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Forensic markers in human blood drawn from *Culex pipens* mosquito (Diptera: Culicidae)

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Abstract
Mosquitoes feed on blood that is a marker in forensic research. *Culex pipens* mosquitoes imbibed human blood and cultured, after ethanol-killing or keeping them alive, for 0, 3, 6, 12, 24, 48 and 72 h and followed by blood count. Red Blood Cells (RBCs) count in living mosquitoes showed reduction at 24 h after blood feeding while in ethanol-killed females the reduction was in a time dependent manner. White Blood Cells (WBCs) count showed a similar pattern in living mosquitoes however, in killed mosquitoes the reduction was rapid between 24-48 h with significant reduction at 48 h. using different blood groups did not affect RBCs and WBCs degradation in living or dead mosquitoes. This indicated that the approximate time after mosquito bite can be determined (without impact from the type of blood group) by the RBCs and WBCs count in living and dead mosquitoes, respectively.

Keywords: Mosquito; Forensic Entomology; Blood Count; *Culex pipens*

1. Introduction
Forensic entomology is a growing field of insect Science, which deals with the relationships between insect related research and investigation of topics related to law especially those related to crimes [1-2]. Forensic entomology can be applied in criminal field and life including investigation of death, drug and poison disclosure, and locating the place where incidents happened [3]. Both terrestrial and aquatic insects can be used in forensic entomology [4-8]. Several insect orders can assist in forensic field including dipterans such as Calliphoridae, Sarcophagidae, and Piophilidae. Coleopteran insects are also contributing in forensic research such as skin beetles (Dermestidae), rove beetles and clown beetles [9-10]. This application is not only restricted to adult stages but also it extends to other developmental stages (the juvenile stages) which are feeding on cadaver and consume a dead body.
Blood is considered as one of the most important biological marks which can be found in crime scenes. This liquid consists of two major components, the plasma and blood cells. Plasma constitute about 55% of blood volume and is composed mainly of water (92%), proteins, glucose, ions, hormones and carbon dioxide [11]. Blood cells are categorized in two main classes which are the Red Blood Cells (RBCs) and the White Blood Cell (WBCs). In adult males, the RBCs count about $5X10^{12}$ cell/ liter while the WBCs are present as about $8X10^{6}$ cell / liter [12].
Members of family Culicidae which are dipteran insects are characterized by their ability to bite, suck and consume blood from human and other vertebrate animals. Adult female mosquitoes search for a blood meal from a vertebrate host and use their mouth parts to suck blood from their veins leading to a complicated digestion process in the abdominal cavity of the mosquito. This process of digestion is achieved in the midgut via synthesis of several proteolytic enzymes. Midgut enzymes hydrolyze blood proteins into free amino acids which are the building blocks of yolk proteins [13-14]. These insects might be found near a crime scene and suck a blood from either the victim and/ or the offender. There are several biochemical and molecular methods to analyze blood from mosquitoes which can provide useful information in crime investigation (Ibrahim *et al.*, unpublished data).
This research aims to use female *Culex pipens* mosquitoes blood meal as forensic markers through counting the number of the white blood cells and the red blood cells in human blood and building a relationship between time after mosquito feeding and number of blood cells in the stomach of the mosquito either in the cases where the mosquito continues alive after blood feeding or it was subject to mortality. Furthermore it addressed the effect of human blood group on the degradation rate of human blood in mosquito.

Material and methods

Insect Culture

This work was performed in summer 2014 under controlled laboratory condition. All experiments were performed using a laboratory strain of *C. pipens*. Adult *C.pipens* were cultured under laboratory condition with 10:14 h light: dark according to a previously described method [15]. Briefly, mosquito eggs were deposited on water surface and covered with green algae. Egg hatched into larva with approximate life span of about 6 days. Larvae developed within this period into pupal stage which changed into the adult stage in about a couple of days. Adults were fed on 10% sucrose solution for two day before using them in blood feeding experiments because the digestive system of mosquitoes completes its development within the first two days after adult emergence.

Experimental Setup

Adults starved for 12 h before experiment. After feeding on human blood for 30 minutes through biting forearm of the donor(s), heads/thoraces were separated from abdomens and blood isolated from abdomens was used in blood count. A total number of 288 females fed on human blood and used in the analysis. After blood ingestion, mosquitoes were divided into four sets.

The first set which was used for temporal count of blood cells in alive insects and was immediately transferred to 28±1 °C and cultured for 3, 6, 12, 24, 48 and 72 h after blood feeding.

