RESEARCH ARTICLE

Effect of Flunitrazepam on Decomposition and Forensically Important Insects Colonization of Pig Carcasses: An Entomology Perspective

[version 1; peer review: 2 approved with reservations]

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Abstract

Background

Determining the post-mortem interval (PMI) through examination of insect activity on deceased bodies is essential in forensic science. Establishing the time of death in cases involving drug ingestion can present difficulties for law enforcement, complicating evidence collection. In unnatural death investigations, a forensic pathology approach is commonly employed, focusing exclusively on samples obtained from the body, which can lead to biases and errors, particularly beyond 72 hours after death. Notably, no research on insect colonization and succession on cadavers has been conducted in Kenya, despite a growing number of unidentified deaths. This research aimed to identify and assess forensically significant insects and to establish the effect of flunitrazepam (Rohypnol®) on carrion-insect successional patterns on pig carcasses.

Methods

Four domestic pigs, averaging 24.8 kg, were used, with one designated as the control and three assigned as the experimental group. The experimental pigs received oral drug administration mixed with 250ml of vodka to simulate drink spiking in a bar. Subsequently, the pigs were euthanized, and their carcasses were placed in cages.

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Adult insects and flightless adult invertebrates were sampled daily until the dry remains stage of decomposition. Existing insect identification keys were utilized for species identification.

Results

The results revealed consistent insect succession patterns across the four carcasses. The mean number of insects across developmental stages decreased as flunitrazepam dosage increased. However, no significant variation among the carcass groups was observed in insect genera. Decomposition was categorized into five stages: fresh, bloated, active decay, advanced decay, and dry, consistent with prior studies. Insect succession included Diptera (e.g., *Chrysomya spp., Lucilia sericata*), Coleoptera (e.g., *Dermestes maculatus*), and Hymenoptera (e.g., *Camponotus sericeus*), with Calliphoridae being most abundant. The flunitrazepam-ingested carcasses showed prolonged decomposition stages compared to control.

Conclusion

These findings highlight the need to consider drugs like flunitrazepam in forensic entomology analyses.

Keywords
Carcasses, Decomposition, Insects, Colonization, Flunitrazepam, Succession

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Introduction

Forensic entomology can be defined as using insects and other arthropods to process criminal cases.[1] In crime scene investigations globally, it helps the practitioner establish the sequence of events leading to death.[2] Forensic entomologists can be helpful as they analyse the insect succession on any remains, helping to reconstruct factors such as the time of death, drug presence, and possible post-mortem body displacement.[3] After death, autolysis commences, and the cells start degrading as the enzymes inside them come into action. This results in the process of decomposition, where bacteria in the gastrointestinal system emit fluids and gases such as hydrogen sulphide, carbon dioxide, methane, ammonia, sulphur dioxide, and hydrogen.[4]

Insects and arthropods are attracted to volatile compounds emitted from a decaying body to feed on the decomposing vertebrate remains known as carrion.[5] These remains host four primary types of insects[6]: necrophagous species, predators and parasites, omnivores, and other species, including springtails and spiders. In forensic entomology, the first two groups, mainly from the Diptera (flies) and Coleoptera (beetles) orders, are significant.[7] The pattern and frequency of arthropod arrival depend on the specific stage of carrion decomposition.[8]

True flies, or Diptera, are crucial in forensic investigations, with the most prevalent species being Calliphoridae (blow flies), Sarcophagidae (flesh flies), and Muscidae (house flies). Calliphoridae and Sarcophagidae usually arrive shortly after death, while Muscidae generally start to colonize during the bloating stage of decomposition.[9] Insect colonizers play a vital role in forensic investigations by helping to estimate the time of death, trace the movement of the corpse, clarify the circumstances and cause of death, link suspects to the crime scene, and determine the post-mortem interval (PMI), which is the period between death and the discovery of the body.[10] However, despite its widespread use globally, forensic entomology is still lagging in Kenya, despite the rising cases of unknown deaths over the years. The estimation of time elapsed since death can be deduced through knowledge of the insect colonization and succession on dead bodies.[11] No research has been conducted on insect colonization and succession on cadavers in Kenya. Furthermore, the presence of drugs in the body significantly impacts insect development and succession.[12] In some cases, the effects of drugs on these insects are determined by their concentration, while in others, their mere presence is enough to influence the insects.[13] Therefore, this study sought to identify the effect of flunitrazepam, describe the baseline insect fauna, and develop insect successional patterns in the upper Kabete region of Kiambu County.

Materials and methods

Research design

This study utilized an experimental case-control research design. Four domestic pigs (*Sus Scrofa domesticus*) weighing 24.6 kg, 24.7 kg, 24.9 kg, and 25.0 kg were used. The pigs were housed in an adequate, well-ventilated pen two weeks before the study commenced. During that period, they were provided fresh food and water and excluded from any medication. One pig was used as a control, and the other three were used as the experimental group. The pigs were selected because they have similar gastrointestinal fauna and skin features to human beings.[14] Also, their size is similar to that of an average human torso. Such experiments are conducted using pigs because of their controlled environment and the fact that they provide a less controversial alternative to human cadavers.[15]

The pigs utilized in this research were sourced from the veterinary farm of the University of Nairobi's Faculty of Veterinary Medicine, a facility dedicated to breeding and maintaining animals for academic and research activities. Therefore, the animals were not privately owned, and their use was approved by the institution in line with established ethical and animal welfare guidelines.

To mimic the effects of drink spiking, the experimental pigs were orally dosed with different doses of flunitrazepam dissolved in 250 ml of vodka (40 per cent ethanol strength) on the evening of June 30, 2021.[16] Particularly, Experimental Group 1 (EXP GR1) was given 1 mg, Experimental Group 2 (EXP GR2) was given 2 mg, and Experimental Group 3 (EXP GR3) was given 3 mg of flunitrazepam. All pigs were euthanised at 5.00 a.m., using the electric stunning method that involves applying electric current through the brain.

The electric stunning method was performed as follows; electrodes were placed on both sides of the pig's head to deliver a current that passes through the brain, causing a rapid loss of sensibility and insensibility to pain. The electrocution was done with sufficient current of 1.3 A at 250V for 20 seconds followed by a second electrocution at 300V on the chest for 5 seconds to fibrillate the heart causing death.[17]

The Directorate of Veterinary Services in Kenya, as well as the American Veterinary Medical Association, approves electric stunning as a humane and fast way of euthanizing the animals in commercial slaughter facilities, and it is thus in compliance with animal welfare regulations.

