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The Statistical Analysis of Meta Morphometric- and Biochemical-Data of *Lucilia cuprina* Immatures Fed on the Muscle Tissues of Clonazepam-Injected Rabbits

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Abstract

The statistical analysis of Meta forensic investigation data is so essential step. It is used to examine, analyze and possibly document forensic investigations results. The met analysis is used to demonstrate the effective immature stage and limited drug dose which are used in forensic investigation. In the current study, the cluster analysis and general estimation equation model were applied to the secondary data of *Lucilia cuprina*, consumed the muscle tissues of drug-injected rabbits. The statistical analysis was done on all morphometric- and biochemical-data of *L. cuprina* immatures. This information can be used to estimate the effective entomotoxicological sample and limited detected dose that will be used in the forensic application. Also, the results showed that the clonazepam dose 6 mg/ml has a unique cluster in almost morphological and biochemical data. Besides that, the prepupal stage can be used as an effective stage in forensic investigation. These findings will enhance the use of insects in forensic investigations.

Keywords: Metadata Analysis; Entomotoxicology; Clonazepam; Statistical Analysis; Oxidative Stress; Developmental Data

Abbreviations: CU-IACUC: Cairo University Institutional Animal Care and Use Committee; HACA: Hierarchical Cluster Analysis; HACA: Hierarchical Cluster Analysis; GEE: Generalized Estimating Equation; AD: Alzheimer's disease.

Introduction

A scientific field called digital forensics deals with gathering, examining, analysing, and maybe documenting objects and reconstructing events in order to offer them as evidence to the court. Although databases typically include evidence, traditional digital investigations frequently excluded them until recently. Despite the fact that the discipline is still in its infancy, due to the growing amount of information that may be used in solving various crimes and the numerous risks linked with the information held on many databases, it is quickly becoming a crucial component of many investigations. The ability to analyse the metadata is crucial nowadays to maximize the utilization of raw data investigations [1,2].

The use of flexible investigative tools, such as forensic entomology, can help with the investigation of the crime investigation. The latter's efficacy was demonstrated in instances involving highly damaged cadavers [3-6]. Numerous species of insects, which comprise the most prevalent and diverse class of arthropods on Earth, can be found near

cadavers [7]. The Callphoridae, and particularly *L. cuprina*, could arrive to carrions shortly after death [6,8,9]. Estimating the post-mortem interval may be aided by their sizes and developmental stages, is so crucial in forensic investigations [10]. Furthermore, in some earlier entomotoxicological experiments, calliphorid flies provided more sensitive results in contrast to human specimens [11,12]. Xenobiotics may impact the rate of decomposition of the human body as well as the growth and aggression of insects [13-16]. These theories led entomologists to conduct additional research investigations on the impact of xenobiotics on the growth rate and biochemical characteristics of the crucial insects for forensics. Cocaine, and diazepam have been successfully detected by dipteran flies [17,18]. Diazepam: According to the Carvalho National Institute on Drug Abuse, the victim percentages grew by over 4.3 times between 2002 and 2015 [19]. Numerous medications have the potential to spread up the food chain, and their effects can be examined inside insect tissues and throughout the insect life cycle [20,21]. The impact of a medicine on insects varies depending on the species, according to the discipline of entomotoxicology [22-24].

Several methods of analysis have been used to determine how drugs and toxins affect crucial insects in forensic investigations. They comprised quantitative studies as well as developmental analyses, which measured the weight, length, and width of immatures as well as the life cycles of insects [25,26]. By the use of HPLC-MS and other analytical techniques, to quantitative analysis [18,27-29]. Additionally, the potential of the blowfly *Callphora sp.* to serve as a forensic sample at a crime scene was confirmed by using a color approach to identify diazepam and other drugs in the bug. The drug was detected from the adult stage to adults in the next generation [30]. However, no studies have examined how insects that ate poisoned or drugged corpses were affected by the bioaccumulation of damaging critical components, as measured by protein carbonyl levels and DPPH. The proposed approaches for measuring antioxidants were affordable, straightforward, sensitive, and selective analyses [31,32]. This might be useful in the field of forensic entomology.

The current study attempted to examine the ability of using statistical analysis of metadata of forensic study to determine the appropriate developmental stage and drug-limited dose in the forensic investigation, especially at clonazepam case.

Materials and Methods

Secondary Data Source

L. cuprina was the Meta data of this research were supplied from a practical study at Entomology department, Faculty of Science, Cairo University, Egypt.