The second set of mosquitoes was used for temporal count of blood cells in dead insects (females were killed by immersing them in 70% ethanol for 2 minutes). Killed females were transferred to 28±1 °C and cultured for 3, 6, 12, 24, 48 and 72 h after blood feeding. Negative control samples consisted of females fed on sucrose solution for 30 minutes while positive control samples consisted of females fed on human blood and their blood counts were performed immediately after feeding.

The third set of mosquitoes fed on human blood of different groups of the ABO system (A, B, AB, and O) followed by direct blood count (control) or incubation for 3, 6, 12, 24, 48, and 72 h at 28±1 °C before blood count (treatment).

The forth set of mosquitoes included samples of ethanol-killed insects after feeding on human blood of the four groups of the ABO system. These samples were incubated for 3, 6, 12, 24, 48, and 72 h at 28±1 °C for blood count. Control samples of this set were made up of immediately killed mosquitoes after human blood feeding for 30 minutes. All counts were performed in 3 independent replicates. In all treatments, human blood was drawn directly from donor, counted and tested statistically against 0 h samples to ensure absence of immediate impact of mosquito ingestion of blood on cell count. Human based treatments are approved by Assiut University research ethics committee in accordance with the code of ethics of the world medical association.

Blood Count

All blood counts were performed immediately after sample collection to avoid degradation of blood cells in long term freezing. Blood cells were counted using hemocytometer (Superior, Germany) according to manufacturer instructions under light microscope (Somatco, Saudi Arabia). This method of manual counting was selected because the amount of blood which can be obtained from each single mosquito was not sufficient to perform automated blood count using automatic machines present in the market. The RBCs or the WBCs in 1 µl blood were counted in all treatments and positive control samples. In samples which failed to supply 1 µl blood due to digestion process, more than one female was used to collect the required volume for blood count.

Data Analysis

All assays were performed in 3 different treatments. Plots were shown as mean ± standard deviation using Medcalc software (Ostend, Belgium). Data were analyzed by one way ANOVA and t-test using version 12 of Medcalc software (Ostend, Belgium).

Results

Temporal changes in blood count following mosquito feeding

RBCs count in alive mosquitoes showed significant reduction from control samples (0 h) ($5.39\pm0.39X10^6$ cell/µl) at 24 (2.31 ± 0.33 $X10^6$ cell/µl) and 48 h (1.1 ± 0.40 $X10^6$ cell/µl) after mosquito feeding (F=73.14; df=6, 14; $P < 0.001$) (Fig. 1A). On the other hand, RBCs count in ethanol-killed mosquitoes showed that the RBCs were significantly reduced in a time dependent manner from 0 h (5.5 ± 0.42 $X10^6$ cell/µl) to 72 h (0.89 ± 0.22 $X10^6$ cell/µl) (F=42.71; df=6, 14; $P < 0.001$) (Fig. 1B). The WBCs count in alive mosquito showed similar pattern to that of the RBCs where number of the WBCs showed significant reduction at 24 h (1.1 ± 0.66 $X10^6$ cell/µl) when compared to 0 h (6.91 ± 1.17 $X10^6$ cell/µl) (F=43.05; df=6, 14; $P < 0.001$) (Fig. 2A). On the contrary, the WBCs count in ethanol killed samples showed different pattern of reduction where the significant reduction from 0 h control samples (6.04 ± 0.2 $X10^6$ cell/µl) was clear at 48 h (0.6 ± 0.24 $X10^6$ cell/µl) after blood feeding by *C. pipens* mosquito (F=44.64; df=6, 14; $P < 0.001$) (Fig. 2B).

Effect of blood group on the reduction rate of blood cells

Temporal changes in blood counts at 0, 3, 6, 12, 24, 48 h were performed using blood samples drawn from *C. pipens* mosquitoes fed on a donor blood of groups A, B, AB, and O. it revealed the same pattern to those represented in (Fig. 1 and Fig. 2). To avoid repetition of data, I have selected the time point 24 h after *Culex* feeding and compared it to control samples collected at 0 h after feeding on human blood. At 24 h after mosquito feeding, all blood groups showed statistical difference from control in the RBCs count in alive insects (Fig. 3A, B, C and D) and ethanol killed insects as well as in the case of the WBCs (Fig. 4A, B, C, and D). The WBCs count in ethanol-killed insects was performed after 48 h after blood feeding and showed the same pattern to those of 24 h in alive mosquitoes.

