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Orijinal araştırma (Original article)

Potential cuticular hydrocarbon biomarkers to estimate the age of the malaria vector, *Anopheles stephensi*

Malaria vektörü *Anopheles stephensi* yaşının potansiyel kutikular hidrokarbon biyolojik işaretleyiciler ile tespiti

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Summary

This paper is aimed at answering whether there is any significant change in cuticular hydrocarbons (CHCs) as female *Anopheles stephensi* mysorensis Liston, 1901 (Diptera: Culicidae) age and become capable of transmitting *Plasmodium* sporozoites. Quantitative GC-MS analyses indicated that the changing trend of some CHCs could be used as a criterion to differentiate various age classes of female *Anopheles stephensi*. Analysis of CHC Annals of Tropical Medicine and Parasitology between two and ten day old females (d_2 & d_{10}) at retention time (RTs) 7.38 and 27.4 (*n*-C_{25}); ten and 21 day old females (d_{10} & d_{21}) at RT 39.6 (*n*-C_{32}); and two and 21 day old females (d_2 & d_{21}) at RTs 6.7 (*n*-C_{12}) and 26.2 (*n*-C_{24}) indicated statistically significant differences. The d_{10} age group could be distinguished from d_2 by a CHC peak at RT 30.7 (3-MeC_{28}), whereas d_2 only showed a trace peak. Separation of d_{10} from d_{21} was also possible by a trace peak at RT 30.6 (4-MeC_{28}) for the former age class but a higher one for the latter. Finally, the d_{21} females could be easily differentiated from the d_2 females by 4-MeC_{28} and 3-MeC_{28} (RTs 30.6 and 30.7). 3-MeC_{28} is assumed to be specific to females who have reached the potentially dangerous age (PDA) and are capable of transmitting *Plasmodium* sporozoites. Under our rearing conditions, d_{10} and d_{21} classes certainly passed the PDA and could be infective from feeding on any gametocyte carrier.

Key words: *Anopheles*, Culicidae, cuticular hydrocarbon, dangerous age, age estimation

Özet

Bu çalışmada, dişi *Anopheles stephensi* mysorensis Liston, 1901 (Diptera: Culicidae)'in yaşına bağlı olarak kutikular hidrokarbonlarında (CHCs) ve Plasmodiumsporozoites taşımasında herhangi bir önemli farklılığın olup olmadığı amaçlamıştır. Kantitatif GC-MS analizleri ile bazı CHCslardaki değişimin *Anopheles stephensi* dişilerinin değişik yaş gruplarının ayrımında bir kriter olarak kullanılabilecekleri saptanmıştır. CHC kütlesinin analizi ile, 2 ve 10 gün yaşlı dişilerin (d2& d10) alıkonma süresi (RTs) 7.38 ve 27.4 (n-C25) de; 10 ve 21 gün yaşlı dişilerde (d10 & d21) RT 39.6 (n-C32) da; ve 2 ve 21 gün yaşlı dişilerde (d2 & d21) RTs 6.7 (n-C12) ve 26.2 (n-C24) de istatistiksel olarak yaş grupları arasında farklılık olduğu ortaya konulmuştur. On gün yaşlı dişilerin (d10) bulunduğu grubun, d2 yaşlıların bulunduğu gruptan RT 30.7 (3-MeC28) de verdiği CHC piki ile ayrılması mümkün olmuştur ki aynı sürede iki gün yaşlıların olduğu grupta sadece hafif bir pik oluşumu saptanmıştır. On gün yaşlı dişilerin d21 yaşlı dişilerden ayrımı da RT 30.6 (4-MeC28) daki hafif bir pik ile mümkün olurken ileriki yaş gruplarında daha kuvvetli pikler görülmüştür. Sonuç olarak d21 yaşlı dişilerin d2 yaşlı dişilerden 4-MeC28 ve 3-MeC28 (RTs30.6 ve 30.7) ile kolaylıkla ayrılabilecekleri belirlenmiştir. 3-MeC28'nin potansiyel tehlikeli yaşa (PDA) ulaşmış ve Plasmodiumsporozoites etmenini taşıyabilecek duruma gelmiş dişilere özel olduğu düşünülmektedir. Böcek üretim koşullarımızda d10 ve d21 gruplarının kesinlikle PDA ya geçtikleri ve her hangi bir hastalık taşıyıcısı ile beslenmesi durumunda hastalık bulaştırabilir duruma geçebilecekleri saptanmıştır.

