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Effects of Tramadol Hydrochloride (an opioid) Injection on the Developmental Rate, Larval Weights and Body Lengths of the *Lucilia sericata* (Meigen) (Diptera: Callophoridae*)* Reared on Rabbit (*Orctylagus cunicunus)* Carrions

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Abstract
This research work was carried out to ascertain the effect of tramadol hydrochloride (an opioid) on the body lengths and weights of the larvae and the developmental rate of the blowfly Lucilia sericata (Diptera: Calliphoridae) reared on rabbit carrions injected with 150ml (50ml/mg) (1/2$LD_{50)}$, 300ml ($LD_{50)}$, 600ml ($2LD_{50)}$ dosages and control (no tramadol). These dosages are the same with those that are normally observed in homicide and abusive cases involving tramadol hydrochloride injection overdose. Mettler Toledo weighing balance (with sensitivity of 0.001-1g) was used to record the weights of the larvae and the pair of compass was used to measure the body length and read with a transparent meter rule while all stages of the insect were monitored and observations recorded. The mean maximum lengths and weights were reached (17.50mm and 0.0930g for $2LD_{50}$ at 96hrs, 14.13mm and 0.0810g for LD_{50} at 96hrs and 13.13mm and 0.0510g for the $LD_{50}/2$ at 108hrs) earlier in those larvae reared on the rabbits injected with tramadol hydrochloride than those of the control (11.38mm and 0.0500g at 108hrs). However the total developmental period appreciated immensely with increased injected dosages and ranged from 362.15hrs in the $2LD_{50,}$ 345.15hrs in the LD_{50} and 309.46hrs in the $LD_{50}/2$. Whereas the control mean developmental period is 280.25hrs. There was significant differences in the mean body length ($H = 0.174$, $P>0.05$) and the mean larval body weight ($H = 0.055$, $P>0.05$) using the non-parametric Kruskal Wallis analysis. Arising from the results therefore, special caution must be taken in the application of the toxicological analysis of insect larvae data of fatal tramadol and other drug related cases.
Keywords: *Entomotoxicology, insects, Lucilia sericata, forensic entomology, carrions*

Introduction
Insects have been around for more than 400 million years and it could be argued that they are the most successful and enduring life form that has ever arisen on the planet. Diverse as well as abundant, insects comprise approximately half of the earth's one and a half million known species (1). There are many more species than those to which we have given names and past estimates have been as high as one hundred million, the majority views nowadays is that we share the planet with between 5 to 15 million species of which insects will be sizeable proportion (1).

Insects are so important to the continued working of the global ecosystem that as long as the wellbeing of the insects is safeguarded the earth should remain habitable for humans (2). This is not overstating the case as herbivores, predators, parasites and as food source for countless species, insects are fundamental in all terrestrial and aquatic food chains. Put simply, without insects, the global ecosystems would disintegrate. They pollinate more than a quarter of a million species of the flowering plants, even from a purely anthropocentric view, without pollinators we will lose one third of all the food we eat. These insects recycle nutrients, enrich soils and dispose of carcasses and dungs. They provide us with silk, honey, wax, medicine and dyes. We use them to control pests mostly insects and weeds. They have been revered as sacred, celebrated in arts and literature and eaten by humans and other animals (2).

The importance of insects to man has given a lot of drive to new concepts in the field of forensic entomology, which is the science of using insects evidence to uncover circumstances of interest to law, often related to crime. Forensic entomology is the application of the knowledge of insects in the investigation of criminal matters. It is not a new science as it was first recorded in China during the 13th century. It was until 1960s' that the discipline began to be accepted. However, it is more accurately divided into three branches; urban, stored-products and medico-legal (3). The last category is referred to as medico-legal or medico-criminal forensic entomology and this is all about the arthropods concerned with felonies such as murder, suicide and rape. Medico-legal forensic entomology has also been used in cases involving physical abuse, child neglect and contraband trafficking (4). The medico-legal forensic entomology lastly is also recently concerned with the application of the insects invading a dead body to detect the cause of death of the organism and it is termed entomotoxicology.

