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Exploring unified methods of killing and storing insect samples for forensic entomotoxicology using diazepam in *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) larvae

Olwen C. Groth [a,*,1], Alexander Strassberger [a], Vera Höft [a], Irina Schusterbauer [a], Susan Rahaus [a,b], Adeyemi D. Adetimehin [c], Matthias Graw [a], Martin H. Villet [d,2]

[a] Institute of Forensic Medicine, Ludwig-Maximilians-Universität in Munich, Munich D-80336, Germany
[b] Department of Forensic Medicine/Aircraft Accident Investigation, German Air Force Institute of Aviation Medicine, Cologne D-51147, Germany
[c] Division of Forensic Medicine and Toxicology, Department of Pathology, University of Cape Town, Cape Town 7700, South Africa
[d] Department of Zoology & Entomology, Rhodes University, Makhanda 6140, South Africa

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ABSTRACT

Forensic entomologists use the maturity of necrophagous larvae to estimate the minimum post-mortem interval (PMI_{min}), ideally taking account of effects that xenobiotics in the corpse may have on insect maturation. Forensic toxicologists may employ larvae to detect drugs in drug-related deaths when human samples are unavailable. Yet current pre-analytical practices of these two professions differ significantly, impeding the successful use of the same samples. Potential benefits of shared pre-analytical practices and opportunities for enhanced collaboration have yet to be fully explored. We employed *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) larvae, raised in the presence of diazepam, to investigate the effects of two standard investigative practices on larvae for drug detection and for quantifying mass and length as proxies of age. Specimens were killed by either blanching or freezing and stored at −20°C for either intermediate or long periods. Blanched larvae showed smaller changes in size and body integrity during storage, thereby producing the most reproducible estimates of PMI_{min}. Consequently, data obtained from blanched larvae were used to evaluate the impact of diazepam on larval development. Diazepam exerted no significant effect on larval mass, and a weak effect on length. Diazepam recovery was significantly higher from blanched larvae, suggesting that freeze-killing causes drug loss. This model system demonstrates the value to forensic entomologists of the standard technique of blanching larvae, followed by storage at −20°C for toxicological analysis. We recommend that forensic toxicologists consider blanching to kill larvae before storage at low temperatures, at least for certain drugs. This approach offers the dual benefit of high-quality specimens for both PMI estimation and drug detection.

1. Introduction

Modern forensic investigation typically draws on diverse disciplines and techniques *ad hoc* to achieve its aims. Transdisciplinary approaches offer the innovation of outcomes that may not have been anticipated from the contributing fields. One such example is entomotoxicology, which combines the disciplines of entomology and toxicology [1–4]. Since xenobiotics in the food source of forensically important arthropods can alter the insects' rates of development [5–8], forensic entomologists should account for such exposure when estimating larval age as a proxy for minimum post-mortem interval (PMI_{min}), or risk making poor estimates [8–10]. For forensic toxicologists, entomological samples may sometimes be a superior, or even the only, option for identifying xenobiotics in drug-related deaths [11–17]. For example, during mass spectrometric detection of substances, the use of carrion-associated insect larvae instead of decomposed human tissue samples may be associated with less-pronounced matrix effects caused by post-mortem degradation products [18]. In two post-mortem forensic cases, some drugs and metabolites were even identified in larvae feeding on the corpses, whereas all the available human specimens tested negative for some of these

* Corresponding author.
E-mail address: olwen.groth@med.uni-muenchen.de (O.C. Groth).
[1] ORCID ID: 0000–0003-4672–041X
[2] ORCID ID: 0000–0002-4335–5667

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substances, possibly due to ante- and/or post-mortem redistribution processes [19].

Although entomotoxicological techniques offer benefits to both forensic entomologists and toxicologists, the field is still largely practised by the two respective professions with independent methods [15]. This is especially reflected in the paucity of unified standards and common guidelines for the collection and storage of carrion-associated insect specimens as evidence for subsequent joint entomological and toxicological analyses [9,15,20]. The topic of standardising such practices has been discussed previously [2,9,15,21], yet with little outcome for experimental work in different laboratories.

Forensic entomologists typically estimate the age of immature insects (*e.g.* larvae) from a corpse or animal carcass to estimate its PMI_{min}. As the size of larvae correlates with their degree of maturity, mass and/or length measurements of larvae allow estimation of their age to reconstruct the times of oviposition and approximate the time since death [8,22]. However, external factors such as the conditions of insect preservation can affect size measurements. The resulting metrological disparities can therefore lead to erroneous PMI_{min} estimates [22,23]. From a toxicological perspective, the size of necrophagous larvae is of limited interest. However, the methods used to collect and store toxicological samples will significantly affect the efficacy of drug detection [24]. In forensic toxicology, it is generally preferable to store biological specimens (*e.g.* human blood and organs) at −20°C or colder until analysis to reduce the effect of toxicant instability on drug detection and quantification [9,15,20,24,25]. However, insect specimens tend to lose shape, body mass, and important morphological features when killed and preserved at such low temperatures [26,27], compromising PMI estimation, so samples of fly larvae are blanched in near-boiling water and subsequently stored in ≥ 70 % ethanol for entomological analyses [15,20,28–31]. This protocol may, in turn, compromise the stability and detectability of drugs or otherwise interfere with toxicological analyses by techniques that couple liquid chromatography and mass spectrometry (LC-MS) [24,32,33]. Some toxicants are susceptible to thermal degradation at higher temperatures [34], and the use of organic solutions to store toxicological samples can be unfavourable, as analytes from the specimen may leach into the preservative solution and even become degraded [35,36].

This study investigated whether unified standardised guidelines would be practical for killing and preserving larvae for both entomological and toxicological forensic applications. We therefore compared the effects of two killing methods and two storage times on two proxies of larval maturity (mass and body length) and drug recovery from larvae, using a model entomotoxicological system involving *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) larvae exposed to diazepam. Several studies have confirmed the forensic relevance of *L. sericata* as a priority model organism for forensic entomo(toxico)logy worldwide [9,37–40] and, like several other benzodiazepines, diazepam is one of the most widely prescribed and abused psychotropic drugs, making it prominent in post-mortem forensic toxicology [41–44]. We addressed the following research questions, using the chronological sampling time of larvae at a constant temperature as a direct measure of their actual physiological age: (a) how are the mass and length of *L. sericata* larvae affected by the (i) killing method, (ii) diazepam concentration in the food source during development, and (iii) intermediate and long-term storage at −20°C and (b) how is the recovery of diazepam from drug-exposed larvae affected by (i) the time of exposure and (ii) the killing method after long-term storage at −20°C?

2. Materials and methods

2.1. Rearing of larvae

Lucilia sericata pupae were purchased from TerraristikShop.net (Düsseldorf, Germany). The identity of emerging adults was first confirmed morphologically using a taxonomic key for European blowflies [45], and then they were used to establish a laboratory colony. Adult flies were maintained in rearing cages (35 ×21 x 21 cm) at room temperature. Sugar cubes and water (in the form of soaked paper towels) were provided *ad libitum*. Minced pork muscle served as a source of protein and a substrate for oviposition.

Third-generation eggs of the same age were incubated together in a Memmert IPP 200 incubator (Schwabach, Germany) at 70(±10)% relative humidity (RH) and 25(±0.5)°C, which falls within the optimal temperature range for the development of *L. sericata* [46]. An artificial light:dark (L:D) cycle of 16:8 h was created using an LED light with automatic timer. Starting approximately 12 h after oviposition, the egg masses were monitored hourly for newly hatched larvae to obtain first instars of uniform age for the experiments.

A stock solution of diazepam (≥ 98 %, Merck, Darmstadt, Germany) was prepared in purified water (Milli-Q Millipore filter system, Bedford, MA, USA) at a concentration of 1 g/L (*m/v*). To improve the solubility of diazepam, hydrochloric acid (37 % *w/w* HCl, Merck, Darmstadt, Germany) was added to the stock solution to obtain a final concentration of 0.04 M. A control mixture (Mixture B) was prepared by dissolving concentrated HCl in purified water to a final concentration of 0.04 M.

Lean, raw, minced pork muscle was used as a breeding substrate to minimise the extent of matrix effects during toxicological analyses. An inert medium was chosen primarily due to the ethical and legislative constraints associated with *in vivo* animal models and particularly post-mortem human tissues [47,48]. Different volumes of the diazepam stock solution were added to batches of raw, minced pork muscle to obtain four concentrations of 0 μg/g, 2 μg/g, 10 μg/g, and 20 μg/g. The range of diazepam concentrations was based on recently published therapeutic concentrations in blood and earlier findings from human intoxication cases [44,49–51]. A total volume of 4.8 mL, consisting of the required volume of diazepam stock solution and Mixture B, was added to each of four 240 g portions of minced pork meat (*e.g.* for the 0 μg/g batch: 0 mL diazepam stock solution + 4.8 mL Mixture B; for the 10 μg/g batch: 2.4 mL diazepam stock solution + 2.4 mL Mixture B). Each batch of minced pork was then mixed in a food processor for 3 min to ensure homogenous distribution of the drug, avoiding cross-contamination between batches of different drug concentrations. Each batch was divided into three 80 g portions, placed in plastic cups (replicates i, ii, and iii; Fig. 1).

