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Effects of different storage and measuring methods on larval length values for the blow flies (Diptera: Calliphoridae) *Lucilia sericata* and *Calliphora vicina*

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ABSTRACT

In forensic entomology, the methods of sampling, killing, and storing entomological samples can affect larval age estimation, and, hence, the estimation of the minimum post-mortem interval. In the existing manuals, there is a certain amount of heterogeneity regarding methods and the recommendations for best practice in forensic entomology are insufficiently validated. This study evaluated three different length-measurement methods for larval stages and examined the influence of different killing and storing methods on the larval length of two forensically important blow flies, *Lucilia sericata* and *Calliphora vicina*.

The three different measuring methods were a) a ruler with a 0.1 mm scale, b) a geometrical micrometer, and c) a computer-aided stereomicroscope. They were used to measure the length of L_1–L_3 *C. vicina* larvae and detect no significant differences. This supports the view that a simple tool like a geometrical micrometer can produce reliable results in forensic entomology.

Newly hatched larvae of *L. sericata* and *C. vicina* were killed with hot water (HW) and divided into two equal subsamples. Lengths of all larvae were measured immediately after killing, then every 24 h until day 4, and once more after 7 days of storage in ≥70%-ethanol.

L. sericata larvae only showed significant changes in length in the HW group stored at room temperature. After 4 and 7 days of storage, these 24-h- and 72-h-old larvae showed a significant decrease in length compared with those in a fridge at 6 °C. This decrease can, however, be considered a negligible natural variation without forensically relevant consequences for larval age estimation of *L. sericata* samples.

For *C. vicina*, an increase in length was observed over time. This was significant only for younger larvae (24-48 h old) stored in 70%-ethanol. This variance in length can lead to a wrong estimation of age; however, only for larvae stored in 70%-ethanol, not for those stored in 96%-ethanol.

Novelty statement: We examined the influence of different killing and storing methods on two forensically important blow flies, *Lucilia sericata* and *Calliphora vicina*. For the latter species we additionally were evaluating three different length measurement methods. The results of both experiments suggest that it is possible to kill and store fly larvae directly in (not hot) ≥70%-ethanol. This simplifies the sampling and storing of fly evidence at the crime scene. We also compared the influence of three different measuring methods for estimating the length of L1–L3 *C. vicina* larvae by using a) a ruler with a 0,1 mm scaling, b) a geometrical micrometer and c) a computer-aided stereomicroscope. No significant differences were detected, supporting the view, that a simple tool like a geometrical micrometer can produce reliable results. This study helps to simplify the sampling and evaluation of entomological evidence and to backup or questioning existing guidelines and best practice recommendations.

1. Introduction

Forensic Entomology is an important tool for establishing the time of death of a person by calculating the window of time between insect colonization of the corpse and its discovery, a period also known as the minimum post-mortem interval (PMImin). This interval is estimated by calculating the age of the oldest life history stage of insects collected from the cadaver or its surroundings. Because the rate of development of an insect is not only species-specific, but also governed by temperature, this estimation involves identifying the insect species found on a corpse and reconstructing the death scene temperatures

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[1]. Blow flies are usually the first group to colonize a body and forensic entomology, therefore, often focuses on these species.

Depending on the method used for the estimation of age, both living and freshly killed specimen samples are collected from the body. The living specimens are reared mainly for identification purposes while the freshly killed specimens can fix the stage of development reached by species at ambient temperatures. Sampling, killing, and storing methods are thus very important in forensic entomology because they may influence the result of the morphologic examination of the larvae collected at the death scene. Larval stages may account for about 50% of the total developmental time of blow flies. Depending on temperature and species, this developmental time can span several weeks [2, 3]. On-site killing and subsequent storage of entomological evidence is very often the preferred way of sampling at a death scene, as this will preserve the developmental stage of insect specimens as found at the very first time of discovery of the corpse and avoid the risk of further larval growth, mortality, or modification of characteristics pertinent to species identification and age estimation.

Despite their importance, the existing manuals on best practice in forensic entomology introduce a certain amount of heterogeneity through the methods recommended therein for sampling and storing insect evidence [2–6]. In order to create a universal standard, Amendt et al. [2] recommend in 2007 the killing of fly larvae by placing them in hot but not boiling water (>80 °C) for at least 30 s and then storing the sample in 70–95% ethanol. There is wide agreement on that practice actually. Catts and Haskell [6], on the other hand, recommend using different solutions for killing and conserving entomological samples: KAAD (a mixture of 95% ethanol, glacial acetic acid, kerosene and dioxane) to kill larval specimens, and Hood's solution (75% ethanol and glycerine) to preserve them. Yet other authors [7] suggest killing the larvae with boiling 70% ethanol and subsequently storing them in the same solution.

