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Detection of Gunshot Residues in Puparia of Blowfly (*Chrysomya albiceps*) and the Influence of Time and Firing Modality

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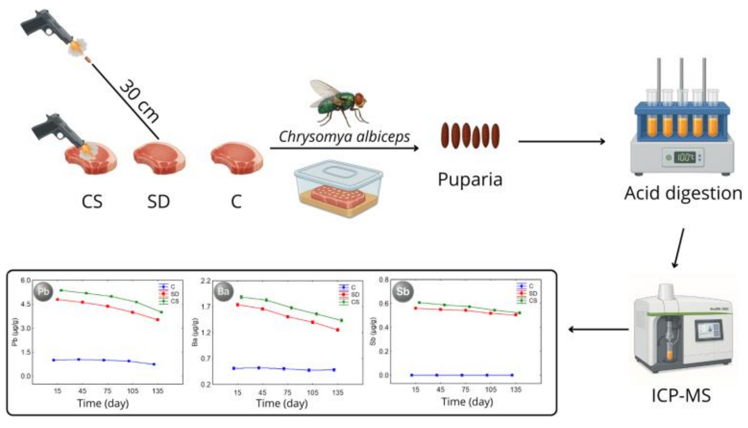
Firearm-related homicides are a global concern, and determining the cause of death in highly decomposed remains a challenge in forensic investigations. This study explores the potential of gunshot residue (GSR) analysis in blowfly (*Chrysomya albiceps*) puparia as a forensic tool, correlating residue levels with firing distance and time since death. Three pork samples were subjected to different shooting conditions: close-range shots, contact shots, and a control (no shots). *C. albiceps* larvae were reared on these samples, and puparia were collected at intervals from 15 to 135 days post-shooting. Inductively coupled plasma mass spectrometry (ICP-MS) analysis revealed significantly higher levels ($p < 0.05$) of lead (Pb), barium (Ba), and antimony (Sb) in puparia from shot samples compared to the control, with contact shots showing the highest concentrations. Additionally, a time-dependent decrease in GSR levels was observed. These findings support the use of forensic entomotoxicology in shooting cases, demonstrating that puparial GSR analysis can help estimate shooting conditions and postmortem interval.

Graphical abstract



Keywords

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1. Introduction

Deaths resulting from firearm projectile injuries are quite frequent worldwide. According to data from the Institute for Health Metrics and Evaluation, it is estimated that in 2016 there were 251,000 deaths caused by firearm discharge, a higher number compared to 1990, when the estimated figure was 209,000 due to the use of this instrument [1]. The same study highlights that Brazil, along with five other countries (the United States of America, Mexico, Colombia, Venezuela, and

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Guatemala), accounted for half of all firearm-related deaths in 2016. More recent data from 2022, published in the 17th Brazilian Yearbook of Public Security (ABSP) [2], indicate that there were 36,300 homicides in Brazil in which a firearm was the instrument used.

Indeed, among international violent deaths, data from the ABSP show that firearms were the most commonly used weapon (76.5%), followed by sharp weapons (15.6%), while the remaining 7.9% involved mainly blunt instruments and asphyxiation [2]. In Mato Grosso do Sul State, Brazil, there was a 0.8% increase in deaths caused by firearms in 2022 compared to data from 2021. In addition, between 2017 and 2022, there was a 196.5% increase in the sale of ammunition in the national market, according to data also published by ABSP [2]. One of the hypotheses for this increase would be the relaxation of public policies for the acquisition of firearms [2].

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In many cases of violent death, there is also an attempt to conceal the bodies. In such instances, when the bodies are found, they are usually located in remote areas with little to no foot traffic, resulting in prolonged exposure before being discovered by authorities. However, determining the cause of death in bodies in an advanced state of decomposition or skeletonization poses a challenge for forensic pathology [3, 4]. In particular, in Mato Grosso do Sul, due to its large territorial extension and low population density (Demographic Census: IBGE 2024), crimes involving the concealment of corpses are more likely to occur.

