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### Impact of adult age on forensic use of *Culex pipiens* mosquito (Diptera: Culicidae)

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#### Abstract

Mosquitoes feed on blood which is a key marker in forensic applications. We identified the impact of female *Culex pipiens* age on person identification using Short Tandem Repeats analysis (STRs) via blood present in their abdomen and established a correlation between mosquito age and Red Blood Cells (RBCs) degradation as indicator about time passed after blood feeding. Human DNA from adults was reduced in samples from 0 and 2 days old than 4-10 days old. These samples failed to supply full STRs profiles (0 and 2 days old females supplied 11 and 10 sites). Older females supplied all of the 14 STRs. Temporal count of RBCs from females with different ages after mosquito feeding revealed faster degradation of RBCs in blood drawn from older females (4-10 days old) than those of younger samples (0 and 2 days old). Age of mosquito has a key impact on forensic use of mosquitoes.

**Keywords:** *Culex pipiens*; STRs; Forensic entomology; DNA genotyping; Blood count

#### 1. Introduction

Female culicid mosquitoes are ectoparasites on human or other vertebrate hosts. They use their piercing and sucking mouth parts to obtain blood necessary for development of their eggs. The life cycle starts with the egg which hatches to larval stage, larvae grow until on water surface for pupal stage. Adults are able to emerge from the mature pupa after floating on water surface<sup>[1]</sup>. Several factors affecting the life span of adults either under laboratory condition or in nature including species, gender, diet, and weather conditions leading to potential adult lifespan ranging from as short as a week to as long as several months<sup>[2-3]</sup>.

Due to their ability to fly and suck human blood, Adult females of mosquito can be used in forensic aspects as blood present in their abdomen can carry useful information in legal investigations. Forensic Entomology is a growing branch of entomological science dealing with the relationships between entomology and legal aspects<sup>[4-5]</sup>. Several insect orders can be applied in forensic entomological investigations especially dipterans such as blowflies, flesh flies and cheese skippers. Members of order Coleoptera also might apply in forensic entomology such as dermastid beetles, rove beetles and clown beetles<sup>[6-7]</sup>. Mosquito can suck blood of the offender or the victim at a crime scene. This blood can supply several information related to crime puzzles as it can identify whether a person was present near a crime scene or not and how long time passed since a mosquito sucked his blood<sup>[8-9]</sup>.

Person identification could be achieved by obtaining human DNA through blood draw from mosquito followed by microsatellites (Short Tandem Repeats, STRs) analysis. STRs are short DNA sequences ranging between 2-5 base pair and present in variable number of copies on human chromosomes. The number of copies is unique for each person as STRs are identical only in identical twins. STRs are used widely as molecular markers in DNA fingerprinting<sup>[10-12]</sup>. These markers are analyzed in a multiplex PCR reaction by several kits present in the market. One of these kits was developed by applied biosystems and it amplifies 15 tetranucleotide loci and a gender marker located on the sex chromosomes (Applied Biosystems, Foster City, CA, USA).

Determination of time passed since mosquito blood ingestion could be achieved by counting physiological markers in human blood like blood cells. Two main categories of blood cells can be found in human blood which are the Red Blood Cells (RBCs) and the White Blood Cell (WBCs). In adult male, RBCs count about  $5 \times 10^{12}$  cell/ liter while WBCs are present as about  $8 \times 10^6$  cell/ liter<sup>[13]</sup>.

In this research, the impact of adult *C. pipiens* mosquito age on the sucking adequacy of

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human blood which is reflected here by amount of human DNA obtained from each single mosquito and the number of STRs amplified in each age. Furthermore, the effect of adult age on the digestion rate of blood is indicated by the temporal rate of degradation of RBCs at different time intervals after mosquito feeding on human blood.

## 2. Material and Methods

### 2.1. Insect Culture and experimental design

Adult *C. pipiens* mosquito were cultured in the laboratory under environmental condition with 10:14 h light: dark and culturing temperature of  $28 \pm 1$  °C according to a previously described method [14]. Briefly, mosquito eggs were deposited on water with green algal cover. Egg hatched into larva stage in water where larvae survived for approximately about 6 days during which they developed into pupae which in turn became adults within a couple of days. Adults fed on 10% sucrose solution during their life span which continued for about 10-14 days.

