranger J (1995) *Control of flies on dairy farms*. Department of Primary Industries, The State of Victoria,
<http://www.dpi.vic.gov.au/dpi/nreninf.nsf/LinkView/7DE0EB86CE9938B8CA256BCF000BBF38B494B54DA7CA01F94A256DEA002753B4>
36. Holloway BA (1991) Identification of third-instar larvae of flystrike and carrion-associated blowflies in New Zealand (Diptera: Calliphoridae). *New Zealand Entomologist* 14:24-28.
37. Wallman JF (2001) Third-instar larvae of common carrion-breeding blowflies of the genus *Calliphora* (Diptera: Calliphoridae) in South Australia. *Invertebrate Taxonomy* 15:37-51.
38. Wallman JF, Adams M (2001) The forensic application of allozyme electrophoresis to the identification of blowfly larvae (Diptera: Calliphoridae) in southern Australia. *Journal of Forensic Sciences* 46:681-684.
39. Nuorteva P, Schumann H, Isokoski M, Laiho K (1974) Studies on the possibilities of using blowflies (Dipt., Calliphoridae) as medicolegal indicators in Finland. 2. Four cases where species identification was performed from larvae. *Annales Entomologici Fennici* 40:70-74.
40. Nuorteva P, Isokoski M, Laiho K (1967) Studies on the possibilities of using blowflies (Dipt.) as medicolegal indicators in Finland. 1. Report of four indoor cases from the city of Helsinki. *Annales Entomologici Fennici* 33:217-225.
41. Meier R (2002) *Combining molecular and morphological data for Sepsidae, Coelopidae, and Diopsidae: More conflict than congruence*. The Schlinger Foundation, Brisbane
42. Stevens J (2002) *The evolution of ectoparasitism in calliphorid blowflies*. The Schlinger Foundation, Brisbane
43. Erzinclioglu YZ (1990) On the interpretation of maggot evidence in forensic cases. *Medicine, Science and the Law* 30:65-66.

44. Meier R, Kotrba M, Ferrar P (1999) Ovoviviparity and viviparity in the Diptera. *Biological Reviews* 74:199-258.
45. Tenquist JD (1998) *Brown Blowfly Life Cycle*. HortResearch - The Horticulture and Food Research Institute of New Zealand Ltd,
<http://www.hortnet.co.nz/publications/hortfacts/hf401046.htm>
46. Williams H, Richardson AMM (1984) Growth energetics in relation to temperature for larvae of four species of necrophagous flies (Diptera: Calliphoridae). *Australian Journal of Ecology* 9:141-152.
47. Kamal AS (1958) Comparative study of thirteen species of sarcosaprophagous Calliphoridae and Sarcophagidae (Diptera). I. Bionomics. *Annals of the Entomological Society of America* 51:261-271.
48. Mackerras MJ (1933) Observations on the life-histories, nutritional requirements and fecundity of blowflies. *Bulletin of Entomological Research* 24:353-362.
49. Norris KR (1959) The ecology of sheep blowflies in Australia; In: A. Keast, R. L. Crocker & C. S. Christian (eds) *Monographiae Biologicae: Biogeography and Ecology in Australia*. Junk, The Hague, pp 514-544.
50. Greenberg B, Kunich JC (2002) *Entomology and the law. Flies as forensic indicators*. Cambridge University Press, Cambridge.
51. Levot G (2002) *Common blowflies that strike sheep in NSW, Australia*. Agnote DAI-152, first edition, March 2000. NSW Agriculture,
<http://www.agric.nsw.gov.au/reader/2632>
52. Johnston TH, Tiegs OW (1922) Notes on the biology of some of the more common Queensland Muscoid flies. *Proceedings of the Royal Society of Queensland* 34:77-104.
53. Callinan APL (1980) Aspects of the ecology of *Calliphora augur* (Fabricus) (Diptera: Calliphoridae), a native Australian blowfly. *Australian Journal of Zoology* 28:679-684.
54. Lee DJ (1968) Human myiasis in Australia. *The Medical Journal of Australia* 170-172.
55. Monzu N (1977) *Coexistence of carrion breeding Calliphoridae (Diptera) in Western Australia*. University of Western Australia, Crawley Ph D.