One of the main challenges faced by forensic experts is that skeletonized bodies lack soft tissues, which severely hinders the determination of the cause of death. Injuries caused by firearms, for example, can no longer be detected [5]. In such cases, radiological techniques can often aid in locating firearm projectiles. However, depending on the type of weapon and ammunition used, the projectile may have passed through the body, making its identification impossible and requiring other methods [4].

Given this, identifying other traces, along with their careful collection and analysis, forms a cornerstone of criminal investigation and can constitute a body of evidence to be used during the judicial process [6]. These traces may be found both near and far from the scene, especially when the site where the body was disposed of does not correspond to the actual crime scene, but rather to the location where the body was discovered [7].

Among biological traces, entomological evidence is important, especially in cases involving bodies in an advanced state of decomposition, as it can help clarify various aspects of a case—for example, assisting in the estimation of the minimum Postmortem Interval (minPMI), as well as the cause of death [8, 9]. Unfortunately, the collection of this type of evidence by forensic experts in Brazil has been neglected due to several factors, such as the lack of standardization, insufficient training of crime scene investigators, and the absence of forensic entomology specialists in the Medical Examiner's Office [10].

Among this entomological evidence, in cases where bodies are in an advanced stage of decomposition, fly puparia can be found months or even years after death [11]. Flies of the order Diptera are considered the most important group of insects in forensic entomology, as they are usually the first to locate the corpse. Shortly after, they begin feeding and then oviposition and/or larviposition, with their immature stages using the body for their development [12, 13]. After the larvae feed and complete the third instar, they leave the body and search for a dry place to pupate, where metamorphosis occurs and adults later emerge, leaving behind only the puparia. These are essentially the "cocoons" of the pupae that remain near the corpses after the adults emerge [8].

Puparia have already been used in the investigation of various causes of death [14-16]. For example, in a case involving the discovery of a mummified woman in her home, drug residues were found in puparial samples near the body, making it possible to infer that the cause of death was likely due to intoxication [14].

When a firearm is discharged while in contact with or at a short distance from a target (up to a maximum of 60 cm), gunshot residues (GSR) become adsorbed in the surface. The greater the distance between the firearm muzzle and the target, the wider the dispersion and the lower the impregnation of residues [17]. Among GSR components, three chemical elements are the most important, as they are present in the primer of the ammunition: lead (Pb), barium (Ba), and antimony (Sb).

Larvae that feed on tissues impregnated with GSR can absorb these three chemical elements, and their concentrations can be determined in pupae and puparia [18-21]. In the study by Roeterdink et al. [16], it was possible to detect gunshot residues (Pb, Ba, and Sb) in post-feeding larvae and pupae of the fly species *Calliphora dubia* (Macquart, 1855), which had fed on meat contaminated with GSR. According to the literature, the simultaneous presence of all three chemical elements in a sample allows us to infer that a firearm was discharged [22-24].

Therefore, although studies are showing that entomological evidence such as puparia can be used to investigate the presence of chemical elements related to the cause of death [14, 16, 25], our literature review did not find any studies that attempted to detect gunshot residues in fly puparia collected a long time after the emergence of the adult. Additionally, no studies were found that compared whether there is variation in the levels of Pb, Ba, and Sb in fly puparia when different shooting modalities are applied (contact shot; close-range shot).

Thus, the objective of this study was to determine the levels of three chemical elements originating from gunshot residues in puparia of the fly species *Chrysomya albiceps* (Wiedemann, 1819) kept in a controlled environment over a time window, and to compare the results according to the shooting distance.

