Original research article

Effect of the killing method on post-mortem change in length of larvae of *Thanatophilus micans* (Fabricius, 1794) (Coleoptera: Silphidae) stored in 70% ethanol

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Abstract

It is recommended that insect larvae collected for forensic purposes should be killed using the same method as was used to create existing models for rate of development. Certain killing methods have been shown to be preferable because they cause less distortion of the specimens, but these are not always practicable in a particular case, and so a method of correcting for effect of killing method is required. Larvae of all instars of *Thanatophilus micans* (Fabricius, 1794) (Coleoptera: Silphidae) were measured and then killed by immersion in ethanol, immersion in hot water or freezing. Samples were re-measured immediately after death, then stored in excess 70% ethanol and re-measured after 1 week and again after 4 weeks. The change in length was significantly different from zero in all samples ($t = -9.07022$, $p < 0.001$). An ANCOVA showed that instar, killing method and storage time all had a significant effect on the change in length. The results showed that *T. micans* larvae have a great potential for change in length during storage, but that the change is not predictable, as the magnitude and sign of the change are variable.

Keywords

Forensic entomology, *Thanatophilus micans*, Silphidae, Killing method, Storage time

Introduction

Forensic entomology is a sufficiently new field that its techniques are still being innovated and refined to make them more accurate and precise. The age of insects on a corpse may be used to estimate the minimum post mortem interval (PMI) of the corpse. While it is preferable to use developmental landmarks to estimate (physiological) age (Dadour *et al*. 2001; Richards *et al*. 2008), this is not always possible. Insect larvae are often collected by non-experts on site or during autopsy (Lord and Burger 1983). This results in dead, preserved samples being delivered to forensic entomologists, preventing the larvae from being reared to the next landmark. As beetle larvae have a longer development period than most flies, accurate predictions of physiological age based on developmental events are not possible from dead samples, resulting in length being used to estimate age in these cases.

Body length and mass can also be used to estimate the age of dead samples, but both are affected by the way in which the insects are killed and preserved as evidence. Mass is particularly subject to change during storage, as lipids can be removed by the storage solution and water content in the samples can change. These two processes can affect the mass of the sample, resulting in flawed data. Changes in a variety of parameters have been recorded in preserved samples, including plankton volume (Ahlstrom and Thrailkill 1962), and length in krill (Miller 1983), fish (Morrison 2004) and insects (Tantawi and Greenberg 1993; Adams and Hall 2003; Amendt *et al.* 2007). Certain killing methods are preferable because they result in less change in length than others (Lord and Burger 1983; Adams and Hall 2003; Amendt *et al.* 2007). To ameliorate this problem, it has recently been correctly recommended that

insect samples collected from crime scenes should be killed by the same method as was used in the construction of the model that the age of the insect larvae will be estimated from (Adams and Hall 2003; Amendt *et al.* 2007). Several authors (Tantawi and Greenberg 1993; Adams and Hall 2003; Amendt *et al.* 2007) have noted that larvae killed in ethanol tend to change length more than those killed by other methods, but these studies refer to fly larvae (maggots), and no data are available on the change of length of beetle larvae. Beetle larvae are generally more sclerotised than maggots, which should mean that any change in length would be different from published dipteran patterns.

Fly larvae are considered to be the most valuable indicators in forensic entomology as they colonise corpses soon after death and can therefore provide an accurate estimate of the PMI. *Thanatophilus micans* adults have been observed on animal carcasses within 24h of death (pers obs), and larvae within 4 days, which coincides with the egg development period (Midgley and Villet submitted). The total development time in *T. micans* is longer than in most flies, which make *T. micans* larvae an important forensic indicator once fly larvae are no longer present on a corpse.

If different killing methods have different but predictable effects on the apparent age of larvae, corrections for the effects can be developed, and then any estimation model would be applicable regardless of the killing method used in a particular investigation. This study thus addresses two aims: to quantify the effects of three popular killing methods on beetle larvae, and to examine the feasibility of developing correction methods for any effects that are found.

