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Succession of three dipteran maggot families on poisoned pig cadavers

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Abstract
Dipteran maggots found on a cadaver are used to estimate its time of death but the time may be incorrect if death is because of ingestion of poison. Decomposition of a zinc phosphide intoxicated and non-intoxicated pigs (*Sus scrofa* Linn.) were evaluated in this study. They were deposited in a forest during a rainy season and repeated during a dry season at Awka, Nigeria. Decomposition durations and stages of decay of the pig cadavers were recorded. Maggots found on the cadavers were collected, reared to adult and were identified. Similar maggot samples were assessed for residues of zinc and phosphorus with Atomic Absorption Spectrophotometer. Both pig cadavers completely decomposed within 10 and 14 days during the rainy and dry seasons respectively. Appearance and development of three dipteran maggot families were observed on the cadavers in a successional pattern. Zinc and phosphorus residues were detected in the maggots that fed on the poisoned cadavers but the residues were not detected in the maggots from the non-poisoned cadavers. The three dipteran maggot families are recommended as forensic entomology specimens for estimation of elapsed time of a questionable death and their bodies will be useful in toxicological analysis in the eastern, Nigeria.

Keywords: forensic entomology; toxicology; dipteran maggots; pig cadavers, Nigeria

Introduction

Insects that feed on cadavers have been studied in different ecosystems [1, 2] in attempt to survey their succession and developmental activities for forensic investigation relating to homicides or any questionable death. The climax of such knowledge by extrapolation is to estimate the possible time of death of any human being [3]. In some circumstances, drugs or poisonous substances may be the cause of the death which may influence the time taken for the cadaver to decompose and may influence insect infestation, succession, necrophagous activities and development hence, culminating in error of estimating post mortem interval (PMI). In such suspected scenario occasioned by man directly or indirectly, toxicological analysis may be carried out on the insect samples instead of the cadaver samples especially when the samples for such analysis are no longer viable. This alternative or augmentative practice of using insect samples for toxicological study is referred to as entomotoxicology [4-6] while using insects fauna in relation to their necrophagous activities and succession on cadavers to estimate PMI is termed forensic entomology [7, 8].

To validate the use of insects in forensic investigation, forensic entomologists have surveyed the entomofauna of different cadavers and in some cases use pigs to mimic real case scenario [9]. In many of the animals used as models, the trend in insects assemblage has remained the same at least at the family taxon. Any difference may show only in the species which has been ecologically suggested to be due to geographical differences that restrict some species to specific locations [10, 11]. It has therefore been suggested that regional climatic conditions have the potential to either expand or contract the entomofauna of any defined ecosystem. This ecological validity has helped in the interpretation of the overlap of insect species of different geographical regions [12].

Different types of pesticide poisons against rodents have been implicated as suicide substances however, zinc phosphide is the most frequently used to commit suicide in Nigeria [23]. In Nigeria, no documented report is available so far in relation to insects associated with poisoned intoxicated cadavers especially the ones that occurred in the forest. Zinc phosphide production dates back to 1740 and was first used as a rodenticide in Italy in 1911 [24]. In Awka Nigeria, it is highly affordable for a common man, readily available in open markets and streets, so it is easily accessed without difficulties. Zinc phosphide is a crystalline compound that is gray in colour [19]. Its mode of action in the body is not clear but it is reported to produce phosphine gas when it mixes with gut fluids upon ingestion by animals. The phosphine gas therefore, enters into the blood stream which is caught up by the liver and lungs leading to death as there are no antidotes for it yet [25].

In Nigeria, communal residency is practiced hence morally devasted individuals often leave the house for a nearby forest to commit suicide by drinking either zinc phosphide or other poisonous substances [23]. Death resulting from such case in the forest decompose and is infested with dipteran maggots for days or weeks before the person could be discovered. In such scenario, the question of when the person died or what killed the person is not always answered because of the dearth knowledge of using cadaveric insects to provide answers of when and what killed such a person. This study therefore, is geared towards evaluating the decomposition duration of pig cadavers sacrificed with a zinc phosphide poison against the decomposition duration in a forest.