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Entomotoxicology is relatively a new branch of forensic entomology that is concerned with the use of insects and other arthropods that invade a dead body in detecting drugs and other toxins in the decomposing tissue when all other basic toxicological specimens such as tissues, blood, and fluids are not available or simply not made available in a criminal scene. Entomotoxicology remains the last option for the investigator in a criminal matter. The careful analysis of the entomo-fauna encountered on a decomposing body or even in an advanced decayed body with the full knowledge of the scene environmental conditions such as temperature and relative humidity can often provide valuable forensic revelations. These techniques include among other things the estimation of the post mortem interval (PMI), movement of the carrion after death, indication of injuries and the presence of drugs and/or toxin.

The pharmacokinetic of drugs in insects depends on the species, the developmental stages as well as their feeding activities (5). Bio accumulation does not only occur in the necrophagous species as it also occurs in parasitoids, predators, parasites and omnivores species (6). At any rate, drug accumulation will not be the same as these species present different feeding behaviours due to their diets and life histories (7). For the entomotoxicological investigations, the uses of the necrophagous species are usually very common and always present on the scene. However the biology and the development are well-known as they are already in use in the forensic entomology to estimate the PMI (8).

Distinctions on the concentration of drugs are noticed in larvae with different feeding habits (9). However, drugs and toxins' metabolite could result from the action of the substrate enzymes (10) and for larval metabolism (11). The malpighian tubules do not only result in the drug excretion during larval stages but are still secreting during the post-feeding stage leading to actively feeding larvae for most of the drugs (12). Immediately the insects are in their pupal stages, the malpighian tubules are degraded and the remaining gut content will only be excreted as meconium during emergence of the adult insects (13), as drugs concentrations seem low in adult life (14). On the detecting of poisons, Utsumi (15) observed that Dipterans were attracted in a different manner by the rat carcasses depending on the poison causing death. Leclercq and Braley (16) showed the presence of arsenic in the Diptera from the families of Piophilidae, Psychodidae and Fanminidae in a case of arsenic poisoning that occurred in France. In a suicidal poisoning, Gunatilak and Goff (17) detected organophosphates (malathion) in maggots of *Chrysomyia megacephala* (Calliphoridae) and *Chrysomia rufuifacies* (Macquart) (Calliphoridae) submitted to toxicological analysis using gas chromatography (GC).

Tramadol's wide use globally, intentionally and unintentional fatal toxicity seems to take place rarely and might be safe as compared to other opioids such as amitriptyline. This may be due to its low abuse potential and because of its less reported respiratory depression (18). It is a synthetic opioid analogue of codeine first synthesized in 1962 by Gunenthel in an attempt to reduce common opioid adverse effects such as respiratory depression (19). The present work is aimed at establishing the roles of tramadol hydrochloride opioid, on the developmental rate of *Lucilia sericata* (Meigen) through the assessment of the larvae body weight, length and the developmental period.

Materials and Methods

The experimental site

The experimental site was located at the Biology Departmental farm of the College of Education Warri, Warri South Local Government Area, Delta State, Nigeria. The study was carried out between September, 24th and October 7th, 2015 in an open fallow plot of the farm. The school is located in Warri on Latitude 5.5432 and Longitude 5.7382, accuracy of 7.1m, Altitude of 10.5, bearing of 61.31^{O}C (information time; 09:38:31(GMT), date; 30/09/2015, provider; GPS). It has a tropical climate characterised by two distinct seasons; the wet season occurs between April and October with a break in August. The dry season lasts from November to April with a cold harmattan between December and January. Warri ranges from 32^{O}C to 37^{O}C at an altitude of 21m, with mean annual rainfall of 2673.8 mm. The natural vegetation is of rainforest, in some areas, the forest is rich in timber tree, as well as fruit trees (20).

The farm lies east of a botanical farm and southeast by a plantation orchard and surrounded by other research crop plots. Grasses, wildflowers, herbs, and weeds cover the field. Measurement of the area approximated 50 × 150m. This size of land is to reduce overlapping olfactory cues.

Experimental Animals

The rabbit (*Orctylagus cunicunus*) has been and continues to be used in laboratory works such as in toxicological and forensic sciences (21; 22; 23; 24), production of antibodies for vaccines and research for human male reproductive system toxicology (25). In 1972, about 450 000 rabbits were used for experiments in the United States alone. The Environmental Health perspective published by the National Institute of Health states that ''rabbit is an extremely valuable model for studying the effects of chemicals and other stimuli on the male reproductive systems'' (25).