Materials and Methods

Thirty larvae of each instar (1^{st}, 2^{nd} and 3^{rd}) of *Thanatophilus micans* (Fabricius, 1794) were collected from a culture maintained at the Department of Zoology and Entomology at Rhodes University. All of the larvae were measured to the nearest 0.1mm using a measurement triangle (Villet 2007). Ten larvae of each instar (Richards and Villet 2008) were killed by immersing them in water at 90˚C for 1min (Amendt *et al.* 2007), freezing at -20˚C for 1h (Amendt *et al.* 2007), or immersing them in 70% ethanol for 1min. The larvae were then immediately remeasured and placed in excess 70% ethanol for storage. The stored larvae were remeasured after 1 and 4 weeks. These times were chosen to mimic typical laboratory analyses, where samples would be measured between 1 and 4 weeks after collection.

The difference between the live length and the three other measurements was then calculated, and converted to a percentage of live length. The arcsine-transformed data were analysed using a one-sample t-test and ANCOVA (with live length as the covariate to control for relative error) in Statistica 7.

Results

Larvae killed in hot water tended to curl tightly, making them difficult to measure, as straightening was required. The bodies in these animals were stiff, in strong contrast to the freeze-killed individuals, whose bodies remained loose and supple. The bodies of the ethanol-killed individuals were of intermediate stiffness. In addition to this, neither the ethanol-killed nor freeze-killed individuals curled.

Plots of the data show more variable change in larger animals, both in terms of total change and proportional change (Fig. 1). There was also a general tendency for smaller and larger individuals to decrease in size more than individuals of intermediate length. While the data for storage time showed a high level of overlap, particularly between 1 and 4 weeks, it is apparent that there was an increase in length as storage time increased.

The ANCOVA results showed significant differences in the change in length for instar, killing method and storage time (Table 1, Fig. 2). First instar larvae showed the most change relative to initial length, while the third instar showed the least. Larvae killed in hot water showed the least change relative to initial length, and those killed by freezing the most, while ethanol-killed larvae showed highly variable changes. The samples shrank initially, but generally extended as storage time passed, with the exception of the ethanol-killed larvae after 4 weeks.

In addition, significant interactions were present between instar and storage time, and between killing method and storage time (Table 1). The interaction between instar and storage time is due to extension of second and third instar larvae over time, while first instar larvae remained the same length. (Newman-Keuls Test, Table 2). The interaction between killing method and storage time is due to strong initial shrinkage in freeze-killed individuals, where other killing methods did not show this pattern as strongly. In addition to this, the extension of ethanol-killed and freeze-killed individuals over time resulted in the 1 week and 4 week specimens from both treatments being different from ethanol-killed individuals after zero weeks and the

ethanol-killed individuals after four weeks being different from all hot-water-killed individuals, which did not change over time (Newman-Keuls Test, Table 3).

Discussion

The results obtained show that length of beetle larvae has a great potential for change after killing, as it does in flies (Tantawi and Greenberg 1993; Adams and Hall 2003). This change cannot be accurately predicted because the magnitude and even the sign of the changes observed differed greatly (Figs 1, 2), an effect also reported in flies by Adams and Hall (2003). The wide range of observed changes shows that the length of preserved larvae cannot be considered to be an accurate indicator of their live length, and is therefore not a reliable indication of larval age. That larvae of intermediate length change less than those of more extreme lengths should also be noted, because this shows a non-linear relationship between initial length and change in length. Corrections based on a linear relationship would result in underestimations of age for small and large individuals. A quadratic or polynomial function would produce a better fit, but could not compensate for the general effect of unpredictable shrinkage or extension, and having no theoretical basis, it is an arbitrary choice.

It has been reported that the killing method that results in the lowest change in length of maggots is immersion in hot water (above 80˚C) (Tantawi and Greenberg 1993; Adams and Hall, 2003). This study showed that this is not necessarily the case for beetle larvae. While the larvae changed very little over time after being killed in hot water, there was still an initial change from live length to the length immediately after death. Only the third instar larvae showed a mean length after being killed that was similar to the initial mean, but there was a high degree of variation around this mean.

This post-killing stability in length is probably due to the muscles being fixed by the heat, or in effect being cooked (Haskell, 1990).

Freezing has been proposed as an alternative to immersion in hot water (Amendt *et al.*, 2007). This study shows that freezing has a strong initial effect on larval length, which lessens over time. Animals that are frozen should therefore be measured after at least one week of storage. The initial shrinkage in frozen animals is probably a behavioural response to growing cold, as the larvae do not die immediately. While hot water and ethanol are relatively quick killing methods, requiring at most 1min in *T. micans*, freezing requires up to 1h. In this time the larvae appear to contract, decreasing their surface area to volume ratio, which improves heat retention. Behavioural thermoregulation has previously been reported in many insects.

