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Immunoassay detection of fly artifacts produced by several species of necrophagous flies following feeding on human blood

David B. Rivers [a, b, *], Gregory Cavanagh [a], Valerie Greisman [a, b], Andrew McGregor [c], Rebecca Brogan [a], Andrew Schoeffield [a]

[a] Department of Biology, Loyola University Maryland, 4501 North Charles Street, Baltimore, MD, 21210, USA
[b] Forensic Studies Program, Loyola University Maryland, 4501 North Charles Street, Baltimore, MD, 21210, USA
[c] Department of Forensic Sciences, School of Graduate and Professional Studies, Stevenson University, Owings Mills, MD, 21117, USA

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ABSTRACT

Foraging behavior of necrophagous flies commonly leads to distortion of human bloodstains and production of artifacts that confound reconstruction efforts at crime scenes. Currently there is no reliable method for detection of fly-derived stains or distinction of the artifacts from human bloodstains. To overcome these deficiencies, a confirmatory test was developed based on immunological detection of cathepsin D found in digestive fluids of *Musca domestica* and *Protophormia terraenovae*. Anti-serum (anti-md3 serum) was generated toward a 17-amino acid synthetic peptide based upon predicted antigenic amino acid sequences for the propeptide and mature enzyme of cathepsin D proteinase from larvae of *M. domestica*. The serum was used to test the hypothesis that digestive artifacts produced by an array of necrophagous flies associated with human decomposition could be detected with the immunoassay. Anti-md3 serum was able to bind artifacts from 27 species of flies representing 9 families. The antiserum reacted with both regurgitate and defecatory stains, but not transfer patterns. Stains from 4 fly species displayed no reactivity with anti-serum in dot blot assays. Anti-md3 serum did not bind to either human or bovine blood stains on filter paper. However, when both types of blood were spiked with synthetic md3 peptide the antiserum was able to bind. Dot blot assays displayed positive reactions with stains produced from larvae and teneral adults of *Sarcophaga bullata*, and with artifacts as old as 7-years after deposition. These observations indicate that the immunoassay permits distinction of artifacts from a wide range of species from human bloodstains, from multiple development stages, and from artifacts that remain at crime scenes for many months to years after deposition. Further work is needed to determine whether the detection of fly artifacts using the antiserum is suitable for non-laboratory conditions.

1. Introduction

The activity of necrophilous insects on human remains has the potential to confound criminal investigations. This is best illustrated by the foraging behavior of several species of calyptrate and non-calyptrate Diptera, which are attracted to human remains, body fluids, feces, and/or saturated substrate under the corpse. During foraging, adult flies walk across the surface of a corpse or through wet body fluids, using gustatory receptors located on tarsi and sponging mouthparts to assess the nutritional value of the fluids and tissues [1]. Applying Locard's Exchange Principle to the interaction between the flies and human remains, evidence of this association will be left behind at the crime scene [2]. For instance, foraging activity is known to cause mechanical disruption of pooled blood and body fluid stains, regardless of whether the fluids are wet or dry [3–5]. Flies can also produce transfer patterns, created by tarsi or other body parts leaving impressions after passing through wet fluids, either at the primary scene or at other sites [6,7]. As adult flies consume human tissues and fluids, they regurgitate and defecate some of the ingested food onto surfaces at or near the crime scene, leading to intermixing of fly artifacts with bloodstains and other human body fluids [6,8]. Artifacts are not restricted to the primary crime scene, as flies display positive phototaxis, and thus

* Corresponding author. Department of Biology, Loyola University Maryland, 4501 North Charles Street, Baltimore, MD 21210, USA.
E-mail address: drivers@loyola.edu (D.B. Rivers).

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are attracted to natural (i.e., windows and doors) and artificial (lamps and ceiling lights) light sources, as well as sources of food (e.g., kitchen), locations in which wet blood may be transferred or artifacts deposited. In essence, false secondary crime scenes are established as a direct consequence of foraging activity on a corpse.

The issues with fly artifacts are magnified by the fact that stains from regurgitation and fecal elimination are virtually indistinguishable from human bloodstains. Fly stains are morphologically very similar in terms of shape, color and size to impact (i.e., forward, back, and mist-like spatter), projected, sneezed, and expirated bloodstains [4], and cannot be reliably distinguished using presumptive or confirmatory tests available for identification of human blood [8,9]. Molecular methods, namely those relying on DNA analysis, are also not effective at providing separation of insect from human stains since DNA profiles can be obtained of an individual from blood consumed by flies [10–12]. Techniques relying on morphological attributes of artifacts and alternate lighting have been reported to be useful in differentiating fly artifacts from human bloodstains [3,4,8,13], but all have limitations that prevent each from being consistently reliable for use in crime scene investigations. For example, one method relies on calculating the ratio of the length of stain tail to the length of stain body, which if greater than one, supposedly excludes bloodstains. The underlying premise that a tail length to body ratio exceeding one excludes all forms of human bloodstains is not correct [8,14,15]. For several fly species, tails are commonly absent from defecatory spots, yielding fecal and regurgitate stains that are indistinguishable from each other [4,7,13]. In reality, artifacts are highly variable in morphology due to unique species behaviors, size of blood meals, and time taken to consume the meal, as well as being dependent on the physical surfaces on which they have been deposited [11].

The recent development of an immunoassay that specifically recognizes fly artifacts offers promise as a confirmatory test to distinguish insect from human bloodstains [16]. Anti-serum (anti-md3 serum) generated toward a unique cathepsin D proteinase in the adult foregut of *Protophormia terraenovae* Robineau-Desvoidy (Diptera: Calliphoridae) reacts with both regurgitate and defecatory stains, however is unable to distinguish between the two types of artifacts [16]. In contrast, dot blot assays demonstrated that antiserum did not bind to transfer patterns (i.e., translocation or tarsal tracks) produced by adult flies in dot blot assays. These observations were not surprising, as anti-md3 serum was predicted to only react with artifacts derived from the digestive tract of *P. terraenovae*, and hence contained cathepsin D proteinase. Importantly, antisera did not react with any type of mammalian blood tested alone [16]. Thus, anti-md3 serum demonstrated a high degree of specificity for fluids/stains containing cathepsin D, and as such offers the potential to be used as a diagnostic tool to recognize fly artifacts present at crime scenes.

The objectives of this study were to evaluate whether the immunoassay could detect regurgitate and defecatory stains from a greater number of forensically important fly species, to test the limits of artifact detection by examining stains produced by multiple fly development stages, and to determine if stains produced by non-carrion insects would react with antiserum, all with the intent to broaden the applicability of the method for use at crime scenes. Additional assays were conducted to test the age limitation of detection with the antiserum by using artifacts that were 3–7-years old from seven species of flies.

2. Materials and methods

2.1. Insect collection and rearing

Unless otherwise indicated, flies were field collected as larvae or adults from decaying piglets placed in galvanized steel animal cages (60 cm × 60 cm x 90 cm) located in Baltimore, Maryland. (47.306732, 4.260684) and Glen Rock, Pennsylvania (39.791745, −76.730040) USA. Piglets were stillbirths provided by the United States Department of Agriculture, Beltsville, MD and weighed 0.68–1.1 kg each. Adult flies were used immediately for artifact collection, while larvae were reared on fresh beef liver (liver was placed on sand in open plastic containers [30 cm × 20 cm x 10 cm]) throughout development at 25 °C, 70–75% RH under a long-day (LD 15:9 h) photoperiod in environmental chambers (Model 1-30BL, Percival Scientific, Boone, IA). Collections occurred over a two-year period to obtain fauna from different seasons (i.e., spring, summer and fall). Adults of *Megaselia scalaris* Loew (Diptera: Phoridae) and *Clogmia albipunctata* (Williston)(Diptera: Psychodidae) were collected inside the Donnelly Science Building at Loyola University Maryland, Baltimore, MD. Species identifications were made using the identification keys of Huckett and Vockeroth [17], Marshall et al. [18], Quate and Vockeroth [19], Rochefort et al. [20], Shewell [21], Wheeler [22], and Whitworth [23]. Voucher specimens of all species are maintained in the Department of Biology, Loyola University Maryland, Baltimore, MD. Identifications occurred after the flies were first used for artifact collections.