2. Results and Discussion

According to our results, the levels of Pb, Ba, and Sb in the puparial samples from larvae that consumed meat shot at close range and at point-blank range were significantly higher than in the puparial samples from larvae that consumed unshot meat ($p<0.05$) (Figure 1). The presence of the detected elements in the puparia can be explained by the fact that part of the material ingested by the fly larvae becomes incorporated into the cuticle or deposited on the external surface, since they are in direct contact with the feeding substrate [27]. In fact, Lagoo et al. [18], using the same analytical methodology as the present study, also detected the same elements in larvae and pupae of the fly species *Lucilia sericata* (Meigen, 1826), which developed on pieces of meat shot with 9mm pistol ammunition; however, in that case, the authors did not analyze the puparia.

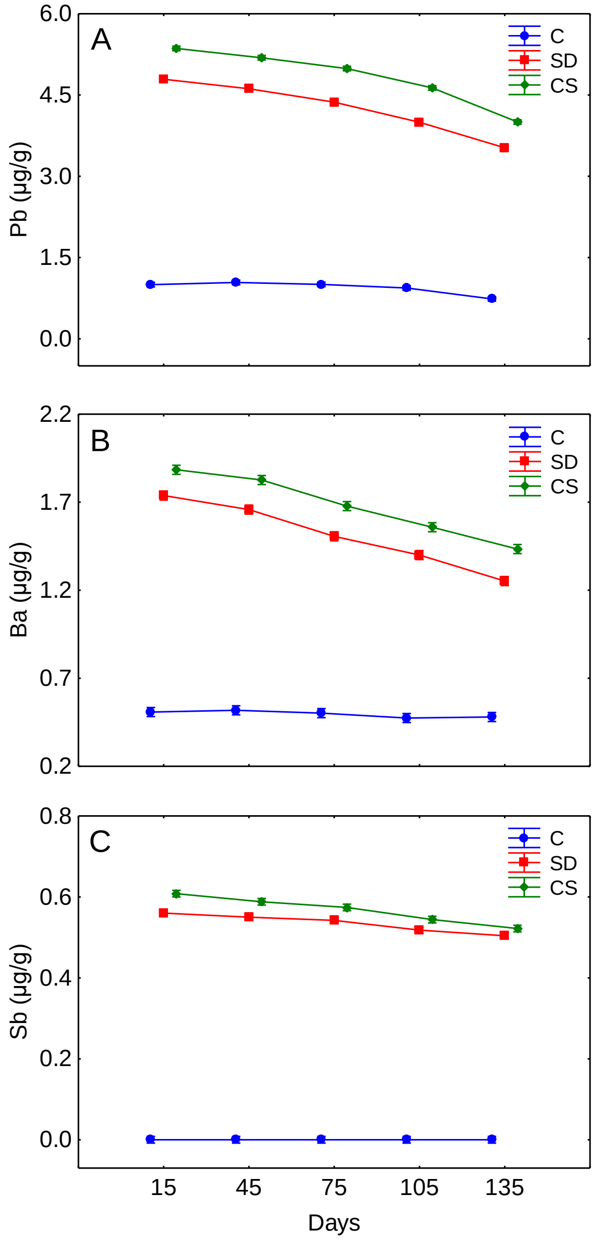

Fig. 1. Mean and confidence intervals of Pb (A), Ba (B), and Sb (C) levels in *C. albiceps* puparia across the control (C), short-distance shot (SD), and contact shot (CS) groups and at the five times (15, 45, 75, 105, and 135 days).

On the other hand, according to Simon et al. [27], during the insect life cycle, part of the metals ingested from a contaminated substrate can be excreted through the meconium during metamorphosis, while another portion may become incorporated into the cuticle, which corresponds to the puparium after adult emergence. Depending on the fly species and the metal analyzed, in some cases the concentration of the chemical element is higher in the larva and the puparium than in the adult [28], since detoxification mechanisms (such as excretion and accumulation in the cuticle) result in lower contaminant levels in the final stage of metamorphosis (the adult). This fact reinforces the importance of using dipteran puparia in the context of entomotoxicology, as they may be the only entomological evidence remaining near a decomposed body and the only means of determining the cause of death.