Moist entomological brushes were used to transfer 100 neonate larvae to each of the 12 cups, once again avoiding cross-contamination between different treatments. The three replicate cups of each concentration were placed into a plastic container (180 ×110 x 125 mm), containing pet litter for pupation and a datalogger (FreeTec V2, Munich, Germany) to measure temperature and humidity (Fig. 1). Each container was covered with a nylon net and all containers were incubated together, starting simultaneously (t_0), in a Memmert IPP 200 incubator (Schwabach, Germany) at 25(±0.5)°C, 70(± 10)% RH and 16:8 h L:D, as described above.

2.2. Sampling and killing of larvae

Larvae were sampled at specific intervals: every four hours until 24 h (t_4, t_8, t_{12}, t_{16}, t_{20}, and t_{24}), then every six hours until 48 h (t_{30}, t_{36}, t_{42}, and t_{48}), then every 12 hours until 96 h (t_{60}, t_{72}, t_{84}, and t_{96}), and finally 24 hours later at 120 h (t_{120}) of exposure (Fig. 1). At each sampling time, two larvae were taken from each triplicate container (i, ii, and iii), one for killing by freezing and the other for blanching (Fig. 1). Larvae were selected at random from each experimental unit. The sequence in which the various treatments was sampled was randomised at each sampling time. The position of each treatment in the incubator was changed after each sampling procedure to minimise the effect of isolative segregation [52].

Larvae to be killed by freezing were thoroughly washed in water at room temperature immediately after selection, and carefully dried on absorbent paper. Each larva was placed in an individual 2 mL

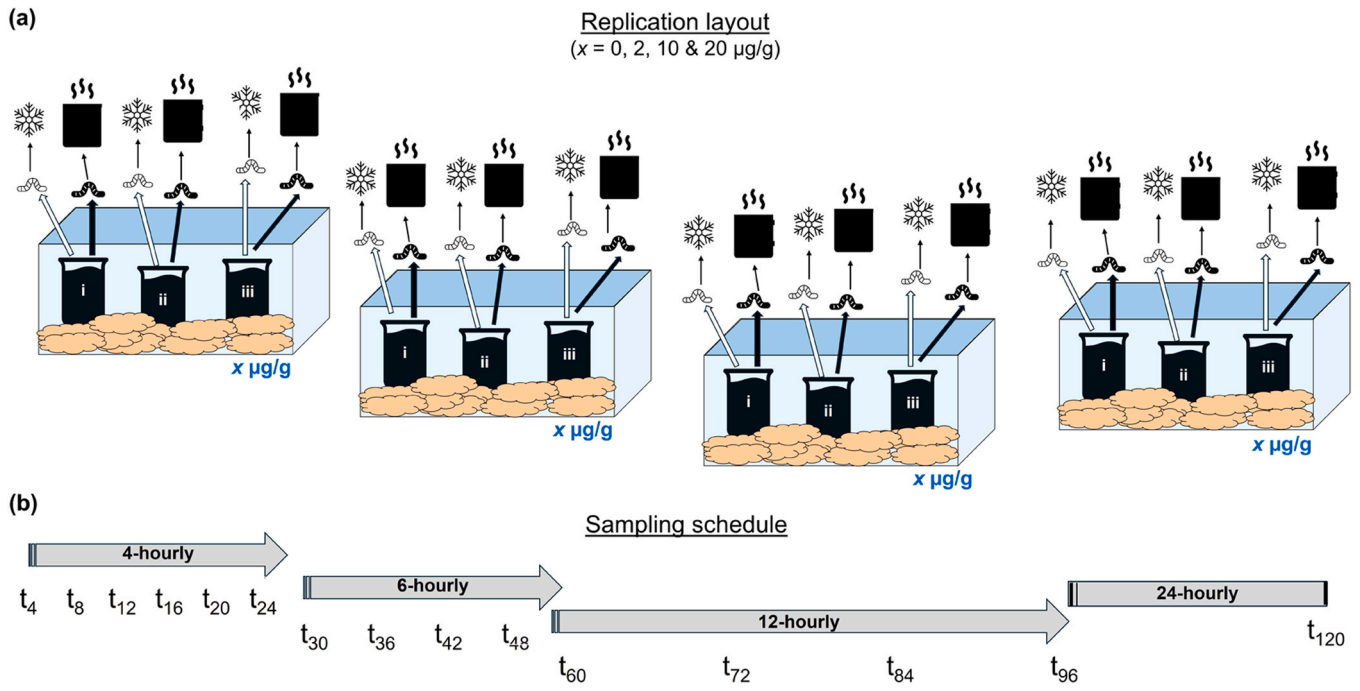

Fig. 1. Diagram of the **(a)** experimental design and **(b)** sampling schedule. The design involves three replicates (i, ii, iii) of four drug concentrations (0, 2, 10, 20 µg/g), housed in separate pupation trays in a single incubator.

microcentrifuge tube and assigned a unique identification code. Microcentrifuge tubes with larvae were left at −20°C overnight (12–24 h) to ensure that larvae killed by freezing were dead before measurement. Larvae killed by blanching, in line with best practice for fly larvae in forensic entomology [26,30,53], were immersed in water at ~85–95°C for ~60 sec immediately after sampling, washed in the same water, and carefully dried on absorbent paper. Each of these larvae was also assigned a unique identification code and stored in a separate 2 mL microcentrifuge tube after the first size measurement [26,54].

2.3. Size measurements and storage of larvae

The size of blanched larvae was measured as soon as they had been dried according to forensic entomological practices. Larvae killed by freezing were thawed after 12–24 h, carefully dried on absorbent paper, and then measured. The choice of freezing larvae for 12–24 h is based on current practices employed in the killing of larvae for toxicological analysis [16,18,19,55]. The mass of individual larvae was measured on a Mettler Toledo MX-5 microbalance (Greifensee, Switzerland) with a resolution of 1 µg. The body length of larvae was measured by gently aligning them along a standard ruler, careful not to damage the larval body during measurement. The length of larvae was read to the nearest 1 mm under a Zeiss Stemi DV4 stereomicroscope (Carl Zeiss, Oberkochen, Germany). Both blanched and frozen larvae were returned to their original microcentrifuge tubes and stored at −20°C immediately after measurement. Storage of larvae at −20°C was chosen because it is commonly used for biological specimens in forensic toxicology [24,25].

Larval length and mass were remeasured after 2 weeks and after 4 months, using the same instruments and methods. These storage intervals correspond to fairly typical storage times for biological specimens used in forensic entomological and toxicological analyses [26].

2.4. Toxicological analysis

2.4.1. Solid phase extraction

The toxicological analysis of larvae was performed five months after initial storage at −20°C. This storage interval corresponds to fairly typical storage times for biological specimens used in forensic entomological and toxicological analyses [26].

An optimised method for extracting, subsequently detecting and quantifying benzodiazepines in larvae was applied (unpublished data). Larvae killed by freezing were analysed separately from blanched larvae to evaluate whether the killing method influenced toxicant stability and/or detectability. Larvae sampled at the respective sampling time points were analysed separately, except for larvae sampled from 4 to 36 h, which were combined to amount to approximately 100 mg per sample. For the extraction of diazepam and its metabolites, approximately 100 mg of larvae from each sampling time point and killing method were placed in a separate 2 mL disposable, reinforced Precellys® (Bertin Technologies, Montigny-le-Brettonneux, France) vial. One hundred microliters of an isotonic NaCl (≥ 99 %, Roth, Karlsruhe, Germany) solution (0.9 % *m/v*) in purified water (Milli-Q Millipore filter system, Bedford, MA, USA) and five stainless steel beads (diameter: 2.8 mm) were added to each vial and larvae were homogenised in a Precellys® 24 tissue homogeniser at 4000 RPM for 90 sec.

After the addition of a deuterated internal standard (IS) mix, containing diazepam-d_5, nordazepam-d_5, oxazepam-d_5, and temazepam-d_5 (Ceriliant, Austin, TX, USA), the homogenate was loaded onto Oasis® PRiME HLB 3cc cartridges (Waters GmbH, Eschborn, Germany) and slowly eluted under positive pressure (Waters Positive Pressure-96 Processor) with 2 % formic acid in acetonitrile (99.9 %, Sigma-Aldrich, Steinheim, Germany). The supernatant was evaporated to dryness at 37°C under a stream of nitrogen and reconstituted in 150 µL of an aqueous ammonium formate solution (5 mM ammonium formate in purified water, ≥ 97 %, Honeywell Research Chemicals, Selzer, Germany), containing 0.01 % formic acid (≥ 95 %, Sigma-Aldrich).