56. Wallman JF, Adams M (1997) Molecular systematics of Australian carrion-breeding blowflies of the genus *Calliphora* (Diptera: Calliphoridae). *Aust. J. Zool.* 45:337-356.
57. Hall MJR, Wall R (1995) Myiasis of humans and domestic animals. *Advances in Parasitology* 35:257-334.
58. Archer MS, Elgar MA (2003) Female breeding-site preferences and larval feeding strategies of carion-breeding Calliphoridae and Sarcophagidae (Diptera): a quantitative analysis. *Australian Journal of Zoology* 51:165-174.
59. Bairnsdale NB (1998) *Sheep Blowflies of Victoria*. Department of Natural Resources and Environment,
[http://www.nre.vic.gov.au/web/root/domino/infseries/infsheet.nsf/50f6cee464308bc94a256512003e92fe/b9a5b616ad46083e4a256553001e017c/\\$FILE/AG0081.pdf](http://www.nre.vic.gov.au/web/root/domino/infseries/infsheet.nsf/50f6cee464308bc94a256512003e92fe/b9a5b616ad46083e4a256553001e017c/$FILE/AG0081.pdf)
60. Levot GW, Brown KR, Shipp E (1979) Larval growth of some calliphorid and sarcophagid Diptera. *Bulletin of Entomological Research* 69:469-475.
61. Cragg JB (1956) The olfactory behaviour of *Lucilia* species (Diptera) under natural conditions. *Annals of Applied Biology* 44:467-477.
62. Barton Browne L (1958) The choice of communal oviposition sites by the Australian sheep blowfly *Lucilia cuprina*. *Australian Journal of Zoology* 6:241-247.
63. Dymock JJ, Forgie SA (1993) Habitat preferences and carcase colonization by sheep blowflies in the northern North Island of New Zealand. *Medical and Veterinary Entomology* 7:155-160.

64. Mackerras MJ, Freney MR (1933) Observations on the nutrition of maggots of Australian blow-flies. *Journal of Experimental Biology* 10:237-246.
65. Levot GW, Shipp E (1983) Interference to egg and larval development of the Australian sheep blowfly by three insect growth regulators. *Entomologia experimentalis et applicata* 34:58-64.
66. Vogt WG, Woodburn TL (1980) The influence of temperature and moisture on the survival and duration of the egg stage of the Australian sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *Bulletin of Entomological Research* 70:665-671.
67. Williams H (1984) A model for the aging of fly larvae in forensic entomology. *Forensic Science International* 25:191-199.
68. Dallwitz R (1984) The influence of constant and fluctuating temperatures on development rate and survival of pupae of the Australian sheep blowfly *Lucilia cuprina*. *Entomologia experimentalis et applicata* 36:89-95.
69. Nishida K, Shinonaga S, Kano R (1986) Growth tables of larvae for the estimation of post mortem interval. *The Ochanomizu Medical Journal* 34:157-172.
70. Newman LJ, Clark J (1926) Trapping blowflies. *Journal of Agriculture, W. A.* 382-391.
71. Williams H (1984) Modification of the West Australian blowfly (Diptera: Calliphoridae) trap for population studies. *Journal of Economic Entomology* 77:806-809.
72. Cole DJW (1996) A further modification of the West Australian fly trap for blowfly studies. *New Zealand Entomologist* 19:87-90.
73. Anderson GS (2000) Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). *Journal of Forensic Sciences* 45:824-832.
74. Dymock JJ, Peters MOE, Herman TJB, Forgie SA (1991) A study of sheep blowflies at Limestone Downs sheep station in the northern Waikato, New Zealand, over two summers. *New Zealand Journal of Agricultural Research* 34:311-316.
75. Fuller ME (1934) Observations on the flies responsible for striking sheep in Western Australia. *Journal of the Council for Scientific and Industrial Research* 7:150-152.
76. Vogt WG, Havenstein DE (1974) A standardized bait trap for blowfly studies. *Journal of the Australian Entomological Society* 13:249-253.
77. Morris MC, Morrison L, Joyce MA, Rabel B (1998) Trapping sheep blowflies with lures based on bacterial cultures. *Australian Journal of Experimental Agriculture* 38:125-130.
78. Wells JD, Kurahashi H (1994) *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) development: Rate, variation and the implications for forensic entomology. *Japanese Journal of Sanitary Zoology* 45:303-309.