Figure 1. The experimental design for the control and the experimental pigs' carcasses. The experimental groups had been administered with 1mg/250 ml Vodka (EXP GR1 = Experimental Group 1), 2 mg/250 ml Vodka (EXP GR2 = Experimental Group 2), and 3 mg/250 ml Vodka (EXP GR3 = Experimental Group 3).

The pig carcasses were promptly transported to the veterinary farm at Upper Kabete Campus. At the site, the carcasses were left in field conditions to decompose naturally until they reached the final stage of decay. The decomposition and insect colonization were monitored for approximately 3 months. Each pig was on open ground and secured against predators with metal cages (Figure 1). These 92 x 92 x 153 cm cages were made of steel-welded frames using 2.5 cm tubing and enclosed with 1.27 cm mesh hardware cloth. They were positioned approximately 100 meters apart at the transition zone between a dense wooded area and an open pasture on the farm.

Ethical approval for animal use was provided by the Biosafety, Animal Use and Ethics Committee of the Department of Veterinary Anatomy and Physiology, Faculty of Veterinary Medicine, University of Nairobi. REF: FVM BAUEC/2019/203.[16]

Study site

The study was conducted at Kanyariri in the upper Kabete region of Kiambu County within the veterinary farm of the University of Nairobi (Kenya).[16] The site was situated in a softwood tree forest. The soil of the site is black, and the tree cover is low, and as a result, the air was humid near the forest floor. The altitude of the study site is 1820M (Latitude -1.2492350 E and Longitude 36.7420570 S), which is a typical montane region. The study site contained remnants of potential natural vegetation, secondary forest species, and other tree species common in evergreen upland forests (Figure 2). The site is also characterized by disturbance and colonized by native invasive tree species. Furthermore, exotic invasive species were also common.

Drug acquisition and sample collection

Insect sampling encompassing adult insects and non-flying invertebrates was performed daily until the carcasses reached the dry remains stage of decomposition. The sampling schedule was as follows: three times daily (7:00 AM, 1:00 PM, and 7:00 PM) for the initial eight days, twice daily (11:00 AM and 5:00 PM) for the subsequent eight days, and once daily (12:00 PM) until the dry remains stage was achieved, which occurred 60 days after death. Each sampling involved capturing arthropods flying near or resting on the carcass using an entomological net. Insects were also collected from natural body cavities (eyes, nose, mouth, and anus) as well as from the cardiac puncture wound. This study concentrated on Sarcophagidae (flesh flies), Calliphoridae (blow flies), Muscidae (house flies), and skin beetles. Adult insects were captured using aerial net sweeps above the carcasses and in the surrounding environment. Flightless insects were removed from the carcasses with forceps. Pitfall traps and plastic cups filled with soapy water were used to capture crawling insects.

Figure 2. Natural vegetation of the study site at Kanyariri in the upper Kabete region of Kiambu County within the veterinary farm of the University of Nairobi (Kenya) constituted a softwood tree forest. The soil of the site is black, and the tree cover is low, and as a result, the air was humid near the forest floor. The altitude of the study site is 1820M (Latitude -1.2492350 E and Longitude 36.7420570 S), which is a typical montane region.

The insects were immersed in boiling water for 30 seconds and then preserved in 75% ethyl alcohol (sup: Scharlau, Cat No: ET00052500). This method halts further development, allowing for the assessment of the insects' developmental stages. Each carcass had two pitfall traps placed approximately 8 cm away from the abdomen; each trap consisted of a plastic cup of soapy water (12 mm diameter and 7 mm deep). All the samples taken were carefully labelled with details such as the geographical location, case number, date and time of collection, name of the collector, and environmental conditions.

Insects identification and data analysis

The insects were identified using a microscope (Model: Leica zoom 2000, Serial No: 1408CX) and standard identification keys. Data analysis was carried out using Microsoft Excel Office 2010 and Statistical Package for the Social Sciences (SPSS) version 25. Correlations between the insect orders that were collected, the number of insects on the decomposing carcasses, the levels of decomposition, and the corresponding periods were depicted in tables. Species diversity, abundance, and distribution in the study were depicted in graphs.

Results

The successional pattern of major forensically important insects and arthropods was analyzed only to identify species' occurrence and density trends. The major emphasis was on the Diptera species that colonized the remains and their succession. It is important to note that only the Diptera species that colonized the remains are represented in the results with a view to their outlook on their application in forensic investigations in Kenya. The findings are presented in chronological sequence for each habitat, and the succession pattern is exhibited as a tabulation. While it is unlikely that the list of species found with the remains is exhaustive, it does indicate the succession patterns of Hymenoptera and Coleoptera.

Identification of the type of insects on the carrion

Generally, three different arthropod orders were isolated from carrion at different stages of decomposition. This study obtained 27 insect species, 20 families, and three orders. The insect species were the same in the four groups; however, the number of insects reduced with the introduction and increased concentration of flunitrazepam. The control had the highest number of insects at 1,259, while EXP GR1, EXP GR2, and EXP GR3 had 1,089, 932, and 852, respectively (Table 1).

Abundance of various insect families

In all four groups (Control, EXP GR1, EXP GR2, and EXP GR3), Calliphoridae was the most abundant, comprising 229 (19.30%), 191 (17.54%), 174 (18.67%), and 148 (17.37%) individuals, respectively (Figure 2). Muscidae followed this in all four groups with 119 (12.63%), 141 (12.95%), 90 (9.66%), and 87 (10.21%) individuals, respectively

Table 1. Number of insects in the four categories of carcasses.