Adults starved for 12 h before experiment except for samples considered as (0 day) after emergence which was used in feeding experiments immediately after emergence. After feeding on human blood for 30 minutes through biting forearm of the donor, heads/thoraces were separated from abdomens and blood obtained from abdomens was used in blood cell count or DNA extraction for STRs determination. A total number of 180 females were used as experimental or control samples. After emergence from pupal stage, adults were kept in cages at  $28 \pm 1$  °C and used for blood feeding at different ages including 0, 2, 4, 6, 8, and 10 days after adult emergence. After blood ingestion, mosquitoes of different ages were divided into two sets; the first set which was cultured for 3 hours and used for human DNA extraction for STRs analysis while the second set was used for temporal count of RBCs in mosquito abdomens at 3, 6, 12, 24, 48 and 72h after blood feeding. For the first set, negative control samples consisted of females fed on sucrose while the positive control data was prepared from blood drawn directly from the donor. On the other hand, negative control samples of the second set consisted of females fed on sucrose solution for 30 minutes while positive control samples consisted of females fed on human blood and their RBCs count was performed immediately after feeding. All experiments were performed in 3 independent replicates. In all treatment human blood was drawn directly from donor, counted and tested statistically against 0h samples. Human based treatments were approved by Prince Sattam bin Abdulaziz University research ethics committee in accordance with the code of ethics of the world medical association.

### 2.2. DNA extraction and human DNA quantification

All DNA extractions contained negative control to ensure absence of contamination in DNA preparation and a direct collection of donor blood as a positive control to analyze STRs profile of the donor. DNA was extracted from either mosquitoes fed on human blood or those fed on sucrose solution by the organic extraction method. Briefly, 500 µl of the digestion buffer (0.01 M Tris HCl, 0.05 M NaCl, 0.01 EDTA, 2% SDS; pH: 8) and 15 µl proteinase K (10 mg/ml) were added to the reaction tubes of both experimental and control samples and were incubated at 56 °C for 12 h. DNA was extracted using phenol chloroform extraction and followed by ethanol precipitation. DNA pellet was washed by 70% ethanol and resuspended in 20 µl sterilized water.

DNA was quantified using Real-Time PCR technology by Quantifiler human DNA quantification kit (Applied Biosystems, Foster, CA, USA) as described by manufacturer instructions. Standards of quantification were first prepared through diluting the stock solution of the kit to the following concentrations: 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/µl. Reactions of quantification were prepared as 25 µl containing (10.5 µl primer mix, 12.5 µl reaction mix and 2 µl of DNA sample or standard DNA sample) in 96 well plate and DNA quantity determination was performed by 7500 real time PCR system (Applied Biosystems, Foster, CA, USA).

### 2.3. Analysis of human STRs

STRs were analyzed in 1 ng human DNA in 10 µl which was mixed with 15 µl of the reaction mixture containing (10 µl Identifiler reaction mixture and 5 µl primer set) and amplified using GeneAmp PCR 2720 thermal cycler (Applied Biosystems, Foster, CA, USA) for 28 cycles. STRs were analyzed using 3110 Genetic analyzer (Applied Biosystems) according to manufacturer instructions. Analysis of electropherograms was performed by GeneMapper Software.

### 2.4. RBCs count

RBCs counts were performed immediately without delay after sample collection to eschew blood cells degradation due to freezing. RBCs were counted using manual hemocytometer (Superior, Germany) according to the instructions provided by manufacturer under light microscope (Somatco, Saudi Arabia). Selection of manual counting was made because the amount of obtained blood from each single mosquito is not enough to perform automated RBCs count using most of automatic machines present in the market. RBCs in 1 µl blood were counted in all treatments and positive control samples.

### 2.5. Data Analysis

All assays were performed in 3 independent treatments. Data was shown as mean  $\pm$  standard deviation using Medcalc software (Ostend, Belgium). Analysis of data was performed by one way ANOVA using version 12 of Medcalc software (Ostend, Belgium).

## 3. Results and discussion

In the present work, adult *C. pipiens* mosquitoes with ages ranging between 0 days and 10 days after emergence were used to study the impact of age on STRs analysis in human blood and digestion rate of RBCs by mosquito digestive tract. This age scale was selected based on our observation of female longevity of the species under investigation at a culturing temperature of  $28 \pm 1$  °C.

After blood ingestion, adults were cultured for 3 hours before DNA extraction. DNA was quantified using Quantifiler human DNA quantification kit using qRT-PCR technology. DNA extracted from adults at 0 day and 2 days after emergence showed statistical difference in concentration from those extracted from the remaining ages (4, 6, 8, and 10 days) (Table 1.). This was associated with clear observation of little blood in female abdomen. In general, feeding success in *Aedes aegypti* mosquito increases with age [15]. In the first 3 days after adult emergence, females of mosquitoes cannot feed efficiently on human blood and even their gut has immature cells to digest it [16-17]. This explains the reason of having little DNA in samples drawn from the first two days after adult emergence when compared to other samples with older ages.

**Table 1:** Concentration of human DNA collected from mosquito-fed human blood. Adult mosquitoes were cultured after emergence at 28± °C. Data in the table represents mean±standard deviation. Letters above means represent statistical differences among means at type error 0.05 (ANOVA).