2.4.2. Analysis by liquid chromatography-coupled mass spectrometry (LC-MS)

Larval extracts were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an Agilent 6495 triple quadrupole (QQQ) system for the detection of diazepam, nordazepam, oxazepam, and temazepam. Chromatographic separation was achieved by injecting 5 µL of each extract onto a Zorbax Eclipse Plus C_{18} column (Rapid

Resolution HD, 2.1×50 mm, particle size: 1.8 µm, Agilent Technologies, Waldbronn, Germany) at 30°C. Mobile phases consisted of 5 mM ammonium formate in water with 0.01 % formic acid (eluent A) and 0.01 % formic acid in methanol (eluent B). A flow rate of 0.4 mL/min was used, with the following gradient for eluent B: 0–1 min: 5–30 %, 1–6 min: 30–60 %, 6–8.5 min: 60–95 %, 8.5–9.5 min: 95 %, 9.5–9.51 min: 95–5 %, and 9.51–12.5 min: 5 % for re-equilibration.

Mass spectrometric analysis was performed in multiple reaction monitoring (MRM) mode with positive electrospray ionisation (AJS ESI+). Data were acquired and processed with the MassHunter software. Identification criteria for diazepam, nordazepam, oxazepam, and temazepam included their retention times relative to those of the corresponding deuterated internal standards, two MRM transitions (diazepam *m/z* 192.9 and 153.9, nordazepam *m/z* 139.9 and 164.9, oxazepam *m/z* 240.9 and 268.9, temazepam *m/z* 254.9 and 282.9), and the expected ion ratio (± 20 %). The limits of detection for diazepam, nordazepam, oxazepam, and temazepam are 13, 5, 11, and 6 µg/kg, respectively. For quantification, the calibration curve for each analyte was created from ten calibration points, using extracts of drug-free larvae that had been spiked with IS and analytes in the corresponding concentrations. Final concentrations in larvae were calculated from the target ion and recalculated for the actual larval mass used for extraction.

2.5. Statistical analyses

Fig. 2 provides a summary of the statistical evaluation, assessing the effect of killing method and storage time at −20°C on parameters essential for entomological analyses, namely larval length and mass (see Section 2.5.1), as well as the effect of killing method and sampling time on drug recovery from the larvae, which are important parameters for toxicological analysis (see Section 2.5.2). To avoid bias in the results, only data from larvae killed by the method with the smallest effect on their length and mass were used to analyse the impact of diazepam on larval development.

2.5.1. Entomological aspects

Larval mass and length were used as dependent variables to investigate the effect of killing method, drug exposure, and storage time on larval size.

Prior to analysis for killing method and storage time, larval mass and length were log-transformed to linearise their allometric growth relationship with sampling time (*i.e.* larval age). Scatterplots of mass or body length against age, generated using Statistica version 14.0.0.15 (2020, TIBICO Software Inc, Palo Alto, CA, USA) and PAST version 4.14 [56], indicated no increase in larval size after 72 h, which marked the end of growth and the start of the post-feeding phase, so the data set was truncated at 80 h to avoid confounding the effect of the covariate (age).

2.5.1.1. Effects of killing method on larval size. The drug concentrations and killing methods were modelled as fixed effects in a two-way analysis of covariance (ANCOVA) of the log-transformed initial masses or body lengths, taking sampling time (*i.e.* growth or aging) into account as a covariate, and interpreted at $\alpha = 0.05$. Tukey's Honest Significant Difference (HSD) test was used for post-hoc analysis in cases where significant differences were found in an ANCOVA.

2.5.1.2. Effect of drug exposure on larval size. Non-log-transformed data of mass and length of blanched larvae were used to analyse the effect of diazepam on larval development. These data were not truncated prior to analysis.

Sampling time and drug concentrations were modelled as fixed effects in a two-way analysis of variance (ANOVA) of the untransformed masses or body lengths, taking sampling time (*i.e.* growth or aging) into account as a covariate, and interpreted at $\alpha = 0.05$.

2.5.1.3. Effect of storage time on larval size. The effects of the drug concentrations and killing methods on untransformed absolute (mg or mm) and relative (%) changes during storage (*i.e.* errors) in the two proxies of actual larval age (*i.e.* their mass and length) after storage for two weeks and four months, respectively, were modelled by two-way

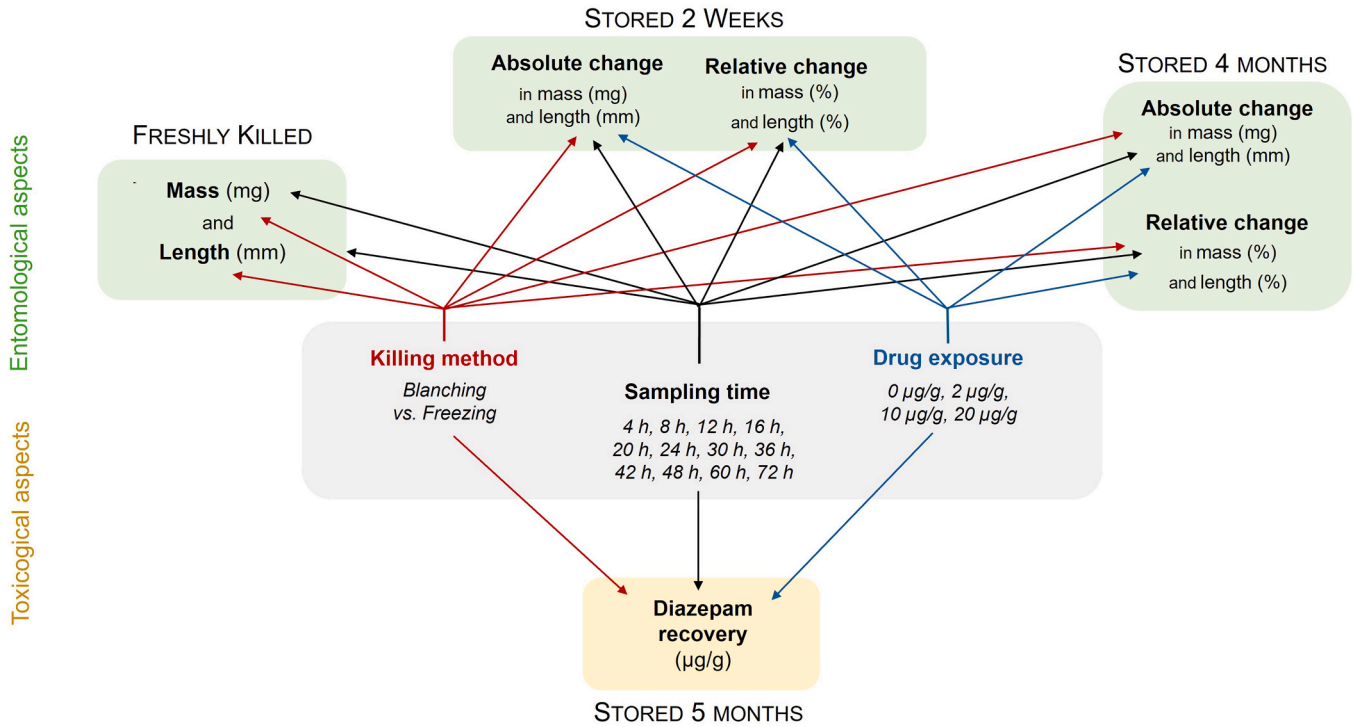

Fig. 2. Summary of the statistical analyses performed to evaluate the effect of different variables on insect size (length and mass) for entomological applications and drug recovery for toxicological applications.

analyses of covariance (ANCOVA) with sampling time (*i.e.* true age) as a covariate, and interpreted with $\alpha = 0.05$. The two-week and four-month storage effects were analysed separately to avoid time-dependent precedence effects in the repeated measures.

2.5.2. Toxicological aspects

2.5.2.1. Effects of killing method and sampling time on diazepam recovery. The effects of the killing method and sampling time on absolute (ppm) and relative (%) recovery of diazepam after storage for five months at −20°C were modelled by two-way analyses of covariance (ANCOVA) with drug dose as a covariate and interpreted with $\alpha = 0.05$.

3. Results

3.1. Entomological aspects

3.1.1. Larval size before storage

3.1.1.1. Effect of sampling time. Sampling time (*i.e.* larval age) was significantly (allometrically) correlated with larval mass (Table S1 in Supporting Information) and larval length (Table S2 in Supporting Information), as expected from the growth process.

3.1.1.2. Effect of killing method. Larvae killed by blanching were generally more rigid and stable than those that were frozen to death, making them easier to handle and measure. Even after several months at −20°C and three freeze/thaw cycles, the physical condition of these larvae remained largely consistent. In contrast, larvae killed by freezing were difficult to handle because they were flaccid and deformable after thawing, and their morphological features were easily damaged during handling. Subjectively, the problem increased after the second freeze/thaw cycle.

Mean larval mass was not statistically significantly affected by the method used to kill the larvae (Table S1 in Supporting Information). However, mean larval length was statistically significantly greater in specimens killed by blanching than in those killed by freezing (Table S2 in Supporting Information, Fig. 3), as expected from previous studies.