Materials and Methods

Study area

The study was carried out in a forest (6º15'15.32N and 7º06'40.22E) during the late rainy season of 2014 (July 13-October 31), and during the early dry season of 2015 (January 11-April 22). The forest is a reserved section of Nnamdi Azikiwe University, Awka, Nigeria managed by the Department of Botany. The forest was characterized with trees, shrubs and grasses. The shrubs include *Chromolaena odarata* (Linn.), *Aspilia africana* (Pers.), *Abelmoschus esculentus* (Linn.), *Andropogon tectorum* (Schaumach. and Thorn.), *Napoleana imperialis* (P.Beauv.), *Elaeis guineensis* (Jacq.) and other perennial trees provided canopy shade over the open floor.

Animal model used and experimental procedures

Eight healthy male domestic pigs (*Sus scrofa* Linn.) that were ten weeks old and weighed 22.3 ± 1.9 kg on the average were purchased from a piggery for the study. During the raining season of 2014, four pigs were isolated in the farm for six hours without food but they were served water. A licensed veterinarian carried out the euthanization of the pigs in anesthetic chamber via the inhalation of 100% of CO_2 in compliance with Animal Ethics Committee as outlined in the veterinary animal care and welfare and medical research literature [20, 33]. Prior to euthanization of the pigs, 50 ml of vegetable oil was used to mix 10 g of zinc phosphide to form a poisonous mixture that was added to a 500 g of moistened spent grain. The poisonous mixture and the moistened spent grain were thoroughly mixed together to form a poisonous feed. Another replicate of the poisonous feed was formulated to obtain two replicates. Two out of the 4 isolated pigs were fed with the two replicates (500 g) of the poisonous feed. The fed pigs were sedated immediately after feeding. The remaining two pigs were also sedated prior to euthanizing them before their necks were fastened with aluminum cable until death occurred. Food poison, over dose inhalation and the fastening of the neck affected the brainstem resulting in immediate unconsciousness and death of the pigs [34]. Their deaths were confirmed by exsanguination by the licensed veterinarian. All the pig cadavers were labelled according to killing processes and transported to the study site. The pig cadavers were deposited on a five-meter square (5 m^2) plot of land at a distance of 2.5 m from one another. Each of the pigs was placed on a sack (16 x 8 cm^2) by 10.56 hours. The sacks served as mats and maggots' collection limit. Each of the pig cadaver was protected with wire gauze cage against vertebrate scavengers. The experimental procedures and processes were repeated during the dry season of 2015.

Collection of ambient temperature, humidity and dipteran fly maggots

Ambient temperature and relative humidity of the forest during the study periods were obtained with portable digital thermo-hygrometer (Mextech TM-1, model). The thermo-hygrometer was hung on a 4 ft. pole in the forest but not under the direct sunlight. It stood for at least 15 minutes before ambient temperature and relative humidity data were recorded for the first 40 days of the study. Observation and recording of changes in decomposition and insects visitation on the pig cadavers commenced immediately they were deposited at the forest till 19.00 hours. From the second day, daily observations and recording of decomposition changes and insects collection were done in the morning (6.00-8.00 hours) and in the evening (16-18 hours). Maggots with similar external morphology found on both the poisoned and non-poisoned pig cadavers were collected on day 4, day 6 and day 8 and were separately placed in clean plastic containers with its screw lids neatly perforated with needle and labelled as first group of maggots. This group of maggots were succeeded by another distinct group of maggots which were first seen on day 8 but were collected on day 10 and day 12, separately placed in containers with perforated lids and labelled as second group of maggots. Another group of maggots that were uniquely different from the first and the second groups of maggots that were first seen on day 12, were collected on day 12, day 14, day 16, day 18 and day 20. They were as well separately placed in containers with perforated lids and labelled accordingly as the third group of maggots. Collection of fly maggots on the pig cadavers started on day 4 and ended on day 20. After the first 20 days, maggots were no longer collected and daily visitation activity changed to once a week (11.00-1.00 hours) for 112 days during the rainy season and 103 days during the dry season. All the maggots irrespective of groups were collected with 5 ml spoon and blunt forceps while adult flies found on and around the cadavers were collected with entomological sweep net.