Pupae of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) and *Protophormia terraenovae* were purchased from Carolina Biological Supply Company (Raleigh, North Carolina) and Fork Tree Ranch (Bonners Ferry, Idaho), respectively. Pupae of *Phormia regina* Meigen (Diptera: Calliphoridae) were kindly provided by Dr. John Stoffolano Jr. (University of Massachusetts-Amherst), and *Chrysomya megacephala* (F.) and *Chrysomya rufifacies* (Macquart)(Calliphoridae) were provided by Dr. Jeffrey Tomberlin (Texas A & M University). Laboratory colonies of *Lucilia illustris* Meigen, *Lucilia sericata* (Meigen), *Lucilia silvarum* (Meigen), *P. regina, P. terraenovae, Calliphora vicina* Robineau-Desvoidy, *Ch. megacephala, Ch. rufifacies, Cynomya cadaverina* Robineau-Desvoidy, all from the family Calliphoridae, and *Sarcophaga bullata* (Sarcophagidae) were maintained as detailed by Denlinger [24]. Adults of all species were reared in wire mesh cages (30 cm × 30 cm x 30 cm) at 25 °C, 70–75% RH under a long-day (LD 15:9 h) photoperiod and fed beef liver and sugar cubes *ad libitum*. Larvae were fed fresh beef liver throughout development under the same conditions as adults in environmental chambers. A colony of *Musca domestica* L. (Diptera: Muscidae) was maintained as described by Rivers [25] under the same conditions detailed for the other fly species.

Nymphs and adults of *Gromphadorrhina portentosa* were purchased from Fluker Farms (Port Allen, Louisiana USA). Cockroaches were maintained in a 10-gallon glass aquarium containing 1 in of wood shavings, water and rat food pellets (Purina Rat Chow, Camanche, Iowa USA). A small colony of *Cimex lectularius* was kindly provided by Dr. Harold Harlan (LTC-Ret, U.S. Army Entomologist) and used immediately to collect fecal artifacts after blood feeding. The colony was maintained for only one generation.

2.2. Collection of fly artifacts

Artifacts were collected from adult flies essentially as described by Rivers et al. [16]. For laboratory reared species, one hundred puparia were transferred to wire mesh cages (30 cm × 30 cm x 30 cm) and maintained as described for adult fly colonies. Fly artifacts were collected 4–7 days after adult emergence from puparia at 25 °C to ensure that meconium from newly emerged flies did not contaminate fly artifacts. Collection of artifacts relied on placement of a single filter paper (Whatman™ No. 4 qualitative disc filter paper [110 mm Ø, GE Healthcare, Buckinghamshire, U.K.]), hung vertically in the center of the cage using a plastic zip tie, so that the

top edge of the paper was 2.5 inches from the inner cage surface (4). Filter paper and zip ties were handled at all times with nitrile gloves to prevent contamination. Following fly exposure to filter paper, each disc was removed from the cage and placed in a plastic zip lock bag and stored in total darkness at 25 °C until dot blot analysis. Preliminary dot blot testing of artifacts on filter paper stored in plain paper bags versus zip lock bags revealed no differences in antiserum reactivity due to method of storage. Analysis of artifacts typically occurred less than 48 h (24–36 h) after artifact deposition. A subset of the collected artifacts was stored for 3, 5, and 7 years at 25 °C in total darkness to test age effects on antiserum binding of artifacts.

Field collected adult flies were immediately placed in wire mesh cages set up for artifact collection and processed as described for laboratory raised flies. The exceptions to this method were for small fly species that could pass through the wire mesh. In those instances, individual flies were placed in petri dishes (100 mm × 15 mm, non-pyrogenic polystyrene, Corning, NY) lined with filter paper (Whatman™ No. 4 qualitative disc filter paper, 110 mm Ø) and with a small plastic weigh boat (1 in x 1 in) containing 1 ml human blood placed on top of the filter paper.

2.3. Collection of larval stains and meconium

To test whether anti-md3 serum would react with larval secretions, *S. bullata* were used to collect larval stains. Larvae were reared on beef liver at 25 °C as described for laboratory colonies and collected as mid third instars. Larval age was estimated based on examination of posterior spiracles using a stereo-dissecting microscope (Zeiss Stemi 2000, Göttingen, Germany) and from measurements of larval weight: mid third stage larvae weighed between 230 and 260 mg [26,27]. The larvae were rinsed in sterile Dulbecco's phosphate buffered saline (w/o $CaCl_2$)(Millipore-Sigma, St. Louis, MO USA), weighed (Mettler Toledo AG204 DeltaRange, Columbus, OH), blotted dry on paper towels, placed individually in a petri dish (100 mm × 15 mm) lined with filter paper, and then maintained at 25 °C for 1 h. Secretion stains/trails were observable within a few minutes of introduction of larvae into petri dishes. A total of 10 larvae were used to collect stains. Following fly exposure to filter paper, each disc was removed from the petri dish and placed individually into a plastic zip lock bag and stored in total darkness at 25 °C until dot blot analysis.

Meconium was collected similarly to artifact collection from adult flies. One hundred puparia of *S. bullata* were transferred to a wire mesh cage lacking water and sugar cubes. Upon adult emergence, meconium was collected on filter paper centered on the bottom of the cage. Following fly exposure to filter paper, the disc was removed from the cage and placed in a plastic zip lock bag and stored in total darkness at 25 °C until dot blot analysis. The experiment was replicated three times using 100 flies per replicate.

2.4. Collection of artifacts from non-carrion species

To determine if anti-md3 serum would react with artifacts from non-carrion insects, artifacts collected from an omnivorous (*G. portentosa*) and hematophagous species (*C. lectularius*) were subjected to immunoassays. Artifacts were collected from adult hissing cockroaches as described for adult flies with the exception that filter paper was placed in the bottom of 10-gallon aquarium. Ten adult cockroaches were placed together in the aquarium with 3 ml of human blood and allowed to feed *ad libitum* over 24 h at 25 °C with a 15:9 h light: dark photoperiod. Artifacts were collected from recently blood-fed *C. lectularius* by placing filter paper strips (0.5 in x 4 in) into the colony for 24 h at 25 °C in total darkness. Following insect exposure to filter paper, each disc or strip was removed and placed individually into a plastic zip lock bag and stored in total darkness at 25 °C until dot blot analysis.

2.5. Generation of antiserum

Antiserum was produced as described in Rivers et al. [16]. In brief, a 17-amino acid synthetic peptide was constructed (Peptide 2.0, Chantilly, VA, USA) based on the amino acid sequence data for the propeptide and mature enzyme of cathepsin D proteinase from larvae of *M. domestica* [28]. The sequence data reported by Padilha et al. [28]. for ppCAD 3 (Accession # ABL84270) for the mature enzyme in larval midguts of *M. domestica* revealed that cyclorrhaphous flies possess a cathepsin D proteinase that lacks a proline loop (of motif DxPxPx (G/A)P). The synthetic peptide (md3) used for this study was predicted to have high antigenic properties for antibody production, and the resulting antiserum (anti-md3 serum) was shown to have a high degree of specificity for regurgitate and defecatory stains produced by *P. terraenovae*, with no reaction with human and other mammalian blood [16]. Antiserum titers as low as 1/500,000 bound synthetic peptide in dot blot assays. Antiserum was stored at −20 °C until used in dot blot assays.

