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Using bacterial and necrophagous insect dynamics for post-mortem interval estimation during cold season: Novel case study in Romania

Lavinia Iancu [a,*], David O. Carter [b], Emily N. Junkins [b], Cristina Purcarea [c]

[a] "Grigore Antipa" National Museum of Natural History, 1, Sos. Kiseleff, Bucharest 011341, Romania
[b] Laboratory of Forensic Taphonomy, Forensic Sciences Unit, Division of Natural Sciences and Mathematics, Chaminade University of Honolulu, Honolulu 96816, HI, USA
[c] Institute of Biology Bucharest, 296 Splaiul Independentei, Bucharest 060031, Romania

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ABSTRACT

Considering the biogeographical characteristics of forensic entomology, and the recent development of forensic microbiology as a complementary approach for post-mortem interval estimation, the current study focused on characterizing the succession of necrophagous insect species and bacterial communities inhabiting the rectum and mouth cavities of swine (*Sus scrofa domesticus*) carcasses during a cold season outdoor experiment in an urban natural environment of Bucharest, Romania. We monitored the decomposition process of three swine carcasses during a 7 month period (November 2012–May 2013) corresponding to winter and spring periods of a temperate climate region. The carcasses, protected by wire cages, were placed on the ground in a park type environment, while the meteorological parameters were constantly recorded. The succession of necrophagous Diptera and Coleoptera taxa was monitored weekly, both the adult and larval stages, and the species were identified both by morphological and genetic characterization. The structure of bacterial communities from swine rectum and mouth tissues was characterized during the same time intervals by denaturing gradient gel electrophoresis (DGGE) and sequencing of 16S rRNA gene fragments. We observed a shift in the structure of both insect and bacterial communities, primarily due to seasonal effects and the depletion of the carcass. A total of 14 Diptera and 6 Coleoptera species were recorded on the swine carcasses, from which *Calliphora vomitoria* and *C. vicina* (Diptera: Calliphoridae), *Necrobia violacea* (Coleoptera: Cleridae) and *Thanatophilus rugosus* (Coleoptera: Silphidae) were observed as predominant species. The first colonizing wave, primarily Calliphoridae, was observed after 15 weeks when the temperature increased to 13 °C. This was followed by Muscidae, Fanniidae, Anthomyiidae, Sepsidae and Piophilidae. Families belonging to Coleoptera Order were observed at week 18 when temperatures raised above 18 °C, starting with Cleridae, Silphidae, and followed by Histeridae, Staphylinidae and Dermestidae. For bacteria, 53 taxa belonging to phyla Proteobacteria (Gammaproteobacteria, Betaproteobacteria), Firmicutes and Bacteroidetes were identified from the mouth cavity (36 OTUs) and rectum cavity (17 OTUs). These shifts in insect and bacterial communities indicated their complementary role in the carcass decomposition process. These results represent the first forensic entomological and microbiological survey in Romania. This study shows the value of forensic entomology as a tool for forensic investigations in Romania and neighboring areas with similar biogeographical characteristics. Moreover, this study represents a novel approach for understanding taphonomy and estimating post-mortem interval during cold season by combining entomological and microbiological methods.

* Corresponding author. Tel.: +40 736430425.
E-mail addresses: lavinia.paul@antipa.ro (L. Iancu), david.carter@chaminade.edu (D.O. Carter), emily.junkins@student.chaminade.edu (E.N. Junkins), cristina.purcarea@ibiol.ro (C. Purcarea).

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1. Introduction

Forensic entomology is a widely recognized, location dependent discipline used in many forensic investigations, particularly for the minimum post-mortem interval (PMI_{min}) estimation [1–4]. Insects have served as physical evidence in numerous cases around the world, such as in the United Kingdom [5], United States of America [6], Canada [7], Italy [8] and Brazil [9], both during the early and extended post-mortem period [10]. Yet, in Romania, a country in southeastern Europe comprising approximately 240,000 km^2 and 20 million inhabitants, forensic entomology is not used as an investigative tool nor do police forces utilize the information from entomological expertise as an aid in death investigations.

Considering that insects are bioregion specific, research in Romania is required before establishing insects as reliable physical evidence. Establishing entomology among the existing Romanian forensic sciences requires an understanding of climatic, geographical, and topographical differences among locations, species-specific methods of attraction and competition [11], as well as the various insects–carcass relationships [12,13]. In addition, insect succession is affected by several other factors, including type and size of carcass, separation of various families and species by direct interactions through feeding preferences, or indirect interactions mediated by the physicochemical environment [14,15].