Sample	Adult age (days)					
	0	2	4	6	8	10
DNA concentration (ng/μl)	0.29 <sup>a</sup> ±0.23	0.27 <sup>a</sup> ±0.19	1.51 <sup>b</sup> ±0.21	1.17 <sup>b</sup> ±0.52	0.0.92 <sup>b</sup> ±0.19	1.25 <sup>b</sup> ±0.61

STRs analysis revealed that at the same time points (0 day and 2 days adult age), 4 STRs sites (D7S820, CSF1PO, D13S317 and D18S51) were missed in amplification reaction of 0 day age adult and in addition to these sites the VGA site was missed at 2 days age adult samples (Table 2.) and this indicates that newly emerged adults sucks less blood than older females, giving rise to lower DNA extracts (Table 1.) and reduced ability to analyze STRs (Table. 2). STRs analysis in human blood drawn from mosquitoes was used previously

in several studies <sup>[8]</sup>, (Ibrahim *et al.*, unpublished data). In these studies, several variables were used to study the effect of time after blood feeding, culturing temperature and type of mosquito on DNA integrity for STRs analysis. In Sicily, human DNA extracted from a mosquito source helped in solving a legal case of a dead person on the beach <sup>[18]</sup>. Interestingly, impact of adult age on its ability to suck enough blood for STRs analysis was not checked. Therefore we tested this variable in our running work.

**Table 2:** Impact of adult *Culex pipiens* mosquito age on STRs analysis from human blood drawn from females.

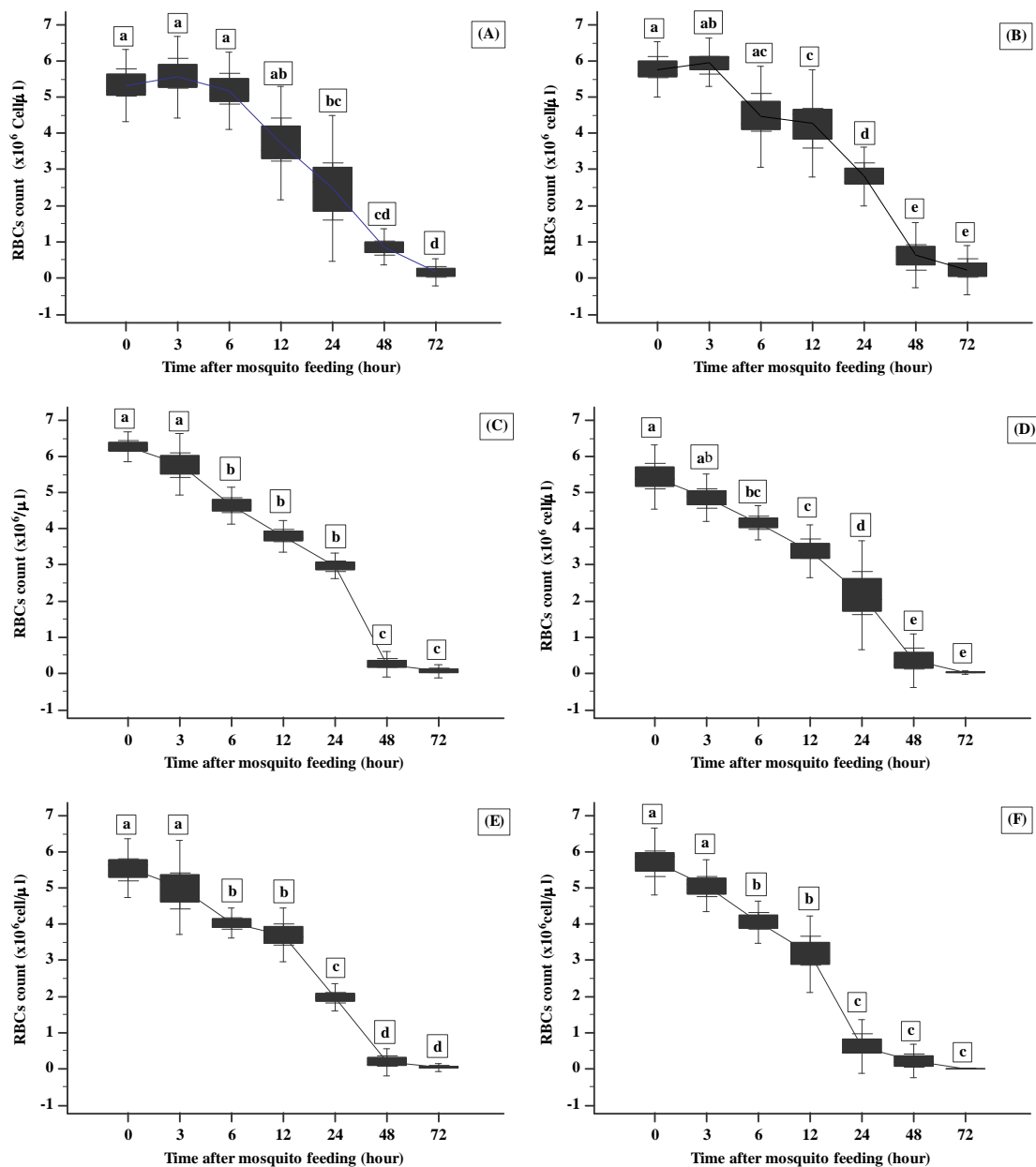
STR Site	Adult age (days)					
	0	2	4	6	8	10
D8S1179	12	12	12	12	12	12
D21S11	30,31,2	30,31,2	30,31,2	30,31,2	30,31,2	30,31,2
D7S820	-	-	8,9	8,9	8,9	8,9
CSF1PO	-	-	9	9	9	9
D3S1358	16	16	16	16	16	16
TH01	6,9	6,9	6,9	6,9	6,9	6,9
D13S317	-	-	9,12	9,12	9,12	9,12
D16S539	12,13	12,13	12,13	12,13	12,13	12,13
D2S1338	16,19	16,19	16,19	16,19	16,19	16,19
D19S433	13	13	13	13	13	13
VWA	16	16	16	16	16	16
TPOX	6,10	6,10	6,10	6,10	6,10	6,10
D18S51	-	-	17	17	17	17
Amelogenin	X,Y	X,Y	X,Y	X,Y	X,Y	X,Y
D5S818	9,12	9,12	9,12	9,12	9,12	9,12
FGA	20,25	-	20,25	20,25	20,25	20,25

