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TECHNICAL NOTE

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Influence of Substrate Tissue Type on Larval Growth in *Calliphora augur* and *Lucilia cuprina* (Diptera: Calliphoridae)*

ABSTRACT: The size of fly larvae is an important variable in the use of these insects to estimate postmortem interval. Furthermore, the nutritional intake of larvae is likely to vary subject to the part of a corpse on which they are feeding. 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, different groups of larvae of *Lucilia cuprina* (Wiedemann) and *Calliphora augur* (Fabricius) were grown on sheep's liver, meat, and brains, and their body lengths compared. Results indicated that the development of larvae fed sheep's liver was adversely affected compared with 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 postmortem 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. We therefore recommend caution in forensic analyses that interpret crime scene data using developmental studies performed with a single type of larval food substrate.

KEYWORDS: forensic science, *Calliphora augur*, *Lucilia* (*Phaenicia*) *cuprina*, postmortem interval, substrate, tissue type, larvae, development, forensic entomology

Nonhuman vertebrate tissues are inevitably used when studying development and succession in carrion breeding fly species, especially Calliphoridae (blowflies), with the aim of applying the data to postmortem interval determinations. Previous developmental studies on fly larvae have used various types of liver as the substrate, including beef (1–3), pork (4), ox (5), lamb (6), an unspecified type (7), and a mix of minced ox liver and jelly meat (8). Liver is a commonly used substrate because it is readily available, relatively inexpensive, and of uniform consistency. Other substrates used include fish (9), pet mince (a mixture of muscle and offal) (10), mammalian muscle (11), (unspecified) meat (12), lean pork (13–16), ground beef (17,18), and mouse carcasses (19). A few studies have compared larval preference for different substrates before rearing (13–16) and only one study appears to have examined the suitability of different substrates for both oviposition and rearing (20).

Uvarov (21) has reviewed insect nutrition and metabolism in general, while Hobson (22–25) conducted various studies on nutrition in blowfly larvae in particular, and Mackerras and Freney (26) observed nutrition and development in maggots of Australian blowflies. Studies on *Calliphora vomitoria* (Wollman in (21)) found that larvae developed much better on brains sterilized at 130°C than on meat sterilized at 115°C. Kozantsikov (in (7)) observed a variation between 29.1 and 75.6 days in the development of *C. 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 (27). Hence, for a large part of their development, such larvae are feeding on brain. A recent study by Kaneshrajah and Turner (28) on *C. vicina* found that an error of up to 2 days might be expected in a postmortem interval estimate using larvae grown on pig's liver compared with brain, heart, kidney, and lungs. 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 Kaneshrajah and Turner (28), reports the results of comparative larval development in two blowflies common in Australia, *Lucilia cuprina* (Wiedemann) and *C. augur* (Fabricius).

Materials and Methods

Larvae in our study were grown on sheep's liver (representative of the commonest fodder type in published developmental studies), superficial sheep's meat 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 (29). *C. 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 (30). *C. augur* is regarded as

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consecutively actively viviparous (31), 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 used by other females of the same species (32). Preferred communal laying sites have high humidity and low illuminance (33). *L. cuprina* does not appear to exhibit precocious egg development (cf *Lucilia sericata* in (34)).

Preparation of Fly Larvae

The flies used in this experiment were from established cultures originating from individuals trapped in Wollongong, NSW, Australia (34°25′S, 150°53′E).

The rearing containers for larvae were square white 2-L ice cream containers, measuring 170 mm wide along each side and 90 mm deep (external measurements). Most of the center of the lid of each container was cut away and replaced with very fine mesh, allowing ventilation but preventing escape of larvae. As larvae of *L. cuprina* were often able to squeeze between the lid and the lip of the rearing container, and thus escape, rearing containers were frequently placed in a moat of tap water during the culturing process to capture and drown escaping individuals. This approach was not necessary for the experiment itself. The bottom of each rearing container was covered with dry wheat chaff to a depth of *c*. 20 mm to provide migrating/wandering larvae with a place to pupate.

Developmental Media

Lamb's fry (liver) and lamb chops were readily obtained from butchers' shops and supermarkets. Sheeps' brains were ordered from a local abattoir. All tissues were purchased together in bulk. Furthermore, they were all fresh, being obtained either on the day of actual slaughter or the following day and were of a standard fit for human consumption. All tissues for experimental use were used on their day of purchase. Each liver was cut into suitably sized portions (*c*. 50 g lots). Half of the portions were frozen at −20°C and the other half were refrigerated at 4°C overnight. All tissues presented to female flies and used in the experiments were thawed and equilibrated to room temperature before use.