Therefore, rabbits were used in this entomotoxicological research work. Sixteen healthy rabbits (*Orctylagus cunicunus*) with mean weight of 2.14±0.18 kg were bought from Ogbuwangue market, along N.P.A express road, Warri in Warri South Local Government Area of Delta State.

The killing method and the experimental layout

The rabbits received tramadol injection of different dosages (intra-muscular); four rabbits were injected with 150mg (50mg/ml) each, four rabbits injected with 300mg (50mg/ml) and four rabbits injected with 600mg (50mg/ml) each (these dosages are the same with those that are normally witnessed in suicidal and abuse cases involving tramadol injection overdoses) while the remaining four rabbits were used as controls. The 150mg, 300mg and 600mg stand for the half LD_{50}, LD_{50} and twice LD_{50} (26) respectively. The treated rabbits died immediately after the injection; the rabbits that received the lowest dosages (150mg) died about 5minutes after receiving the injections while the other treated rabbits (300mg and 600mg) died immediately after receiving the injections. The control rabbits were killed by cervical dislocation. They were placed into heavy thrash bags and taken from the killing centres to the study centre through a car. The rabbit carrions were guarded against vertebrate scavengers with wire gauze that permits entrance of all the insects and other arthropods. The wire gauze was used to form cylindrical cages of height and width of 30 and 20cm respectively supported with iron rods for each of the rabbit carrions. An inter carcass distance of at least 20m was maintained to minimise interruption of flies from adjacent colonies (27).

Arthropods sampling and data collection.

Sampling for the entomotoxicological studies was carried out. Second instar larvae were collected (according to Dyar's Rule) from the decaying carrions and the larvae from each carrion were bred in transparent plastic containers with depth of 15cm and width diameter of 11.5 cm at 25^{O}C, each (with muslin cloth covering and rubber bands to permit ventilation and to hinder the escape of the insects) containing saw-dust and part of the decaying carrion-remains to feed the immature insects, the second instar larvae were reared till adult stage. Times for the emergence of all the stages of the insects were recorded.

Length and weight measurement

At regular intervals of 6 to 12h, larvae were randomly sampled from each carrion representative (the rearing plastic container), and killed immediately in the boiled water (28). The lengths and weights were duly measured and recorded for further analysis. The remaining larvae were allowed to complete their development. Pupation, emergence and eclosion were investigated at 6h to 12h intervals in order to find out the time it took each sample's flies to complete their life cycles; the emergence and eclosion time were recorded also. Mettle Toledo electrical weighing balance with sensitivity (of readability) of 0.001g -1g was used to measure the weight of the larvae. For each weighing; the mettle Toledo balance was calibrated with standard weight of 200g before weighing the larvae while the length of the fly larvae from the second instar stage were obtained by a pair of compass and read on transparent metre rule. Obtained data were recorded accordingly for further analysis.

Results

The effects of tramadol on the mean body lengths of the larvae.

The mean body length obtained is presented in Fig.1. The results showed that as tramadol hydrochloride concentration increased, there was also an increase in the sampled mean larval body length. The colonies with the highest concentration ($2LD_{50}$) showed the highest increment while the control colonies recorded the least mean body length of the larvae.