Ethanol-killed individuals show the most erratic changes, but the mean change of these individuals was less than that under the other two methods. If large samples of individuals are taken, then a mean length calculated after ethanol-killing is the best estimate of live length in silphid larvae, in contrast to what has been found regarding maggots (Tantawi and Greenberg 1993; Adams and Hall 2003). Amendt *et al.* (2007) made a general recommendation about all insect larvae, but this study shows that beetle larvae should be treated differently to fly larvae. This is probably because, while the entire integument of maggots is relatively unsclerotized and deformable, only the intersegmental membranes of silphid larvae are deformable to any significant degree, and they constitute less than a quarter of the overall length of the body.

The relative change at each instar shows a pattern, with first instar larvae shrinking the most, then second instar larvae shrinking less and third instar larvae showing a slight extension. Tantawi and Greenberg (1993) reported that maggots of the same length but different ages underwent different amounts of shrinkage, but these comparisons were made relative to lengths after death, not live length. It is likely that the pattern found in this study is also due to age difference, accounting for some of the variation within each group, as well as the difference between instars. The effect of age may be mediated through the accumulation of lipids as the larvae mature, since these would be dissolved and leached by ethanol over time, causing differential shrinkage related to relative lipid content and therefore to age.

Length is a widely used surrogate for age, despite the limitations of this method, particularly in young specimens (Dadour *et al.* 2001; Gaudry *et al.* 2001; Richards *et al*. 2008). Small specimens could be young or stunted by competition or low temperatures (Richards *et al*., 2008; Midgley and Villet, submitted), but length measurements cannot take this into account (Gaudry *et al.* 2001). Even when these effects are absent, there is natural variation in body length at any given age (Dadour *et al*. 2001; Midgley and Villet submitted), resulting in sampling error in length measurement, and larvae of the same species moult at different lengths, meaning that reaching a given length does not trigger moulting, and is therefore not a direct surrogate for age. However, with dead samples, it is perhaps the best indicator available.

Due to the changes observed in all killing methods, it is recommended that beetle larvae be measured while still alive, whenever possible. The range in magnitude and

sign of length change makes it impossible to employ corrections, as confidence in corrected data would be low, even for large samples. It is recommended that instead of using corrections, samples of 25-30 of the largest larvae are measured and a 'window of variation' analogous to the PMI window described by Catts (1992) be calculated, taking into account the instars and killing and preservation methods. The empirical results presented here (Fig. 1) indicate that this window is about 25-30% of live length, depending on which statistical method is used to summarise the variation , and that it is often asymmetrical relative to live length. The derived estimates of a PMI window can be expected to follow a similar pattern, depending on the sample size.

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Legends to Figures

Figure 1: A diagram of the length change of *Thanatophilus micans* larvae, (a), (b) and (c) show the change in millimetres, while (d), (e) and (f) show the change as a proportion of live length. Freeze-killed larvae are shown in figures (a) and (d), ethanol -killed larvae in figures (b) and (e) and hot-water -killed larvae in figures (c) and (f). Measurements after 0 weeks are indicated by (●), 1 week by (■) and 4 weeks by (◊).

Figure 2: The mean change (± 95% confidences interval) in *Thanatophilus micans* larval length (proportion of live length), showing significant changes for (i) killing method, (ii) storage time and (iii) larval instar.

Table 1: ANCOVA results for the change over time in length of *T. micans* larvae of different instars following killing by various methods. Bold values are statistically significant ($\alpha = 0.05$).

	SS	d.f.	MS	F	p
Intercept	0.106	1	0.106	**19.469**	**0.000**
Live Length	0.185	1	0.185	**33.900**	**0.000**
Instar	0.333	2	0.166	**30.543**	**0.000**
Killing Method	0.078	2	0.039	**7.195**	**0.001**
Time	0.246	2	0.123	**22.584**	**0.000**
Instar * Killing Method	0.050	4	0.013	2.299	0.060
Instar * Time	0.080	4	0.020	**3.688**	**0.006**
Killing Method * Time	0.242	4	0.061	**11.107**	**0.000**
Instar * Killing Method * Time	0.044	8	0.005	1.003	0.435
Error	1.319	242	0.005		

Table 2: Newman-Keuls tests for the interaction between storage time and instar, after an ANCOVA of change in length over time in *Thanatophilus micans* larvae showed significant differences occurred. Bold values indicate significant differences.