In each of the three groups of the maggots collected from both the poisoned and non-poisoned pig cadavers, 10 maggot samples were collected from each of them exactly on day 4 for first group, day 8 for second group and day 12 for third group. These specific days were marked as the days new distinct maggots were observed on the cadavers. The 10 maggot samples collected from each of the groups were reared to adults in a wooden cupboard placed at the forest that was covered with a muslin cloth in the form of tent and protected against rainfall. The rearing of the maggots is to estimate the day, they will emerge into adults, counting from the day the pig cadavers were sacrificed and deposited at the forest. During rearing of the maggots, they were placed in clean containers (9.0 cm depth and 6.5 cm width) half-filled with a sterile mixture of sand and rice husks to serve as moisture absorbent and pupation medium for the maggots. The containers were separately covered with muslin cloth and held in place with rubber band, while the maggots were introduced in the containers with the sterile mixtures and were fed with pork that was bought fresh from the market the day the pigs were sacrificed. The pork was kept in a clean container without any preservation but was covered with a muslin cloth and held in place with a rubber band to prevent flies from accessing the pork. This is to mimic a feeding source similar to the decomposition status of the pig cadavers where the maggots were collected from. For clarity purposes, both the adult flies collected with a sweep net and the emerged flies reared in the cupboard at the forest were morphologically compared and matched. They were processed, pinned and identified with reference to insects of Nigeria checklist and bibliography [26]. The identified flies were sent to a taxonomist at the Institute of Agricultural Research, Ahmadu Bello University Zaria, Nigeria for verification and validation.

Assessment of maggot samples with Atomic Absorption Spectrophotometer (AAS)

Maggot samples collected basically on days 4, 6, 8, 10, 12, 14, 16, 18 and day 20 respectively from which the three

groups were previously formed were killed on those stated days with hot water and preserved with 70 % ethanol. One gram of each of the preserved maggots for each day representing the three dipteran maggot groups was washed with distilled water. The washed maggots in a beaker were digested with 10 ml of 70 % Chloric acid ($HOCl_4$) and 10 ml of concentrated Nitric acid (HNO_3) by indirectly heating the beaker with a water bath at 60 $^{\circ}$C for one hour in a fume cupboard. The filtered solution of the digested maggots was analyzed with *Atomic Absorption Spectrophotometer* (AAS–model: BUCK Scientific 210GP) to assess the zinc and phosphorus contained in the maggots collected during the rainy season. This processes and procedures were repeated for the dry season study of 2015.