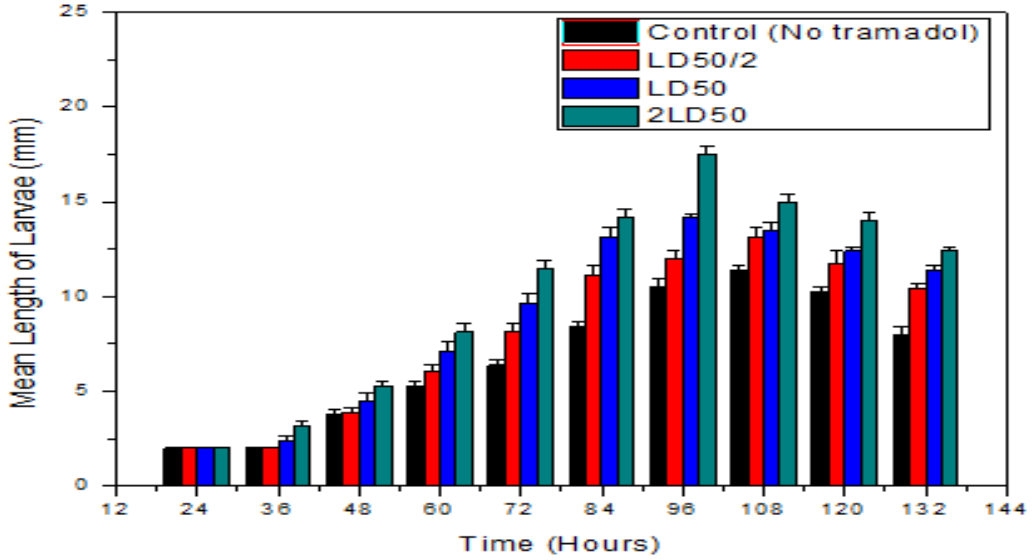

Fig. 1: Graphical representation of the mean length of larvae bred from carrion intoxicated with tramadol injection of different concentration.

While the $2LD_{50}$ increased in the mean larvae body length to 17.50mm at 96hrs post emergence, the LD_{50} increased to 14.13mm and the $LD_{50}/2$ increased to 13.13mm compared to 10.50mm recorded for the control colonies within the same period (Fig.1). However, their mean larval body length decreased after 96hrs for colonies of $2LD_{50}$ to 12.38mm at 132hrs, whereas the LD_{50} colonies' mean larval body length decreased to 11.38mm compared to the control colonies that increased to 11.38mm (Fig.1). There was significant difference between the mean body lengths using the non-parametric Kruskal Wallis.

The Effects of the Tramadol on the Larval Weight.

The mean body weight of the larvae sampled from different concentrations of the tramadol hydrochloride and the control are presented in Fig. 2.

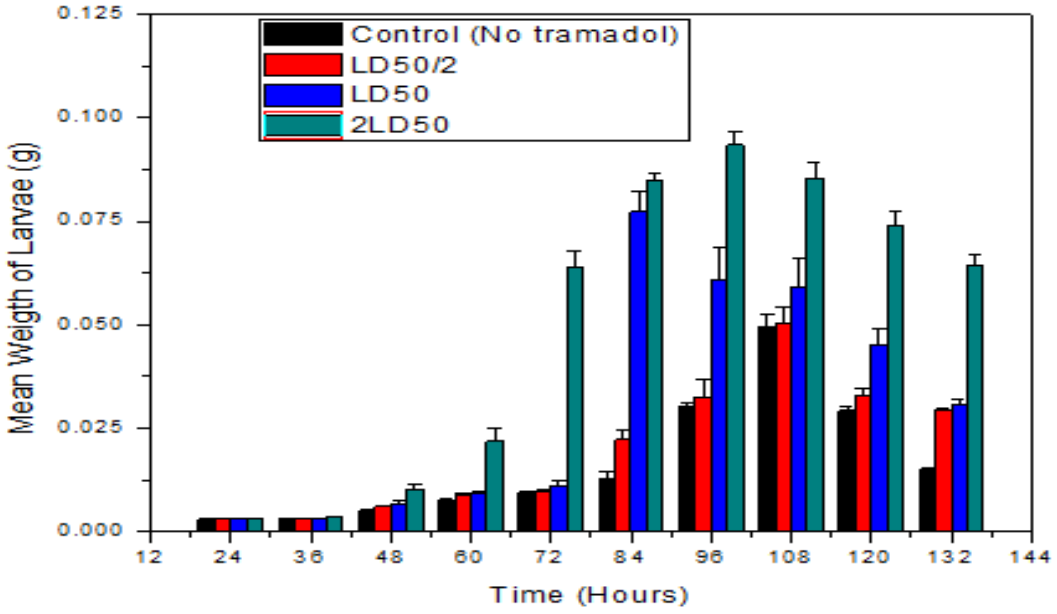

Fig. 2 Graphical representation of the mean weight of larvae bred from carrions intoxicated with tramadol injection of different concentration.