Although there is some evidence underpinning the effectiveness of current collection methods, the scientific basis requires more work to become fully established. In this regard it is worth of mentioning that the courts are increasingly demanding validated methods in forensic science [8]. In forensic entomology, in particular, there is a strong need for validated sampling, killing and, storing methods which guarantee the best result for the majority of the possible taxa and ages.

Tantawi and Greenberg [9], while studying the blow flies *Protophormia terraenovae* Robineau-Desvoidy 1830 (Diptera: Calliphoridae) and *Calliphora vicina* Robineau-Desvoidy 1830 (Diptera: Calliphoridae), realized that the method of preservation affects the results of age determination in the early larval period only. Adams and Hall [10] found length differences between larvae that were killed in hot water and then stored in 10% formalin, 80% ethanol, or 95% ethanol. Their recommendation was to kill larvae by immersion in 80 °C hot water for 30s and to then store them in 80% ethanol. Day and Wallman [11] studied the effect of seven different preservative solutions (100% EtOH; 10% formalin and Kahle's solution: 95% ethanol, formaldehyde, glacial acetic acid and water) on the larvae of the blow flies *Calliphora augur* Fabricius 1775 (Diptera: Calliphoridae) and *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae). They found that 10% formalin and Kahle's solution were the only preservatives that did not induce significant changes in the body length of the larvae. However, formalin hampers DNA recovery and is, therefore, unsuitable as a preservative for entomological evidence [3].

In this study, the influence of different killing and storing methods on the offspring of the two most forensically important blow flies in Europe, *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) and *C. vicina*, was examined. For the latter species, the efficiency of three different length-measurement methods was additionally evaluated.

The long-term aim of our research is a harmonization of standards and recommendations for methods of sampling and storing entomological evidence, as we believe that a forensic discipline needs to follow precise methodological guidelines and have to stand on a sound scientific fundament to be able to provide acceptable testimony for the courtroom.

2. Materials and methods

2.1. Fly stocks

Several hundred adult *Calliphora vicina* and *Lucilia sericata* were held in separate rearing cages (120 × 60 × 60 cm) at room temperature (average temperature approximately 18 °C, 60% RH) and a 12:12 L:D cycle. They were provided with water and sugar *ad libidum*. Every two days, bovine blood was offered as a source of protein and, once a week, to maintain the cultures, beef liver was supplied as an oviposition medium.

2.2. Oviposition and rearing

Fresh beef liver served as a medium for oviposition for both fly species. After oviposition, the liver and deposited eggs were placed in a rearing chamber (25 °C) for about 24 h. The hatched larvae were then transferred to a plastic cup filled with ground meat (half beef, half pork) as a food source, *ad libidum*, and placed in a container (12 × 12 × 7.5 cm) sealed with a perforated lid.

2.2.1. Lucilia sericata

Fifteen plastic cups with ground meat, containing 20 larvae each, were placed in an incubator (Jumo Imago 500) at 20 °C. On the following three days, at intervals of 24 h, five cups were taken from the incubator, so that three sample groups, each comprised of 100 larvae, were obtained. The first batch of larvae were killed after 24 h of feeding (group A); the second, after 48 h of feeding (group B); and the third, after 72 h (group C). Each group was divided in two equal subsamples: the first subsample was killed by hot, but not boiling, water (≤80 °C: HW), and then stored in 75% ethanol; the second subsample was killed in boiling 75% ethanol and left in this killing solution (HE). Half the specimens from each subsample were then stored at room temperature (HW1A-C and HE1A-C), while the other half were stored in a fridge at +6 °C (HW2A-C and HE2A-C).

Larval lengths were measured with a geometric micrometer immediately after killing (time 0), 24 h after killing (time 1), 48 h after killing (time 2), 72 h after killing (time 3), 96 h after killing (time 4), and 7 days after killing (time 5).

The described procedure was repeated three times, i.e., for a total of 900 larvae.

2.2.2. Calliphora vicina

Six plastic cups with ground meat, containing 20 larvae each, were placed in an incubator (Jumo Imago 500) at 23 °C. On the following three days, at intervals of 24 h, 2 cups were taken, so that three sample groups, each comprised of 40 larvae, were obtained. The first batch of larvae were killed after 24 h of feeding (group A); the second, after 48 h of feeding (group B); and the third, after 72 h of feeding (group C). The larvae were killed in hot, but not boiling, water (≤80 °C) and subsequently divided in two equal subsamples of 20 larvae each: a first subsample was stored in 70% ethanol, a second subsample in 96% ethanol. Both subsamples were stored at room temperature. Larval lengths were measured immediately after killing (time 0), 24 h after killing (time 1), 48 h after killing (time 2), 72 h after killing (time 3), 96 h after killing (time 4), and 7 days after killing (time 5).

After the first 7 days, the larvae were measured with a geometric micrometer at intervals of one week, for four weeks.

The described procedure was repeated five times, i.e., for a total of 600 larvae.