3.1.1.3. Effect of drug treatment. Sampling time had a significant effect on larval mass ($p = 0.000$, partial $\eta^2 = 0.918$) (Table S5 in Supporting Information) and length ($p = 0.000$, partial $\eta^2 = 0.944$) (Table S6 in Supporting Information). Diazepam concentration had a significant effect on larval length ($p = 0.009$, partial $\eta^2 = 0.093$), but no significant effect on larval mass ($p = 0.087$, partial $\eta^2 = 0.053$). Fig. 4 depicts the mean mass and length of blanched larvae that had been exposed to different diazepam concentrations as a function of sampling time.

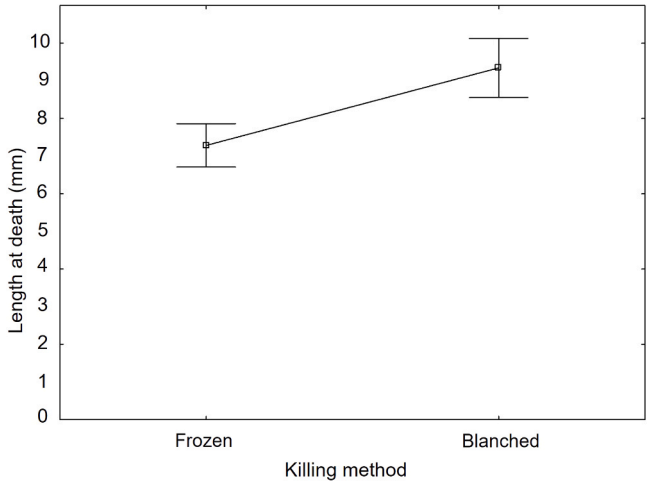

Fig. 3. Mean length of larvae before storage as a function of killing method. Vertical bars denote 95 % confidence intervals.

3.1.2. Larval size after storage

The effects of sampling time (*i.e.* larval age), diazepam concentration, and killing method on mean absolute and relative changes (*i.e.* estimate errors) in larval mass and length after medium-term (two weeks) and long-term (four months) storage at −20°C are summarised in Table 1.

3.1.2.1. Two-week storage at −20°C. Mean absolute change in larval mass after two weeks of storage was not significantly affected by sampling time, killing method or diazepam concentration, but only very narrowly so in the case of sampling time (Table 1).

Mean absolute change in length was significantly affected only by sampling time and killing method (Table 1). Blanched larvae showed no significant change in length and the variation within each sampling time included 0.0 (*i.e.* no change in length, Fig. 5a). Younger larvae killed by freezing showed similar responses, but increasing degrees of shrinkage occurred in larvae older than 24 h (Fig. 5a).

Mean relative change in mass was significantly affected by sampling time, killing method, and diazepam concentration. Larvae younger than 42 h showed larger relative errors, especially in those killed by freezing (Fig. 5b). Among the diazepam concentrations, Tukey's test showed that the mean relative change in mass in the 2 μg/g treatment was significantly ($p < 0.05$) higher than in the 10 μg/g treatment (Fig. 6a).

Mean relative change in length was significantly affected by sampling time and killing method (Table 1). Blanched larvae showed no significant change in length, unlike larvae killed by freezing (Fig. 6b).

3.1.2.2. Four-month storage at −20°C. The absolute change in larval mass during long-term storage was significantly affected by time of sampling, but not by diazepam concentration or killing method (Table 1).

Mean change in absolute length of larvae was significantly affected only by sampling time and killing method (Table 1). There was no significant change in the length of the blanched larvae, but frozen larvae became significantly shorter in storage (Fig. 7a).

Mean relative larval mass was significantly affected by sampling time and diazepam concentration, but not by killing method (Table 1). Tukey's test showed that the relative change in larval mass was significantly greater in the 2 μg/g treatment than in the 10 μg/g treatment (Fig. 7b).

Mean relative change in length of blanched larvae was significantly affected only by sampling time and killing method (Table 1). Young larvae tended to be proportionately longer after storage, irrespective of killing method (Fig. 7c). Older larvae that were killed by freezing were often relatively shorter than before storage, unlike older blanched larvae, which showed little change at all (Fig. 7c).

3.2. Toxicological aspects

3.2.1. Absolute recovery of diazepam from larvae

Diazepam concentrations in larvae ranged from 12 μg/kg to 1600 μg/kg, irrespective of killing method, sampling time, and diazepam concentration in the food source.

The effects of diazepam concentration in the food source, killing method, and sampling time (*i.e.* larval age) on the mean absolute recovery of diazepam from larvae after storage for five months at −20°C are detailed in Table S3 in the Supporting Information.

Diazepam concentration in the diet ($p < 0.000$) had a significant effect on absolute drug recovery from larvae, as expected. No significant effect of killing method ($p = 0.102$) was observed on absolute diazepam recovery. Sampling time had a significant effect ($p < 0.000$) on absolute

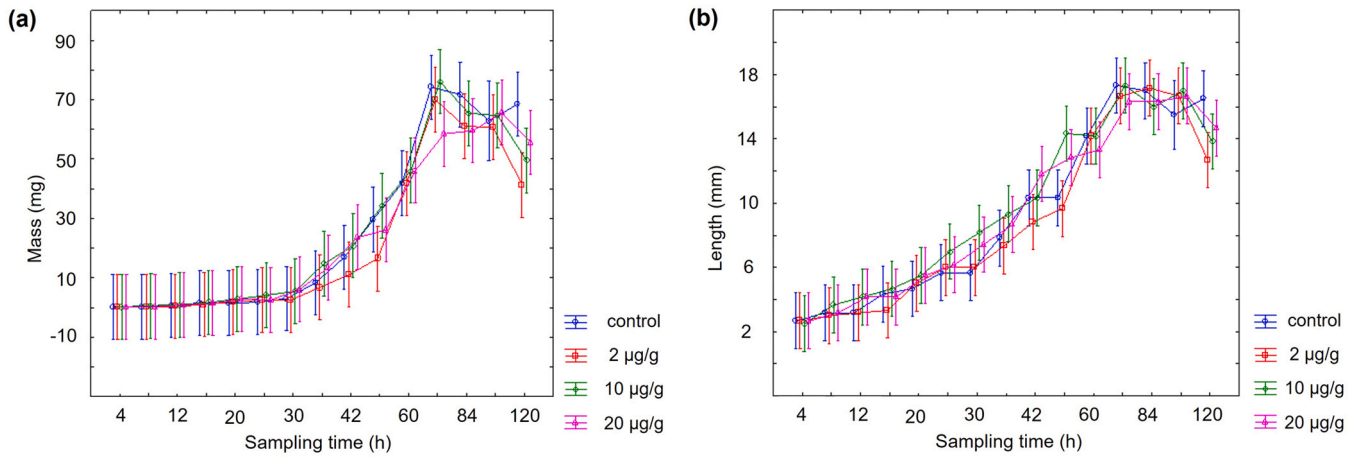

Fig. 4. **(a)** Mean mass and **(b)** length of *L. sericata* larvae that had been killed by blanching after exposure to different concentrations of diazepam (0, 2, 10, and 20 µg/g), as a function of sampling time until 120 h of development. Vertical bars denote 95 % confidence intervals.

drug recovery from larvae, with significantly lower absolute concentrations of diazepam detected in larvae older than 60 h compared to larvae sampled at 42 h (Fig. 8a).

3.2.2. Relative (proportional) recovery of diazepam from larvae

The effects of diazepam concentration in the food source, killing method, and sampling time (*i.e.* larval age) on the relative recovery of diazepam from larvae after storage for five months at - 20°C are detailed in Table S4 in the Supporting Information.

Diazepam concentration in the diet ($p = 0.001$), sampling time ($p < 0.000$), and the killing method ($p = 0.002$) all had a significant effect on the relative drug recovery from larvae. Significantly higher recoveries were obtained for larvae sampled from 36 to 48 h compared to older larvae (Fig. 8b). The relative recovery of diazepam was significantly lower for larvae that were killed by freezing (Fig. 8c).

4. Discussion

4.1. Overview of the parameters investigated

This study aimed to identify methods to successfully use the same necrophagous insect larvae for the entomological estimation of PMI and forensic toxicological analyses. We evaluated the effect of two factors on the mass and length of *L. sericata* larvae as proxies for true larval age, as such changes may lead to misinterpretation of larval maturity and thus introduce errors in PMI estimates during actual forensic casework. Similarly, the impact of three variables on the absolute and relative recovery of diazepam from larvae was evaluated for the successful application of immature necrophagous insects for toxicological analyses to identify drugs previously consumed by a deceased person. Absolute recovery is an important indicator of detection thresholds when applying larvae for drug detection. Relative recovery expresses drug recovery as a function of the drug concentration in the medium and is therefore important for estimating the dose in the food source.