Experimental Analysis of Origin Data

Clonazepam (C1277) Sigma-Aldrich was dissolved in purified distilled water at four concentrations: 0 mg/mL, 0.7 mg/mL, 1 mg/mL and 6 mg/mL were injected into four Netherlands rabbits, Oryctolagus cuniculus domesticus. The rabbits were euthanized by decapitation according to the method of Close et al. [33], and the approval of Cairo University Institutional Animal Care and Use Committee (CU-IACUC) under the number CU-I-F-47-19. The rabbits' muscle tissues were used in this experiment as the insect's foodstuff. The experimental groups including the following treatment: An egg batch (~140 eggs) of *L. cuprina* El-Bassiony [7] was placed on the foodstuff, and the hatched larvae were allowed to reach the pupal stage, in a 100 ml plastic container covered with fabric mesh. The negative control groups were fed on the muscle tissues of control rabbit (not injected with clonazepam nor saline), while the positive control groups were fed on the muscle tissues of rabbit injected with saline. Each control or treated group was replicated three times. The immatures were dried, and morphologically measured by using a digital caliper (MITYTOUO, digital Vernier) and a digital balance (RADWAG, WTB200). The biochemical analysis included HPLC-MS to determine the clonazepam concentration inside the insect was determined according to the methodology of Rojas, et al. [34]. Also, the determination of the protein oxidative damage informing of protein carbonyls amount, was performed according to Levine, et al. [35], the total protein concentration of samples was determined spectrophotometrically according to the method of Bradford [36]. While, the α,α-diphenyl-β-picrylhydrazyl (DPPH), was determined according to Blois [37].

Statistical Analysis

All experimental groups were subjected to a non-parametric analysis using a Kruskal-Wallis revealing test with a p-value of a 0.05. Hierarchical Cluster Analysis (HACA) based on agglomerative statistics using Ward's Method was performed to analyze the data. The use of cluster analysis is essential to find the potential clusters or groups among the observational units based on levels of similarity and differentiation is the aim of HACA [38]. The cluster's average similarity is calculated at each level. It is computed how different each example in a cluster is from that average similarity. All morphological and biochemical metadata were investigated using the Generalized Estimating Equation (GEE). All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 17.0. Armonk, NY: IBM Corp.).

Results and Discussion

The non-parametric analysis, using Kruskal-Wallis test, clarified significant ($p < 0.05$) differences in most of the

morphological data of the treated larval, prepupal and pupal stages of *L. cuprina*, in comparison with the two control groups (ϰ2 = 9.9, 13.5, 13.5; df =4, 4, 4; and p value<0.05, <0.05, <0.05, respectively). A generalized estimated equation (GEE) showed that the different concentrations of Clonazepam (0.7, 1, and 6 mg/ml) influenced significantly the morphological (weight, length, width), the analytical (HPLC-MS), and the biochemical measures, in form of DPPH inhibition percentage and protein carbonyls amount, of different developmental stages (first, second, third larval instars, and prepupa) and puparium of *L. cuprina* (Table 1).

Categorical variables	Chi-square (χ^2)	df	*QIC	p value
Intercept				
Weight	1802	1	50.13	<0.0001
Width	6657.8	1	59.5	<0.0001
Length	8645	1	109.9	<0.0001
HPLC	1709.9	1	264.3	<0.0001
DPPH	4607.5	1	45.3	<0.0001
PC	5264.9	1	47.4	<0.0001
Effect of different insect developmental stages				
Weight	4473	4	50.13	<0.0001
Width	3679	4	59.5	<0.0001
Length	3338.8	4	109.9	<0.0001
HPLC	341.1	4	264.3	<0.0001
DPPH	73.9	4	45.3	<0.0001
PC	50.5	4	47.4	<0.0001
Effect of different clonazepam concentration				
Weight	15.11	4	50.13	<0.05
Width	7.3	4	59.5	<0.05
Length	5.2	4	109.9	<0.05
HPLC	79.7	5	264.3	<0.0001
DPPH	815.2	5	45.3	<0.0001
PC	886.6	5	47.4	<0.0001
Effect of interaction between different insect developmental stages & clonazepam concentration				
Weight	77.3	16	50.13	<0.0001
Width	97.11	16	59.5	<0.0001
Length	54.48	16	109.9	<0.0001
HPLC	110.79	15	264.3	<0.0001
DPPH	657.2	15	45.3	<0.0001
PC	179.3	15	47.4	<0.0001

Table 1: Testing the interaction of different concentrations of clonazepam (negative control, positive control, 0.7, 1, and 6 mg/ml) on different developmental stages (first, second, third larval instars, prepupa, and puparium) of *Lucilia cuprina*, in terms of morphological measurements and, analytical and biochemical analyses, using Generalized Estimating Equation (GEE).
*QIC is the quasi-likelihood under the independence model criterion and is a derivation of Akaike's information criterion for GEE.