Significant differences ($p<0.05$) were also observed between the levels of chemical elements in SD and CS, with higher values in the samples collected from the piece of meat that was shot at point-blank range. When a firearm is discharged with the muzzle in contact with a target, there is a greater deposition of secondary elements from the shot [29]. As the distance between the firearm and the target increases, there is a progressive decrease in the concentration of secondary elements reaching the target, and beyond 70 cm, only the projectile reaches it [30]. Merli et al. [31] collected samples from the skin of pigs around gunshot wounds caused by a Beretta 9 mm semi-automatic pistol fired from different distances, and found that the levels of chemical elements decreased as the distance between the firearm and the pig's skin increased.

The element with the highest concentration in the pulverized samples was Pb, followed by Ba and then Sb (Figure 1). This result is consistent with the data reported by Dalby et al. [32], as well as by Costa et al. [21], Bessa et al. [33], Motta et al. [24], and Roeterdink et al. [16], all of whom found these elements in the same order of concentration as observed here, though in larval and pupal samples. Specifically, Costa et al. [21], investigating two real cases involving the discovery of bodies in an advanced stage of decomposition, quantified Sb in fly larvae in only one of the cases. In the other case, the element was below the detection limit. This led the authors to conclude that it was not possible to confirm whether the body in the second case had been shot at close range or point-blank.

This finding supports our results, as Sb was also below the detection limit in the samples from the control group. The absence of detection in this group may also be explained by the possibility that the meat sample came from an animal that had not lived in an environment contaminated by this chemical element. Studies conducted in agricultural regions near industrial or mining areas have shown soil and plant contamination by Sb, and consequently, the risk of its accumulation in animals and humans that consume these plants as part of their diet [34-35].

It should also be emphasized that the levels of chemical elements observed in the larvae may be related to the insect species used in the study. Abd Rashid et al. [18], studying *Chrysomya megacephala* (Fabricius, 1794) larvae that fed on meat contaminated with GSR, found higher levels of Ba (1.18 µg g^{-1}) compared to Pb (0.5 µg g^{-1}), which differs from our results. The authors suggest that the elimination of Ba is slower than that of Pb in *C. megacephala*, because Ba is similar to Ca, which plays an essential role in larval development.

To explain the higher concentration of Pb compared to the other elements, it is important to consider that the most common projectiles are composed solely of this metal [36]. However, at the moment of firing, there is intense friction between the projectile and the inner surface of the barrel, resulting in a large amount of Pb being expelled through the muzzle. When analyzing the levels of the three chemical elements in the primers of 38 caliber revolver ammunition, the same authors observed that Sb had the lowest concentration. This fact explains why the level of this element is lower than that of Pb and Ba.

It is important to highlight that the elements can be detected because they are present due to contamination throughout the pig's life [18, 37]. In fact, the literature includes studies that discuss the presence of these elements in pork. Zhang et al. [38] measured the levels of four chemical elements, including Pb, in pork samples in China and found that Pb levels were higher than those of the other elements analyzed. Other studies, such as those by Bilandžić et al. [39] and Song et al. [40], also reported the presence of this element in pork and beef sold in markets and on farms. This is a consequence of environmental contamination, mainly due to anthropogenic activities.

During the period of our study, over the days analyzed, the levels of all three elements in the SD and CS groups showed a decreasing trend (Figure 1). This decrease occurred progressively; however, even the last puparial samples collected (Day 135) did not show concentrations below the detection limit. We can infer that at a certain point during the skeletonization phase, the residue levels in the puparia may no longer contribute to determining gunshot occurred in a real case.

On the other hand, Roeterdink et al. [16] did not find significant concentrations of gunshot-related chemical elements in puparia of the fly species *Calliphora dubia* (Macquart, 1855), using a methodology and sampling design similar to that of our study. A possible explanation for this is that we analyzed a greater number of puparia and at different time points after adult emergence, which may have increased the detection capacity for these elements. However, we must also consider the hypothesis that *C. dubia* metabolizes these chemical elements differently, to the extent that they are not found in significant concentrations in the puparia [27-28].