For this, we used a model system of *L. sericata* larvae, raised in the presence of the benzodiazepine drug diazepam, which was supplied to the immature insects in the form of spiked minced meat. Although the use of whole animal carcasses may resemble natural conditions more closely than homogenised, spiked food sources do, larvae from the same treatment group of such experimental designs are likely to be exposed to uneven, uncontrolled concentrations of the toxicant [9,15], necessitating much larger sample sizes to attain adequate statistical power. The use of solid liver tissues from animals that have received an ante-mortem dose of a drug may also not result in uniform delivery to larvae, as tropism in drug levels may occur in the liver [57,58], depending on the spatial scale of feeding of individual larvae. Furthermore, liver and *in situ* models may contain enzymes that produce metabolites in addition to the particular drug under investigation. These metabolites may exert their own effects on larval growth, which may not be accounted for by experimental design. Some studies have also shown inhibited larval growth on liver diets compared to other tissues [8]. It would therefore be prudent to first establish the absolute concentration-dependent effect of a toxicant prior to applying animal models or parts of animal models (*e.g.* liver).

Our statistical analyses confirmed that sampling time has a significant effect on larval size, as expected because developing larvae grow exponentially in mass and length as they age. Another obvious finding that was anticipated, was that significantly higher (absolute and relative) recoveries of diazepam were generally observed from larvae that were exposed to higher concentrations of the drug. Observing these predicted outcomes gives confidence in the remaining results.

Parameters that were previously largely unknown for *L. sericata* include the methods of processing entomological specimens that may be (in)compatible with toxicological methods and *vice versa*.

4.2. Entomological aspects

4.2.1. Effect of larval size on its measurement

Sampling time (*i.e.* true larval age) had a significant relationship with the absolute and relative changes (*i.e.* measurement errors) in larval mass and length during storage at −20°C, except for the absolute change in mass after two weeks, for which $p = 0.054$, *i.e.* only very marginally not significant (Table S1 in Supporting Information). Since true larval age is the variable that forensic entomologists are interested in estimating, it is problematic if it is also the source of estimation errors, so it is vital to understand the underlying mechanism(s).

These effects appear to be partially due to the relative precision of the measurements: the resolution of any particular measuring instrument is (ideally) constant, so its resolution constitutes a bigger proportion of smaller measurements than of larger measurements, particularly when the resolution is at a similar scale to the smallest measurements [22]. The effect of relative precision is expected to create a hyperbolic relationship, which is what was found in our analyses of relative errors (Figs. 5b, 7c), supporting this explanation. Forensic entomologists therefore need to choose measuring instruments with resolutions that are at least an order of magnitude finer than the size of the measurements that they are making to avoid this source of metrological inaccuracy when estimating PMIs [26].

Even when measurements can be made perfectly accurately, changes in larval size that originate during storage will propagate as errors in

Table 1
Summary of effects of sampling time (*i.e.* larval age), diazepam concentration, and killing method on mean absolute and relative changes (*i.e.* errors) in larval mass and length after medium- (two weeks) and long-term (four months) storage at −20°C.

Storage time	Size	Independent effect	F	*p*-value	partial η^2
Absolute error (mg or mm)					
2 weeks	Mass	Sampling time	$F_{1,280}$ = 3.73	0.054	0.0132
		Drug concentration	$F_{3,280}$ = 1.06	0.366	0.0112
		Killing method	$F_{1,280}$ = 0.28	0.597	0.0001
	Length	Sampling time	$F_{1,279}$ = 105.17	< **0.001**	0.2738
		Drug concentration	$F_{3,279}$ = 0.81	0.491	0.0086
		Killing method	$F_{1,279}$ = 20.144	< **0.001**	0.0673
4 months	Mass	Sampling time	$F_{1,275}$ = 51.98	< **0.001**	0.1590
		Drug concentration	$F_{3,275}$ = 0.83	0.475	0.0090
		Killing method	$F_{1,275}$ = 0.06	0.802	0.0002
	Length	Sampling time	$F_{1,276}$ = 138.26	< **0.001**	0.2913
		Drug concentration	$F_{3,276}$ = 1.40	0.243	0.0150
		Killing method	$F_{1,276}$ = 57.79	< **0.001**	0.1731
Relative error (%)					
2 weeks	Mass	Sampling time	$F_{1,280}$ = 302.97	< **0.001**	0.5197
		Drug concentration	$F_{3,280}$ = 4.09	**0.007**	0.0420
		Killing method	$F_{1,280}$ = 15.12	< **0.001**	0.0512
	Length	Sampling time	$F_{3,280}$ = 4.09	< **0.001**	0.3241
		Drug concentration	$F_{1,279}$ = 133.76	0.111	0.0212
		Killing method	$F_{1,279}$ = 5.34	**0.021**	0.0188
4 months	Mass	Sampling time	$F_{1,345}$ = 438.47	< **0.001**	0.5596
		Drug concentration	$F_{3,345}$ = 2.96	**0.032**	0.0251
		Killing method	$F_{1,345}$ = 2.29	0.131	0.0066
	Length	Sampling time	$F_{1,346}$ = 237.17	< **0.001**	0.4067
		Drug concentration	$F_{3,346}$ = 1.43	0.232	0.0123
		Killing method	$F_{1,346}$ = 70.59	< **0.001**	0.1694

calculations of the true age of larvae and therefore in the estimated PMI. In this study, these relative errors generally tend to zero in larger larvae, except for measurements of length in freezer-killed larvae (Figs. 5b, 7c). This strongly supports previous recommendations that fly larvae intended for estimating PMI should be killed by blanching [20,30,54]. These recommendations are at odds with those for the preservation of toxicological samples by freezing [9,15,20,24,25] that have led to recommendations in the literature that separate, earmarked samples should be collected for entomological and toxicological analyses [53].

Although the mass of smaller larvae can usually be determined more accurately than their length, younger larvae have a greater surface-to-volume ratio, thus resulting in greater relative loss of mass during storage. In contrast, errors in length measurements due to relative (im) precision are more likely to occur in smaller – and therefore younger – larvae [22], which is reflected in the relationships of sampling time to measurement errors (*e.g.* Fig. 7c). Frozen larvae showed a deviation even for the older larvae, which is an effect of the killing method in addition to relative measurement precision.

With these caveats in mind, it is still no surprise that sampling time (*i.e.* larval age) is allometrically correlated with mass and length (Tables S1, S2 in Supporting Information). It was therefore analytically strategic to use a covariate to control for the effects of sampling time, as we did when considering the experimental effects of killing method and diazepam exposure.

The quantification of effect sizes (Tables S1, S2 in Supporting Information) also helps to put the following discussions in perspective.

4.2.2. *Effect of killing method on larval size*

Killing method had a more pronounced effect on the length of larvae (Fig. 3) than on their mass (Tables S1, S2 in Supporting Information).

Blanched larvae were generally longer (Fig. 3) and straighter than those killed by freezing, which was observed at all measuring events (*i.e.* immediately after blanching, and after two weeks and four months of storage at −20°C). This effect comes from the distention of the air sacs in the respiratory system as the larvae are heated, and the subsequent fixation of the distended posture when the muscles denature. For precisely this reason, blanching has been established in forensic entomology as a procedural standard for fly larvae [26,30,53] that is designed to preserve larvae at their maximum extended body length [30], maximising standardization of the length measurement, while freezing kills larvae in unstandardized postures and extensions.

Larval length is used more commonly than mass for age estimation, because mass is characteristically more difficult to measure accurately in very young larvae that may be near the detection threshold of the available balances [22]. At least in principle, mass should not be affected by killing method if the larval body remains intact. A significant effect of killing method on larval mass was observed only for the relative change in mass after two weeks of storage at −20°C. Here, larvae killed by freezing lost a significantly higher proportion of their original mass than blanched larvae.

Frozen larval tissues are susceptible to mechanical disruption by ice crystals that gradually grow inside the cells. Freezing can also lead to the rapid disintegration of fat body tissue [59] and to perforation of the intestine, into and from which haemolymph may leak. Thawed larvae may therefore have significantly reduced turgor, making them shrunken and flaccid, which seems to increase with the number of freeze/thaw cycles they experience. In addition, fluids from damaged tissues may leak from the larval body via the intestine when thawed, which may cause a loss of body mass. However, the physical appearance of our freezer-killed larvae and the results of our statistical analyses suggest that the loss of mass is not as problematic as the loss of turgor in unblanched larvae. For blanched larvae, change in larval mass and length is less pronounced, likely because blanching stabilises the supporting muscle tissues during storage at −20°C [8,30]. Similar effects were observed in other studies where shrinkage of larvae in preservative solutions (*e.g.* 70 % ethanol) was prevented by first heating the larvae in near-boiling water [26,28,30].

For forensic entomological applications, blanching of blowfly larvae prior to storage in preservative solution is generally recommended to ensure reliable and reproducible larval lengths [20,30,53]. On the other hand, for toxicological applications, storage in organic solution can be unfavourable.

Our results suggest that storage of blanched larvae at −20°C can be applied as a common practice for preservation of fly larvae in forensic entomology, as these larvae showed no significant change in mass and length after storage [26,53].