79. Szelagiewicz M, Michalski M, Sokol R (1995) The influence of selected disinfectants on the development of *Calliphora vomitoria*. *Medycyna Weterynaryna* 51:284-285.
80. Higley LG, Haskell NH (2001) Insect development and forensic entomology; In: J. H. Byrd and J. L. Castner *Forensic Entomology. The Utility of Arthropods in Legal Investigations*. CRC Press, Boca Raton, pp 287-302.
81. Lord WD, Burger JF (1983) Collection and preservation of forensically important entomological materials. *Journal of Forensic Sciences* 28:936-944.
82. Tantawi TI, Greenberg B (1993) The effect of killing and preservative solutions on estimates of maggot age in forensic cases. *Journal of Forensic Sciences* 38:702-707.
83. Byrd JH, Castner JLE (2001) *Forensic entomology. The utility of arthropods in legal investigations*. CRC Press, Boca Raton.

84. Green AA (1951) The control of blowflies infesting slaughterhouses. 1. Field observations on the habits of blowflies. *Annals of Applied Biology* 38:475-494.
85. Johl HK, Anderson GS (1996) Effects of refrigeration on development of the blow fly, *Calliphora vicina* (Diptera: Calliphoridae) and their relationship to time of death. *Journal of the Entomological Society of British Columbia* 93:93-98.
86. Tessmer JW, Meek CL, Wright VL (1995) Circadian patterns of oviposition by necrophilous flies (Diptera: Calliphoridae) in southern Louisiana. *Southwestern Entomologist* 20:439-445.
87. Greenberg B (1990) Nocturnal oviposition behaviour of blow flies (Diptera: Calliphoridae). *Journal of Medical Entomology* 27:807-810.
88. Singh D, Bharti M (2001) Further observations on the nocturnal oviposition behaviour of blow flies (Diptera: Calliphoridae). *Forensic Science International* 120:
89. Payne JA (1965) A summer carrion study of the baby pig *Sus scrofa* Linnaeus. *Ecology* 46:592-602.
90. Micozzi MS (1986) Experimental study of postmortem change under field conditions: effects of freezing, thawing, and mechanical injury. *Journal of Forensic Sciences* 31:953-961.
91. Cianci TJ, Sheldon JK (1990) Endothermic generation by blow fly larvae of *Phormia regina* developing in pig carcasses. *Bulletin of the Society for Vector Ecology* 15:33-40.
92. Erzinclioglu YZ (1986) Areas of research in forensic entomology. *Medicine, Science and the Law* 26:273-278.
93. Grassberger M, Reiter C (2002) Effect of temperature on development of the forensically important holarctic blow fly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae). *Forensic Science International* 128:177-182.
94. Wall R, French N, Morgan KL (1992) Effects of temperature on the development and abundance of the sheep blowfly *Lucilia sericata* (Diptera: Calliphoridae). *Bulletin of Entomological Research* 82:125-131.
95. Nuorteva P (1971) Duration of development of *Calliphora alpina* (Zett.) (Dipt., Calliphoridae) in subarctic northern Finland. *Annales Entomologici Fennici* 37:209.
96. Marchenko MI (2001) Medicolegal relevance of cadaver entomofauna for the determination of the time of death. *Forensic Science International* 120:89-109.
97. Byrd JH, Butler JF (1998) Effects of temperature on *Sarcophaga haemorrhoidalis* (Diptera: Sarcophagidae) development. *Journal of Medical Entomology* 35:694-698.
98. Byrd JH, Allen JC (2001) The development of the black blow fly, *Phormia regina* (Meigen). *Forensic Science International* 120:79-88.
99. Byrd JH, Butler JF (1996) Effects of temperature on *Cochliomyia macellaria* (Diptera: Calliphoridae) development. *Journal of Medical Entomology* 33:901-905.
100. Byrd JH, Butler JF (1997) Effects of temperature on *Chrysomya rufifacies* (Diptera: Calliphoridae) development. *Journal of Medical Entomology* 34:353-358.
101. Wells JD, LaMotte LR (1995) Estimating maggot age from weight using inverse prediction. *Journal of Forensic Sciences* 40:585-590.
102. Greenberg B, Tantawi TI (1993) Different developmental strategies of two boreal blow flies (Diptera: Calliphoridae). *Journal of Medical Entomology* 30:481-484.