Discussion

This study focused on the use of insects in forensic Science and specifically on using culicid mosquitoes in determination of the approximate time at which a person was present near a crime scene via counting of human blood cells and comparing the count values to the reference control to establish a correlation between time after mosquito feeding and number of blood cells present in the gut of the mosquito. So far, forensic use of mosquitoes is limited only to molecular analysis of human blood for identification purposes using either PCR technology, DNA dot blot or Short Tandem Repeats (STRs) analysis [16-23]. Although using mosquito-drawn human blood in molecular analyses can supply useful information in legal aspects up to person identification, however, determination of time passed after mosquito bite near crime scenes might be of more importance in some forensic cases, so this study was designed to determine approximate time after mosquito bite to human via establishing the correlation between time and number of blood cells.

The RBCs and WBCs were reduced at 24 h after blood feeding

by alive culicid mosquito female of the genus *Culex*. This reduction continued for 48 h and 72 h too. The RBCs lysis is the initial step in blood digestion by mosquitoes as they contain the major blood protein hemoglobin [24]. Reduction in blood cells can be related also to the level of digestive enzymes in mosquito digestive tract. Activity of digestive enzymes in mosquito gut can be considered as a key marker for blood digestion rate. In the mosquito, *Culex nigripalpus,* which has a very similar physiology to our model, digestive enzymes in midgut and fat body reach peak at 22-25 h after sucking a blood meal and significantly decreased to the minimum level at 30 h in fat body and 58 h for midgut [25]. Peak of trypsin and aminopeptidase in hindgut of *Anopheles stephensi* Liston was at 30 h while it decreases to the minimum at 60 h [26]. Similarly, blood meal induced a trypsin like proteinases in *Aedes aegypti* in about 8-12 h after blood feeding [27-28]. Mosquitoes use human blood protein content in production of yolk proteins, in *Aedes aegypti*, egg maturation ends in 63 h, which need all blood components are degraded by the mosquito at this time. This explains the small number of cells present in all samples at 72 h.

In ethanol-killed mosquitoes, the RBCs count showed time dependant degradation at all time points while the WBCs showed a significant reduction at 48 and 72 h after mosquito feeding. Although digestion process is stopped by killing the mosquitoes, however the nature of each cell type affected the degradation rate of the cells. *In vitro*, when WBCs, incubated for several days in the anticoagulant buffer at room temperature, their count remained stable [29-30] but this was not the case for the RBCs which are easily shrink and disintegrate in unsuitable media. Different blood groups of the ABO system did not affect the lysis rate of human blood drawn from *C. pipens* mosquito. Physiological and chemical condition of human blood is affecting the host mosquito in terms of feeding preference and quantity of blood intake. Anaemic blood negatively influenced egg production and fecundity of the mosquito *Aedes aegypti* [31]. Although, it was reported that *Aedes aegypti* mosquitoes prefer blood group (O) in feeding [32] and *Anopheles stephensi* prefers AB group [33], our data did not show any difference between blood groups in degradation rate of blood cells.

Using mosquitoes in forensic research helped in solving many puzzles related to crimes. Such use is limited by the factor of the ability of mosquitoes to travel from place to place but also keeping into account that there is a possibility of mosquito death in the crime scene or in the case of tightly closed crime scene might support our trial. Recently we have found that temperature of mosquito culture, time after blood feeding, and even mosquito species affect the human blood DNA originating from mosquito (Ibrahim *et al.*, unpublished data). Insect has been previously used in time determination of post-mortem intervals through several genera and developmental stages of insects. Dipteran insect families Calliphoridae, Muscidae, Fanniidae, Sarcophagidae, and Phoridae are of importance in postmortem intervals [34]. In most cases this is done based on the age of the developmental stage of the insect and the abiotic condition. Callophorid insects colonize in a later time on indoor cadavers than outdoor [35]. 16 Sarcophagid species, 3 fanniids, 11 calliphorids, and one phorid were reported in Brazil to have a potential use in forensic research [36].

Conclusion

This study revealed that the RBCs count in alive *C. pipens* can be used as a marker for forensic determination of the approximate time passed after mosquito bite to human. On the contrary, the WBCs were more stable in dead *C. pipens* and had more preference than the RBCs in killed or dead mosquitoes. Furthermore, it concluded that different blood groups did not change the degradation rate of blood cells either in alive or in dead mosquitoes.