Treatment	1st *0 Week	1st *1 Week	1st *4 Weeks	2nd *0 Week	2nd *1 Week	2nd *4 Weeks	3rd *0 Week	3rd *1 Week
1st *1 Week	0.882							
1st *4 Weeks	0.351	0.328						
2nd *0 Week	0.949	0.872	0.277					
2nd *1 Week	**0.000**	**0.000**	**0.004**	**0.000**				
2nd *4 Weeks	**0.000**	**0.000**	**0.011**	**0.000**	0.627			
3rd *0 Week	0.591	0.509	0.560	0.343	0.001	0.003		
3rd *1 Week	**0.001**	**0.002**	**0.038**	**0.002**	0.403	0.424	0.021	
3rd *4 Weeks	**0.000**	**0.000**	**0.000**	**0.000**	0.221	0.201	0.000	0.058

Table 3: Newman-Keuls results for the interaction between killing method and storage time, after an ANCOVA of length change over time in *Thanatophilus micans* larvae showed significant differences occurred. Bold values indicate significant differences. (HWK: hot water killed, Etoh: Ethanol killed, Cold: Frozen)

Treatment	Cold*0 Week	Cold*1 Week	Cold*4 Weeks	Etoh*0 Week	Etoh*1 Week	Etoh*4 Weeks	HWK*0 Week	HWK*1 Week
Cold*1 Week	**0.000**							
Cold*4 Weeks	**0.000**	0.166						
Etoh*0 Week	**0.000**	0.435	0.047					
Etoh*1 Week	**0.000**	0.707	0.151	0.479				
Etoh*4 Weeks	**0.000**	0.066	0.513	0.010	0.092			
HWK*0 Week	**0.000**	0.705	0.069	0.988	0.645	**0.014**		
HWK*1 Week	**0.000**	0.819	0.079	0.995	0.723	**0.015**	0.939	
HWK*4 Weeks	**0.000**	0.829	0.063	0.993	0.708	**0.010**	0.964	0.854