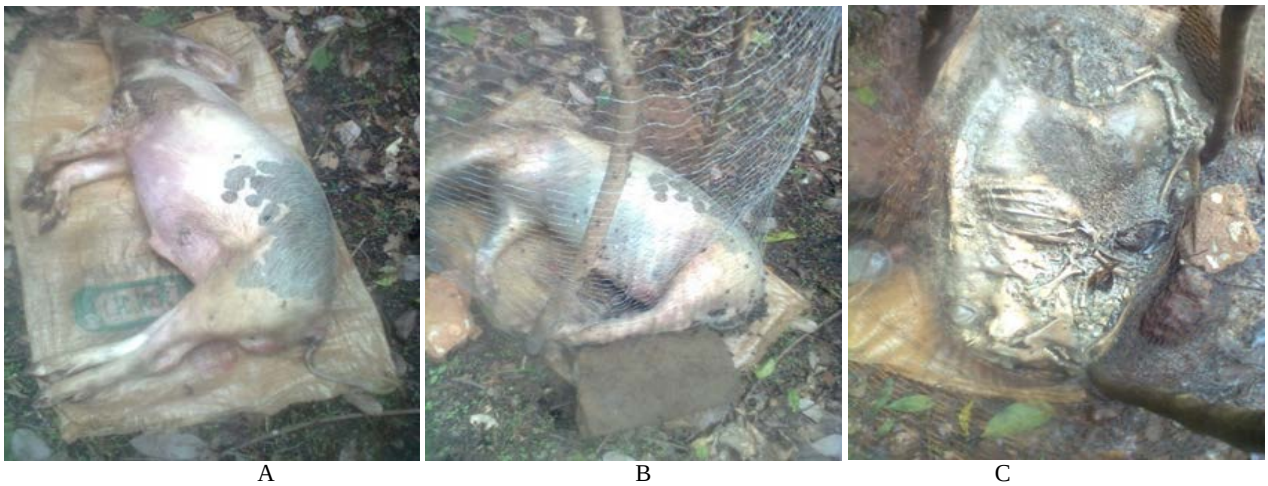
Results

The mean temperature and relative humidity recorded at the forest for the first 40 days during the decomposition of the cadavers and skeletonization period during the rainy season of 2014 were 27.2 °C and 81.9 % respectively. There was no difference between the decomposition durations of the poisoned and non-poisoned pig cadavers which lasted for 10 days during the rainy season. Three decomposition stages; fresh body, bloated decay and wet decay, (figure- 1) were observed on the pig cadavers irrespective of their killing procedures. The fresh body lasted for 1 day while the bloated decay stage commenced on the second day and lasted for 2 days. The wet decay stage commenced on the late evening of the third day and lasted for 7 days, thus decomposition stages were completed in 10 days (figure- 2). The same types of maggots were seen on the pig cadavers irrespective of the killing procedures.

During the dry season of 2015, the mean temperature and relative humidity recorded for the first 40 days during the decomposition and skeletonization period of the pig cadavers were 30.8 °C and 41.8 % respectively. There was no difference between the decomposition durations of both the poisoned and the non-poisoned pig cadavers similar to that of the rainy season. However, there was a difference between the mean temperature and the humidity of the rainy season against that of the dry season hence, the decomposition duration was longer during the dry season. Thus, the pig cadavers completely decomposed in 14 days during the dry season against the 10 days decomposition duration of the pig cadavers observed during the rainy season (figure- 2). Therefore, there was a difference between the decomposition duration of the cadavers during the rainy season and that of the dry season.

The first group of maggots which were collected on day 4 during the wet decay stage and reared, emerged as adults of three species of blow fly on day 8. They were identified as *Chrysomya albiceps* (Weid.), *Chrysomya chloropyga* (Weid.) and *Chrysomya regalis* (Rob-Desv.) in the family, Calliphoridae. The second group of maggots that succeeded the blow fly species, that were collected on day 8 which coincided with the emergence date of the blow fly species and were also reared, emerged as adults on day 22 as a different fly species, identified as *Chrysomyza africana* (Hendel) in the family, Ulidiidae. Third group of the maggots that succeeded the ulidiid fly on day 12 but collected on day 14 for rearing, emerged on day 33 as a singleton species, identified as black soldier fly (*Hermatia illucens* Linn.) in the family of Stratiomyidae.

Interestingly, the same time at which the same types of maggot species appeared on the cadavers and similar pattern of succession observed on the cadavers during the rainy season were also observed during the dry season. Similarly, same emergence dates observed during the rainy season in all the three fly species were also observed during the dry season. Rearing of the dipteran maggot species were successful in both rainy and dry seasons irrespective of the killing method of the pig cadaver (table- 1).



A B C

The three decomposition stages of the pig cadavers during the rainy season, July-October, 2014

A B

C D

The four decomposition stages of the pig cadavers during the dry season, January-April, 2015

Fig 1: Decomposition stages of the pig cadvers irrespective of killing procedures at the forest of Nnamdi Azikiwe University Awka, Nigeria 2014/2015