2.6. Dot blot analysis of fly artifacts

Dot blot assays were used for detection of cathepsin D in fly artifacts in the form of regurgitate and feces (defecatory or fecal stains) produced by flies fed human blood. Human whole blood (O^+, adult male) was purchased from BioChemed Services (Winchester, VA. USA) and stored frozen at −80 °C until use. Three milliliters of freshly thawed blood were placed in a small polystyrene Petri dish (60 × 15 mm, non-pyrogenic, Falcon Brand, Corning, NY, USA) and placed in the center of a wire mesh cage with 100 adult flies (4–7 days after emergence at 25 °C), along with water and sugar cubes. A filter paper disc was introduced into the cage immediately afterward as described above. Feeding and deposition of artifacts were allowed to occur *ad libitum* over 24 h at 25 °C with a 15:9 h light: dark photoperiod [7]. Filter paper containing fly artifacts was cut into strips (0.5″ x 4.0”) and placed into sterile 15 ml conical tubes (Falcon Brand). Binding with anti-md3 serum was performed as described in the Promega Technical Manual for ProtoBlot®II AP System with Stabilized Substrate using the modifications detailed in Rivers et al. [16]. In brief, seconday antibody (Ab) (goat anti-rat IgG H & L chains conjugated to alkaline phosphatase)(Millipore-Sigma, St. Louis, MO USA) was allowed to react with anti-md3 serum that had bound to artifacts, and was visualized colorimetrically by the addition of stabilized substrate. The secondary Ab was selected for use due to it being highly cross-absorbed and showing no reaction to serum proteins from human, bovine, chicken, goat, guinea pig, horse, mouse, rabbit and sheep when tested by the manufacturer. Rivers et al. [16] demonstrated no reaction between the secondary Ab and whole blood from human, bovine, rat, swine, canine and feline. For most species tested, 4–10 collections of fly artifacts with at least 10–15 stains assayed per collection (for a minimum of 40–150 artifacts/species) were analyzed by dot blot assays. With some of the field collected species, only one or two adults were collected, and thus, dot blot analysis was of a single artifact collection. All membranes were digitally captured using a ChemiDoc MP imaging system (BioRad, Hercules, CA) equipped with ImageLab image analysis software (BioRad, v. 5.0, Hercules, CA).

For comparisons, anti-md3 serum was tested against human (male O^+) and bovine blood, and with blood spiked with md3 synthetic peptide, Dulbecco's phosphate buffered saline, and no sample. Two microliter aliquots of each control sample were pipetted onto filter paper (Whatman #4), allowed to air dry, and

then subjected to dot blot analysis as described. The final concentration of each synthetic peptide used was 10 μg protein per blot.

2.7. Statistical analysis

Percentage data were arcsine transformed before analyses to yield normal distributions. One- and two-way analyses of variance were performed using GraphPad InStat statistical software (v. 3.0a for Macintosh, GraphPad Software, San Diego, CA., USA). Means were compared using Student Newman Keul's multiple comparisons tests with $\alpha = 0.05$.

3. Results

3.1. Fly collections

A total of 31 species of flies from 11 different families were used to test the efficacy of the dot blot assays (Table 1). Of these, 25 species were field collected across three seasons, and two species (*L. sericata* and *P. regina*) were collected from multiple geographic regions. For all of the laboratory-reared species except *D. melanogaster*, four types of artifacts were deposited by adult flies following consumption of human blood: regurgitate, defecatory stains, translocation and tarsal tracks. Fig. 1 shows a representative collection of artifacts from *S. bullata* following consumption of human blood that displays all four types of artifacts. Only regurgitate and defecatory stains were derived from the fly digestive tracts, and thus predicted to bind anti-md3 serum [16]. Translocation and tarsal tracks resulted from flies walking or landing in wet blood and then leaving an imprint of a body part on a non-bloodied surface (filter paper): tarsal tracks were impressions of tarsi (pulvilli) while translocation represented non-symmetrical strains produced by dragging the abdomen, leg, or some other body part through wet blood. For the 11 species of laboratory

Table 1
Reaction of artifacts from different fly species with anti-md3 serum.

Family	Species	Origin	n	Adult food source			
				human	bovine		mouse
				blood	blood	liver	carcass
Anthomyiidae							
	Anthomyia illocata	Glen Rock, PA	1	+	nt	nt	nt
Calliphoridae							
	Calliphora vicina	Baltimore, MD	1000	+	+	+	+
	Calliphora vomitoria	Baltimore, MD	3	+	nt	nt	nt
	Chrysomya megacephala	College Station, TX	1000	+	+	+	nt
	Chrysomya rufifacies	College Station, TX	1000	+	+	+	nt
	Cynomya cadaverina	Baltimore, MD	1000	+	nt	nt	nt
	Lucilia illustris	Glen Rock, PA	1000	+	+	+	+
	Lucilia sericata	Baltimore, MD	1000	+	+	+	+
		Glen Rock, PA	300	+	nt	nt	nt
	Lucilia silvarum	Baltimore, MD	500	+	+	+	nt
	Phormia regina	Baltimore, MD	1000	+	+	+	+
		Glen Rock, PA	1000	+	+	+	+
		Amherst, MA	1000	+	+	+	+
	Pollenia sp.	Glen Rock, PA	3	+	nt	nt	nt
	Protophormia terraenovae	Bonners Ferry, ID	1000	+	+	+	+
Dolichopodidae							
	Unidentified sp.	Glen Rock, PA	1	+	nt	nt	nt
Drosophilidae							
	Drosophila sp.	Glen Rock, PA	1	+	nt	nt	nt
	Drosophila melanogaster	Raleigh, NC	50	−	nt	nt	nt
	Unidentified sp.	Glen Rock, PA	1	+	nt	nt	nt
Muscidae							
	Hydrotaea aenescens	Glen Rock, PA	3	+	nt	nt	nt
	Hydrotaea ignava	Glen Rock, PA	5	+	nt	nt	nt
	Musca sp.	Glen Rock, PA	1	+	nt	nt	nt
	Musca domestica	Raleigh, NC	1000	+	+	+	+
	Phaonia sp.	Glen Rock, PA	2	+	nt	nt	nt
Phoridae							
	Megaselia sp.	Glen Rock, PA	1	+	nt	nt	nt
	Megaselia scalaris	Baltimore, MD	100	+	nt	nt	nt
Piophilidae							
	Piophila casei	Glen Rock, PA	1	+	nt	nt	nt
		Glen Rock, PA	2	−	nt	nt	nt
	Prochyliza xanthostoma	Baltimore, MD	1	−	nt	nt	nt
Psychodidae							
	Clogmia albipunctata	Baltimore, MD	1	−	nt	nt	nt
Sarcophagidae							
	Ravinia sp.	Glen Rock, PA	3	+	nt	nt	nt
	Sarcophaga sp.	Glen Rock, PA	1	+	nt	nt	nt
	Sarcophaga bullata	Cambridge, MA	1000	+	+	+	+
Sepsidae							
	Sepsis sp.	Glen Rock, PA	2	+	nt	nt	nt
		Glen Rock, PA	3	−	nt	nt	nt
Sphaeroceridae							
	Unidentified sp.	Glen Rock, PA	1	−	nt	nt	nt

n = number of flies tested in a total of 3–10 replicates (100 flies/replicate) for each age of artifact. nt = not tested. For some species, replicates Artifacts were collected from adult flies fed human blood for 24 h at 25 °C, 15:9 h light: dark cycle, and then maintained in plastic bags at room temperature in total darkness until used in dot blot assays.

reared flies, regurgitate stains were the dominant type of stain deposited ($X \pm SEM = 48.9 \pm 2.9\%$, $n = 1100$, $df_{11,\ 1100}$, $F = 66.3$, $P < 0.001$), followed by defecate ($39.6 \pm 1.3\%$), and tarsal tracks ($10.9 \pm 2.0\%$). Translocation stains were not common for any of the species ($0.6 \pm 0.1\%$), but when they were produced, it was most often by *S. bullata* ($47.9 \pm 2.1\%$) and *Cy. cadaverina* ($44.9 \pm 3.4\%$).