Order	Family	Genus/species	Control Group	Experimental Group 1	Experimental Group 2	Experimental Group 3
Diptera	Calliphoridae	*Chrysomya bezziana*	28	28	22	20
		Chrysomya megacephala	17	17	17	18
		Chrysomya chloropyga	42	33	26	24
		Chrysomya vomitoria	54	36	36	23
		Calliphora vicina	23	21	20	15
		Lucilia sericata	47	30	28	29
		Protophormia terraenovae	32	26	25	19
	Sarcophagidae	*Parasarcophaga ruficornis*	46	32	23	25
		Sarcophaga Inzi	34	25	20	25
	Muscidae	*Musca domestica*	98	81	38	37
		Stomoxys evanida	61	60	52	50
	Sphaeroceridae	*Leptocera sp.*	56	58	52	35
	Sepsidae	*Allosepsis indica*	55	42	31	22
	Micropezidae	*Mimegralla albimana*	49	38	31	30
	Stratiomyidae	*Ptecticus melanurus*	15	10	10	11
	Phoridae	*Megaselia scalaris*	29	24	26	20
	Fanniidae	*Fannia canicularis*	74	49	27	26
Coleoptera	Formicidae	*Pheidole megacephala*	89	80	69	67
	Nitidulidae	*Nitidulidae sp.*	24	24	24	38
	Staphylinidae	*Staphylinidae violaceous*	20	22	24	20
	Carabidae	*Angionychus lividus*	23	20	20	20
	Bostrichidae	*Bostrichidae sp.*	45	45	45	43
	Histeridae	*Hister monitor*	66	65	59	51
	Silphidae	*Thanatophilus sinuatus*	21	21	22	14
	Dermestidae	*Dermestes maculatus*	44	45	44	45
	Cleridae	*Necrobia rufipes*	88	81	71	62
Hymenoptera	Formicidae	*Camponotus sericeus*	48	45	45	38
	Apidae	*Trigona carbonaria*	31	31	25	25
Total			**1259**	**1089**	**932**	**852**

The table shows the number of insect species collected in the four categories of carcasses, both the control and experimental groups. The insect species were the same in the four groups; however, the number of insects reduced with the introduction and increased concentration of flunitrazepam. The control had the highest number of insects at 1,259, while EXP GR1, EXP GR2, and EXP GR3 had 1,089, 932, and 852.

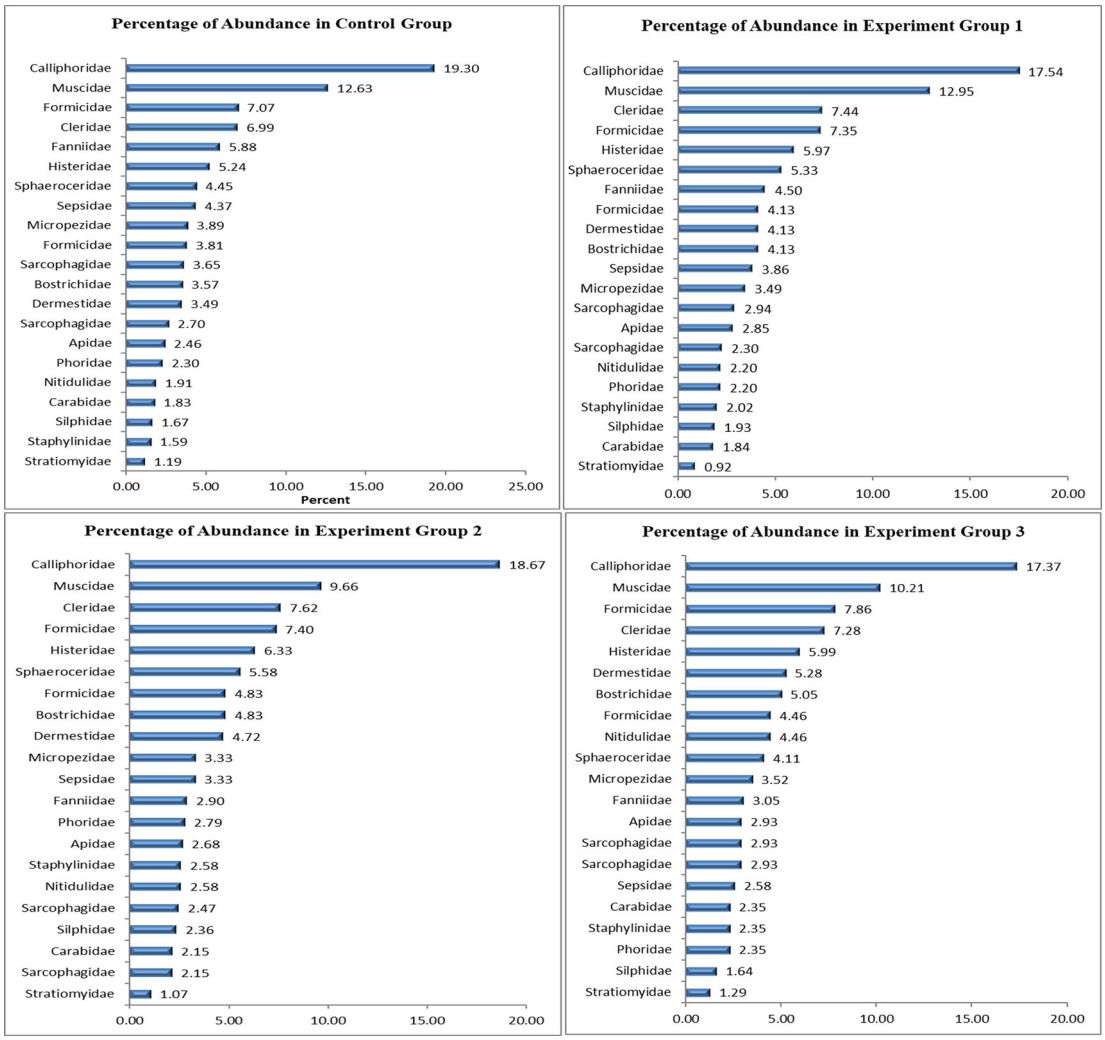

Figure 3. The Percentage of abundance of insects in the four groups (control and experiment groups). In all four groups (Control, EXP GR1, EXP GR2, and EXP GR3), Calliphoridae was the most abundant, comprising 229 (19.30%), 191 (17.54%), 174 (18.67%), and 148 (17.37%) individuals, respectively. Muscidae followed this in all four groups with 119 (12.63%), 141 (12.95%), 90 (9.66%), and 87 (10.21%) individuals.

(Figure 3). The least abundant family in all four groups was the family Stratiomyidae. These results provide insight into the relative abundance of different insect families within each experimental group, highlighting the consistent dominance of Calliphoridae across all groups.

Species of arthropods associated with each stage of decaying pigs

Different species of arthropods were associated with various stages of pig decay. However, the number of insects decreased with the introduction of flunitrazepam and its concentration.