Sample Generation

Cages containing gravid females and males of each fly species were presented with a portion of sheep's liver at room temperature for 1 h to permit females to oviposit or larviposit. First-instar *C. augur* larvae were transferred to the different tissue 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 *c*. 25 h 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 of between 1 and 11 days. A total of 33 rearing containers were used for each species. The day and substrate type to which a set of larvae was allocated was determined by lottery to randomize 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 ± 3.5°C (range 24.0–28.5°C) and ambient humidity. All rearing containers were left undisturbed until the allocated time had elapsed.

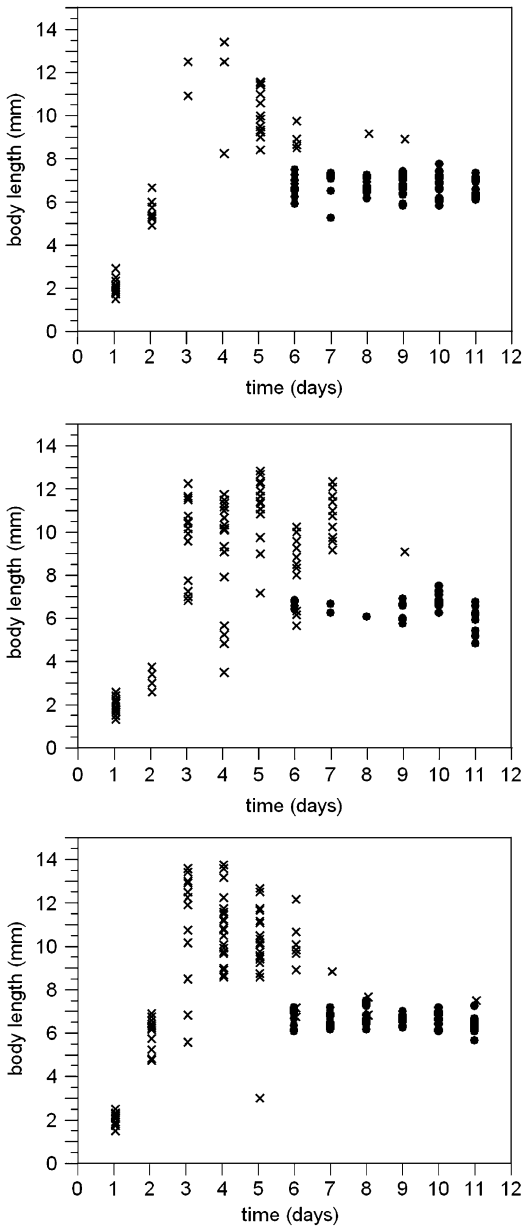

FIG. 1—*Growth of* Lucilia cuprina *larvae on different sheep tissues—raw data shown.*

Sample Collection, Handling, and Preservation

The time spent on searching for larvae in each sample was limited to 15 min so as to standardize the age of the larvae. It was felt that, as searching until all larvae had been recovered could take up to 40 min, especially in brain, that extended searching might introduce unwanted variability in larval size. Collected larvae were killed immediately by immersion in boiling water, dried with paper towel and preserved in 80% ethanol (EtOH). All larvae were placed into vials posteriad. Body length, growth stage (i.e., pre- or postfeeding) and/or instar were recorded. Where larval growth stages differed behaviorally (i.e., feeding third instars and wandering third instars) the different types observed were

TABLE 1—*Mean body length (mm) ± SD of larval instars and growth stages, by day, of* Lucilia cuprina *grown on different sheep tissues.*