103. Davies L, Ratcliffe GG (1994) Development rates of some pre-adult stages in blowflies with reference to low temperatures. *Medical and Veterinary Entomology* 8:245-254.
104. Fowler J, Cohen L (1990) *Practical Statistics for Field Biology*. John Wiley & Sons Ltd, West Sussex.

105. Adams ZJO, Hall MJR (2003) Methods used for the killing and preservation of blowfly larvae, and their effect on post-mortem larval length. *Forensic Science International* 138:50-61.
106. Hanksi I (1976) Breeding experiments with carrion flies (Diptera) in natural conditions. *Annales Entomologici Fennici* 42:113-121.
107. Grassberger M, Reiter C (2001) Effect of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen- and isomorphen-diagram. *Forensic Science International* 120:32-36.
108. Introna F, Altamura BM, Dell'Erba A, Dattoli V (1989) Time since death definition by experimental reproduction of *Lucilia sericata* cycles in growth cabinet. *Journal of Forensic Sciences* 34:478-480.
109. Sachs JS (1998) A maggot for the prosecution. *Discover* November:103-108.
110. Bornemissza GF (1957) An analysis of arthropod succession in carrion and the effect of its decomposition on the soil fauna. *Australian Journal of Zoology* 5:1-12.
111. Catts EP (1991) Analyzing entomological data; In: E. P. Catts and N. H. Haskell *Entomology and death: a procedural guide*. Joyce's Print Shop Inc, Clemson, pp 124-137.
112. Barratt BIP, Ferguson CM, Heath ACG, Logan RAS (2001) Relative abundance and seasonality of Calliphoridae and Sarcophagidae (Diptera), potential vectors of rabbit haemorrhagic disease virus (RHDV) in the South Island of New Zealand. *New Zealand Journal of Zoology* 28:417-428.
113. Hanski I, Kuusela S (1980) The structure of carrion fly communities: differences in breeding seasons. *Annales Entomologici Fennici* 17:185-190.
114. Heath ACG, Appleton C (2000) Small vertebrate carrion and its use by blowflies (Calliphoridae) causing ovine myiasis (flystrike) in New Zealand. *New Zealand Entomologist* 22:81-87.
115. MacLeod J (1949) The climatology of blowfly myiasis. II. Oviposition and daily weather indices. *Bulletin of Entomological Research* 40:179-201.
116. Wells JD, King J (2001) Incidence of precocious egg development in flies of forensic importance (Calliphoridae). *Pan-Pacific Entomologist* 77:235-239.
117. Smeeton WMI, Koelmeyer TD, Holloway BA, Singh P (1984) Insects associated with exposed human corpses in Auckland, New Zealand. *Medicine, Science and the Law* 24:167-174.
118. Goff ML (1992) Problems in estimation of postmortem interval resulting from wrapping of the corpse: a case study from Hawaii. *Journal of Agricultural Entomology* 9:237-243.
119. Leclercq M, Vaillant F (1992) Forensic entomology: an original case. *Annales de la Societe Entomologique de France* 28:3-8.
120. Byrne AL, Camann MA, Cyr TL, Catts EP, Espelie KE (1995) Forensic implications of biochemical differences among geographic populations of the black blow fly, *Phormia regina* (Meigen). *Journal of Forensic Sciences* 40:372-377.
121. Moribayashi A, Shudo C, Kurahashi H (2001) Latitudinal variation in the incidence of pupal diapause in Asian and Oceanian populations of the flesh fly, *Boettcherisca peregrina* (Diptera: Sarcophagidae). *Medical Entomology and Zoology* 52:263-268.
122. Goff ML, Omori AI, Goodbrod JR (1989) Effect of cocaine in tissues on the development rate of *Boettcherisca peregrina* (Diptera: Sarcophagidae). *Journal of Medical Entomology* 26:91-93.
123. Goff ML, Brown WR, Omori AI (1992) Preliminary observations of the effect of methamphetamine in decomposing tissues on the development rate of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae) and implications of this effect on the estimations of postmortem intervals. *Journal of Forensic Sciences* 37:867-872.

124. Bourel B, Hédouin V, Martin-Bouyer L, Bécart A, Tournel G, Deveau M, Gosset D (1999) Effects of morphine in decomposing bodies on the development of *Lucilia sericata* (Diptera: Calliphoridae). *Journal of Forensic Sciences* 44:354-358.