Note; A = Fresh body, B = Bloated decay, C = Wet decay, D = Dry decay

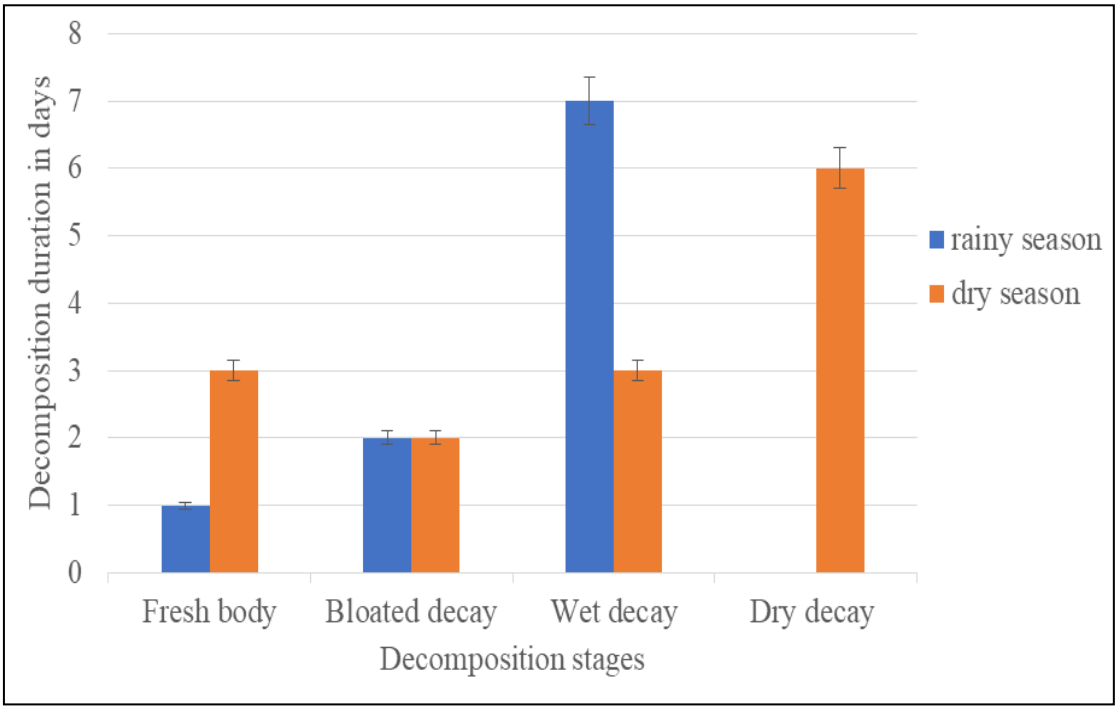


Fig 2: Decomposition stages of the poisoned and strangled pig cadavers in days during the rainy and dry seasons of 2014 and 2015 respectively at Nnamdi Azikiwe University Awka, Nigeria

Table 1: Succession and emergent dates of the three dipteran fly species collected on the pig cadavers at Nnamdi Azikiwe University Awka, Nigeria

Days of collection		Rainy season	Dry season
Day 4	Sample collected	Fly maggots	Fly maggots
	No. collected	10	10
	No. emerged	10	10
	Day of emergent	Day 8	Day 8
	Fly species	*Chrysomya albiceps*	*Chrysomya albiceps*
		C. chloropyga	*C. chloropyga*
		C. regalis	*C. regalis*
Day 8	Sample collected	Fly maggots	Fly maggots
	No. collected	10	10
	No. emerged	7	10
	Day of emergent	Day 22	Day 22
	Fly species	*Chrysomyza africana*	*Chrysomyza africana*
Day 14	Sample collected	Fly maggots	Fly maggots
	No. collected	10	10
	No. emerged	8	5
	Day of emergent	Day 33	Day 33
	Fly species	*Hermatia illucens*	*Hermatia illucens*

The assessment of the zinc and phosphorus (the active ingredients of the rodenticide) on the maggots collected on both the poisoned and non-poisoned pig cadavers with atomic absorption spectrophotometer (AAS), showed that during the rainy season, all the maggots collected on the zinc phosphide intoxicated pigs bio-accumulated the zinc in their bodies. Evidence from the reared maggots showed that the ones collected between day 4 and day 8 belong to the family Calliphoridae. Zinc residues in the body of this group of maggots were 0.04 mg, 0.058 mg and 0.079 mg which depicted the maggots collected on days 4, 6 and 8 respectively. The maggots collected on days 10 and 12 belong to the family Ulidiidae. Zinc residue detected on the maggots collected on day 10 was 0.084 mg while the ones collected on day 12 were not used for zinc examination because the quantity was less than I g hence, the 0 mg was actually a nil result. Other zinc residues recorded include, 0.06 mg, 0.06 mg and 0.062 mg which depicted the maggots collected on days 14, 18, and 20 respectively, they belong to the family Stratiomyidae. Maggots collected on day 16 were as well less than 1 g for zinc examination hence the 0 mg represented a nil result. Phosphorus residue was very minute; it was detected in the maggots collected only on day 4 which was 0.0018 mg against the 0.04 mg of zinc residues in the maggots collected the same day. Other subsequent maggot samples collected between days 6 and 20, did not show any trace of phosphorus (figure- 2). All the maggot samples collected on the non-poisoned pig cadavers did not show any trace of zinc and phosphorus respectively.