	Life Stage								
Day	1st	1st–2nd	2nd	2nd–3rd	3rd	WP	LBP	BP	*p*
1									
Meat	1.95 ± 0.30	–	–	–	–	–	–	–	0.6944
Brain	1.97 ± 0.23[†1]	–	–	–	–	–	–	–	
Liver	1.88 ± 0.33	–	–	–	–	–	–	–	
2									
Meat	–	–	6.02 ± 0.69	–	–	–	–	–	0.1620
Brain	–	–	5.59 ± 0.57	–	–	–	–	–	
Liver	2.75 ± 0.28[§]	3.35 ± 0[§]	3.71 ± 0.02[§]	–	–	–	–	–	
3									
Meat	–	–	–	5.53 ± 0[§]	11.53 ± 2.11	–	–	–	0.0310
Brain	–	–	–	–	11.69 ± 1.09[§]	–	–	–	
Liver	–	–	–	–	9.75 ± 1.84	–	–	–	
4									
Meat	–	–	8.62 ± 0[§]	–	10.93 ± 1.35	–	–	–	0.0204
Brain	–	–	–	–	11.36 ± 2.73[§]	–	–	–	
Liver	–	–	5.60 ± 0[§]	–	8.92 ± 2.70	–	–	–	
5									
Meat	–	–	–	–	10.22 ± 1.27[†1]	–	–	–	0.0009
Brain	–	–	–	–	10.04 ± 1.00	–	–	–	
Liver	–	–	–	–	11.58 ± 1.11[†1]	–	–	–	
6									
Meat	–	–	–	–	9.36 ± 1.77	–	–	6.68 ± 0.40	0.2944
Brain	–	–	–	–	8.94 ± 0.55	–	–	6.81 ± 0.34[†1]	
Liver	–	–	–	–	8.22 ± 1.58	–	–	6.7 ± 0.21	0.4710[‖]
7									
Meat	–	–	–	–	8.83 ± 0[§]	–	–	6.65 ± 0.30	0.4470[‖]
Brain	–	–	–	–	–	–	–	6.90 ± 0.75	
Liver	–	–	–	–	10.76 ± 1.09[§]	–	–	6.53 ± 0.26	
8									
Meat	–	–	–	–	7.21 ± 0.63[§]	–	–	6.86 ± 0.39	0.4096[‖]
Brain	–	–	–	–	9.14 ± 0[§]	–	–	6.76 ± 0.29	
Liver	–	–	–	–	–	–	–	6.09 ± 0[§]	
9									
Meat	–	–	–	–	–	–	–	6.65 ± 0.22	0.0097[‖]
Brain	–	–	–	–	8.85 ± 0[§]	–	–	6.78 ± 0.41	
Liver	–	–	–	–	9.08 ± 0[§]	–	–	6.26 ± 0.48	
10									
Meat	–	–	–	–	–	–	–	6.72 ± 0.37	0.5150[‖]
Brain	–	–	–	–	–	–	–	6.80 ± 0.57	
Liver	–	–	–	–	–	–	–	6.92 ± 0.40	
11									
Meat	–	–	–	–	–	–	–	6.45 ± 0.36	0.0023[‖]
Brain	–	–	–	–	–	–	–	6.73 ± 0.40	
Liver	–	–	–	–	–	–	–	5.99 ± 0.68	

Where SD = 0, $n = 1$.
[†]Outliers removed,
[‡]Number removed,
[§]Excluded from analysis,
[‖]Pupae compared.
WP, white pupae; LBP, light brown pupae; BP, brown pupae.

preserved in separate vials. Pupae were killed by direct immersion in 80% EtOH.

Measurement, Data Handling, and Statistics

The length of the larvae and pupae was measured with the aid of a dissecting microscope and Mitutoyo Absolute digimatic digital calipers (Mitutoyo, Kawasaki, Japan). Larval body length was measured, viewed laterally, as the distance between the most distal parts of the head and the last abdominal segment. The ambient temperature within the temperature-controlled room was monitored with small data loggers (iButtons). Data entry and analysis were performed using JMP® (SAS Institute Inc., Cary, CN).

Results

Growth of L. cuprina *Larvae on Different Sheep Tissues*

The average sample sizes of larvae recovered from the different tissues were 13 for brain (an average of 65% of the total), 11 for liver (56%) and 17 for meat (85%). The fact that the number of larvae recovered was almost always lower than the number initially placed in each rearing container is not an indication of mortality, but rather the difficulty of recovering the larvae from the tissue (see Methods). 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 Fig. 1. The final data set with outliers removed is summarized in Table 1.