The clustering, of *L. cuprina* developmental stages, revealed that there was a separate cluster group between negative and positive control in weight, HPLC-MS and protein carbonyls analyses (Figure 1 A,D,F). However, there was a separate

cluster between 6 mg/ml clonazepam treated group in the rest parameters (Figure 1 B,C,E). Both HPLC-MS and protein carbonyls analyses declared an obvious dissimilarity pattern between the control and 6mg/mL clonazepam treated group (Figure 1A-F). There was a similarity pattern between the prepupa and pupa stages, and also between the second and third larval stages in all morphological data (weight, length, and width) (Figure 2 A-C). Moreover, similar pattern occurred between the empty puparium and pupa regarding the concentration of clonazepam (mg/ml) in insect's tissues using HPLC-MS, α, α-diphenyl-β-picrylhydrazyl (DPPH) inhibition percentage (%) and protein carbonyls amount (Figure 2 D-F). The study by Carvalho, et al. [39] revealed a direct correlation between the weight characteristics of *C. albiceps* and *C. putoria* larvae and the diazepam drug concentration, which is consistent with our findings. Different flies fed on ethanol Tabor, et al. [40], anticholinergic Oliveira, et al. [41], cocaine de Carvalho, et al. [39] and methamphetamine all displayed the same reactions [42]. Contrarily, diazepam inhibited the growth of larvae in *L. cuprina* treated with larger doses of the medication and in *C. albiceps* [43,44].

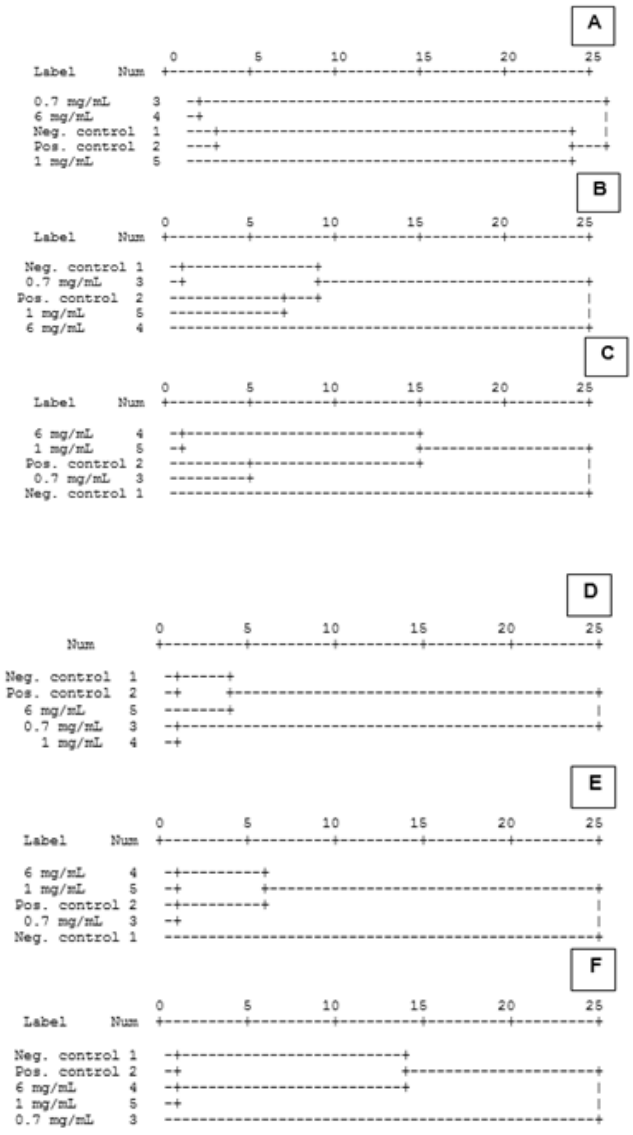

Figure 1: Dendrogram of the cluster analysis (using Ward's Method) to evaluate the similarity between different clonazepam concentrations (negative control, positive control, 0.7, 1, and 6 mg/ml), which has been fed to the developmental stages of *Lucilia cuprina*, and the (A) weight (mg), (B) length (mm), (C) width (mm), (D) concentration of clonazepam (mg/ml) in insect's tissues using HPLC-MS, (E) α,α-diphenyl-β-picrylhydrazyl (DPPH) inhibition percentage (%) and (F) protein carbonyls amount in insect's tissues using (OD/ mg protein/ min).