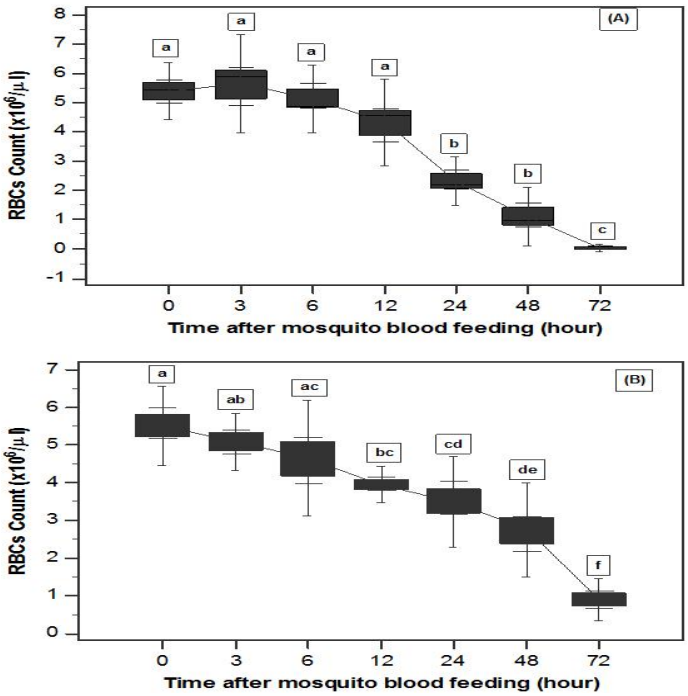

Fig 1: RBCs count in human blood samples collected from adult *Culex* sp Adult mosquitoes fed on human blood for 30 minutes and immediately transferred to 28±1 for Zero hour (immediate count), 3, 6, 12, 24, 48, and 72 hours after mosquito feeding. Cells were counted in 1 µl blood using haemocytometer. Samples of 48 h and 72 h failed to supply 1 µl blood, so several females served as a source of blood (A) Alive *Culex* sp (B) Ethanol-killed *C. pipens* Error bars represent standard deviations of three measurements. Different letters above error bars represent significant difference among means at type error = 0.05 (ANOVA).

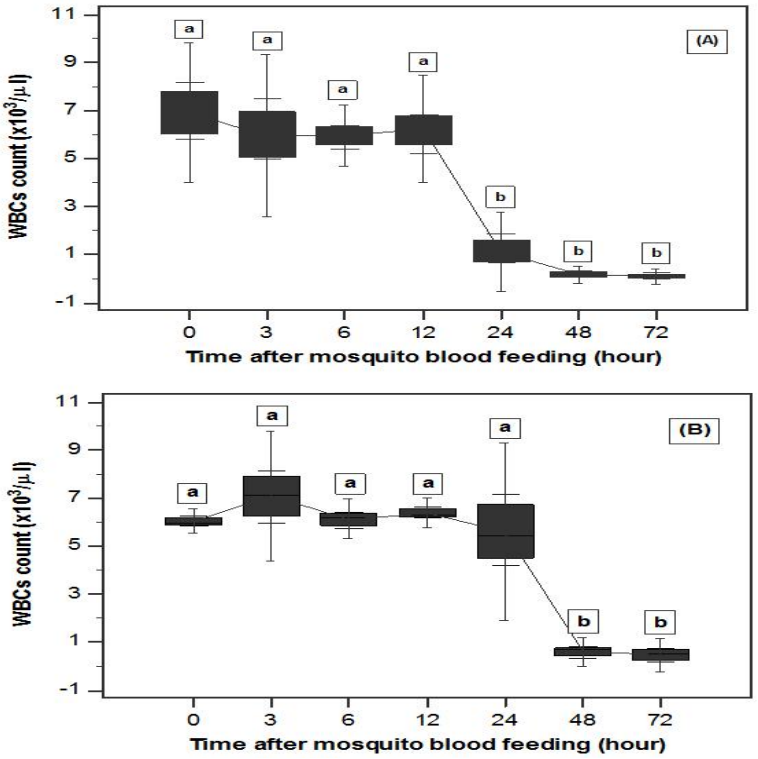

Fig 2 : WBCs count in human blood samples collected from adult *Culex* sp Adult mosquitoes fed on human blood for 30 minutes and immediately transferred to 28±1 for Zero hour (immediate count), 3, 6, 12, 24, 48, and 72 hours after mosquito feeding. Cells were counted in 1 µl blood using haemocytometer. Samples of 48 h and 72 h failed to supply 1 µl blood, so several females served as a source of blood (A) Alive *Culex* sp (B) Ethanol-killed *C. pipens*. Error bars represent standard deviations of three measurements. Different letters above error bars represent significant difference among means at type error = 0.05 (ANOVA).