Among the field collected flies, *Piophila caesi*, *Prochyliza xanathstoma*, *C. albipunctata, Sepsis* sp. *Drosophila* sp. *Megaselia* sp., *A. illocata* and the dolichopidid and sphaerocerid were not observed producing regurgitate stains or displaying bubbling behavior. The only type of artifact collected for each fly was defecate. None of these species were able to be reared in the laboratory due to the small number of specimens collected for each type.

3.2. Dot blot assays of fly artifacts

For each species of fly tested by dot blot analysis, artifacts were collected from adults following *ad libitum* feeding on human blood for 24 h at 25 °C under a 15:9 h light: dark regime. Consequently, the number of artifacts deposited on filter paper by each species varied, which is reflected in the variation in total artifacts tested for each species as shown in Fig. 2. Despite these differences, the vast majority ($96.8 \pm 2.4\%$, $n = 1256$) of artifacts assayed for all species reacted positively with anti-md3 serum (Table 1, Fig. 2). A positive reaction indicated that the anti-md3 serum bound specifically to an artifact, which was detected by blue to purple color formation in the presence of alkaline phosphatase conjugated secondary antibody and substrate. Artifacts from 27 species of flies representing 9 families bound anti-md3 serum (Table 1). Based on the morphological appearance of the artifacts, positive dot blot detection was of regurgitate and defecatory stains (Fig. 2). For two species of calliphorids (*P. regina* and *L. sericata*), colonies were established from multiple geographical locations, and artifacts collected from each bound anti-md3 serum (Table 1).

Despite the positive dot blot assays, the intensity of color development was not identical for all species or for all artifacts produced by a given fly species (Fig. 2A–J). For adults of *P. casei*, *Sepsis* sp., and the dolichopodid, the few artifacts deposited yielded weak positive reactions as evident by faint blue color development.

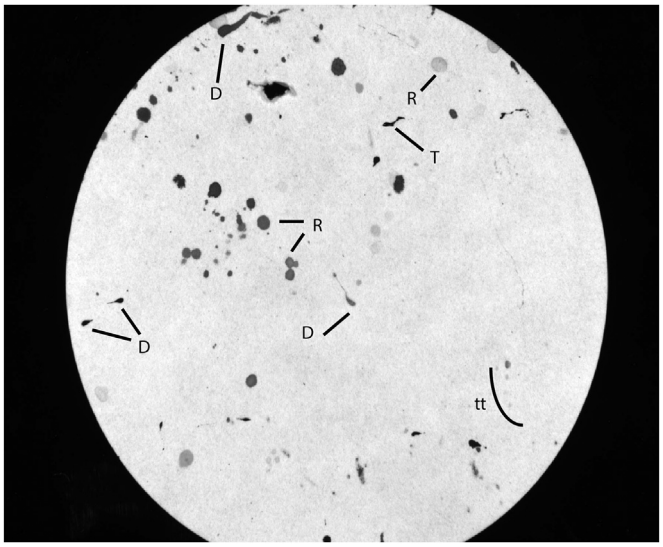

Fig. 1. Representative artifact collection from necrophagous flies after consumption of human blood. Artifacts were produced by 100 adults of *Sarcophaga bullata* over 24 h at 25 °C in which adults fed *ad libitum* on human blood, sugar cubes and water. R = regurgitate stains, D = defecatory stains, T = translocation, and tt = tarsal tracks.

In the case of *P. casei* and *Sepsis* sp. these observations were in contrast to negative reactions from separate tests of other field collected specimens (Table 1, Fig. 3). A similar trend was observed with the three drosophilids, in that the 2 field collected species (*Drosophila* sp. and one unidentified) produced artifacts that yielded positive reactions in dot blot assays, yet stains generated by the laboratory reared *D. melanogaster* tested negative (Table 1, Fig. 3A). In total, artifacts from 6 species (*D. melanogaster*, *P. casei*, *P. xanathostoma*, *C. albipunctata*, *Sepsis* sp., and the sphaerocerid) displayed no reactivity with anti-serum in dot blot assays (Table 1, Fig. 3A, B, and E).

Anti-md3 serum did not bind to human and bovine blood samples pipetted onto filter paper (Fig. 3D and G), but when both types of blood were spiked with synthetic md3 peptide, the blood samples reacted positively to the antiserum (Fig. 3H). There was no evidence of false positives, meaning samples binding anti-md3 serum in the absence of fly artifacts.

3.3. Dot blot assays of non-carrion artifacts

To test the species specificity of anti-md3 serum, dot blot assays were performed with artifacts collected from two non-dipteran insects. Artifacts were collected from *G. portentosa* following *ad libitum* feeding on human blood and from *C. lectularius* after parasitic blood feeding. For adults of *C. lectularius*, the only type of artifact produced was the result of fecal elimination. In contrast, adults of *G. portentosa* produced two types of artifacts: defecatory stains and numerous transfer patterns. Regardless of species or artifact type, no color development occurred in dot blot assays using anti-md3 serum with artifacts produced by either species (Fig. 3C and F).

3.4. Dot blot assays of aged fly artifacts

Artifacts were collected from adult flies following *ad libitum* feeding on human blood for 24 h, and then stored for several years to test whether antiserum could recognize aged fly stains. Regurgitate and defecatory stains collected from *L. illustris*, *L. sericata*, *P. regina*, *P. terraenovae*, *M. domestica* and *S. bullata* that had been stored for 3, 5, and 7 years at 25 °C all bound anti-md3 serum in dot blot assays (Table 2). Similarly, 3-year-old artifacts from blood-fed adults of *C. vicina* and *C. vomitoria* also reacted positively in dot blot assays. Based on the intensity of color development in dot blot assays, there did not appear to be any diminished binding of the antiserum with fly artifacts that were 3- (Fig. 4A and B), 5- (Fig. 4C and D), or 7-years-old (Fig. 4E and F) by comparison to those freshly collected (Fig. 2A–J).

3.5. Dot blot assays of larval stains and meconium

To test whether anti-md3 serum could detect fly stains other than regurgitate and defecate, artifacts were collected from feeding third stage larvae and newly emerged adults of *S. bullata*. The resulting larval stains were presumed to reflect both secretions from the mouth opening and excreta. All larval stains tested positive in dot blot assays using anti-md3 serum (Fig. 5A). Similarly, the antiserum reacted positively with meconium collected from newly emerged adults (Fig. 5B). Positive reactions demonstrated variable color development, suggesting differences in cathepsin D proteinase concentrations both in each type of stain, and among the same kind of artifact i.e., larval stains versus meconium.