The results showed that as the tramadol hydrochloride concentration increased, there was also an increase in the sampled larval body weight. While the $2LD_{50}$ increased in the larval body weight to 0.0930g at 96hrs post emergence, the LD_{50} increased to 0.081g and the $LD_{50}/2$ increased to 0.0320g compared to 0.0300g recorded for the control colonies within the same period. However, the mean body weight of the larvae decreased after 96hrs for colonies of the $2LD_{50}$ to 0.0640g at 132hrs, whereas the LD_{50} mean larval body weight decreased to 0.031g and the $LD_{50}/2$ decreased to 0.0290g compared to the control colonies that increased to 0.0500g at 108hrs but decreased to 0.0150g at 132hrs (Fig.2). There was significant difference ($P > 0.05$) between the mean body weights using the non-parametric Kruskal Wallis analysis

The effects of tramadol hydrochloride injection on the mean developmental periods of the different stages of the L.sericata at different concentrations

The colonies with the highest concentration had longer pupal and adult stages, the mean pupal time of emergence ranged from 161.15hrs for $2LD_{50}$ to 142.40hrs for the control colonies.

Also on the adult emergence, the adults of the control colonies emerged at a mean period of 280.25hrs, followed by the $LD_{50}/2$ at 309.46hrs and LD_{50} at 348.15hrs while the $2LD_{50}$ emerged at a mean period of 362.15hrs indicating a reverse of what was obtained at the larval stage. The eggs were witnessed at the same time of 6.25hrs in all the colonies and the larvae were deposited at a mean period of 24hrs, the pupal and adults emergence were not the same.

Tramadol has significant effects on the total emergence of the adult *L. sericata* and it is dependent on the concentration of the tramadol. The $2LD_{50}$ length of emergence was 362.41hrs (15.1days), the LD_{50} was 348hrs (14.5days), the $LD_{50}/2$ was 309.6hrs (12.9days) and the control was 280hrs (11.67days). The obtained data was presented in Fig. 3.

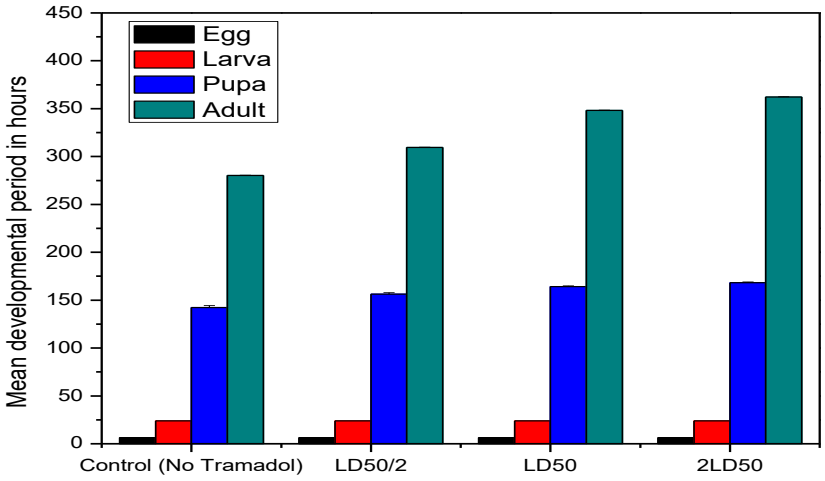

Fig.3 : The graphical representation of the mean developmental periods of the different stages of the dipteran flies of different concentration

Discussion

This research observed that the presence of tramadol in the rabbit tissues increased the larval feeding growth rate but also increased the larval developmental period. This result is in line with Goff *et al., (*22) who carried a research on the effect of heroine on the development of *B. peregrine.* They found that more rapid development of maggots in all the treated colonies until maximum sizes were attained. Similar results were reported for methamphetamine (23) and in amitriptyline (29). This result was also in line with the work of Lamia *et al.,(*30) on the acceleration of the larval developmental rate from the colonies with the highest concentration to the control and the acceleration of the pupal and adult emergence periods from the control to the colonies with the highest concentration but contrary to the Lamia *et al.,(*30), on the result of larval growth at the 24hrs was the same here, instead of being at different time as reported by the authors at the 24hrs (Fig. 3).