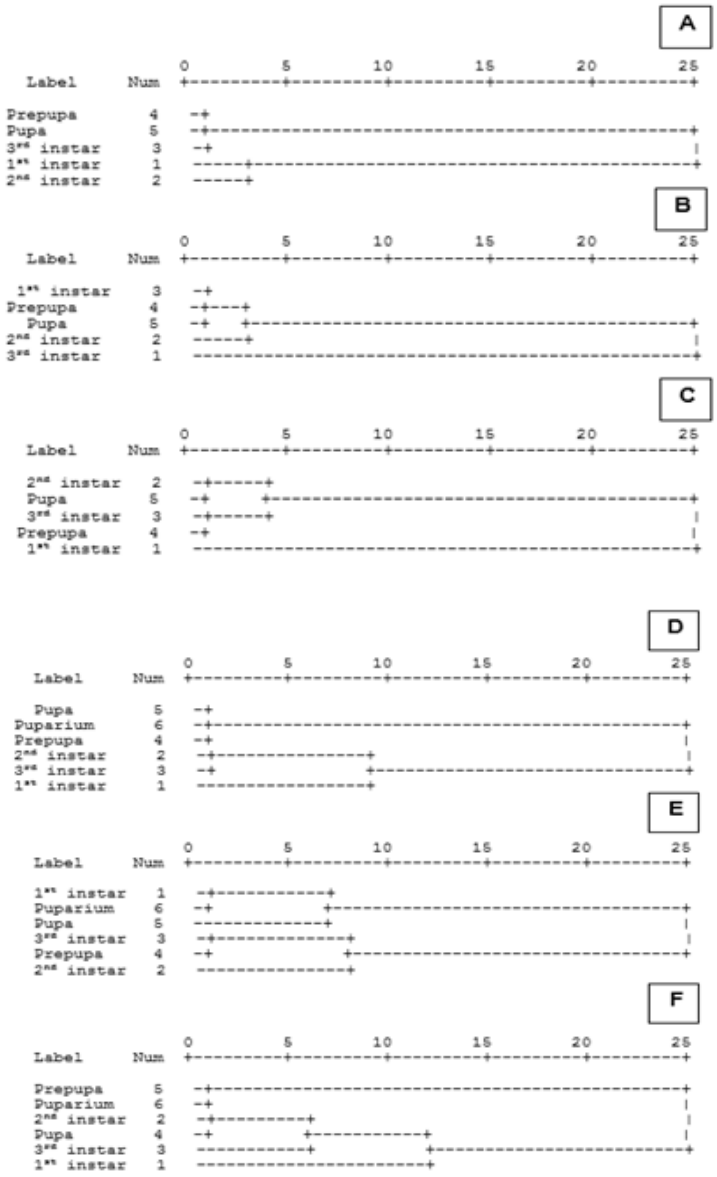

Figure 2: Dendrogram of the cluster analysis (using Ward's Method) to evaluate the similarity effect between the developmental stages (second, early third, and late third larval instars and prepupa) of *Lucilia cuprina*, which fed on different clonazepam concentrations (negative control, positive control, 0.7, 1, and 6 mg/ml), and the (A) weight (mg), (B) length (mm), (C) width (mm), (D) concentration of clonazepam (mg/ml) in insect's tissues using HPLC-MS, and E) α,α-diphenyl-β-picrylhydrazyl (DPPH) inhibition percentage (%) (F) Protein carbonyls amount in insect's tissues using (OD/ mg protein/ min).