Fresh stage: At the fresh stage of decay, the recovered insects included *Pheidole megacephala, Chrysomya bezziana, Chrysomya megacephala, Chrysomya chloropyga, Chrysomya vomitoria, Calliphora vicina, Lucilia sericata, Protophormia terraenovae, Parasarcophaga ruficornis, Musca domestica, Stomoxys evanida*, and *Sarcophaga Inzi.* There were also a few specimens of *Leptocera sp.*

Bloat stage: The bloat stage was characterized by the presence of *Pheidole megacephala, Chrysomya bezziana, Chrysomya megacephala, Chrysomya chloropyga, Chrysomya vomitoria, Calliphora vicina, Lucilia sericata, Protophormia terraenovae, Parasarcophaga ruficornis, Musca domestica, Stomoxys evanida, Sarcophaga Inzi, Leptocera sp., Bostrichidae sp., Staphylinidae violaceous, Angionychus lividus, Necrobia rufipes,* and *Trigona carbonaria.*

Active decay stage: Insects included *Chrysomya bezziana, Chrysomya megacephala, Chrysomya chloropyga, Chrysomya vomitoria, Calliphora vicina, Lucilia sericata, Parasarcophaga ruficornis, Musca domestica, Stomoxys evanida, Sarcophaga Inzi, Leptocera sp., Allosepsis indica, Mimegralla albimana, Megaselia scalaris, Fannia canicularis, Pheidole megacephala, Nitidulidae sp., Staphylinidae violaceous, Angionychus lividus, Hister monitor, Dermestes maculatus, Necrobia rufipes, Camponotus sericeus*, and *Trigona carbonaria.*

Advance decay stage: Was characterized by the presence of *Stomoxys evanida, Leptocera sp., Allosepsis indica, Mimegralla albimana, Ptecticus melanurus, Megaselia scalaris, Fannia canicularis, Pheidole megacephala, Nitidulidae sp., Staphylinidae violaceous, Bostrichidae sp., Hister monitor, Thanatophilus sinuatus, Dermestes maculatus, Necrobia rufipes. Additionally, other species like Musca domestica, Calliphora vicina, Chrysomya bezziana, Lucilia sericata, Chrysomya chloropyga, Chrysomya vomitoria*, and *Chrysomya megacephala* were also present, although their numbers had significantly reduced.

Dry stage: The main species included *Dermestes maculatus, Bostrichidae sp., Hister monitor, Pheidole megacephala, Necrobia rufipes, Nitidulidae sp., Chrysomya chloropyga*, and *Allosepsis indica.* However, there were only a few specimens of *Musca domestica, Mimegralla albimana, Ptecticus melanurus, Calliphora vicina, Megaselia scalaris*, and *Thanatophilus sinuatus*.

Entomological succession at different stages of decomposition

The succession of insects in different stages of decomposition among the four groups of carcasses (Control, EXP GR1, EXP GR2, and EXP GR3) was consistent.

Fresh stage: In the Diptera order, species collected during the fresh stage of carcasses in all four groups included *Chrysomya bezziana, Chrysomya megacephala, Chrysomya chloropyga, Chrysomya vomitoria, Lucilia sericata, Protophormia terraenovae, Parasarcophaga ruficornis, Musca domestica*, and *Stomoxys evanida.* In the Coleoptera order, the species found in the fresh stage included *Pheidole megacephala*, while in the Hymenoptera order, *Camponotus sericeus* was found.

Bloating stage: In the bloating stage, species in the Diptera order found in all four groups (control, experiment group one, experiment group two, and experiment group three) included *Chrysomya bezziana, Chrysomya megacephala, Chrysomya vomitoria, Calliphora vicina, Lucilia sericata, Protophormia terraenovae, Parasarcophaga ruficornis, Sarcophaga Inzi, Musca domestica, Stomoxys evanida, Fannia canicularis*, and *Ptecticus melanurus.* In the Coleoptera order, species found included *Pheidole megacephala, Staphylinidae violaceous, Angionychus lividus, Bostrichidae sp., Camponotus sericeus, Necrobia rufipes*, and *Trigona carbonaria.*

Active decay stage: In the Control, EXP GR1, EXP GR2, and EXP GR3 groups, most of the categorized insect species were found, except for a few. For instance, *Ptecticus melanurus, Bostrichidae sp.*, and *Thanatophilus sinuatus* were not found in this stage in any of the four groups of pigs. Additionally, *Allosepsis indica* and *Mimegralla albimana* species were present in very low numbers. Abundant species in this stage included *Chrysomya bezziana, Chrysomya megacephala, Chrysomya chloropyga, Chrysomya vomitoria, Calliphora vicina, Lucilia sericata, Parasarcophaga ruficornis, Sarcophaga Inzi, Musca domestica, Stomoxys evanida, Leptocera sp., Fannia canicularis, Pheidole megacephala, Nitidulidae sp., Staphylinidae violaceous, Angionychus lividus, Hister monitor, Dermestes maculatus, Necrobia rufipes, Camponotus sericeus*, and *Trigona carbonaria.*

Advanced decay stage: New insects began to appear while others disappeared at this stage. For instance, *Chrysomya bezziana, Chrysomya megacephala, Parasarcophaga ruficornis, Sarcophaga Inzi, Angionychus lividus*, and *Camponotus sericeus* disappeared in this stage. Additionally, the *Megaselia scalaris* species had very few insects during this stage. Therefore, species in the advanced stage included *Trigona carbonaria, Musca domestica, Stomoxys evanida, Leptocera sp., Allosepsis indica, Mimegralla albimana, Ptecticus melanurus, Megaselia scalaris, Fannia canicularis, Pheidole megacephala, Nitidulidae sp., Staphylinidae violaceous, Bostrichidae sp., Hister monitor, Thanatophilus sinuatus, Dermestes maculatus, Necrobia rufipes, Chrysomya chloropyga, Chrysomya vomitoria, Calliphora vicina*, and *Lucilia sericata.*

Dry stage: Species from the Diptera and Hymenoptera orders significantly decreased in all four groups of decomposing pigs (control, experiment group one, experiment group two, and experiment group three), leaving species from the Coleoptera order. The remaining Diptera order species included *Chrysomya chloropyga, Musca domestica, Allosepsis indica, Mimegralla albimana, Ptecticus melanurus*, and *Megaselia scalaris.* The remaining Coleoptera order species included *Pheidole megacephala, Nitidulidae sp., Bostrichidae sp., Hister monitor, Dermestes maculatus*, and *Necrobia*

Table 2. Independent samples test for control and experimental group one.