Regarding the larval growth, larvae from all colonies fed on the tramadol treated colonies were larger and attained their maximum length earlier than those from the control colonies. Bourel *et al.,* (31), reported that when the presence of morphine in the tissue is not considered, an underestimation of the PMI is possible for the larvae of *L. sericata* measuring 8 to 14mm total length. Also Hedouin *et al*., (24) demonstrated the potential underestimation of the PMI based on the developmental analysis of the necrophagous flies larvae of the *L. sericata* fed on the tissues of the deceased rabbits previously perfused with various concentrations of morphine.

The error in the estimation of the PMI in this study could be as much as in the error of 81.80hrs. Insects and their products could be used as dependable specimens for toxicological analysis in the absence of tissues and body fluids normally sampled for forensic purposes. In cases of advanced or skeletonised bodies, analyses of collected carrion feeding insects may provide the most authentic and dependable measurable credible sources of toxicological information. This research work suggests that caution must be taking in the calculation of the PMI and in the interpretation of the entomo fauna style of development in the situations where toxins and drugs could be involved in the criminal acts.

Conclusion

This study has shown that drugs such as tramadol can affect the developmental style of the arthropods and as such can influence the colony of such insects. The length and the weight of larvae can indicate drugs intoxication of the specimen as compared to the control, suggesting that entomotoxicology can be involved in criminal investigations.

References

1. McGavin, GC: Expedition Field Techniques: Insects and other Arthropods, The centre for supporting field research, exploration and outdoor learning. Royal Geographical Society with IBG, 1 Kensington Gore, London SW72AR. ISBN 0-907649742. 2007.
2. Berenbaum, MR: Bugs in the systems: Insects and Their Impacts on Human Affairs, Addisson Wesley. and Castner, eds. CRC Press, Boca Raton. 1995.