Characterization of DNA in mosquito gut is one of the most powerful research tools to help investigators in solving crime puzzles using both DNA dot blot or PCR based technology <sup>[19-26]</sup> however, analysis of human STRs in insects is not only restricted to adult mosquitoes but also it extends to other developmental stages where STRs can be analyzed in the gut content of larvae feeding on decomposing bodies through total DNA extractions <sup>[27-28]</sup>.

Degradation rate of blood cells varied among different ages of adult *C. pipiens* (Fig. 1). At 0 day and 2 days old females, blood started clear reduction in RBCs count at 24 and 12 hours after mosquito feeding respectively. This reduction continued at 48 and 72 hours after feeding (Fig 1. A, B). On the other hand, the remaining ages of females (4 days and more started significant reduction in RBCs count as early as 6 hours after mosquito feeding (Fig 1. C, D, E, and F) which clearly indicate that after 4 days from adult emergence the digestive system of *C. pipiens* is functioning better than the younger

ages.

Blood count can be useful in determination of time passed after a person was present near a crime scene via comparing number of blood cells to the reference control in order to establish a direct correlation between time passed after mosquito feeding on human blood and number of blood cells present in the digestive tract of the mosquito <sup>[9]</sup>. However, in the running work the impact of adult age on the degradation rate of red blood cells was addressed to supply useful information regarding which age of females would be better for forensic application in temporal determination experiments. Based on results indicated here, it is clear that older ages (4 days and more) adults have a higher rate of RBCs degradation than younger age and this is possibly due to full development of their digestive system at this age.



**Fig 1:** Effect of adult female *Culex* age on temporal pattern of degradation of RBCs in human blood drawn from mosquito with adult age (A) Zero days after adult emergence, (B) 2 days, (C) 4 days, (D) 6 days, (E) 8 days, (F) 10 days. After blood feeding for 30 minutes, mosquitoes were cultured in 28±1 °C and RBCs were counted at 0 (immediate count), 3, 6, 12, 24, 48 and 72 hour after blood ingestion. Error bars represent standard deviations of three measurements. Different letters above error bars represent significant difference among means at type error = 0.05 (ANOVA).

Application of mosquitoes in forensic Science is a promising field in forensic entomology due to the ability of females to pierce human skin and suck blood which might happen before or after a crime or in legal-related situations. Blood source might come from either the victim or the offender so these insects can be considered as a link between the suspect and crime scene or the sufferer and crime scene. This blood can be used for STRs analysis up to several days after ingestion [8] or it may be help through blood parameters determination in timing information. Interestingly, mosquitoes tend to stay after blood meal in the same place for few hours and they prefer the same host for another blood meal [29-30]. This phenomenon increases the specificity of mosquito toward a single blood source and reduces the probability of different source blood

contamination while processing samples.

#### 4. Conclusion

The main goal of the present study was to determine mosquito age impact on the potency of STRs analysis of DNA samples isolated from human blood sucked by *C. pipens* after ingestion human blood for 30 minutes and culturing for 3, 6, 12, 24, 48, 72h. It also supported the possible use of *C. pipens* in timing-related forensic applications.

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