4. Discussion

Rivers et al. [16] recently developed an immunoassay that

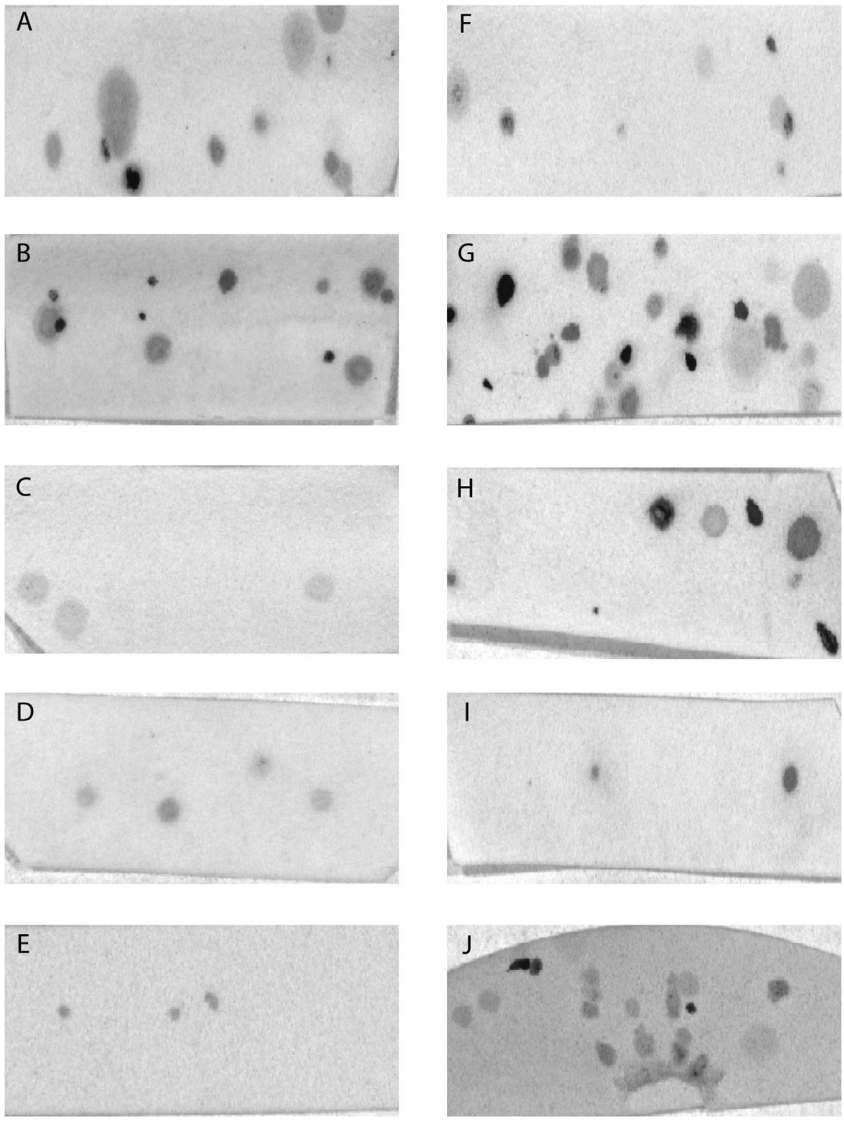

Fig. 2. Detection of artifacts from different fly species using anti-md3 serum. Positive dot blot assays for artifacts from (A) *Calliphora vicina*, (B) *Phormia regina*, (C) *Pollenia* sp., (D) *Hydrotaea ignava*, (E) *Megaselia scalaris*, (F) *Protophormia terraenovae*, (G) *Cynomya cadaverina*, (H) *Sarcophaga bullata*, (I) *Piophila casei*, and (J) *Lucilia sericata* following consumption of human blood.

permitted discernment between artifacts produced by *P. terraenovae* and human bloodstains. Here, we extend the range of fly species tested with the immunoassay using anti-md3 in dot blot assays. Artifacts from 27 species of flies representing 9 families (calyptrate and non-calyptrate) bound anti-md3 serum. Twenty of the species (flies from the families Calliphoridae, Sarcophagidae, Muscidae, Phoridae and Piophilidae) are known to commonly colonize or feed on human remains in the United States, while the remaining species (or families), though less common, associate with some aspect of human decomposition [29,30]. The broad reactivity of anti-md3 serum with artifacts from the flies tested in this study is consistent with the view that many, if not all, species of necrophagous and saprophagous cyclorrhaphous Diptera possess digestive cathepsin D proteinases [31]. This speculation is based on the fact that such flies have an extremely acidic midgut as larvae and/or adults, presumed to be an evolutionary adaptation to feeding on a diet (i.e., a corpse) containing a high load of microorganisms [32]. An acidic midgut favors the lytic activity of lysozymes and digestive cathepsin D proteinases [31]. Surprisingly, some of the artifacts testing positive in dot blot assays were from non-necrophagous or non-saprophagous species (e.g., *A. illocata*, and a dolichopodid). This suggests that the range of flies possessing cathepsin D in digestive fluids extends beyond just those species feeding on bacteria-rich diets [33,34].

Regurgitate and defecatory stains produced positive dot blot reactions, whereas transfer patterns did not. The antiserum did not distinguish between artifacts resulting from the fly digestive track (i.e., regurgitate vs. defecate). The immunoassays also did not permit detection of transfer patterns (i.e., translocation and tarsal tracks). However, this was expected based on previous observations of artifacts from *P. terraenovae* [16]. Transfer patterns were created by the adult flies interacting with wet blood and then leaving body impressions on filter paper. As a consequence, tarsal tracks and translocation stains were essentially identical in chemical composition to the food source (blood) and thus were devoid of cathepsin D. During dot blot assays, the presence of transfer patterns intermixed with regurgitate and defecatory stains can lead to the incorrect interpretation that false negatives exist, when in reality

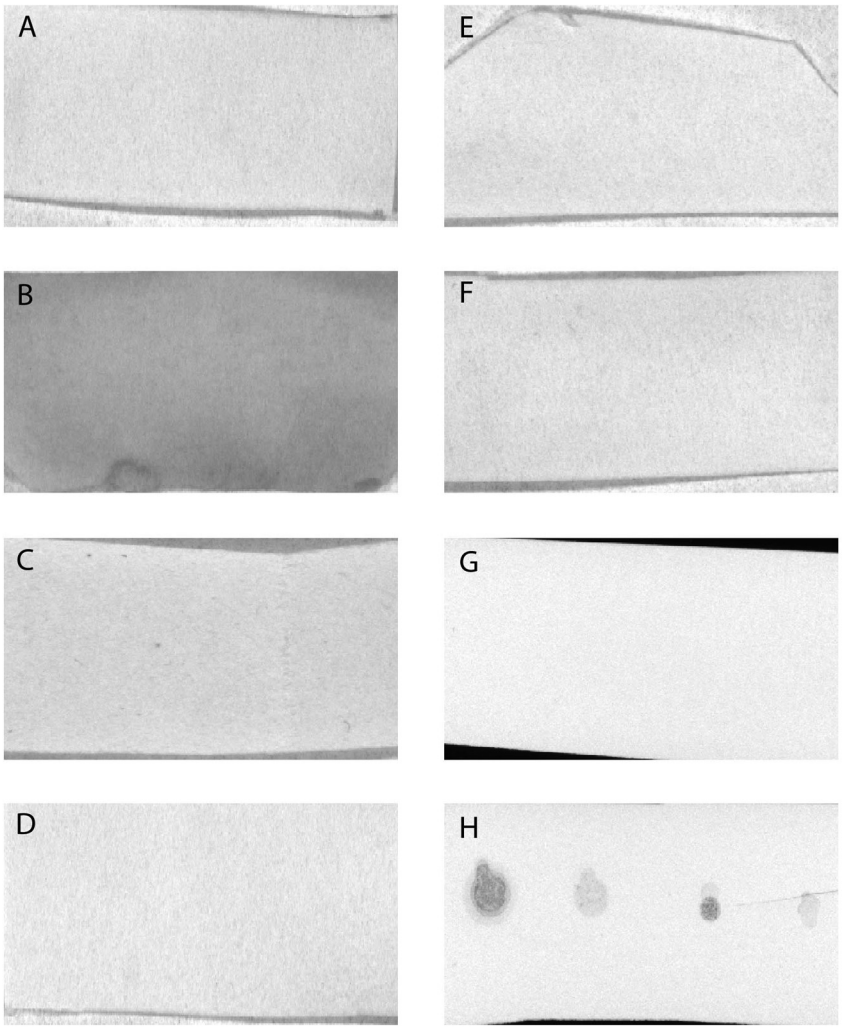

Fig. 3. Detection of artifacts from different fly species using anti-md3 serum. Negative dot blot assays for artifacts from (A) *Drosophila melanogaster*, (B) *Sepsis* sp., (C) *Cimex lectularius*, (E) *Piophila casei*, and (F) *Gromphadorhina portentosa*. Blood from (D) human and (G) bovine also did not bind anti-md3 serum, but (H) human (left two dots) and bovine blood (right two dots) spiked with synthetic md3 peptide did test positive in dot blot assays.