3. Catts, EP, Goff, ML: Forensic entomology in criminal investigation. Annual Review of Entomology. 37:257-272. 1992.
4. Benecke, MR: Child neglect and forensic entomology. Forensic Science International 120:155-159. 2001.
5. Nolte, KB, Pinder, RD, Lord, WD: Insects' larvae used to detect cocaine poisoning on a decomposed body. Journal of Forensic Sciences. 37:1179-1185. (1992).
6. Faria, LDB, Tranca, LA, Godoy, WAC: Larval production by *Chrysomyia albiceps*, *Cochiomyia macellaria*, *Chrysomyia megacephala* and *Chrysomyia putoria*. Entomol. Exp. Applic. 90:149-155. 1999.
7. Miller, ML, Lord, WD, Goff, B, Donnely, ET, Mcdonough, JC: Alexis, Isolation of amitriptyline and nortriptyline from fly puparia (Phoridae) and beetle exuviae (Der- mestidae) with mummified human remains. J. Forensic Sci. 39:1305–1313. 1994.
8. Kintz, P, Tracqui, A, Margin, P: Toxicology and fly larvae on a putrefied cadaver. Journal of Forensic Science Soc. 30:243-246. 1990.
9. Gosselin, M, Ramirez-Fernandez, MDM, Willie, SMR, Samyn, N, Boech, GD, Bourel, B: Quantification of methadone and its metabolite 2-Ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine in the third instar larvae of *L.sericata* (Diptera: *Calliphoridae*) using liquid chromatography-mass spectrometry, Tandem. Journal of Annual Toxicology. 34:1-7. 2010.
10. Kharbonche, H, Augsburger, M, Cherix, D, Sportkert, F, Giround, C, Wyss, C, Champed, D, Mangne, P: Codeine accumulation and elimination in larvae, pupae, and stages of the Blowfly *L.sericata* and its effects on its development. International Journal of Legal Medicine. 122:205-211. 2008.
11. Wood, M, Laloup, M, Pien, KV, Samyn, N, Morris, M, Mark, RAA, De-Bruijin, EA, Macs, VI, De Boeck, G: Development of a rapid and sensitive method for the quantification of *Bemodia vepines* in *Calliphora vicina* larvae and puparia by LCMS-MS. Journal of Anal. Toxicology. 27:500-512. 2003.
12. Sadler, WD, Richardson, J, Haigh, S, Bruce, G, Poinder, DJ: Amitriptyline Accumulation and Elimination in *Calliphora vicina*. Am. Journal of Forensic Medical Pathology. 18: 397-403. 1997.
13. Parry, S, Linton, SM, Francis, PSO, Dennel, MJ, Toop, J: Accumulation and excretion of Morphine by *Calliphora stygia*, an Australia blowfly species of Forensic importance. Journal of Insects' Physiology. 57(1):62-73. 2011.
14. Nuorteva, P, Nuorteva, SL: The fate of mercury in sarcosaprophageous flies and in eating them, Ambio 11:34–37. 1982.
15. Utsumi, K: Studies in arthropods congregating to animal carcasses, with regards to the estimation of the post-mortem interval. Ochanomizu Medical Journal. **7**:207-223. 1958.
16. Leclercq, M, Braley, G: Entomologue et medicare legale; datation dela mort. Journal of Médical / Legal Science. 28:271-278. 1985.
17. Gunatilake, K, Goff, ML: detection of organophosphate poisoning in a putrefying body by analysing arthropod larvae. Journal of Forensic Science. 34:714-716. 1989.
18. Tjaderbor, M, Johnson, SH, Hagg, S, Ahler, J: Fatal unintentional intoxication with tramadol during 1995-2005. Forensic Science Int. 173:107-111. 2007.
19. Shipton, EA: Tramadol: Present and Future. Anaesth. Intensive Cares. 28: 363-374. 2000.
20. Egborge, ABM: Water pollution in Nigeria :Biodiversity and chemistry of Warri River, Warri**.** Ben Miller Books Nigeria Limited. ISBN 0-999-768, pg 275-288. 1994.
21. Goff, ML, Omori, AI, Godbrod, JR: Effect of cocaine in tissues on the development rate of *Boettcherisca peregrine* (Diptera: *Sarcophagidae*). Journal of Medical Entomology. 26:91-93. 1989.
22. Goff, ML, Lord, WD: Entomotoxicology. American Journal of Forensic Medicine and Pathology. 15:51-57. 1991.
23. Goff, ML, Brown, WA, Hewadi Karana, KA, Omori, AI: Effect of heroin in the decomposing tissue on the development of *Boettecherisca peregrine* (Diptera: *Sarcophagidae*) and the implications of the effect on the estimation of the post-mortem interval using arthropod development patterns. Journal of Forensic Sciences. 36:537-542. 1999.
24. Hedouim, V, Bourel, B, Martin-Bouyer, L, Becart, A, Tournel, G, Deveaux, M, Gosset, A: Determination of drugs levels in the larvae of *L. sericata* (Diptera: *Calliphoridae*) in reared rabbits carcasses containing morphine. Journal of Forensic Science. 44:351-353. 1999.
25. Jodie, K, Margaret, S, Williams, S: Alternative to Animal Testing and Experiment (AATEX) 14, Special Issue, Proc. 6th World Congress on Alternative and Animal use in Sciences, August 21-25, Tokyo, Japan, pg 163-165. 2006.
26. Matthesien, T, Wohrmann, T, Coogan, TP, Uragg, H: The experimental toxicology of tramadol: An overview: Toxicol. Lett. 95:63-71. 1998.

27. Mahat, NA, Zafarana, Z, Jayaprakash, PT: The influence of rain and Malathion on the oviposition and development of the blowfly *L.sericata* (Diptera: *Calliphoridae*) ingesting rabbits carcasses in Kelatain, Malaysia. Forensic Science international. 192:19-28. 2009.
28. Adams, ZJ, Halls, MJ: Methods used in the killing and presservation of blowfly larvae and their effects on the PMI length. Forensic Science International. 138:50-61. 2003.
29. Goff, ML, Brown, WA, Omori, AI, Raporte, D: Preliminary observation of the effects of *Parasarcophaga ruficornis* (Diptera: *Sarcophagidae*) and implications on the effects on the estimation of the PMI. Journal of Forensic Sciences. 38:316-322. 1993.
30. Lamia, ME, Zeinab, AE, Hassan, MM: Effects of Tramadol on the Development of *L. sericata* and the Detection of the Drug Concentration in the Post-mortem Rabbit Tissues and Larvae. Journal of Entomology. 8:353-365. 2011.
31. Bourel, B, Hedouim, V, Martin-Bouyer, L, Becart, G, Deveaux, M, Gosset, D: Effects of morphine in the decomposing bodies on the development of *L. sericata* (Diptera: *Calliphoridae*). Journal of Forensic Science. 44:354-358. 1999.