		Levene's test for equality of variances		t-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% confidence interval of the difference	
									Lower	Upper
Number of Insects	Equal variances assumed	.403	.529	.584	38	.562	8.20000	14.03483	-20.21204	36.61204
	Equal variances not assumed			.584	36.419	.563	8.20000	14.03483	-20.25259	36.65259

Table 2 compares the mean insect numbers between the control and experimental groups. The results reveal no significant difference ($p > 0.05$) in insect numbers between these two groups.

Table 3. Independent samples test for the control and experimental group two.

		Levene's test for equality of variances		t-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% confidence interval of the difference	
									Lower	Upper
Number of Insects	Equal variances assumed	1.699	.200	1.230	38	.226	16.00000	13.00821	-10.33374	42.33374
	Equal variances not assumed			1.230	32.609	.227	16.00000	13.00821	-10.47745	42.47745

Table 3 compares mean insect numbers between the control and experimental group two. The results indicate no significant difference ($p > 0.05$) in insect populations between these groups.

Table 4. Independent samples test for the control and experimental group three.

		Levene's test for equality of variances		t-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% confidence interval of the difference	
									Lower	Upper
Number of Insects	Equal variances assumed	2.408	.129	1.563	38	.126	19.70000	12.60337	-5.81419	45.21419
	Equal variances not assumed			1.563	30.439	.128	19.70000	12.60337	-6.02397	45.42397

Table 4 compares the mean insect populations between the control and experimental group three. The results suggest no significant difference ($p > 0.05$) in insect numbers between these groups.

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rufipes. This information provides insights into the arthropod species' changing composition at different pig decomposition stages.

Differences in insect populations among the four categories of pig carcasses

An independent sample t-test was used to assess these differences. Table 2 compares the mean insect numbers between the control and experimental groups. The results reveal no significant difference ($p>0.05$) in insect numbers between these two groups.

Table 3 compares mean insect numbers between the control and experimental group two. The results indicate no significant difference ($p>0.05$) in insect populations between these groups.

Table 4 compares the mean insect populations between the control and experimental group three. The results suggest no significant difference ($p>0.05$) in insect numbers between these groups.

Discussions

Pig carcass decomposition was classified into five stages: fresh, bloated, active decay, advanced decay, and dry. These stages correspond with the five primary decomposition stages described by Eberhardt and Elliot[18] fresh, bloated, active decay, post-decay, and skeletal.[19] They also match the classification by Moura,[20] which includes fresh, bloated, decaying (combining active and advanced decay), and dry stages.

The current study results differ from Odo's findings,[21] which reported the fresh stage lasting from day 0 to day 1, the bloated stage occurring from day 2 to day 3, the active decay stage spanning from day 3 to day 6, the advanced decay phase starting on day 7 and continuing until day 15, and the dry decay stage beginning on day 16 and ending on day 60.[22] These differences in decomposition rates may be attributed to temperature variations. Temperature is one of the extrinsic factors crucial for bacterial development, affecting decomposition rates. It plays a pivotal role in decomposition, and its fluctuations can significantly affect the observed decomposition stages.[23] It is important to mention that temperature was not measured directly in this study, but in future studies, it may be useful to add temperature data as a variable.[24] This would give a better idea of the role of temperature changes, daily and seasonal, in the course of decomposition. Additional factors that affect decomposition are age, constitution, cause of death, ventilation, and humidity.[21]

The four carcass groups contained insects of the orders Diptera, Coleoptera and Hymenoptera. This observation is consistent with that made by Vitta[25] on the decomposition of pig carcasses in which two insect orders were observed. However, they differ from Moura's findings,[20] which included three orders of insects: Diptera, Coleoptera, and Hymenoptera. Throughout all decomposition stages, the observed insect species included *Chrysomya bezziana, Chrysomya megacephala, Chrysomya chloropyga, Chrysomya vomitoria, Calliphora vicina, Lucilia sericata, Protophormia terraenovae, Parasarcophaga ruficornis, Sarcophaga Inzi, Musca domestica, Stomoxys evanida, Leptocera sp., Allosepsis indica, Mimegralla albimana, Ptecticus melanurus, Megaselia scalaris, Fannia canicularis, Pheidole megacephala, Nitidulidae sp., Staphylinidae violaceous, Angionychus lividus, Bostrichidae sp., Hister monitor, Thanatophilus sinuatus, Dermestes maculatus, Necrobia rufipes, Camponotus sericeus*, and *Trigona carbonaria.* These findings support Vitta[25] observation of species such as *Chrysomya rufifacies, Chrysomya megacephala, Musca domestica, Fannia canicularis, Parasarcophaga ruficornis, Piophila casei, Dermestes maculatus*, and *Necrobia rufipes* during pig carcass decomposition.

Among all the collected insects, Calliphoridae were the most abundant, followed by families Muscidae, Formicidae, Cleridae, Sarcophagidae, Fanniidae, Histeridae, Sphaeroceridae, Sepsidae, Micropezidae, Formicidae, Bostrichidae, Dermestidae, Apidae, Phoridae, Nitidulidae, Carabidae, Silphidae, Staphylinidae, and Stratiomyidae. These findings are consistent with Eberhardt and Elliot's observations[18] of the primary colonizers being Calliphoridae, Muscidae, Formicidae, Cleridae, Sarcophagidae, Fanniidae, Histeridae, Micropezidae, and Formicidae. They also align with discovery of adult specimens from eight Diptera families: Calliphoridae, Muscidae, Sarcophagidae, Phoridae, Piophilidae, Fanniidae, Sphaeroceridae, and Anthomyiidae during five stages of decomposition.[26]

The insect succession during different decomposition stages among the four carcass groups (control, experiment group one, experiment group two, and experiment group three) was consistent.[27] In the fresh stage, Diptera species such as *Chrysomya bezziana, Chrysomya megacephala, Chrysomya chloropyga, Chrysomya vomitoria, Lucilia sericata, Protophormia terraenovae, Parasarcophaga ruficornis, Musca domestica*, and *Stomoxys evanida* were observed. Coleoptera species, including *Pheidole megacephala*, and Hymenoptera species like *Camponotus sericeus*, were also found.[28] These findings agree with Odo's observation[21] of main species during the fresh stage, which included *Chrysomya albiceps, Lucilia sericata, Musca domestica, Stomoxys evanida, Sarcophaga inzi*, and *Camponotus sericeus.*

They also align with Heo identification of species[29] like *Chrysomya rufifacies, Chrysomya megacephala, Parasarcophaga ruficornis*, and *Musca domestica* during the fresh stage.