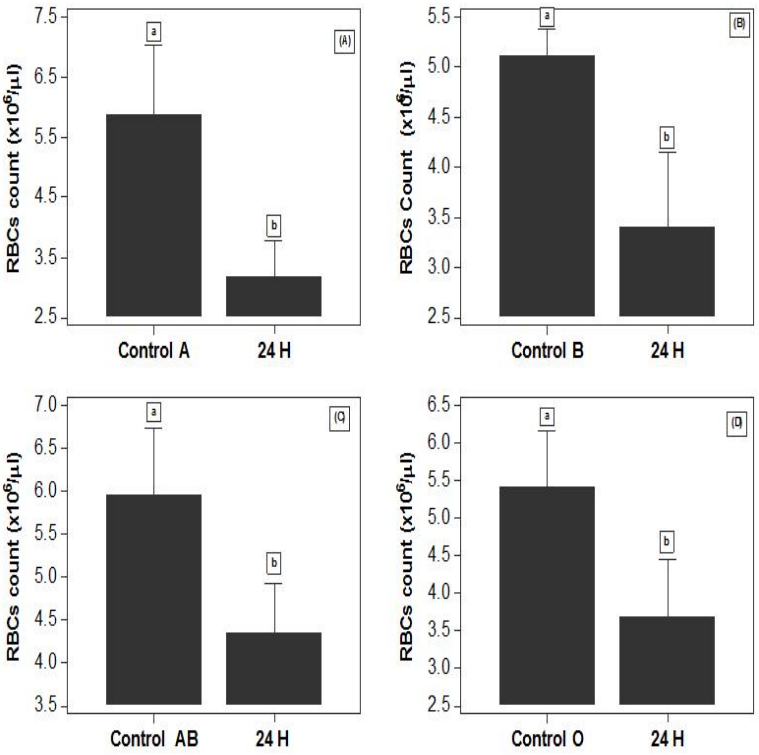

Fig 3: Effect of blood group type on the number of the RBCs collected from mosquito abdomens after feeding on human blood for 30 minutes and culturing for 24 h at 28±1 °C. Samples were collected from donors with blood. (A) Group A, (B) Group B, (C) Group AB, (D) Group O. Error bars represent standard deviations of three measurements. Different letters above error bars represent significant difference between means at type error = 0.05 (t-test). Data of this graph is a part of time course experiments performed at 3, 6, 12, 24, 48 and 72 h after blood feeding. The time point selected in the graph is 24 h after mosquito feeding which represent the significant point in reduction of RBCs count.

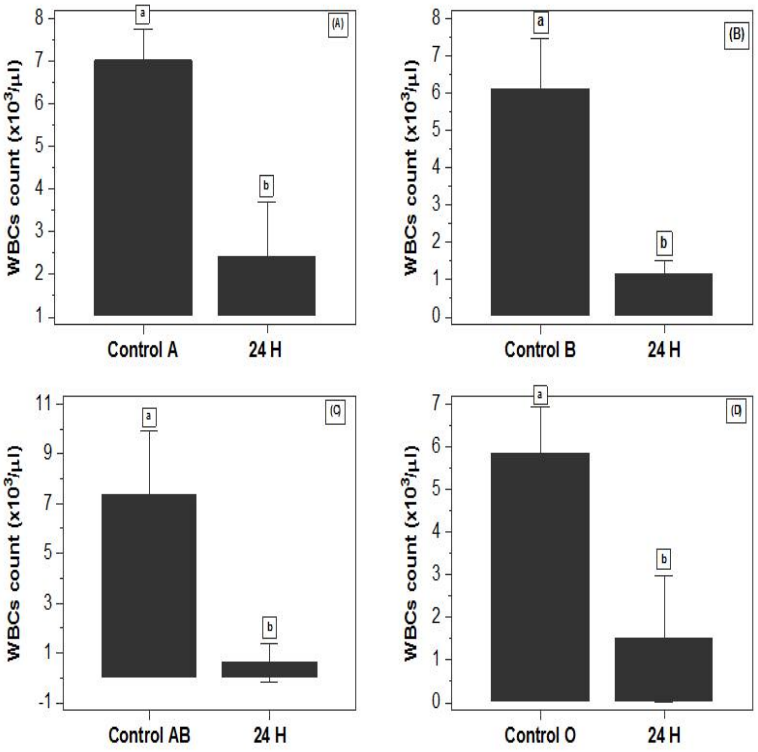

Fig 4: Effect of blood group type on the number of the WBCs collected from mosquito abdomens after feeding on human blood for 30 minutes and culturing for 24 h at 28±1 °C. Samples were collected from donors with blood group: (A) Group A, (B) Group B, (C) Group AB, (D) Group O. Error bars represent standard deviations of three measurements. Different letters above error bars represent significant difference between means at type error = 0.05 (t-test). Data of this graph is a part of time course experiments performed at 3, 6, 12, 24, 48 and 72 h after blood feeding. The time point selected in the graph is 24 h after mosquito feeding which represent the significant point in reduction of the WBCs count

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