Anahtar sözcükler: Anopheles, Culicidae, kutikular hidrokarbon, tehlikeli yaş, yaş tahmini

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Introduction

Malaria is the most devastating vector-borne disease. Southeastern Iran accounts for about 60% of the country's annual cases (Basseri et al., 2008). Malaria is transmitted by females of a number of *Anopheles* mosquitoes. *Anopheles stephensi* Liston, 1901 is one of the main vectors of malaria in south and southeastern Asia (Manouchehri et al., 1992) and among the most important vectors in the world (Zahar, 1988). Three strains of *Anopheles stephensi* are recorded from the south of Iran (Basseri et al., 2004), but *An. stephensi* strain mysorensis is the dominant vector in southeastern Iran (Basseri et al., 2008). It is an efficient vector of malaria in Baluchistan region, especially in the low coastal plateaus (Manouchehri et al., 1976; Yaghobi, 2005).

A female *Anopheles* has the capacity to be a vector when the fully-grown sporozoites, which are an asexual invasive form of the parasite, become established in her salivary glands (Knell, 1991). One of the most important indices in malaria transmission is the vector longevity since it guarantees the extrinsic cycle (Warrel & Gilles, 2002). Vector longevity is a single parameter in the more complex vectorial capacity equation. The dangerous age of a malaria vector at which they can carry sporozoites begins right after completion of a sporogonic cycle, is not a fixed term and depends on the *Anopheles* and *Plasmodium* species and ambient temperature (McDonald, 1957; Warrel & Gilles, 2002). Therefore, age estimation of malaria vectors in an endemic region plays an important role in planning any control program (Motabar, 1971). Age determination/estimation can be done through a variety of clues, e.g., mating plugs (Gillies, 1956), coiling of the trachea in the ovary (Detinova, 1962) and follicle remnants (WHO, 1959). Another method which has gradually developed in the last two decades is based on the qualitative and quantitative properties of cuticular hydrocarbons (CHCs). This method was first applied by Chen et al. (1990) on *Culex quinquefasciatus.*

The external surface of an insect is covered by a special non-cellular layer, known as cuticle, which is chemically changed during growth and molting phases (Chapman, 1998). The wax layer is the outermost part of the cuticle and is composed of hydrocarbons, in particular n-alkanes, alkenes, and methylated branched alkanes (Schal et al., 1998). CHCs are involved in species-recognition, chemical communication, nestmate recognition, dominance and fertility and provide a barrier against dehydration (Blomquist et al., 1987; Montooth & Gibbs, 2003; Rasoolian et al., 2008; Rasoolian & Nikbakhtzadeh, 2009). These compounds have been used to separate the closely-related groups of mosquitoes, such as siblings within the *An. gambiae* complex (Carlson & Service, 1979; Carlson & Service, 1980; Anyanwu et al., 2000; Anyanwu et al., 2001), strains of *An. stephensi* (Anyanwu et al., 1993) and *Aedes albopictus* (Krugher & Pappas, 1993). The relative abundance of CHCs has been recently used to determine whether *An. stephensi* is old enough to transmit malaria (Brei et al., 2004). In the present work, CHC variations among different age groups of adult female *An. stephensi* were sequentially studied to see if the technique has enough sensitivity to discriminate between different age groups. The study was particularly focused on the CHC patterns of those individuals most likely to have passed the dangerous age limit and become capable of transmitting the *Plasmodium* sporozoites that cause malaria.

Materials and Methods

Mosquito rearing

Specimens of *Anopheles stephensi* mysorensis from Baluchistan, Iran, were reared in the insectary of Iranshahr Research Station at 28°C, 80% RH and 13L:11D photoperiod. Six day old females were provided human blood via an artificial feeding apparatus (Nasirian & Ladonni, 2006). The larvae were fed ground Tetramin® fish food (Tropical Flakes, Tetra Holding, Inc. Blacksburg, VA, USA). Adults were separated by sex and maintained on 10% sucrose solution. Unmated female specimens were periodically collected at three age classes of 2 day old (d_2), 10 day old (d_{10}) and 21 day old (d_{21}), and then immediately killed by freezing at -20°C for 30 min.