During the bloating stage, Diptera species such as *Chrysomya bezziana, Chrysomya megacephala, Chrysomya vomitoria, Calliphora vicina, Lucilia sericata, Protophormia terraenovae, Parasarcophaga ruficornis, Sarcophaga Inzi, Musca domestica, Stomoxys evanida, Fannia canicularis*, and *Ptecticus melanurus* were observed. Coleoptera species like *Pheidole megacephala, Staphylinidae violaceous, Angionychus lividus, Bostrichidae sp., Camponotus sericeus, Necrobia rufipes*, and *Trigona carbonaria* were also found. These findings correspond with Odo's observation of species[21] during the bloated stage, including *Chrysomya chloropyga, L. sericata, Chrysomya vomitoria, Musca domestica, Stomoxys evanida, Trigona carbonaria, Melipona beecheii, Camponotus sericeus, Camponotus perrisii, Monomorium minimum, Crematogaster sp, S. violaceous*, and *Necrobia rufipes*. They also align with Vitta identification of species[25] like *Fannia canicularis, Chrysomya rufifacies, Chrysomya megacephala, Parasarcophaga ruficornis*, and *Musca domestica* during the bloated stage.

Most insect species from the four groups were present in the active decay stage, except for *Ptecticus melanurus, Bostrichidae sp.*, and *Thanatophilus sinuatus.*

Additionally, *Allosepsis indica* and *Mimegralla albimana* were scarce. Abundant species during this stage included *Chrysomya bezziana, Chrysomya megacephala, Chrysomya chloropyga, Chrysomya vomitoria, Calliphora vicina, Lucilia sericata, Parasarcophaga ruficornis, Sarcophaga Inzi, Musca domestica, Stomoxys evanida, Leptocera sp., Fannia canicularis, Pheidole megacephala, Nitidulidae sp., Staphylinidae violaceous, Angionychus lividus, Hister monitor, Dermestes maculatus, Necrobia rufipes, Camponotus sericeus*, and *Trigona carbonaria.* Odo also observed insects during the active decay stage of pig carrion decomposition, including *L. sericata, Chrysomya chloropyga, Chrysomya vomitoria, Musca domestica, Stomoxys evanida, Sarcophaga inzi, Necrobia rufipes, Coelus ciliata, Hister monitor*, and *D. maculatus*, among others.[21] These findings are consistent with Abd El-Gawad et al.[30] identification of species like *Chrysomya megacephala, Parasarcophaga ruficornis*, and *Piophila casei* during the active decay stage.

In the advanced decay stage, new insect species appeared while others disappeared. Species like *Chrysomya bezziana, Chrysomya megacephala, Parasarcophaga ruficornis, Sarcophaga Inzi, Angionychus lividus*, and *Camponotus sericeus* disappeared in this stage. Additionally, the *Megaselia scalaris* species were scarce during this stage. The species present in the advanced stage included *Trigona carbonaria, Musca domestica, Stomoxys evanida, Leptocera sp., Allosepsis indica, Mimegralla albimana, Ptecticus melanurus, Megaselia scalaris, Fannia canicularis, Pheidole megacephala, Nitidulidae sp., Staphylinidae violaceous, Bostrichidae sp., Hister monitor, Thanatophilus sinuatus, Dermestes maculatus, Necrobia rufipes, Chrysomya chloropyga, Chrysomya vomitoria, Calliphora vicina*, and *Lucilia sericata.* These findings are like Odo's observations of species during the advanced decay stage of pig carrion decomposition, including *C. vomitoria, Chrysomya chloropyga, Chrysomya albiceps, Lucilia sericata, Stomoxys evanida, Musca domestica, Thanatophilus sinuatus, H. monitor, N. rufipes, Coelus ciliatus, Harmonia axyridis, Messor galla*, and *Melipona beecheii.*[21] They also align with Vitta identification of species like *Parasarcophaga ruficornis, Dermestes maculatus, Hister sp.*, and *Necrobia rufipes* during the advanced decay stage.[25]

In the dry stage, species from the Diptera and Hymenoptera orders significantly decreased in all four groups of decomposing pigs (control, experiment group one, experiment group two, and experiment group three), leaving species from the Coleoptera order.[31] Remaining species from the Diptera order included *Chrysomya chloropyga, Musca domestica, Allosepsis indica, Mimegralla albimana, Ptecticus melanurus*, and *Megaselia scalaris.* Species from the Coleoptera order that remained included *Pheidole megacephala, Nitidulidae sp., Bostrichidae sp., Hister monitor, Dermestes maculatus*, and *Necrobia rufipes.* These findings correspond with Odo's observation of species during the dry decay stage of pig carrion decomposition, including *M. domestica, Chrysomya chloropyga, L. sericata, Monomonium minimum, Solenopsis molesta, N. rufipes, H. monitor*, and *Zootermopsis augusticollis.*[21] They also align with Packard and Dabbs' identification of species like *Dermestes maculatus and Trox sp.* during the dry stage.[32]

Compared to the control, the experimental pig carcasses showed extended durations in each of the five decomposition stages (fresh, bloated, active, advanced, and dry).[22] The extension increased with the concentration of flunitrazepam in experimental groups one, two, and three. These findings align with a study by the National Institute of Justice,[33] which found that drugs, especially cancer drugs, tend to lower the decomposition rate of human corpse. The insect species were the same in all four groups, including the control. However, the number of insects decreased with the introduction and concentration of flunitrazepam for EXP GR1, EXP GR2, and EXP GR3, respectively. The control had the highest number of insects at 1,259, followed by EXP GR1 with 1,089 insects, then EXP GR2 with 932 insects, and the least was EXP

GR3, which had 852 insects. This suggests that the introduction and concentration of flunitrazepam affects the abundance of insects colonizing the corpse.