It is worth noting that the levels of the three analyzed chemical elements were detected in the puparia from the CS and SD groups collected 135 days after the start of the experiment. Considering that puparia may be the only entomological evidence found on a skeletonized body [41], we emphasize the importance of these results in contributing to the determination of the cause of death.

3. Material and Methods

3.1 Rearing and Collection of Specimens

Adult flies of the species *C. albiceps* were collected on the campus of the State University of Mato Grosso do Sul, in the municipality of Dourados, Brazil (22º 11' S, 54º 55' W), during the first half of September 2021. The adult flies were attracted using decomposing meat (offal and pieces of beef) exposed outdoors in a plastic tray measuring 65 x 30 cm and captured by active collection using an entomological net. The collected flies were placed in an insect-rearing cage measuring 40 cm³, with a wooden frame and base, and sides and top covered with mesh. They were then transferred to the Forensic Research Center (NUPEFO) at the UEMS campus in Dourados, MS, Brazil, for subsequent sorting and identification.

For species identification, the identification keys proposed by Carvalho and Mello-Patiu [42] and Carvalho and Ribeiro [43] were used. To facilitate identification, the flies were placed in a freezer at a low temperature for 1 minute to anesthetize them, thus making handling easier for identification under a stereomicroscope. Specimens of *C. albiceps* were then placed in a BOD incubator model MA 415 (Marconi, Brazil) with controlled temperature at 25 ± 1ºC and a photoperiod of 12:12 (L:D) hours. The insects were kept in a rearing cage for mating, following the methodology with adaptations from Paula et al. [44]. For the maturation of the ovarian follicles, bovine liver was offered for 5 days [45], and the insects were continuously fed with sugar and water. For female oviposition, 50 grams of ground beef was offered after 5 days. We decided not to use eggs from the first generation because we needed a large number of ovipositions, and since these flies were wild, we could not rule out the possibility that they had already oviposited in their natural habitat.

After oviposition, 0.05 g of eggs were collected using a fine brush and deposited onto 200 grams of ground beef placed in a 350 mL plastic container, which was then placed in the center of a larger 500 mL container filled with sawdust as a pupation substrate to obtain adults of the first generation. Immediately after adult emergence, thirty flies, 15 males and 15 females, were placed in a rearing cage with the same characteristics described above for mating and ovarian maturation. Eggs from this second generation were collected using a fine brush, divided into 3 batches with the same mass (0.3 g) to be used in the subsequent phase of the experiment with pieces of pork meat.

3.2 Experiment on Pieces of Pork Meat

Three pieces of pork meat, with an average mass of 1.149 ± 35 g each, were used in the experiment and taken to the ballistics sector of the Regional Unit of Forensics and Identification of Dourados city, Mato Grosso do Sul, Brazil (URPI-Dourados). On one of the pieces, three gunshots were fired from a distance of 30 cm from the surface of the meat; this group was designated the "short-distance shot" treatment (SD). On another piece, three shots were fired with the gun barrel pressed directly against the meat, designated as the "contact shot" treatment (CS). The third piece received no shots and was considered the control (C). The firearm used was a Taurus revolver with .38 caliber ammunition manufactured by Companhia Brasileira de Cartuchos. The choice of firearm and ammunition type was based on the fact that this type of weapon is more commonly used by police and criminals [35, 46].

Each piece of meat was individually placed in a plastic container measuring 40 x 25 cm, with a lid that had a voile-type fabric opening to allow airflow. The bottom of each container was covered with a layer of approximately 1.0 cm of sawdust to allow pupation of post-feeding larvae. The batches of eggs collected in the previous step (0.3 g) were deposited onto each piece of meat, maintaining the same ratio between the mass of eggs and the meat used in the previous phase. The plastic containers were then placed in a BOD incubator model MA 415, at a constant temperature of 25 ± 1 ºC and a photoperiod of 12 hours light and 12 hours dark.