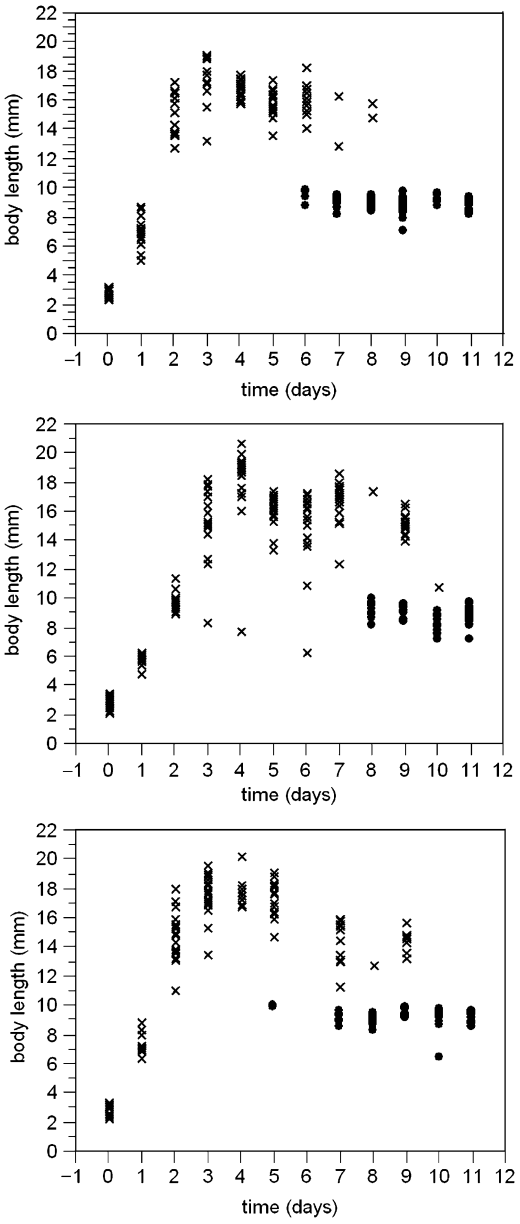

FIG. 2—*Growth of* Calliphora augur *larvae on different sheep tisses—raw data shown.*

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 wheat 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.

Significant differences were not detected between the groups on day 1, the day of egg hatching. The nonsignificant 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 1). 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 color 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 to reduce their length 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 constitutes the 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, the resultant pupae may nonetheless achieve a similar size to those originating on meat or brain.

Growth of C. augur *Larvae on Different Sheep Tissues*

The average sample sizes of larvae recovered from the different tissues were 17 for brain (an average of 86% of the total), 17 for liver (84%) and 18 for meat (89%). Our earlier remark, that recovery numbers should not be interpreted as an indicator of larval mortality, is also applicable here. 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 Fig. 2. The final data set, with outliers removed, is summarized in Table 2.

Transitional forms, first to second and second to third instars, were observed on liver but not on the groups of larvae 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. In the group grown on brain not all individuals migrated on any 1 day; pupae were sometimes observed in the drying meat as well as in the wheat chaff. Three days were recorded on which all larvae had migrated from meat, and 2 days were recorded on which all larvae had migrated from liver.

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 but it was not possible to statistically compare this single 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 nonsignificant difference for day 2 indicates that, at that time, the group grown on meat was not significantly different to the group grown on brain. 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 in the chaff and

TABLE 2—*Mean body length (mm) ± SD of larval instars and growth stages, by day, of* Calliphora augur grown on different sheep tissues.

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

Where SD = 0, $n = 1$.
[†]Outliers removed,
[‡]Number removed,
[§]Excluded from analysis,
[‖]Pupae compared.
WP, white pupae; LBP, light brown pupae; BP, brown pupae; ND, no data.

most of the larvae from the brain indicates that there were probably 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 another occasion. The nonsignificant difference for measurement of pupae at day 8 suggests that while larvae of *C. augur* grown on liver seem to lag developmentally, pupae can eventually achieve a similar size to those originating on other tissues.

Discussion

The developmental lag in larvae grown on liver, compared with meat and brain, was seen in both fly species examined, and largely reflects the results of Kaneshrajah and Turner (28). We only observed significantly smaller pupae from larvae grown on brain once for each species, whereas Kaneshrajah and Turner (28) observed that larvae grown on brain and heart showed a marked wet weight loss during the postfeeding stage, leading to pupae of reduced weight and size. We did, however, observe

significantly smaller pupae from larvae grown on liver 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 (24). Hobson (23) found that tryptase is present in *Lucilia* larvae reared aseptically on sterilized brains 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 (24). Mackerras and Freney (26) 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 *Chrysomya rufifacies*, sometimes strongly so. A study by Constable (35), which included both *C. augur* and *L. cuprina*, found evidence to suggest that proteolytic enzymes have an important role in larval survival.

It therefore seems that the main factors contributing to the observed differences in growth rate are tissue structure, activity of proteolyic enzymes and the amount of soluble protein, but other nutrients may also play a role. Using United States Department of Agriculture data on food composition (29), a comparison of nutrients in the tissues examined by us 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 (21)) showed that a larva is able to build fat from proteins and accumulate it in the fat bodies, to be freely spent during metamorphosis, whereas a pupa builds no further fat. Generally, fatty substances are used and stored for energy. It follows that larvae consuming more fat will be expending less energy on metabolism than those feeding on substances which are purely proteinaceous. These larvae can thus, in principle, direct more energy into growth.

The liver acts as a filter and accumulates and metabolizes 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.

In conclusion, larvae of both blowfly species grown on sheep's liver were often smaller than those grown on brains and meat. They also molted 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 when estimating postmortem interval. Our results suggest that there may be limitations in the forensic application of such data if they derive from a type of animal tissue other than that on which larvae at a death scene have been feeding. We therefore repeat the advice of Kaneshrajah and Turner (28) 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.

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