4.2.3. *Effect of diazepam on larval development*

The effect of diazepam on larval development was assessed by examining differences in length and mass between larvae that were treated with varying concentrations of the drug in their food source. To avoid demonstrated imprecision arising from the killing method on

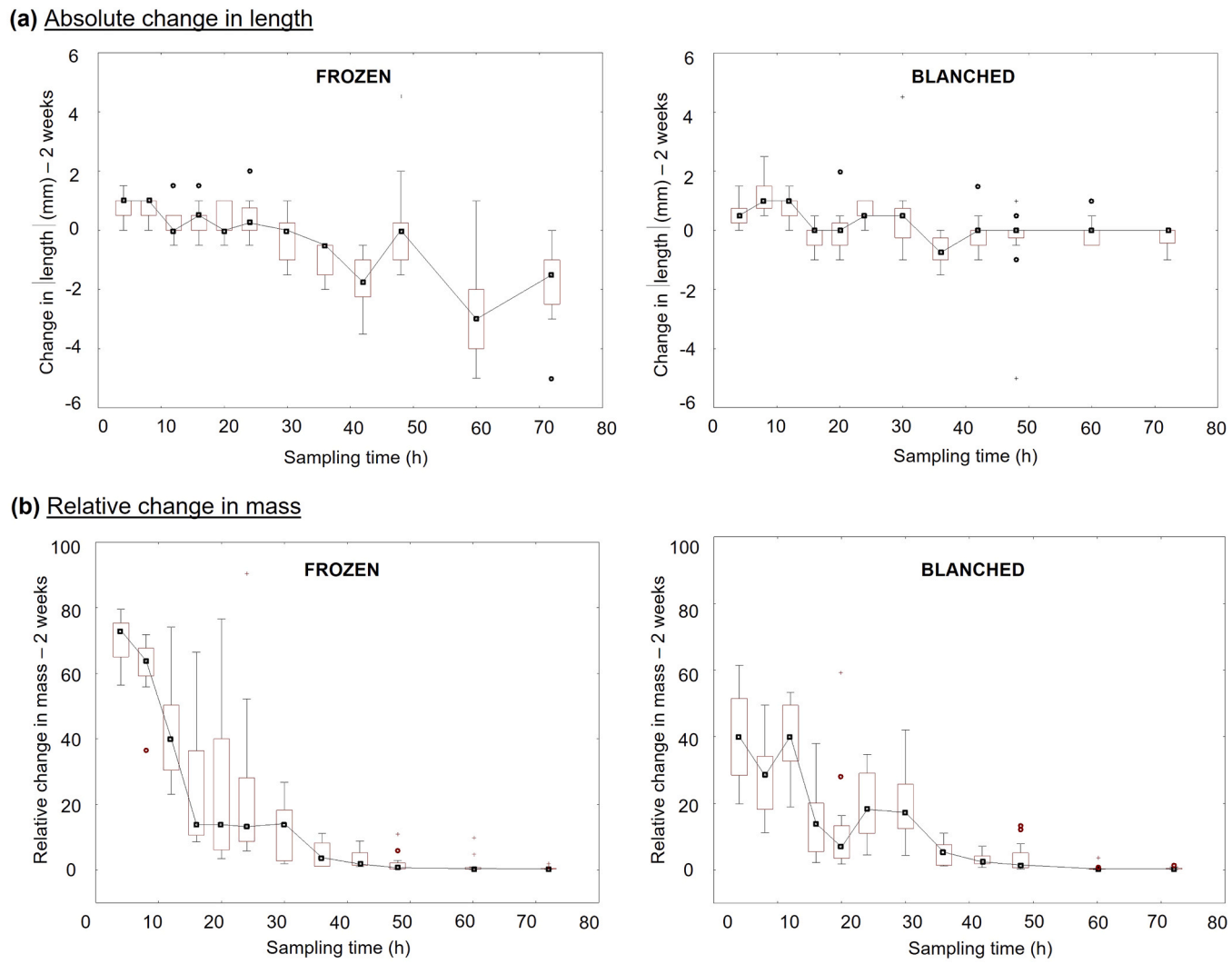

Fig. 5. Box plots showing **(a)** the absolute change in length and **(b)** the relative change in mass of frozen and blanched larvae, respectively, after storage at −20°C for two weeks as a function of sampling time (*i.e.* larval age).

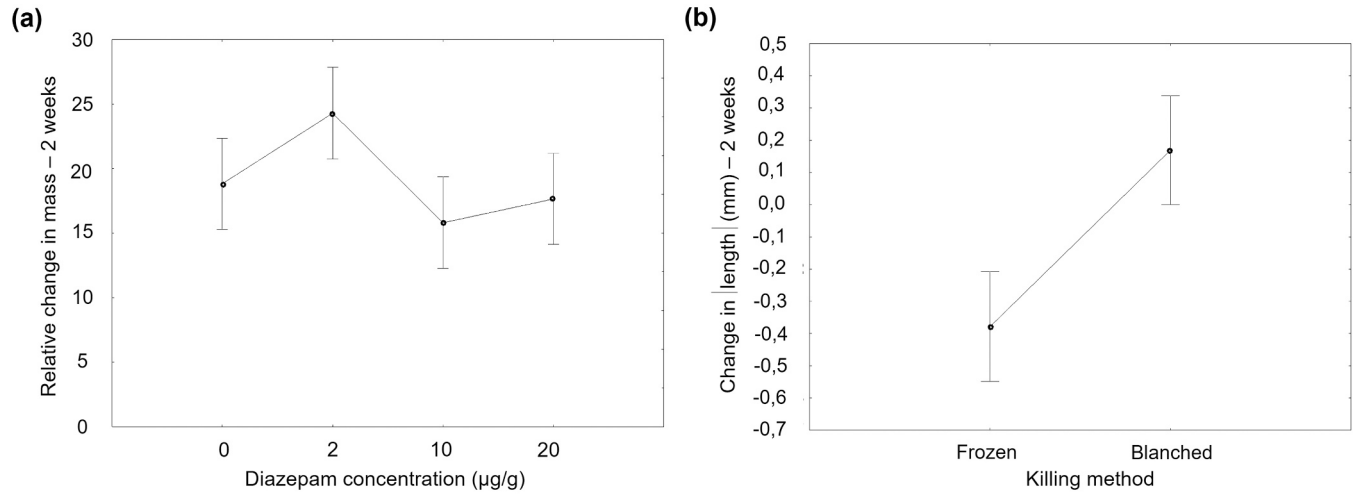

Fig. 6. Relative change in **(a)** larval mass as a function of diazepam concentration and **(b)** larval length as a function of killing method after two weeks at −20°C.

larval length and mass (see Section 4.2.2), larvae that were killed by freezing were excluded from these analyses.

Diazepam concentrations in post-mortem blood from authentic cases typically range from 25 µg/L to 3000 µg/L (≙ 0.025–3 µg/g) [51]. Diazepam concentrations in drug-related fatalities, particularly in solid human tissues like liver and muscle, are not well documented. Older