During the dry season, similar observations made during the rainy season were repeated. All the maggot samples collected on the zinc phosphide intoxicated pigs showed presence of zinc residues. Maggots in the family of Calliphoridae, collected between day 4 and day 8 showed zinc residues in the ranges of 0.041 mg, 0.052 mg and 0.08 mg. They depicted the maggots collected on days 4, 6 and 8 respectively. The maggots collected on days 10 and 12 belong to the family Ulidiidae. Zinc residues detected on them were 0.087 mg and 0.081 respectively. Others zinc residues were, 0.063 mg, 0.0 mg, 0.0 mg and 0.06 mg which depicted the maggots collected on days 14, 16, 18, and 20 respectively and they belong to the family Stratiomyidae. Maggots collected on days 16 and 18 respectively were as well less than 1 g for zinc examination hence the 0 mg assigned to them were actually nil results. Phosphorus residue as recorded during the rainy season was also detected but only in the maggots collected on day 4. It was also very small 0.002 mg against the 0.041 mg of zinc residues detected in the maggot samples that were collected the same day. Subsequent maggot samples collected between days 6 and 20, did not as well show any trace of phosphorus (figure- 3) whereas, all the maggot samples collected on the non-poisoned pig cadavers did not also show any trace of zinc and phosphorus respectively.

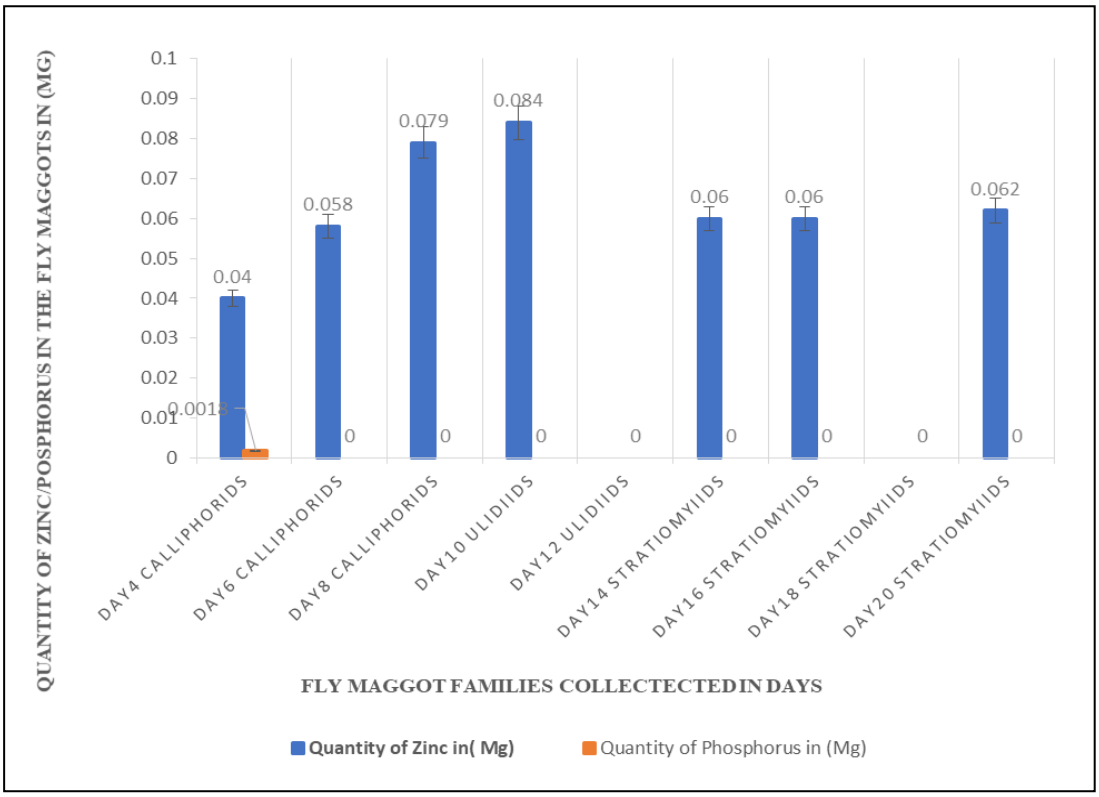


Fig 3: Quantity of zinc and phosphorus in (Mg) contained in the Maggot families collected on different days during the rainy season at Nnamdi Azikiwe University Awka, Nigeria