125. Williams H, Richardson AMM (1983) Life history responses to larval food shortages in four species of necrophagous flies (Diptera: Calliphoridae). *Australian Journal of Ecology* 8:257-263.
126. Martin MD, Kinnear JF, Thomson JA (1971) Developmental changes in the late larva of *Calliphora stygia*. IV. Uptake of plasma protein by the fat body. *Australian Journal of Biological Sciences* 24:291-299.
127. Greenberg B (1985) Forensic entomology: case studies. *Bulletin of the Entomological Society of America* 31:25-28.
128. Nishida K (1984) Experimental studies on the estimation of postmortem intervals by means of fly larvae infesting human cadavers. *Japanese Journal of Legal Medicine* 38:24-41.
129. Myskowiak J-B, Doums C (2002) Effects of refrigeration on the biometry and development of *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) and its consequences in estimating post-mortem interval in forensic investigations. *Forensic Science International* 125:254-261.
130. Upton MS, Norris KR (1980) *The collection and preservation of insects and other terrestrial arthropods*. The Australian Entomological Society Inc., Brisbane.
131. Byrd JH (2001) Laboratory rearing of forensic insects; In: J. H. Byrd and J. L. Castner *Forensic Entomology: The Utility of Arthropods in Legal Investigations*. CRC Press, Boca Raton, pp 121-142.
132. Haskell NH (1991) Procedures in the entomology laboratory; In: E. P. Catts and N. H. Haskell *Entomology and death: a procedural guide*. Joyce's Print Shop Inc, Clemson, pp 111-123.
133. Johnson MD (1975) Seasonal and microseral variations in the insect populations in carrion. *American Midland Naturalist* 93:79-90.
134. Kneidel KA (1984) Influence of carcass taxon and size on species composition of carrion-breeding Diptera. *The American Midland Naturalist* 111:57-63.
135. Kuusela S, Hanski I (1982) The structure of carrion fly communities: the size and the type of carrion. *Holarctic Ecology* 5:337-348.
136. Payne JA, King EW, Beinhart G (1968) Arthropod succession and decomposition of buried pigs. *Nature* 219:1180-1181.
137. Tullis K, Goff ML (1987) Arthropod succession in exposed carrion in a tropical rainforest on O'ahu Island, Hawai'i. *Journal of Medical Entomology* 24:332-339.
138. Wells JD, Greenberg B (1994) Resource use by an introduced and native carrion flies. *Oecologia* 99:181-187.
139. Kaneshrajah G, Turner B (2004) *Calliphora vicina* larvae grow at different rates on different body tissues. *International Journal of Legal Medicine* 118:242-244.
140. Uvarov BP (1928) Insect nutrition and metabolism. A summary of the literature. *Transactions of the Entomological Society of London Part II*:255-343.
141. Hobson RP (1931) Studies on the nutrition of blow-fly larvae. I. Structure and function of the alimentary tract. *Journal of Experimental Biology* 8:109-123.
142. Hobson RP (1932) Studies on the nutrition of blow-fly larvae. II. Role of the intestinal flora in digestion. *Journal of Experimental Biology* 9:128-138.
143. Hobson RP (1932) Studies on the nutrition of blow-fly larvae. III. The liquification of muscle. *Journal of Experimental Biology* 9:359-365.
144. Hobson RP (1932) Studies on the nutrition of blow-fly larvae. IV. The normal role of microorganisms in larval growth. *Journal of Experimental Biology* 9:366-377.
145. Holden J (2002) *USDA Nutrient Database for Standard Reference*. Nutrient Data Laboratory, United States Department of Agriculture, http://www.nal.usda.gov/fnic/foodcomp/Bulletins/ndl_info.html

146. Day DM, Wallman JF (In press.) *Width as an alternative measurement to length for postmortem interval estimations from Calliphora augur (Diptera: Calliphoridae) larvae.*
147. Constable SA (1994) A comparison of proteases produced by larvae of *Lucilia cuprina* (Wiedemann), *L. sericata* (Meigen), *Calliphora augur* (F.) and *C. stygia* (F.) (Diptera: Calliphoridae). *Journal of the Australian Entomological Society* 33:203-210.
148. Kashyap VK, Pillay VV (1989) Efficacy of entomological method in estimation of postmortem interval: a comparative analysis. *Forensic Science International* 40:245-250.