Chemical extraction

Each sample included a pool of ten specimens of the same age class; extraction was by surface immersion in 800 µl pure n-Hexane, followed by gentle agitation on a shaker for five minutes to obtain enough cuticular wax without being contaminated by the internal lipids (Desena et al., 1999; Rasoolian & Nikbakhtzadeh, 2009). Extracts were concentrated to 40 µl by evaporating the hexane under nitrogen flow and resuspended by vortexing for 15 seconds.

Quantitative gas chromatography- mass spectrometry

One microliter of each concentrate was analyzed with a GC-MS Varian Star 3800 instrument linked to a Varian Saturn 2000 Mass Selective Detector, set to monitor *m/z* 40-400 at 70 eV with a scanning speed of 1 scan/sec. The samples were analyzed at a split ratio of 40% using a bounded phase, fused silica capillary column (50 m × 0.25 mm ID, 0.12 µm FT) coated with CP-Sil8cb (5% phenyl, 95% polydimethylsiloxane, non-polar). The carrier gas was helium at 1 ml/min. The injector port and detector temperatures were set at 300˚C. The oven temperature was started at 32°C and ramped at 2°C/min to 82°C, followed by a ramp at 10°C/min to 285°C. CHCs of a single extract of each specimen were measured ten times by quantitative GC-MS. A blend of 0.25 ppm n-pentadecane ($C_{15}H_{32}$, 10% in ethyl benzene), n-pentacosane ($C_{25}H_{52}$, 10% in ethyl benzene) and n-dotriacontane ($C_{32}H_{66}$, 1% in ethyl benzene) was used as an external standard. Authentications of these hydrocarbons were obtained from Accu Standard, New Haven, CT, USA.

CHC peaks were autointegrated and their relevant mass calculated with the Saturn® GC/MS Workstation computer package (Saturn View™ version 5.2.1, 1989-1998, Varian Associates, Inc.). To quantify the relative mass of each CHC peak, n-pentadecane was applied in five ascending concentrations (Fig. 1) to provide a regression equation (y= 2447754x– 4.051096, R^2: 0.96). To ensure that none of the detected CHCs were a result of sample contamination, regular negative controls (HPLC-grade n-Hexane, 97% purity, Qualigens, India) were injected between runs.

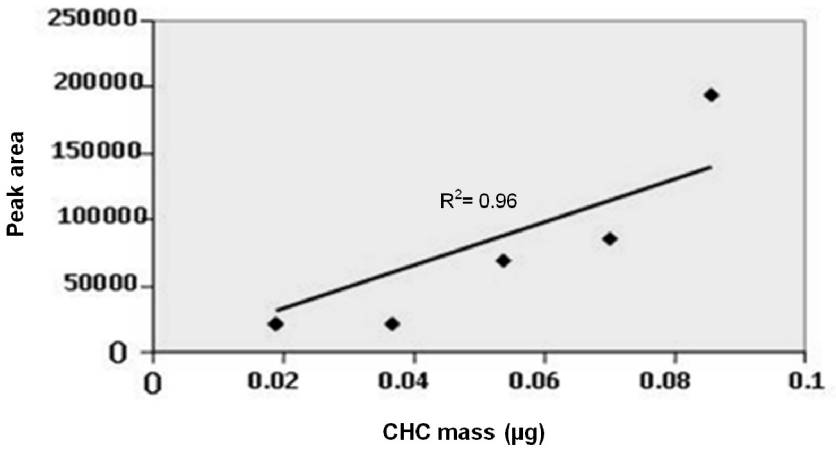

Figure 1. Linear regression analysis of peak area v.s. ascending concentrations (µg) of n-pentadecane, y= 2447754x– 4.051096

Chemical identification

Identification was done using spectra from the NIST library (NIST Mass Spectrometry, 2007). Calculation of the equivalent chain lengths (ECL) was based on the comparison of relative retention time and mass spectra of all GC peaks with the standard compounds.