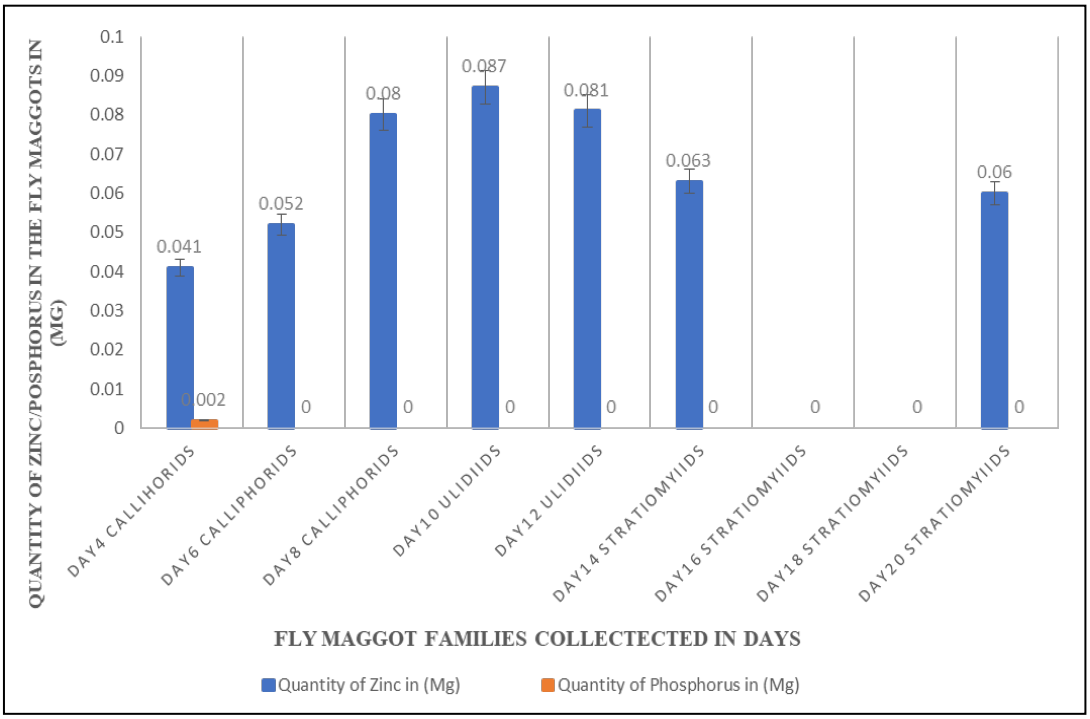


Fig 4: Quantity of zinc and phosphorus in (Mg) in the Maggot families collected on different days during the dry season at Nnamdi Azikiwe University Awka, Nigeria

Discussion

Using the knowledge of decomposition of cadavers and insects associated with them is one of the ways entomology contributes to the field of forensic science by estimating the postmortem interval (PMI) of a cadaver that has been decomposed and infested with insects. Decomposition of a cadaver commences with autolysis immediately after death [7] though the process may not be physically noticed but the insects attracted to the body may be the evidence that decomposition has commenced [13, 14, 15]. Because some insects especially dipteran fly species are synonymous with cadavers, their presence on cadavers have been used to provide their PMI [7, 10]. However, such estimated PMI may be wrong if the natural process of the cadaver decay has been altered. The alteration may be that the cadaver was either preserved with chemical or wrapped with thick cloth and/or that the cause of death was due to ingestion of poison [22].

In this study, decomposition of a zinc phosphide intoxicated pigs and non-poisoned pigs approximately decomposed completely within each time frame during the rainy and dry seasons respectively. The difference as regards the duration and stages of decay during the two seasons was linked to the temperature and humidity differences of the two seasons. The mean temperature and the humidity at the forest during the rainy season ephemerally characterized a hot humid weather. There was copious rainfall though not recorded but as reported, Awka ecologically lies in the Guinea Savanna of Nigeria and experiences approximately 1800 mm of rain annually [28] thus, the rain returned the would-be dry decay to wet decay. During the dry season, the temperature and the humidity at the forest transiently characterized an arid climate with a remarkable dry wind depicting a harmattan weather which delayed putrefaction in the cadavers. The effects of the seasonal changes in temperature and humidity on the duration and stages of decay of the pig cadavers were in agreement with the reports of Anderson [21] who stated that season has a major influence on weather of every region while Hobischak *et al*. [27] reported that there was no difference on pig cadaver decomposition placed under the direct sunlight against the pig cadaver placed in a shade within same season.