149. Turchetto M, Lafisca S, Costantini G (2001) Postmortem interval (PMI) determined by study sarcophagous biocenoses: three cases from the province of Venice (Italy). *Forensic Science International* 120:28-31.
150. Anderson G (1997) The use of insects to determine time of decapitation: a case-study from British Columbia. *Journal of Forensic Sciences* 42:947-950.
151. Benecke M (1998) Six forensic entomology cases: description and commentary. *Journal of Forensic Sciences* 43:797-805.
152. Goff ML, Lord WD (1994) Entomotoxicology. A new area for forensic investigation. *The American Journal of Forensic Medicine and Pathology* 15:51-57.
153. Anderson G (1995) The use of insects in death investigations: an analysis of forensic entomology cases in British Columbia over a five year period. *Canadian Society of Forensic Sciences Journal* 28:277-292.
154. Sukontason K, Sukontason K, Vichairat K, Piangjai S, Lertthamnontham S, Vogtsberger RC, Olson JK (2001) The first documented forensic entomology case in Thailand. *Journal of Medical Entomology* 38:746-748.
155. Hall RD, Anderson PC, Clark DP (1986) A case of human myiasis caused by *Phormia regina* (Diptera: Calliphoridae) in Missouri, USA. *Journal of Medical Entomology* 23:578-579.
156. Lord WD, Rodriguez WC (1989) Forensic entomology: the use of insects in the investigation of homicide and untimely death. *The Prosecutor* 22:41-48.
157. Lane RP (1975) An investigation into blowfly (Diptera: Calliphoridae) succession on corpses. *Journal of Natural History* 9:581-588.
158. Lee HL, Marzuki T (1993) Preliminary observation of arthropods on carrion and its application to forensic entomology in Malaysia. *Tropical Biomedicine* 10:5-8.
159. Rodriguez WC, Bass WM (1983) Insect activity and its relationship to decay rates of human cadavers in East Tennessee. *Journal of Forensic Sciences* 28:423-432.
160. Wolff M, Uribe A, Ortiz A, Duque P (2001) A preliminary study of forensic entomology in Medellin, Columbia. *Forensic Science International* 120:53-59.
161. Shean BS, Messinger L, Papworth M (1993) Observations of differential decomposition on sun v. shaded pig carrion in coastal Washington State. *Journal of Forensic Sciences* 38:938-949.
162. Wallman JF (2004) The application of entomology to criminal investigations; In: J. Horswell *The practice of crime scene investigation*. CRC Press, Boca Raton, pp 347-359.
163. Day DM, Wallman JF (2005) A comparison of frozen/thawed and fresh larval nidus in development of *Calliphora augur* (Diptera: Calliphoridae) larvae.
164. Day DM, Wallman JF (In press) *Width as an alternative measurement to length for postmortem interval estimations from Calliphora augur (Diptera: Calliphoridae) larvae.*
165. Wallman JF, Leys R, Hogendoorn K (2005) Molecular systematics of Australian carrion-breeding blowflies (Diptera: Calliphoridae) based on mitochondrial DNA. *Invertebrate Systematics* 19:1-15.

166. Erzinclioglu YZ (1990) Letter to the Editor. *Forensic Science International* 45:191-192.
167. Schander C, Halanych KM (2003) DNA, PCR and formalinized animal tissue - a short review and protocols. *Organisms Diversity and Evolution* 3:195-205.
168. Early M, Goff ML (1986) Arthropod succession patterns in exposed carrion on the island of O'Ahu, Hawaiian Islands, U.S.A. *Journal of Medical Entomology* 23:520-531.
169. Goff ML (1991) Comparison of insect species associated with decomposing remains recovered inside dwellings and outdoors on the island of Oahu, Hawaii. *Journal of Forensic Sciences* 36:748-753.
170. Mann RW, Bass WM, Meadows L (1990) Time since death and decomposition of the human body: variables and observations in case and experimental field studies. *Journal of Forensic Sciences* 35:103-111.
171. Deonier CC (1940) Carcass temperatures and their relation to winter blowfly populations and activity in the southwest. *Journal of Economic Entomology* 33:166-170.
172. Weston SA, Parish CR (1990) New fluorescent dyes for lymphocyte migration studies. *Journal of Immunological Methods* 133:87-97.