After hatching, the larvae fed exclusively on the pieces of pork meat and developed until the post-feeding stage, at which point they left the meat in search of sawdust for pupation. After six days, adult flies emerged, leaving empty puparia in the sawdust.

To evaluate whether it is possible to detect gunshot residues, 30 puparia were collected from each container (SD, CS, and C), initially 15 days after the start of the experiment, corresponding to 72 hours after adult emergence. From this initial collection, samples of 30 puparia from each plastic container were collected every 30 days, with the last collection performed on day 135. All collected puparia were immediately frozen.

3.3 Sample Preparation and Analysis

To obtain samples for analysis by inductively coupled plasma mass spectrometry (ICP-MS), the frozen puparia were ground using a mortar and pestle. After preliminary tests, a mass of 350 mg of puparia was standardized to obtain better readings. The method used was adapted from Costa et al. [21]. The 350 mg of puparia was added to 4.0 mL of concentrated HNO_3, previously purified, and 3.0 mL of 30% P.A. H_2O_2. The heating program consisted of a 10-minute ramp, with a 5-minute dwell time at 100°C. Subsequently, using ultrapure water, the samples were brought to a final volume of 10.0 mL.

For the ICP-MS analyses, a NexIon 300D instrument (PerkinElmer, Brazil) was used. Operational conditions were: plasma gas flow rate of 16 L min^{-1}, nebulizer gas flow rate of 0.96 L min^{-1}, auxiliary gas flow rate of 1 L min^{-1}, RF power of 1350 W, and isotopes ^{121}Sb, ^{138}Ba, ^{208}Pb. The analyses were performed in quintuplicate.

The analytical curve was constructed with eight points, with concentrations ranging from 0 to 10 μg L^{-1}. To evaluate the elements, the limit of detection (LOD) and limit of quantification (LOQ) presented in Table 1 were used.

Table 1. Limits of Detection (LOD) and Limits of Quantification (LOQ) in ng L^{-1} for the chemical elements.

	Pb	Ba	Sb
LOD	0.093	0.219	0.105
LOQ	0.310	0.730	0.350

3.4 Statistical Analysis

The influence of the three treatments (CS, SD, and C) was evaluated with the levels of chemical elements in the fly puparia samples, using a factorial Analysis of Variance (ANOVA), respecting the assumptions of this method. Subsequently, the Tukey test was applied after confirming a statistically significant effect of any factor. For statistical analyses, the software Statistica 14 [47] was used, considering a significance level of 0.05.

4. Conclusions

The levels of Pb, Ba, and Sb were higher in that fed on meat containing firearm discharge residues compared to the control group. Pb showed the highest concentration among the samples, followed by Ba and then Sb, with the latter not detected in the control group. The highest concentrations were found in the CS group (contact shot) compared to the SD group (short-distance shot).

These levels progressively decreased over time in both the CS and SD groups. Further studies with older pupae will help determine the point at which the quantification limits of these elements are reached, thereby establishing an appropriate time frame for pupae collection in real cases, providing valuable support in criminal investigations.

Our results may support the adoption of protocols in forensic units recommending the collection and analysis of pupae at human remains recovery sites. A limitation for applying this type of analysis would be in cases of using non-toxic ammunition, as the chemical elements Pb, Ba and Sb do not make up the primer.

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Author Contributions

GVG = Conceptualization, Methodology, Investigation, and Writing - Original Draft; TLAC = Visualization, and Writing - Review & Editing; MCPS = Methodology, and Writing - Review & Editing; SELJ = Formal analysis, and Visualization; WFAJ = Methodology, Funding acquisition, and Writing - Review & Editing; CALC = Validation, Investigation, Resources, and Writing - Review & Editing.

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