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TECHNICAL NOTE

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A comparison of frozen/thawed and fresh food substrates in development of *Calliphora augur* (Diptera: Calliphoridae) larvae

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Abstract 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 paper reports on the results of such a study using larvae of *Calliphora augur* (Fabricius) on sheep's liver. Approximately 20 first-instar larvae were collected on sheep liver and subsequently transferred to paired treatments of fresh and frozen/thawed liver equilibrated to room temperature. They were then left undisturbed for 1–10 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. We conclude that freezing and thawing of a developmental medium of sheep's liver has no significant effect on growth of *C. augur* larvae.

Keywords Forensic entomology · *Calliphora augur* · Larvae · Development media · Freezing · Postmortem interval

Introduction

While Catts [1] 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 [2–12]. To date, the only study examining the effects of freezing and thawing on the growth of larvae feeding on carrion has been that of Micozzi [13], who found differences in the interior of 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 freshly killed animals than in frozen/thawed ones but the sequence is the same and that freezing/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.

The use of frozen and thawed tissues, rather than whole carcasses, for studies on oviposition and development in carrion flies is also quite common [14, 15], but there have been no published studies comparing larval growth on fresh vs frozen/thawed tissues. If freezing a developmental substrate weakens connective tissues and facilitates aerobic decay, it may be the case that larvae feeding on such tissues may have an accelerated growth rate. We present results from an experiment examining the effect of freezing and thawing the developmental medium on development in the blow fly, *Calliphora augur* (Fabricus). Our objective was to assist with standardisation and quality assessment of techniques [16].

Materials and methods

C. augur was selected because it is relatively abundant and, locally in New South Wales, the most commonly collected member of the genus *Calliphora* from bodies of deceased persons, especially those located inside buildings [17]. A laboratory culture of *C. augur* was established from individuals trapped in the Woonona area of Wollongong, New South Wales (34°20′ S, 150°54′ E) and maintained with mixed ages of females and males.

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First-instar larvae were collected on sheep's liver over a 1-h period. We then allocated approximately 20 new larvae to paired samples of approximately 50-g pieces of fresh and frozen/thawed sheep's liver. During the preparation of each whole liver, before 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 before use.

Paired samples and their positions in the laboratory were chosen by lottery. After sample allocation, the larvae were each allowed to develop undisturbed for 1–10 days until their allocated time had elapsed. Developing larvae were kept on shelving in an Axyos (Brisbane, Australia) 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 [18, 19]. Larvae were placed into vials posteriad to prevent head curling [20]. 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 10 days in preservative, the length of the larvae and pupae was measured with the aid of a dissecting microscope and Mitutoyo (Kawasaki, Japan) absolute digimatic digital callipers (accuracy ± 0.02 mm, resolution 0.01 mm). 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, accuracy ±1.0°C, resolution 0.5°C). Data entry and analysis were done using JMP (SAS Institute, Cary, NC, USA) and Excel (Microsoft Corporation, Redmond, WA, USA).

Results and discussion

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 Fig. 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 at 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.

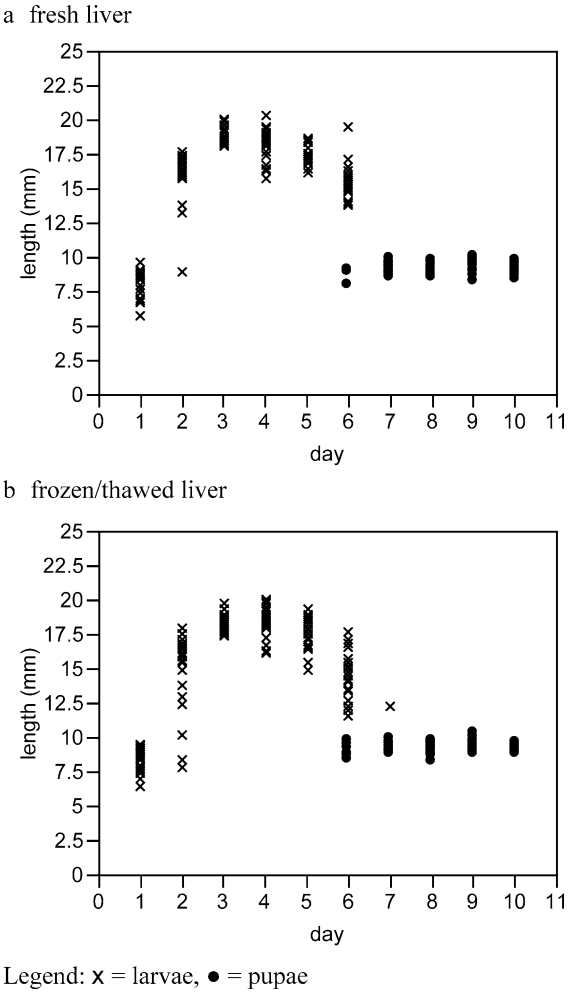

Fig. 1 Growth of *C. augur* larvae (length in millimetre) over time on **a** fresh sheep's liver and **b** frozen/thawed sheep's liver. x, larvae; ●, pupae

Both groups of larvae moulted from first instar to second instar within 24 h and from second instar to third instar within 48 h. 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. 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 [21]. Pupae were only observed on days 8, 9 and 10. Descriptive data for each group are shown in Table 1.

Table 1 Summary growth data of *C. augur* larvae on fresh vs frozen/thawed sheep liver (mean body lengths over time)

Time (days)	0	1	2	3	4	5	6	7	8	9	10
Fresh											
Life stage	First	Second	Third	Third	Third	Third, M^{9a}	Third/P^{8a}	Third/P^{1a}	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
SD		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	First	Second	Third	Third	Third, C^{2a}	Third, M^{1a}	Third/P^{3a}	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
SD		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

P Mature (dark brown) pupae, *C* number migrated to chaff (as superscript), *M* number still on meat (as superscript)
[a]Excluded from analysis

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. *C. augur* is regarded as consecutively, facultatively viviparous [22] and does not lay eggs. The experiment was not repeated due to the low expected cohort size of 50 maggots [21] 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 [13] 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 our study, which used a single type of tissue butchered and tested for human consumption, i.e. with most of the surrounding connective tissue removed.

Conclusion

We conclude 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 our conclusions have the wider applicability they suggest. For now, workers in forensic entomology may have improved confidence that a frozen food substrate probably does not significantly affect development of fly larvae.

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