these artifacts should not bind antiserum [16]. Collectively, these observations are not viewed as limitations of the immunoassay for at least two reasons. First, the primary need at a crime scene is to differentiate fly artifacts from human bloodstains in a reliable and quantifiable manner, not to determine the exact type of fly stain. Far less information is derived from knowing precisely what type of fly artifact is present [14]. Second, translocation and tarsal tracks are not nearly as common following foraging on human blood as regurgitate and defecatory stains [6,7]. They are also far easier to discern based on morphological features than other types of fly artifacts [7].

Among the fly species that produced artifacts that reacted with antiserum, the total number of artifacts produced by each species and the binding efficiencies of anti-md3 with stains were not equal for all species tested by dot blot assays. Rivers and McGregor [7] have previously demonstrated species-specific differences in total artifact production, morphology and type of stains produced with 5 of the species tested in this study. Their study showed that variation among species was heavily influenced by diet, and not as much by body size differences of adult flies. Similar observations were made by Striman et al. [13] when comparing artifact morphology of *L. sericata* and *C. vicina* following consumption of human blood. Undoubtedly, species preferences for feeding on liquid diets in general and human blood specifically accounts for the artifact differences observed in this study. The differential binding of the antiserum with stains among and between species is likely

Table 2
Reaction of aged artifacts from different fly species with anti-md3 serum.

Family	Species	n	Age of artifacts (years)		
			3	5	7
Calliphoridae					
	Calliphora vicina	400	+	nt	nt
	Calliphora vomitoria	3	+	nt	nt
	Lucilia illustris	600	+	+	nt
	Lucilia sericata	800	+	+	+
	Phormia regina	800	+	+	+
	Protophormia terraenovae	800	+	+	+
Muscidae					
	Musca domestica	600	+	+	+
Sarcophagidae					
	Sarcophaga bullata	800	+	+	+

n = number of flies tested in a total of 4–8 replicates (100 flies/replicate) for each age of artifact. nt = not tested. Artifacts were collected from adult flies fed human blood for 24 h at 25 °C, 15:9 h light: dark cycle, and then maintained in plastic bags at room temperature in total darkness until used in dot blot assays.

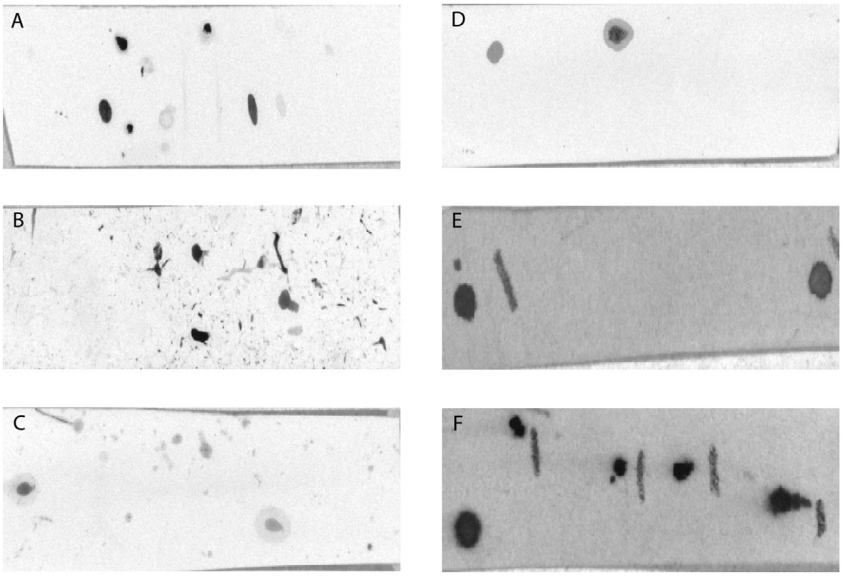

Fig. 4. Detection of aged artifacts from different fly species using anti-md3 serum. Artifacts were stored at room temperature in total darkness for 3–7 years after collection from adult flies fed human blood. Positive dot blot assays for 3-year old artifacts from (A) *Phormia regina* and (B) *Sarcophaga bullata*, for 5-year old artifacts from (C) *Protophormia terraenovae* and (D) *Phormia regina*, and for 7-year old artifacts from (E) *Protophormia terraenovae* and (F) *Sarcophaga bullata*. Dark vertical lines in (E) and (F) are incidental pencil marks and did not react with antiserum.

accounted for by artifacts possessing variable concentrations of cathepsin D in relation to one other. Such differences could have occurred at the time the fluid was produced/released by the fly or could be attributed to digestion/degradation of the enzyme in exogenous fluids. However, the strong binding reactions of aged artifacts with antisera would argue against the latter scenario. Similarly, pepsin-like enzyme activity (i.e., digestive cathepsin D) is readily detected in regurgitate stains produced by *P. terraenovae* [35]. It is also possible that in some instances the appropriate epitope of the enzyme was not fully exposed for efficient binding of the antiserum. This condition can be substrate dependent i.e., influenced by the material that the stain is located, which may also impact the affinity of antigen binding to the substrate [36]. Species-specific differences in the secondary or tertiary structure of cathepsin D would conceivably contribute to either of these scenarios [37–39].

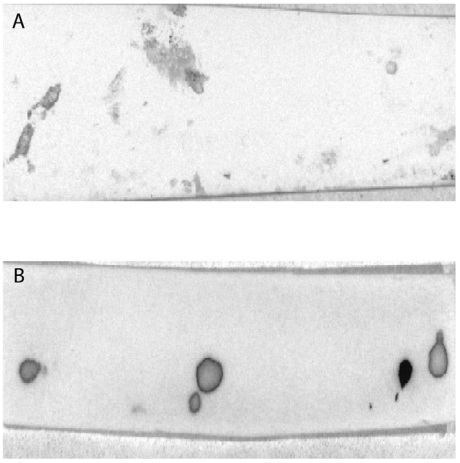

Fig. 5. Detection of (A) larval stains and (B) adult meconium from *Sarcophaga bullata* in dot blot assays using anti-md3 serum. Larval stains were collected from early third stage larvae and meconium was collected during the first 24-h of adult emergence at 25 °C.

One or multiple of these explanations may account for the observations that artifacts from some individual *P. casei* and *Sepsis* sp. reacted positively with the antiserum, yet stains produced by other adults of the same species apparently did not react positively anti-md3 serum. There is also the possibility that enzyme was present in these latter artifacts but at a concentration that was below the lower limits of the colorimetric detection used in the immunoassays. Any of these scenarios could also explain the observations that 4 species (*D. melanogaster*, *P. xanthostoma*, *C. albipunctata*, and the sphaerocerid) of saprophagous/necrophagous flies produced artifacts that did not react with anti-md3 serum. Further work will continue to improve the detection limits of the dot blot assays.