Figure 1

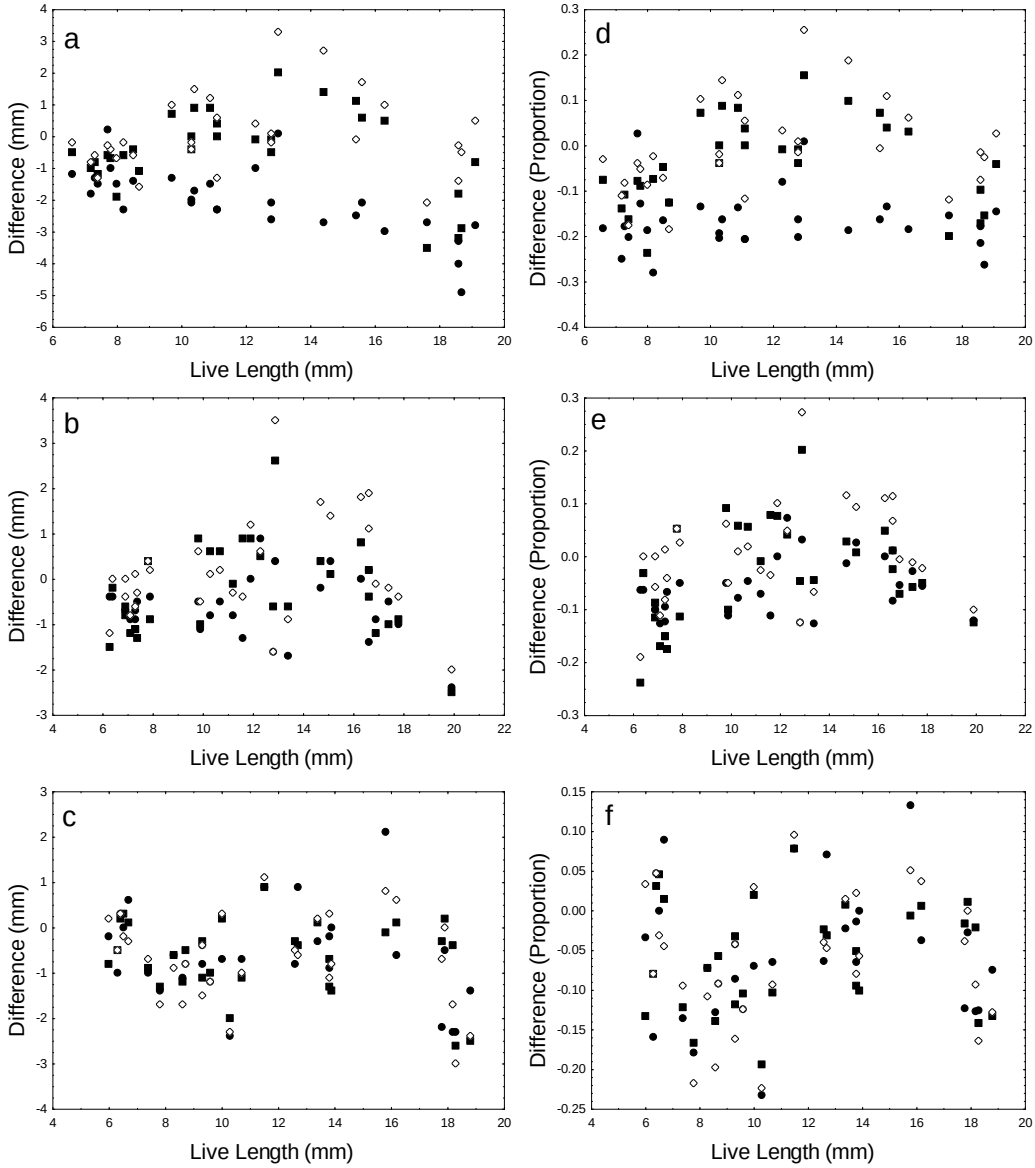
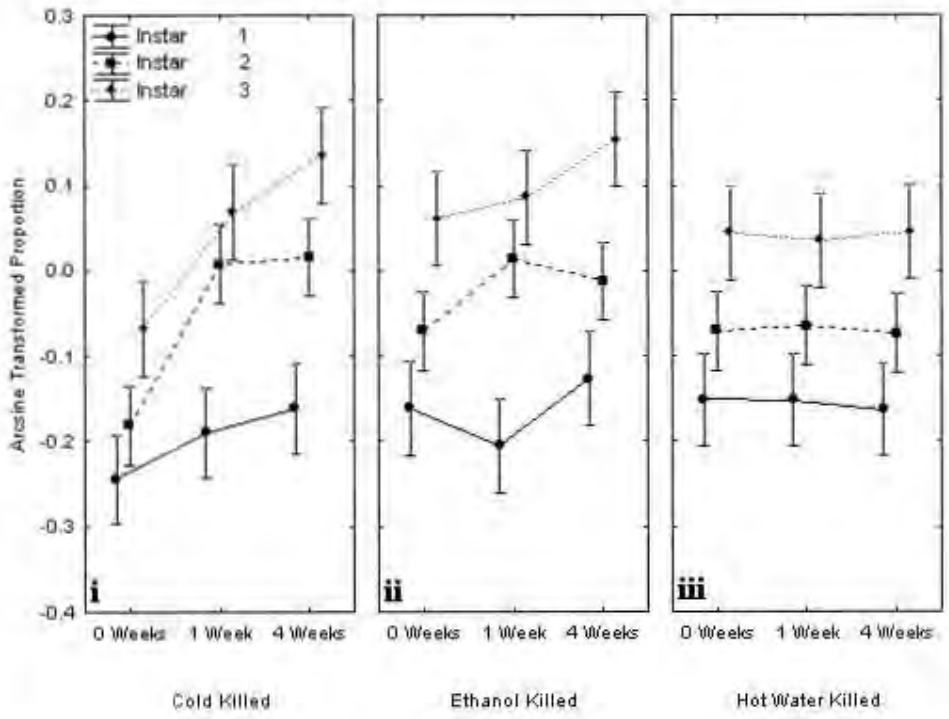

a
Difference (mm)
Live Length (mm)
d
Difference (Proportion)
Live Length (mm)
b
Difference (mm)
Live Length (mm)
e
Difference (Proportion)
Live Length (mm)
c
Difference (mm)
Live Length (mm)
f
Difference (Proportion)
Live Length (mm)

Figure 2


Instar 1
Instar 2
Instar 3
Arcsine Transformed Proportion
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
i
ii
iii
0 Weeks
1 Week
4 Weeks
Cold Killed
Ethanol Killed
Hot Water Killed