Nonetheless, it is important to mention this study's limitations to ensure readers do not misunderstand the results. Climatic differences constituted one of the major constraints, with conditions regulating the speed of decomposition. Moreover, although the sample size of this research is large, it might not be sufficient to capture the diversity of conditions that exist in a setting where forensic investigators work.[34] Such limitations must be considered during the analysis of the findings and should be improved in the future.

Temperature is one of the elements that defines decomposition and affects the phases observed.[35] Temperature was not controlled in this study or even measured as a variable, but treating it as a potential variable in future studies would be helpful. This would assist in knowing how temperature changes, either daily or seasonally, affect decomposition.

Other than temperature, other factors such as air movement and populations of microbes in the context of decomposition can also affect the decomposition rate and the process's various phases. For example, the oxygen and bacterial activity during the process may vary widely depending on the burial depth and soil type.[36] The moderating effect of these factors on the relationship between decomposition stages and these factors can also be determined through further research.[37]

The presence and activity of specific insect species have been known to influence decomposition rates. For instance, in the fresh stage, the predominance of particular species, like *Chrysomya bezziana* and *Chrysomya megacephala*, suggests their significant role as early colonizers.[38] Their feeding activities accelerate decomposition during this phase. Future studies could delve deeper into the ecological dynamics of insect species during decomposition, shedding light on their distinct contributions to the process.

Although this study was done on flunitrazepam, it is important to note that other drugs can also react with the process of decomposition. Future studies ought to broaden the search by exploring an extended array of substances typically connected with forensic cases. Such a thorough approach will allow forensic scientists to make better judgments in instances where drugs are suspected.

Conclusions

This paper shows that increasing concentrations of flunitrazepam led to an elongation of the five decomposition phases. Further, the insect succession trends during these periods were similar among all four groups of carcasses (control, EXP GR1, EXP GR2, and EXP GR3). The total population of insects in each stage, however, decreased as the concentration of flunitrazepam increased. Despite these observations, no statistically significant differences in insect numbers were detected among the four carcass groups.

While this study focused on flunitrazepam, it is crucial to acknowledge that other drugs may also interact with decomposition processes. Future research should expand the scope to investigate a broader range of substances commonly associated with forensic cases. This comprehensive approach will enable forensic scientists to make more informed assessments when drugs are suspected to be involved. Further research should also consider using human corpses.

Ethics and consent

The Biosafety, Animal Use and Ethics Committee of the Department of Veterinary Anatomy and Physiology, Faculty of Veterinary Medicine, University of Nairobi, provided ethical approval for animal use. REF: FVM BAUEC/2019/203 on 12/03/2019.[16]

Software and code

SPSS version 25 and Microsoft Excel Office 2010.

Reporting guidelines

Mendeley Data: ARRIVE 2.0 checklist for Effect of Flunitrazepam on Decomposition and Forensically Important Insects Colonization of Pig Carcasses: An Entomology Perspective. https://doi.org/10.17632/mnt66z9tks.1.[40]

Data availability

Underlying data

Mendeley Data: Effect of Flunitrazepam on Decomposition and Forensically Important Insects Colonization of Pig Carcasses with Corresponding Meteorological Records: https://doi.org/10.17632/4r4sp2vzn9.1.[39]

This project contains the following underlying data

- Data file 1. Insect Colonization.xlsx
- Data file 2. Kabete Climate Data.xlsx
- Data file 3. Figures and Tables for the Decomposition of Pig Carcasses Data.docx

Acknowledgment

The authors would like to thank Mr. Caleb Musango, Mr. Richard Ochieng, and Mrs. Phyllis Mwai for their unwavering support and the Department of Human Anatomy, Department of Biochemistry, and Department of Biology (Entomology section), University of Nairobi, for providing the study site and all the required equipment and reagents for this research. The authors would like to extend their appreciation to Phoebe and Richard Ochieng for the identification of insects. The above-mentioned individuals and departments have granted permission to be acknowledged for their contribution to this research.

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Open Peer Review

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Version 1

Reviewer Report 27 November 2025

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?

Sapna Sharma
Maharshi Dayanand University, Haryana, India

The manuscript has been written based on the study conducted on the effects of flunitrazepam on decomposition rate and insect succession using pig carcasses. The topic is definitely much needed especially no such study has been carried out in Kenya. But the most basic feature of such studies i.e the temperature is missing which limits its relevance. The manuscript has the a number of flaws according to me which have been discussed heading wise as under:

Background
I didn't understand the meaning and context of growing number of unidentified deaths in terms of Forensic entomology.

Introduction
Forensic entomology also includes civil cases, so the opening definition is incomplete.
How are growing number of unidentified deaths relevant to forensic entomology? Aren't the pathological methods enough? There needs to be a justification for its usage in Kenya.
The closing statement of the introduction is poorly structured. Reframe.
Any specific justification for choosing this drug? Include in the introduction.
Any specific reason for selecting Vodka? If yes, give a context and reference in the introduction.
What were the criteria for dose selection? They can't be random.
Weren't the pigs still under the effect of flunitrazepam when stunned?
The last sentence about the ethical approval can be merged into the 2nd para of the methodology with a reframing.

Study site
There is no mention of temperature or season and humidity which are the backbone of such studies.

Drug acquisition and sample collection
Were 60 days common for all carcasses as mentioned in line 4 of this heading?
Adult Flying insects will be better in the 7th line.

Figure 2: the description is already given so it is needed here.

Insects identification and data analysis
Insects' instead of Insects
There is no mention of the identification keys used with relevant references.
When the aim was to study succession, there's no reason to concentrate solely on Diptera.

Identification of the type of insects on the carrion
Why is this a carrion? Which carrion is being talked about here?
Isolated is not an apt term here.
Table 1 has a wrong order assigned to Formicidae.
I don't see any point of being descriptive under tables and Figures.
Table 1's caption should be reframed.
Figure 3: Percentage of what? What is on the Y axis in these figures?
Please be consistent with the groups/ categories. Use "groups" and keep it consistent in the captions and text.

Species of arthropods associated with each stage of decaying pigs
Whose categorisation of decomposition stages have you followed? Cite. It should come here instead of being in the discussion.
It makes no sense when the days of each stage aren't mentioned. Moreover, which stages of the insects were collected at each decomposition stage?
Some species are very specific to some temperature ranges so without temperatures all these species don't hold any value.
Is *Pheidole megacephala* a coleopteran? That is a huge mistake especially since it is mentioned time and again.
Keep consistency in the way of writing various groups. Its confusing and casual to write it differently.