Artifacts produced from 7 species of forensically important necrophagous flies that had been stored 3–7 years after collection and then subjected to the immunoassays tested positive in reactivity with antiserum. Undoubtedly the enzyme is concentrated as liquid evaporates from the stain, consistent with the evaporative cooling and food concentration mechanisms purported for bubbling behavior of Diptera [40,41]. These observations also suggest that cathepsin D does not degrade quickly when expelled from the fly in regurgitate or feces. The latter indicates that confirmatory testing for fly stains could occur many days to months after a crime scene has been discovered and processed, provided that artifacts have not been modified by cleaners, photobleaching or some other exogenous material [42]. Research is currently underway to examine the impact of common cleaners used at a crime scene on the utility of the immunoassay to distinguish fly artifacts.

Anti-md3 serum reacted with artifacts produced by other developmental stages, in the form of oral secretions and excreta from third stage larvae as well as meconium expelled by teneral adults of *S. bullata*, but not to human or bovine blood tested alone. When mammalian bloodstains were spiked with synthetic md3 peptide, the antiserum in turn reacted to the mixtures. Thus, anti-md3 serum demonstrates a high degree of specificity for stains containing digestive cathepsin D. These findings represent significant progression toward the development of a diagnostic tool that permits reliable distinction of fly artifacts from human bloodstains.

Anti-md3 serum did not react with artifacts produced by two

non-carrion species. This observation for *G. portentosa* is consistent with the diet and gut environment of hissing cockroaches lacking the conditions that favor digestive cathepsin D [31]. Similarly, defecatory stains of *C. lectularius* also demonstrated negative reactions in dot blot assays. This later finding is interesting in that *C. lectularius* is hematophagous and several other species of hemipterans have been shown to possess cathepsin aspartic proteases [43]. Digestive cathepsin D is present in the midguts of *Rhodnius prolixus* (Hemiptera: Reduviidae) and *Dysdercus peruvianus* (Pyrrhocoridae) [31,44], while adults of *Triatoma infestans* (Reduviidae) were found to possess two distinct structural forms of cathepsin D (with and without a proline loop) in the midgut [45]. The lack of anti-md3 serum binding to bed bug artifacts argues that digestive cathepsin D is not present in cimicids like *C. lectularius* or that dipteran cathepsin D is structurally unique from those produced by hemipterans and possibly other insects. Additional research is being conducted to determine if anti-md3 serum can recognize artifacts of other hematophagous species of Hemiptera, especially those that are known to occasionally frequent human remains or are suspected of causing death.

5. Conclusions

Dot blot assays using anti-md3 serum demonstrated positive reactions between the antiserum and artifacts produced by 27 species of flies representing 9 families. Only 4 fly species produced artifacts that did not bind the antiserum, and this may simply reflect concentrations of antigen (i.e., cathepsin D) below the detection limits of the colorimetric detection used in the dot blot assays. By contrast, two species of flies that were not necrophagous or saprophagous in terms of foraging behavior did react with anti-md3 serum. Additional research is needed to understand the full range of flies possessing cathepsin D in digestive fluids, and hence that produce artifacts that would be detected with the confirmatory test described in this study. Importantly, human and bovine blood did not react with the antiserum. Thus, the immunoassay displays high specificity for distinguishing fly artifacts from human blood and blood from other mammals. The fact that dot blot assays also bound artifacts from larvae and teneral adults (meconium) of *S. bullata*, and reacted with artifacts that were 3–7-years old indicates that anti-md3 serum has tremendous promise to be used in a confirmatory test that reliably detects fly contaminants at crime scenes. The next steps need to be testing the immunoassay with artifacts deposited on materials commonly found at crime scenes and to perform validation studies.

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Conflict of interest

None.

References

[1] J.K. Tomberlin, R. Mohr, M.E. Benbow, A.M. Tarone, S. VanLaerhoven, A roadmap for bridging and applied research in Forensic Entomology, Annu. Rev. Entomol. 56 (2011) 401–421.
[2] S.H. James, J.J. Nordby, S. Bell (Eds.), Forensic Science: An introduction to scientific and investigative techniques, fourth ed., CRC Press, Boca Raton, 2014.
[3] A. Fujikawa, L. Barksdale, D.O. Carter, *Calliphora vicina* (Diptera: Calliphoridae) and their ability to alter the morphology and presumptive chemistry of bloodstain patterns, J. Forensic Ident. 59 (2009) 502–512.
[4] A. Fujikawa, L. Barskdale, L.G. Higley, D.O. Carter, Changes in the morphology and presumptive chemistry of impact and pooled bloodstain patterns by *Lucilia sericata* (Meigen)(Diptera: Calliphoridae), J. Forensic Sci. 56 (2011) 1315–1318.
[5] R.M. Zuhu, M. Supriyani, B. Omar, Fly artifact documentation of *Chrysomya megacephala* (Fabricius)(Diptera: Calliphoridae)-a forensically important blowfly species in Malaysia, Trop. Biomed. 25 (1) (2008) 17–22.
[6] M.A. Parker, M. Benecke, J.H. Byrd, R. Hawkes, R. Brown, Entomological alteration of bloodstain evidence, in: J.H. Byrd, J.L. Castner (Eds.), Forensic entomology: the utility of using arthropods in legal investigations, second ed., CRC Press, Boca Raton, 2010, pp. 539–580.
[7] D.B. Rivers, A. McGregor, Morphological features of regurgitate and defecatory stains deposited by five species of necrophagous flies are influenced by adult diets and body size, J. Forensic Sci. 63 (1) (2018) 154–161.
[8] M. Benecke, L. Barksdale, Distinction of bloodstain patterns from fly artifacts, Forensic Sci. Int. 137 (2003) 152–159.
[9] A. Durdle, R.J. Mitchell, R.A.H. van Oorschot, The use of forensic tests to distinguish blowfly artifacts from human blood, semen, and saliva, J. Forensic Sci. 60 (2) (2015) 468–470.
[10] A. Durdle, R.J. Mitchell, R.A.H. van Oorschot, The change in human DNA content over time in the artefacts of the blowfly *Lucilia cuprina* (Meigen)(Diptera: Calliphoridae), Forensic Sci. Int. 3 (2011) e289–e290.
[11] A. Durdle, R.A.H. van Oorschot, R.J. Mitchell, The morphology of fecal and regurgitation artifacts deposited by the blow fly *Lucilia cuprina* fed a diet of human blood, J. Forensic Sci. 58 (4) (2013) 897–903.
[12] G. Kulstein, J. Amendt, R. Zehner, Blow fly artifacts from blood and putrefaction fluid on various surfaces: a source for forensic STR typing, Entomol. Exp. Appl. 157 (2015) 255–262.
[13] B. Striman, A. Fujikawa, L. Barksdale, D.O. Carter, Alteration of expirated bloodstain patterns by *Calliphora vicina* and *Lucilia sericata* (Diptera: Calliphoridae) through ingestion and deposition of artifacts, J. Forensic Sci. 56 (2011) S123–S127.
[14] D.B. Rivers, T. Geiman, Insect artifacts are more than just altered bloodstains, Insects: Adv. Forensic Entomol. 8 (2) (2017) 37. https://doi.org/10.3390/insects8020037.
[15] S.V. Langer, M. Illes, Confounding factors of fly artefacts in bloodstain pattern analysis, J. Can. Soc. Forensic. Sci. 48 (5) (2015) 215–224.
[16] D.B. Rivers, G. Acca, M. Fink, R. Brogan, D. Chen, A. Schoeffield, Distinction of fly artifacts from human blood using immunodetection, J. Forensic Sci. 63 (6) (2018) 1704–1711.
[17] H.C. Huckett, J.R. Vockeroth, in: J.F. McAlpine, B.V. Peterson, G.E. Shewell, H.J. Teskey, J.R. Vockeroth, D.M. Wood (Eds.), Manual of nearctic Diptera: Muscidae, vol 2, Agriculture Canada, Ottawa, 1987, pp. 1115–1131.
[18] S.A. Marshall, T. Whitworth, L. Roscoe, Blow flies (Diptera; Calliphoridae) of eastern Canada with a key to Calliphoridae subfamilies and genera of eastern North America, and a key to the eastern Canadian species of Calliphorinae, Luciliinae and Chrysomyiinae, Can. J. Arthrop. (2011), https://doi.org/10.3752/cjai.2011.11. Ident. No. [11].
[19] L.W. Quate, J.R. Vockeroth. Manual of nearctic Diptera: Psychodidae, vol 1, In: J.F. McAlpine, B.V. Peterson, G.E. Shewell, H.J. Teskey, J.R. Vockeroth, D.M. Wood, eds., Agriculture Canada, Ottawa, p. 293–300.
[20] S. Rochefort, M. Giroux, J. Savage, T.A. Wheeler, Key to forensically important Piophilidae (Diptera) in the Nearctic Region, Can. J. Arthrop (2015), https://doi.org/10.3752/cjai.2015.27. Ident. No. 27.
[21] G.E. Shewell, G.E. Manual of nearctic Diptera: Calliphoridae, vol 2, In: J.F. McAlpine, B.V. Peterson, G.E. Shewell, H.J. Teskey, J.R. Vockeroth, D.M. Wood, eds., Agriculture Canada, Ottawa, p. 1133–1146.
[22] M.R. Wheeler. Manual of nearctic Diptera: Drosophilidae, vol 2, In: J.F. McAlpine, B.V. Peterson, G.E. Shewell, H.J. Teskey, J.R. Vockeroth, D.M. Wood, eds., Agriculture Canada, Ottawa, p. 1011–1018.
[23] T. Whitworth, Keys to the genera and species of blow flies (Diptera: Calliphoridae) of America North of Mexico, Proc. Entomol. Soc. Wash. 108 (3) (2006) 689–725.
[24] D.L. Denlinger, D. L, Induction and termination of pupal diapause in *Sarcophaga* (Diptera: Sarcophagidae), Biol. Bull. 142 (1972) 11–24.
[25] D.B. Rivers, Evaluation of host responses as means to assess ectoparasitic pteromalid wasp's potential for controlling manure-breeding flies, Biol. Control 30 (2004) 181–192.
[26] D. Gennard, Forensic entomology: An introduction, second ed., Wiley-Blackwell, West Sussex, U.K., 2012.
[27] D.B. Rivers, T. Ciarlo, M. Speilman, R. Brogan, Changes in development and heat shock protein expression in two species of flies [*Sarcophaga bullata* (Diptera: Sarcophagidae) and *Protophormia terraenovae* (Diptera: Calliphoridae) reared in different sized maggot masses, J. Med. Entomol. 47 (4) (2010) 677–689.
[28] M.H.P. Padilha, A.C. Pimentel, A.F. Ribeiro, W.R. Terra, Sequence and function of lysosomal and digestive cathepsin D-like proteinases of *Musca domestica* midgut, Insect Biochem. Mol. Biol. 39 (2009) 782–791.
[29] J.H. Byrd, J.L. Castner, Insects of forensic importance, in: J.H. Byrd, J.L. Castner (Eds.), Forensic entomology: The utility of arthropods in legal investigations, second ed., CRC Press, Boca Raton, 2010, pp. 39–126.