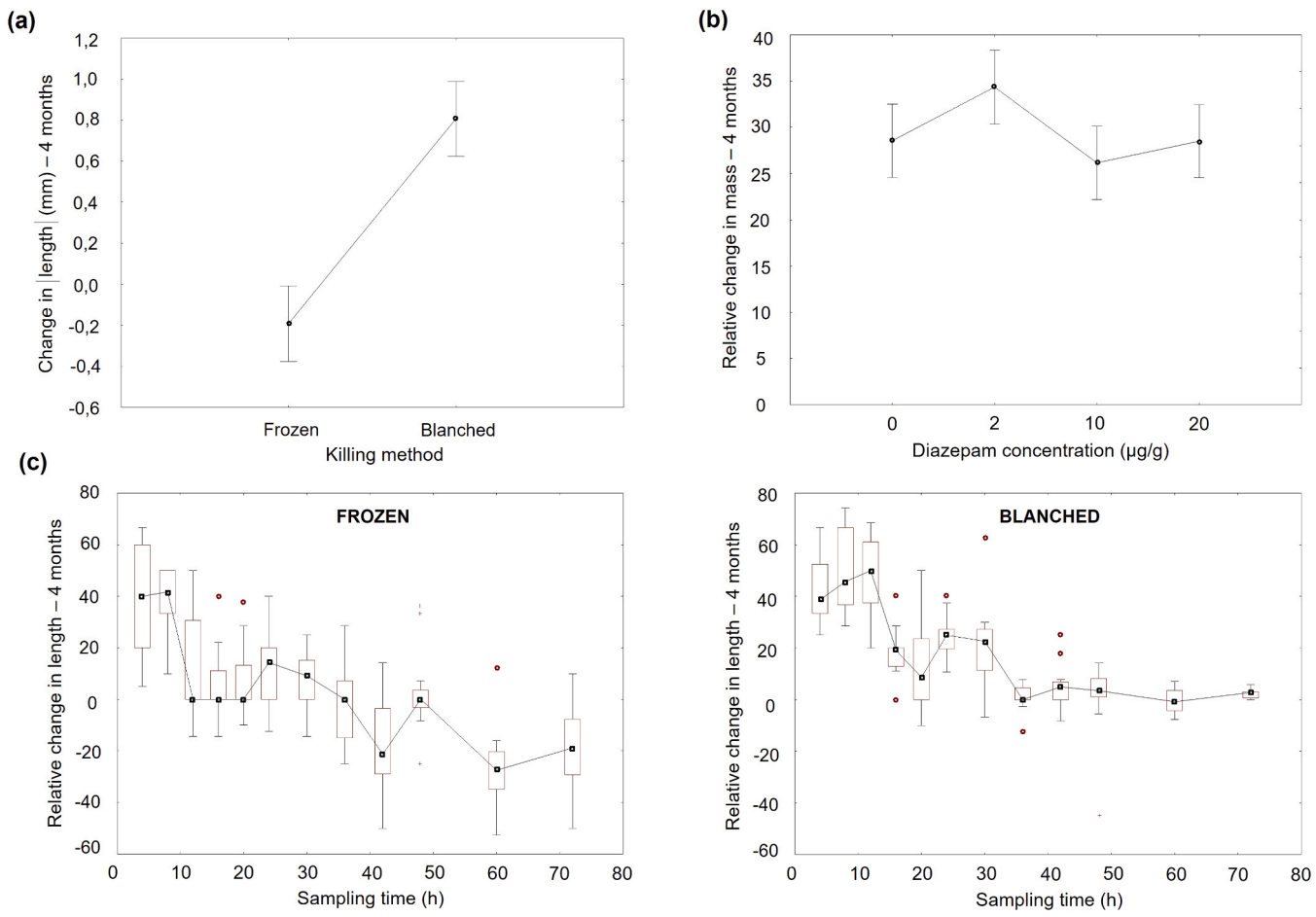

Fig. 7. **(a)** Mean absolute change in larval length as a function of killing method, **(b)** mean absolute change in larval mass as a function of diazepam concentration, and **(c)** box plots, showing the relative change in length as a function of sampling time for frozen and blanched larvae, respectively, after storage at −20°C for four months.

reports of diazepam-related fatalities involved mean blood diazepam concentrations of approximately 20 000 µg/L (≙ 20 µg/g) and liver diazepam concentrations between 1 µg/g and 20 µg/g [44,49,50]. We therefore chose 2, 10, and 20 µg diazepam per gram of food source (*i.e.* 2–20 µg/g) to obtain a concentration range representative of real post-mortem cases.

Our results show that, even in very high doses, diazepam exerts no significant effect on the mass of *L. sericata* larvae (Table S5 in Supporting Information). In contrast, the diazepam dose apparently has a significant effect ($p = 0.009$) on the length of the larvae. Larvae treated with 10 µg/g diazepam were significantly longer than larvae from the two lower doses (*i. e.* 0 µg/g and 2 µg/g) at 48 hours of development, and larvae treated with 2 µg/g were significantly shorter than control larvae at 120 h of development (Fig. 4b). However, when comparing the partial eta-squared-values (Table S6 in Supporting Information) of the other fixed effects in the analysis, *i.e.* sampling time (partial $\eta^2 = 0.944$) and its interaction with dose (partial $\eta^2 = 0.253$), to that of the effect of diazepam dose (partial $\eta^2 = 0.093$) on larval length, it becomes clear that the latter has a weak power. Furthermore, the pattern follows a transient trend (Fig. 4b), so this effect can be viewed as negligible.

Apparently, no developmental studies of *L. sericata* larvae fed with diazepam were published by 2019 [9,15] and we found none more recently, but studies involving other species are summarised in Table 2. The focus of these studies varies with respect to the insect species, the concentration of diazepam, the dietary matrix, killing method, and the reporting of rearing temperatures. It is therefore difficult to make quantitative generalization, but qualitative points of consistency were that larvae fed diazepam grew faster and heavier (Table 2), sometimes with dose-dependency. Although the study involving *Wohlfahrtia nuba* (Wiedemann, 1830) (Diptera: Sarcophagidae) larvae [60] found results consistent with other studies (Table 2), its forensic relevance is unclear because it used approximately 1000-fold more diazepam than other studies [44,49–51]. This outlying dose may also explain the lack of effects of sampling times and diazepam concentrations on larval growth in that study.

A further crucial factor to consider when comparing data from different growth studies is the methodology employed to kill larvae prior to mass and length measurements. In the studies listed in Table 2, in which larval development was monitored by mass and length measurements, the method of killing larvae before measurement was either not specified, larvae were weighed alive, and/or larval length was determined after blanching and preservation in organic solvent. Our findings of the effect of killing method on larval size indicate that a unified, standardised method for killing larvae prior to length and mass measurements during developmental studies is required, because the killing method may significantly alter larval size.

In summary, earlier studies on the effects of drugs on insect development mainly vary concerning the insect species and the experimental set-up, including differences in toxicant concentration, temperature, and killing method. Furthermore, any concentration-dependent effects may be non-monotonic, *e.g.* hysteresis [9,15]. It has also been suggested that different species (some in different families) may respond differently to the same xenobiotic substance [9,15].

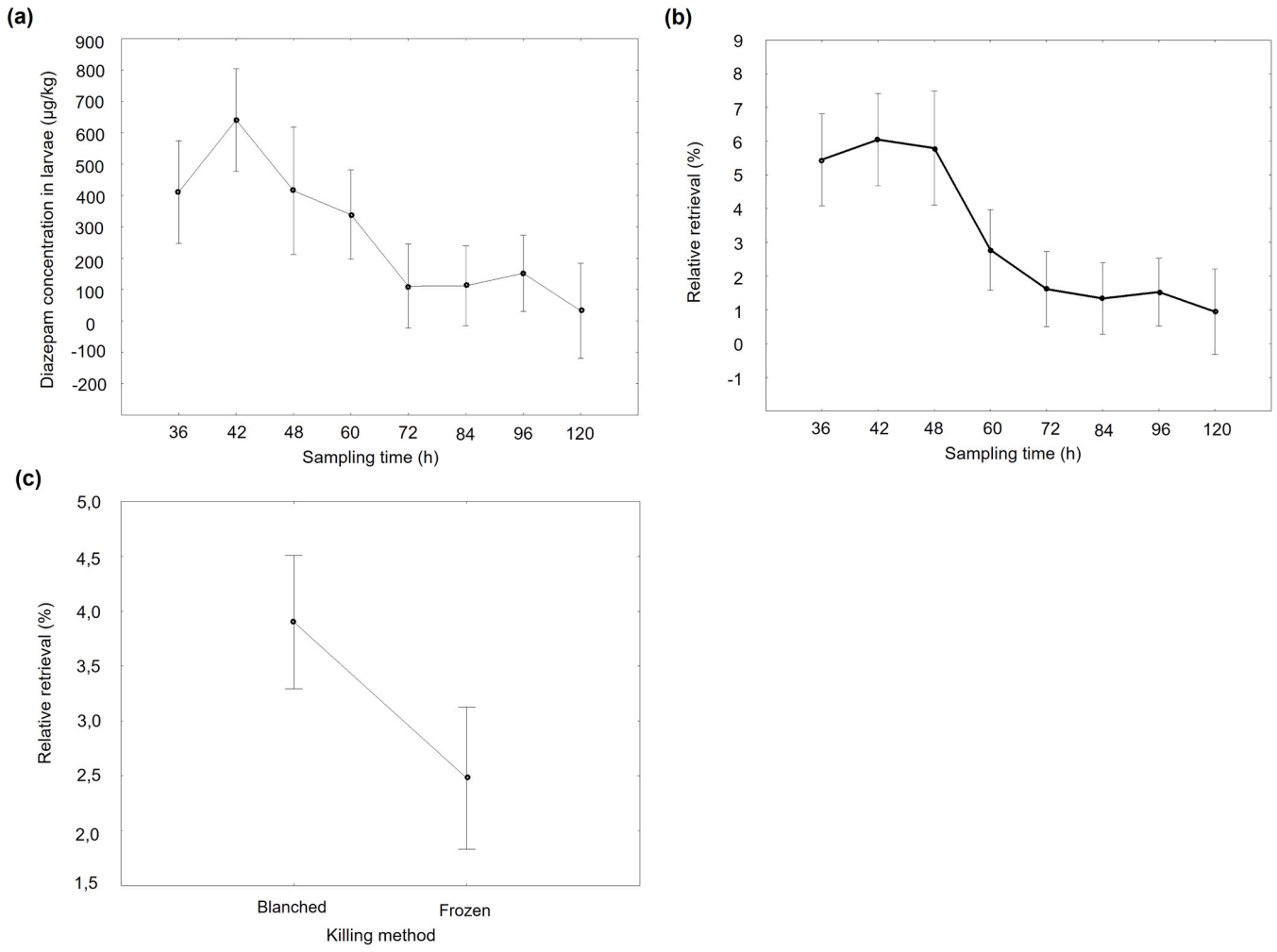

Fig. 8. Effect of sampling time on the **(a)** absolute (µg/kg) and **(b)** relative (%) recovery of diazepam from larvae, and **(c)** the effect of killing method (*i.e.* blanching *vs.* freezing) on the relative (%) recovery of diazepam (killing method had no significant effect on absolute recovery of diazepam).