When insects were used as biomarker agents, the identification of pharmaceuticals in insects not only aided medico-legal forensic applications but also environmental biomonitoring [32,45]. Previous data proved the ability of HPLC-MS to detect several drugs in insects. Morphine was analyzed by HPLC-MS in C. albiceps, and the drug could be detected in the feeding and post-feeding larvae [46]. Recently, the effect of Viagra overdose plus diazepam on the third larval stage, pupae, and adult of *C. albiceps* were tested by using HPLC-MS, and the results ensured the presence of the two tested drugs in all developmental instar of the insect [43]. In the current research, the clonazepam concentration recorded significant increasing in all treated groups ($\varkappa 2$ =11.76, 13.2, 13.7; df= 4; p-value <0.05). The different similarity pattern of the instar stages might imply that the drug converted into its metabolite form, 7-acetamido-clonazepam. When the larvae consumed the smallest amount of the drug (0.7 mg/mL), the drug concentration reached

its peak at the start of the second instar. This demonstrates that the medication bioaccumulates in the tissues of the insect [22]. The developmental stage of the insect, its eating environment, and/or the physiochemical stability of a drug in this insect were all known to affect the bioaccumulation of medicines in necrophagous insects [22,47].

The other biochemical analysis is protein carbonyls analysis, which has been used to identify pesticides in freshwater fish Channa punctata (Bloch). Protein carbonyls analysis is thought to be an indicator of oxidative stress factors [48-51]. The phenolic chemicals found in plants Renault, et al. [52], or even the usual concentrations of environmental toxins in insects [53]. In addition to being inexpensive and easy to use, Dalle-Donne, et al. [48] advocated this study as an efficient method since protein carbonyls develop and are more stable than other oxidative chemicals. The current investigation demonstrated that compared to controls, most treated groups and developmental stages had greater levels of damaged protein, as measured by protein carbonyls of *L. cuprina* (x2= 13.4, 12.8; df= 4; p-value 0.05, respectively). Similarly, clonazepam was associated with Alzheimer's disease (AD), which was distinguished by a high level of protein carbonyl quantity [54].

The current data demonstrated a steady decrease in the protein carbonyls amount from one stage to the next, particularly in the pupal stage, and this may be associated to the synthesis of clonazepam metabolites or as a characteristic of the inactive pupal stage. However, after giving the treated instars access to the muscle tissues with the highest medication concentration (6 mg/mL), the amount of protein carbonyls considerably decreased in all treated instars compared to the other treated groups (0.7 and 1 mg/mL). According to (Abdelfattah, et al.; Costa, et al.; Boguszewska-Makowska et al.) [32,55,56] and other researchers, this could mean that the drug is having both enzymatic and non-enzymatic effects. It could also mean that the equilibrium between protein oxidation and the redox regulation of proteolysis is shifting. Additionally, it can demonstrate that the medicine was absorbed less quickly by the insect than it was eliminated [22]. Besides that, utilizing DPPH inhibition % in comparison to the negative control (i.e. the naive carcass that died without any injections) showed that the drug was successfully detected in all phases and in the empty puparium. This demonstrated the potential for employing *L. cuprina*'s empty puparium in the event of a late discovery of a corpse. Additionally, the first, second, and third larval instars' antioxidant levels considerably increased in comparison to the positive control, in all drug's concentrations. A lot of studies used DPPH in antioxidant's analyses, for example, assessing various pharmaceuticals activities Parmar, et al. [57], investigating of polyphenols in detoxification of free radicals Wei, et al. [58], and assessing the antioxidant ability of the Malathion-exposed black soldier fly [32].

Conclusion

The concentration of clonazepam in injected rabbits directly affected *L. cuprina* growth rate, and significantly increased their weight, length, and width. Moreover, the biochemical analyses, such as HPLC-MS and protein carbonyls amount were effective in detecting the drug in all insects' developmental stages. DPPH was a very sensitive and efficient method to record the biochemical changes in the drug-injected rabbits, though the insect immatures feeding habits on carcasses. Also, the empty puparial case of *L. cuprina* has proven its validity to detect clonazepam along the different concentrations, and in case of delayed corpses discovery. In the current study, we demonstrated that the ability to use the secondary data of *L. cuprina* (Wiedemann 1830), which consumed the muscle tissues of rabbits given drugs, was subjected to cluster analysis and the general estimate equation model. In the forensic application, this information can be utilized to estimate the limited detected dose and the effective entomotoxicological sample.

Ethical Approval

This study was approved by Cairo University Institutional Animal Care and Use Committee (CU-IACUC) under the number CU-I-F-47-19.

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Author Contributions

FMA investigation, data calculation, statistical analysis, and writing the original document. EAA and GME conceptualization, investigation, supervision, and editing of the original manuscript. EEA writing the final manuscript. This manuscript is a part of FMA M.Sc. thesis. All authors contributed to the article and approved the submitted version.

Competing Interests

The authors declare that they have no conflict of interest.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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