Differences in insect populations among the four categories of pig carcasses
This could remain part of the previous section. It is lending no value to the text here. Separate heading is unnecessary, and these tables are of no value.

Discussions
Where did these days suddenly appear from? I didn't find any such mention in the results, so how can it be compared?
Temperature has been mentioned as being important but there is no mention of it in the study design which is a huge flaw. The discussion here is about the results that haven't been reported.
Para 3 is very casually written. Read and rewrite properly. It's just a repletion of the results.
Para 4 describes the figure which was self-explanatory. Where is the discussion? Where did Odo and Heo conduct their studies and at what temperature?
Para 6: where is the discussion? Right through page 12 till the last paragraph it is San discussion.
Last Para on page 12: Th results have no mention of the duration of the decomposition stages, so how was this conclusion about extended duration drawn?
But flunitrazepam is not a cancer drug. Compare with the same class of drugs.
The constraints of the study are confusing in terms of your claim that the sample size was large.
The study of the climatic conditions especially the ones relevant to the placement of carcasses (in and around) forms the crux which is absent here. If that the temperature wasn't controlled it

wouldn't have mattered much but it not being measured is a massive flaw as most such studies take it as one of the most important factors.
The discussion about air movement and microbes is irrelevant here.

Conclusions
The conclusions drawn should be based on the results which are missing in the context of elongation of the decomposition stages.
No statistically significant differences make the value of the study zero. The authors should do away with this statistical tool because it isn't rendering any merit here.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Forensic Entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 17 September 2025

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?

Swaima Sharif
Aligarh Muslim University, Aligarh, India

The study aims to examine the effects of flunitrazepam on decomposition rate and insect succession using pig carcasses as human analogues. The topic is of potential forensic relevance, but the current manuscript suffers from fundamental design weaknesses, analytical limitations, and presentation problems that severely limit the reliability and interpretability of the findings.

Major concerns:
1. The rationale for selecting 1–3 mg flunitrazepam doses and their relevance to human forensic scenarios is not provided.
2. Flunitrazepam was mixed with vodka, yet no **vodka-only control** was included. Ethanol can itself alter decomposition and insect colonization, creating a confounding variable.
3. Independent-sample t-tests were applied to groups with n=1, violating fundamental assumptions of independence and normality.
4. Reported “no significant differences” are therefore meaningless, and the conclusions regarding insect abundance or decomposition stage extension are unsupported.
5. The results and discussion sections contain extensive duplication**:** Stage-by-stage species lists appear almost verbatim in both sections.
Abundance patterns (e.g., dominance of Calliphoridae) are repeatedly described despite being clear in tables and figures.
6. The discussion focuses largely on restating descriptive results rather than providing mechanistic interpretation (e.g., how flunitrazepam might physiologically delay decomposition).
7. Table 1 lists *Pheidole megacephala and Camponotus sericeus* under Coleoptera: Formicidae, whereas Formicidae belong to Hymenoptera. Such errors cast doubt on the accuracy of species identification, which is critical in forensic entomology.
8. Several typographical inconsistencies (species names, spacing, stage descriptions) further reduce clarity.
9. Temperature and humidity are primary drivers of decomposition and insect colonization, yet no on-site measurements of ambient or carcass temperature, humidity, or rainfall were recorded or analyzed. The authors themselves note temperature as a critical factor but rely only on general site descriptions. Without continuous meteorological data, the observed delay in decomposition in flunitrazepam-treated carcasses cannot be distinguished from natural variation in weather or microclimate, undermining the core interpretation of a drug effect.
Future work should employ data loggers at each carcass site and incorporate these abiotic variables into the statistical analysis to properly isolate treatment effects.
10. Although the abstract and conclusion claim that flunitrazepam prolonged decomposition and reduced insect numbers, these assertions are not statistically supported. The observed patterns could easily reflect uncontrolled environmental variation or stochastic differences among carcasses.

If increasing the number of pigs is not possible for ethical or institutional reasons, the authors cannot treat this as an inferential experiment.

Instead, they should reframe the entire paper as an exploratory/descriptive case study and present their results accordingly.

Suggested Approach for the Authors
1. Reframe the Study Objective
Present the work as a pilot or observational case study exploring how flunitrazepam might influence decomposition and insect colonization under Kenyan field conditions. Avoid any claims of "effects," "dose–response," or statistical significance.
State clearly that the findings are hypothesis-generating, not hypothesis-testing.
2. Change the Language Throughout
Replace phrases like "significant difference," "effect of flunitrazepam," or "dose-dependent reduction" with neutral descriptions such as:
"We observed a lower insect count in the carcasses receiving higher flunitrazepam doses, but replication was insufficient to test statistical significance."
"These observations suggest potential trends that warrant further investigation."
Emphasize qualitative patterns (e.g., order of insect arrival, decomposition stages) rather than numerical comparisons.
3. Adjust the Statistical Treatment
Remove invalid t-tests and any p-values.
Provide only descriptive statistics: counts, percentages, means ± ranges, graphs of trends.
Use visual representations (bar plots, time-series plots) to communicate trends rather than claiming inferential outcomes.
4. Highlight Ethical Constraints
Clearly state that animal welfare considerations limited the number of carcasses.
Explain that the aim was to establish baseline data for Kenya and to guide future, more controlled research.
5. Strengthen Environmental Context
Even if continuous measurements were not made, incorporate any available regional climate data (e.g., from a nearby weather station) to provide context for decomposition rates.
Explicitly discuss how lack of temperature and humidity data limits interpretation.
6. Focus the Discussion
Concentrate on:
Species inventory (new or notable records for the region).
Stage-wise insect succession patterns under Kenyan conditions.
Observed—but untested—differences among carcasses with and without flunitrazepam.
Avoid repeating long species lists; summarize key ecological insights.
7. Taxonomic Accuracy
Correct the Formicidae misclassification and have all identifications reviewed by a trained entomologist.
Provide a voucher specimen statement to strengthen credibility.
With only four carcasses, the paper can still make a **valuable contribution** as a first regional report of insect succession on drug-treated remains, provided it is explicitly presented as **descriptive baseline data**.
This reframing will protect the authors from overinterpretation and make the manuscript ethically and scientifically sound.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Forensic entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.