Table 2
Published studies on effects of diazepam and one study of its metabolite nordazepam on necrophagous fly development (*not spec* = not specified).

Source	Fly species	Drug concentration (µg/g)	Matrix	Temp Killing method	Larval mass or length Larval growth rate
[61]	*Calliphora vicina* (Robineau-Desvoidy, 1830, Diptera: Calliphoridae)	nordazepam 0.5 1.0 2.0	Spiked beef heart	18–24°C Weighed alive; Boiled and conserved in ethanol:acetic acid (75:25) for length measurement	heavier[c] unaltered length unaltered
[62]	*Musca domestica* (Linnaeus, 1758, Diptera: Muscidae)	1.5 2.0 3.0	Spiked diet *not spec*	26.2(±0.5)°C No mass or length measurements	*not spec* faster
[63]	*Chrysomya albiceps* (Wiedemann, 1819) (Diptera: Calliphoridae)	18[a]–25[b]	whole rabbit	30(±4)°C No mass or length measurements	*not spec* 7 h slower
[64]	*Ch. albiceps*	5900	rabbit liver	*not spec* *not spec*	heavier faster
[64]	*Ch. putoria* (Wiedemann, 1819) (Diptera: Calliphoridae)	5900	rabbit liver	*not spec* *not spec*	heavier faster
[60]	*Wohlfahrtia nuba* (Wiedemann, 1830) (Diptera: Sarcophagidae)	10 000 17 000	rabbit liver	24(±1)°C Presumably weighed alive	heavier faster
[65]	*Ch. albiceps*	32000[a]–470000[b]	whole rabbit	Outdoors: 21.8–31.8°C No mass or length measurements	*not spec* faster

[a] The lowest concentration was detected in rabbit skeletal muscle
[b] The highest concentration was detected in rabbit liver
[c] Observed only for larvae from the 1 µg/g treatment and only on days 4 and 6

4.2.4. Effect of diazepam on larval size during storage

As may be expected, the analyses of covariance show that diazepam exposure in the food medium generally did not affect the accuracy of measurements following storage (Table S1 in Supporting Information). When considering all larvae, irrespective of the killing method, relative change in larval mass after storage was significantly higher for larvae from the 2 μg/g treatment compared to those from the 10 μg/g treatment, whereas no significant difference was observed for larvae from the control group. When considering the partial eta-squared (η^2) values (Table S1 in Supporting Information), it is evident that the impact of drug concentration on larval mass is only moderate during intermediate storage (partial $\eta^2 = 0.08$) and small during long-term storage (partial $\eta^2 = 0.02$). The small effect sizes and the absence of a significant difference from control larvae suggest that the effect of diazepam concentration on the relative change in mass during storage can be disregarded.

4.3. Toxicological aspects

4.3.1. Sampling time on diazepam recovery

Toxicological analyses of individual very young larvae of the same age were not feasible due to their low body masses. To obtain the minimum mass required for sample extraction of approximately 100 mg per sample, larvae collected between 4 and 36 h of development were pooled for toxicological analysis. As a result, a detailed evaluation of diazepam pharmacokinetics during the early stages of larval development, *i.e.* a 4-hourly evaluation until 24 h and a 6-hourly evaluation until 36 h of development, was not feasible.

Notwithstanding this limitation, the time at which the larvae were sampled (*i.e.* larval age) had a significant effect on both the absolute and relative recoveries of diazepam from the insects. The highest diazepam concentrations were recovered from larvae sampled at 42 h, after which diazepam concentrations gradually decreased (Figs. 8a, 8b). This suggests that the absorption of diazepam in *L. sericata* at 25℃ is highest relative to its rate of elimination at approximately 42 h of age.

In general, the extent of drug accumulation in a biological system is determined by the relationship between the rates at which the drug is absorbed into bodily fluids and tissues, metabolised by the organism, and excreted from the organism [9]. In the case of necrophagous larvae, age plays a significant role in this process, as larval feeding habits and metabolic activity are highly dependent on the stage of development, which in turn affects the extent of drug accumulation in the body. During the active feeding stage, necrophagous larvae typically ingest substantial quantities of food and therefore also the drugs contained in the food. A significant amount of ingested drug is stored with the food in the crop, rather than in the actual larval tissues [66,67]. The gradual decline in diazepam concentrations after 42 h, which roughly corresponds with the cessation of feeding and the beginning of the post-feeding stage in *L. sericata* larvae at 25℃ [46], is thus likely associated mostly with the gradual emptying of the gastro-intestinal tract once feeding ends. This would explain the significantly lower percentage recoveries from larvae sampled at 60, 72, 84, 96, and 120 h in comparison to younger, actively feeding larvae (Fig. 8b).

The metabolism of diazepam to nordazepam, oxazepam, and temazepam in *L. sericata* larvae in our study (data not shown) undoubtedly contributed to the observed decline in diazepam recovery from the larvae over time after 42 h. However, this process was likely to have a lesser effect than larval feeding rates, as described above. It has been demonstrated that necrophagous larvae, including *L. sericata*, metabolise drugs, resulting in the same hydroxylated and demethylated derivatives that are observed in the human body during metabolism [17,61,68,69]. Drug metabolism in necrophagous insects should thus be considered by forensic toxicologists when interpreting entomotoxicological data from real cases [9,15].

It has long been established that the toxicological analysis of larvae from a corpse would provide little information about the dosage consumed by the deceased [70]. In real forensic cases, the drug quantity detected in a larva depends on the amount consumed and absorbed by the deceased before death, tissue tropism at the feeding site, the decomposition of the body, metabolism, sequestration, and excretion in the larva, and subsequent forensic handling [9]. Most of these variables are also time-sensitive. Our diazepam concentrations in larvae are significantly higher than those from real cases (3,3–6,7 μg/kg), as described by De Aguiar França *et al.* [16], but fall in the same range as was found for its metabolite nordazepam by Tracqui *et al.* [70] (21–770 μg/kg) in real cases and by Wood *et al.* [17] (311–467 μg/kg), who fed 1 μg/g of nordazepam to larvae of *C. vicina*.

4.3.2. Killing method on diazepam recovery

The significant effect observed for killing method on relative recovery of diazepam from larvae (Fig. 8c) suggests that the application of the standard entomological method to kill necrophagous larvae can (sometimes) also be successfully applied during toxicological practices. Blanched larvae yielded significantly higher recoveries of diazepam, even after five months of storage at −20℃.

Larvae intended for drug analysis are typically washed in water at room temperature after sampling to remove any external contaminants, and then frozen [19,61,65,71]. Our observations for diazepam suggest that this procedure may result in loss of analyte from the insects during storage. It is possible that tissue damage and consequent loss of larval contents during freezing (see Section 4.2.2) may also lead to a significant leakage of analyte from larvae. Due to the stabilising effect of hot water on larval muscles, blanching and subsequent storage at −20℃ seems to be a good alternative for optimal drug recovery, at least for diazepam. Furthermore, the use of near-boiling water during blanching also serves as a washing step, thus avoiding an additional step during sampling and storage. The successful application of this method for the entomotoxicological detection of thermolabile drugs requires further investigation.

5. Conclusion

Although forensic entomotoxicology can be seen as the interface between forensic entomology and toxicology, the practices of these two disciplines often differ. With the aim of improving collaboration between forensic entomologists and toxicologists, we investigated whether it would be reasonable to formulate unified methods for killing and preserving necrophagous larvae for forensic entomotoxicological applications.

We have recommended methods that produce preserved larvae that can be used effectively for both PMI estimation and at least some toxicological analyses. Blanching, followed by storage at −20℃, resulted in fly larvae suitable for medium- and long-term storage, PMI estimation, and diazepam detection.

Next, insect size should be measured with equipment with a threshold of precision that is at least an order of magnitude smaller than the smallest specimen.

Finally, our results suggest that even the presence of very high levels of diazepam in a corpse does not necessitate consideration when applying *L. sericata* larvae for PMI_{min} estimations.

CRediT authorship contribution statement

Martin H. Villet: Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Matthias Graw:** Supervision, Resources, Funding acquisition. **Adeyemi D. Adetimehin:** Writing – review & editing, Data curation, Conceptualization. **Susan Rahaus:** Resources, Investigation, Formal analysis. **Irina Schusterbauer:** Investigation, Formal analysis. **Vera Hoeft:** Writing – review & editing, Investigation, Formal analysis. **Alexander Strassberger:** Writing – review & editing, Investigation, Formal analysis. **Olwen C. Groth:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation,

Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2024.112255.

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