[30] K.G.V. Smith, A manual of forensic entomology, London and Cornell University Press, London, U.K, 1986.
[31] W.R. Terra, C. Ferreira, Biochemistry and molecular biology of digestion, in: L.I. Gilbert (Ed.), Insect molecular biology and biochemistry, vol. 1, Academic Press-Elsevier, London, 2012, pp. 365–418.
[32] S.H. Bowen, Mechanism for digestion of detrital bacteria by the cichlid fish *Sarotherodon mossambicus* (Peter), Nature 260 (1976) 137–138.
[33] F.P. Espinoza-Fuentes, A.F. Ribeiro, W.R. Terra, Microvillar and secreted digestive enzymes from *Musca domestica* larvae. Subcellular fractionation of midgut cells with electron microscopy monitoring, Insect Biochem. 17 (1987) 819–827.
[34] F.J.A. Lemos, W.R. Terra, Properties and intracelular distribution of a cathepsin D-like proteinase active at the acid region of *Musca domestica* midgut, Insect Biochem. Mol. Biol. 21 (1991) 457–465.
[35] D.B. Rivers, G. Acca, M. Fink, R. Brogan, A. Schoeffield, Spatial characterization of proteolytic enzyme activity in the foregut region of the necrophagous fly, *Protophormia terraenovae*, J. Insect Physiol. 67 (2014) 45–55.
[36] H. Towbin, J. Gordon, Immunoblotting and dot immunobinding – current status and outlook, J. Immunol. Methods 72 (1984) 313–340.
[37] D.A. Knecht, R.C. Mierendorf, R.L. Dimond, Immunological recognition of modifications on functionally related proteins, Methods Enzymol. 98 (1983) 159–166.
[38] F.K. Tamaki, M.H.P. Padilha, A.C. Pimentel, A.F. Ribeiro, W.R. Terra, Properties and secretory mechanism of *Musca domestica* digestive chymotrypsin and its relation with *Drosophila melanogaster* homologs, Insect Biochem. Mol. Biol. 42 (2012) 482–490.
[39] J. Leyria, L.L. Fruttero, R. Ligabue-Braun, M.S. Defferrari, E.L. Arrese, J.L. Soulages, B.P. Settembrini, C.R. Carlini, L.E. Canavoso DmCatD, a cathepsin D-like peptidase of the hematophagous insect *Dipetalogaster maxima* (Hemiptera: Reduviidae): Purification, bioinformatics analyses and the significance of its interaction with lipophorin in the internalization by developing oocytes, J. Insect Physiol. 105 (2018) 28–39.
[40] J. Hendrichs, S.S. Cooley, R.J. Prokopy, Post-feeding bubbling behaviour in fluid-feeding Diptera: concentration of crop contents by oral evaporation of excess water, Physiol. Entomol. 17 (2) (1992) 153–161.
[41] G. Gomes, R. Köberle, C.J. Von Zuben, D.V. Andrade, D.V. Droplet bubbling evaporatively cools a blowfly, Sci. Rep. 8 (1) (2018) 5464, https://www.nature.com/articles/s41598-018-23670-2.
[42] R. Li, Forensic biology: identification and DNA analysis of biological evidence, first ed., CRC Press, Boca Raton, 2009.
[43] J.G. Houseman, A.E.R. Downe, Cathepsin D-like activity in the posterior midgut of hemipteran insects, Comp. Biochem. Physiol. Part B: Comp. Physiol. 75 (3) (1983) 509–512.
[44] A.C. Pimentel, F.J. Fuzita, G. Palmisano, C. Ferreira, W.R. Terra, Role of cathepsin D in the midgut of *Dysdercus peruvianus*, Comp. Biochem. Physiol., B 204 (2017) 45–52.
[45] C. Balczun, J. Siemanowski, J.K. Pausch, S. Helling, K. Marcus, C. Stephan, H.E. Meyer, T. Schneider, C. Cizmowski, M. Oldenburg, S. Höhn, Intestinal aspartate proteases TiCatD and TiCatD2 of the haematophagous bug *Triatoma infestans* (Reduviidae): sequence characterisation, expression pattern and characterisation of proteolytic activity, Insect Biochem. Mol. Biol. 42 (4) (2012) 240–250.