The succession of the three families of the dipteran maggots associated with the decomposition of the pig cadavers were unique but the time of appearance and disappearance of their adults were not discerned due to rapid decomposition of the pig cadavers. This remarkable observation was the same for the rainy and dry seasons, irrespective of the killing procedures. Therefore, the pattern of succession of the dipteran fly maggots showed that the three blow fly species (*Chrysomya albiceps*, *Chrysomya chloropyga* and *Chrysomya regalis*) were the first colonizers of the pig cadavers. The succession sequence of the three dipteran maggot families (calliphorids, ulidiids and stratiomyids) on the pig cadavers was interesting as each maggot family appeared and disappeared during a particular decay stage of the cadavers. These maggots have been assigned necrophagous role on cadavers by Smith [10], hence they contributed immensely to the faster decomposition of the pig cadavers irrespective of their killing procedures and season of decomposition. This finding concurred with the report of Campobasso *et al*. [29] that rapid decomposition of cadavers was due to presence of necrophagous insect community and that elevated temperatures reduced the development time of dipterans. Galloway *et al*. [30] also noted pre-skeletonization at day 7 after death at a temperature above 39° C and that onset of decomposition and mummification was faster for cadaver placed outdoor than indoor cadaver. Despite seasonal rotation, Rodriguez and Bass [31] noted that blow fly species dominated first wave of insects on cadavers and that rapid skin dehydration was typical of dry windy climates which is in agreement with our finding in this study.

The allogenicity of the fly maggots made them to feed on the poisoned pig cadaver without being deterred. Hence, they bio-accumulated the components of the poison (zinc and phosphorus) that killed the pigs as they voraciously fed on the poisoned pig cadaver in agreement with Fisher *et al.* [14]. The two components of the poison are heavy metals that are toxic to animals especially when consumed in excess. The toxicity of the poison on the pigs was as a result of the phosphorus component which turned into phosphine gas. Phosphine gas is reported to cause acute kidney and liver damage as well as heart failure [17-19]. Zinc phosphide mode of action is unclear but it is suggested that upon ingestion, it would react with gastric acid of the stomach to liberate unstable phosphine gas which has a high lethal effect [18, 19]. The phosphine is rapidly absorbed and cause the inhibition of C oxidase, causing mitochondrial morphology, and oxidative respiration impairment at a cellular level. Because of its damage to the heart and the lungs, the victims are lost in the early stage [19, 25, 32]. The three dipteran fly families collected from the zinc phosphide intoxicated pig cadavers showed traces of zinc residue why phosphorus residue was not detected except on the blow fly family collected only on day 4. We suggest that there was a minute remnant of the zinc phosphide on the pig cadaver tissues within the first 4 days of the decomposition hence, the detection of the phosphorus residues only on day 4. On the contrary, both the zinc and phosphorus residues were not detected on the three dipteran fly families collected on the non-poisoned pig cadavers. We have no evidence base explanation on the biochemistry of the zinc phosphide on the pig cadavers but we suggest that the phosphine gas evaporated after day 4. Therefore, it is assumed that the zinc phosphide remaining in the pig cadavers may not have had a direct toxic effect on the dipteran fly maggots. This may suggest why the three families of the dipteran fly activity and the decomposition of the poisoned and the non-poisoned pig cadavers were the same.

Conclusion

The predictable succession sequence of the adult stages of the three dipteran fly families on the pig cadavers was not discerned due to faster decomposition of the pig cadavers irrespective of their killing procedures and seasons. We therefore, report only the dipteran maggot families because of their predictable time of appearance on the pig cadavers and their remarkable time of their emergence as adult flies. Also, their continued dominance on the cadavers would present them as useful samples for toxicological analysis. We therefore, recommend that the three dipteran maggot families should be a valuable tool in forensic entomology and entomotoxicology in the eastern, Nigeria as this is a first baseline report in the region.

Declarations

Ethical clearance

All relevant international guidelines for the care and use of animals were followed in the study.

Conflict of interest

The authors declared that there is no conflict of interest

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