Experimental models in forensic entomology have been developed in various locations worldwide, including Europe [16,17], Canada [18,19], United States of America [4,20], Africa [21], Brazil [22], Australia [23], in different seasons [24–26] and at different temperatures [27,28]. These studies employed a morphological description and identification of necrophagous insect species, both adults and larvae [29,30]. Some of these studies also identified decomposition stages and the corresponding role of necrophagous insects [25,31]. These approaches can be complemented with molecular biology, such as cytochrome c oxidase I (COI) DNA barcoding [32–36], which can facilitate an accurate and reliable identification of forensically important insects from human or animal carcasses.

In addition to forensic entomology, forensic microbiology represents a more recent research direction that could contribute to a more accurate estimation of PMI [37–40]. In particular, chronological and spatial variation of microbial communities could provide data to complement entomological evidence or perhaps, with additional research, serve as physical evidence in cases where necrophagous insects are less active in low temperature conditions (during night time and/or winter season), and in unforeseen circumstances that prevent the cadaver colonization such as freezing, depth of burial or plastic bags [41–43]. So far, few studies have considered the possibility of using post-mortem microorganisms to estimate PMI [37–39], but the identification of microorganisms from the soils associated with corpses has begun [37,40]. This is most commonly achieved by analyzing the 16S rRNA gene [37,38] and 16S rRNA gene FLX amplicon pyrosequencing [39]. Until now, most of these studies focused on warm seasons [20,25], with few data obtained during cold seasons [44].

The current study was developed from a scenario of a suspicious death during cold season in an outdoor urban park. The aim was to better understand the structure of insect and bacterial communities during the colder months when a body is not immediately colonized by insects due to constant low temperature. The objectives of the current study were (1) to monitor gross decomposition, (2) to monitor the climatic parameters, (3) to characterize insect community structure, (4) to characterize bacterial community structure from the mouth and rectum, because these regions are typically first colonized by insects, and (5) to identify the bacteria found both in larvae and carcass tissue. The ultimate outcome was to corroborate all these data to prove useful for PMI_{min} estimation.

The current data report the first experimental investigation into the carcass decomposition process in Romania during a cold season by using corroborated entomological and microbiological approaches. Thus, this experiment represents a pioneering study aiming to introduce forensic entomology as an official expertise in forensic investigations in Romania and to identify potential bacterial markers of decomposition.

2. Material and methods

2.1. Experimental protocol

Swine carcasses were used as the experimental model and they were acquired from a swine farm in close proximity to Bucharest in accordance with Law No. 205/2004 regarding animal protection rights in Romania. Domestic swine (*Sus scrofa domesticus*) are a commonly used analog for human remains due to biochemical tissue similarities and body mass [45], which is helpful since there are few studies using human cadavers [44,46–48] due to ethical reasons. Swine also attract similar necrophagous insect species that can be statistically compared with those associated with a decaying human body [47].

Three swine carcasses, two females and one male, weighed approximately 15 kg each and being about 3 months old, were acquired from the farm. The swine belonged from a lot deceased by natural causes and disease-free, according to the veterinarian. The carcasses were placed in sealed plastic bags immediately after death, and transported to the experimental site within 30 min post-mortem, ambient temperature being approximately 13 °C.

The experimental site was located in an exposed urban location in Bucharest, Romania (44°27′10″ N 26°05′04″ E). Carcasses were placed directly on the ground in an 11,500 m^2 park type area of the *Grigore Antipa* National Museum of Natural History, and were protected from vertebrate scavengers by a 1 m × 0.5 m wire cage with 3 cm wide metal mesh, with no roof (Fig. 1A). Each carcass was positioned at approximately 10 m distance from each other. The nearby tree vegetation consisted of *Aesculus hyppocastanum*, *Tilia* sp., and *Fraxinus* spp. After 17 weeks exposure, the carcasses were surrounded by low vegetation composed of grass, flowers and shrubs (Fig. 1B).

The ambient temperature and relative humidity were recorded every 4 h using a button logger thermo-hygrometer (Ecotone, Poland) placed in close proximity (10–15 cm) of the cages, half a meter above the ground, in a plastic jar with holes. These parameters were also simultaneously recorded during each sampling with a thermo hygrometer P 330 HVAC (Dostmann Electronic, Ireland). The soil temperature was measured in the surrounding area of the carcasses at 10 cm depth, twice a day (9 a.m. and 3 p.m.) using a Voltcraft DET-3R stem thermometer (Voelkner, Germany). The average weekly precipitation values were obtained from Băneasa weather station of National Meteorological Administration (http://www.meteoromania.ro) located at 8.5 km from the experimental site. In Romania the average annual temperature is approximately 11 °C in the south and 8 °C in the north.

During summer the average temperatures in Bucharest rise to, and sometimes above, 28 °C, while in winter, the average temperatures are below 2 °C.

Precipitation is regular, with 750 mm per year only on the highest mountains, and approximately 600 mm in Bucharest (http://www.meteoromania.ro).

2.2. Sampling

The sampling methods used provided qualitative, presence/absence of data that were not suitable for quantitative statistical analyses but, nevertheless, revealed the temporal association of