Statistical analysis

Variance homogeneity of data was verified with Levene's test ($P < 0.05$) and the Kolmogorov-Smirnov test was used to verify the normal distribution of data. Because the data were normal, the parametric paired t-test was applied to test for statistical differences (n=10, 95% confidence level). All statistics were calculated using SPSS Ver. 16.00 (Statistical package, Chicago, Illinois 60606, USA).

Results

Characterized cuticular hydrocarbons

Nineteen hydrocarbon peaks were detected within the retention time (RT) range of 6.6-39.6 min for the purified cuticular extracts of *An. stephensi* from which 16 peaks could be chemically characterized using mass spectrometry (Table 1).

Table 1. CHCs of female *Anopheles stephensi* identified using gas chromatography-electron impact mass spectrometry and mean ± SEM of calculated mass (µg) of 19 chemically-characterized CHCs in the GC profile of three age classes of *An. stephensi* females, n= 10, d_2: young females (2 days old), d_{10}: mid-age females (10 days old), d_{21}: old females (21 days old)

RT[1]	*Peak Identity*	*ECL*[2]	*Diagnostic MS Ions (m/z)*	*Mean$_{d2}$ ± SEM*	*Mean$_{d10}$ ± SEM*	*Mean$_{d21}$ ± SEM*
6.6	*n*-C_{10}	10.00	41, 142	1.7518 ± 0.001	1.7568 ± 0.004	1.7549 ± 0.002
6.7	*n*-C_{12}	12.00	43, 170	20.03 ± 0.411	21.237 ± 0.942	21.375 ± 0.474
7.3	N/C[3]	-	-	2.5315 ± 0.017	2.5871 ± 0.041	2.6015 ± 0.028
7.38	N/C	-	-	1.7466 ± 0.003	1.7578 ± 0.003	1.7526 ± 0.012
7.5	N/C	-	-	1.7527 ± 0.002	1.7556 ± 0.005	1.7554 ± 0.002
7.8	*n*-C_{14}	14.00	57, 198	2.2305 ± 0.011	2.2695 ± 0.026	2.2583 ± 0.016
8.1	4,6-diMeC_{14}	14.36	57, 198	2.2440 ± 0.011	2.2820 ±0.026	2.2773 ± 0.016
8.79	*n*-C_{15}	15.00	57, 212	1.7760 ± 0.003	1.7811 ± 0.003	1.7760 ± 0.005
24.5	*n*-C_{23}	23.00	324	T[4]	T	T
26.1	5-MeC_{23}	23.55	85, 281	T	T	T
26.2	*n*-C_{24}	24.00	338	1.7764 ± 0.010	1.7264 ± 0.008	1.7124 ± 0.005
27.4	*n*-C_{25}	25.00	352	1.7794 ± 0.007	1.7425 ± 0.004	1.7571 ± 0.014
28.5	3-MeC_{26}	26.70	351	1.7116 ± 0.002	1.7042 ± 0.001	1.7134 ± 0.0
29.7	5-MeC_{27}	27.50	281, 309	T	T	T
30.6	4-MeC_{28}	28.58	365	T	T	1.7121 ± 0.002
30.7	3-MeC_{28}	28.75	379	T	1.7075 ± 0.0	1.7157 ± 0.006
31	*n*-C_{29}	29.00	408	T	T	T
32.7	*n*-C_{30}	30.00	422	T	T	T
39.6	*n*-C_{32}	32.00	57, 450	1.978 ± 0.11	1.7234 ± 0.007	2.1096 ± 0.082

[1] RT: retention time (minutes)
[2] ECL: Equivalent Chain Length
[3] N/C: could not be characterized by MS
[4] T: trace

Average mass of each eluted CHC was calculated with an n-pentadecane regression curve (Table 1). Also included in this table are the equivalent chain lengths and the most useful diagnostic mass spectral ions. All identified hydrocarbons were n-alkanes, monomethylalkanes and dimethylalkanes. These included 10 n-alkanes ranging from C_{10-32}; the most abundant one was n-dodecane (2nd eluted compound in the chromatogrames of all ages).

Quantitative differences of CHCs among age classes

Paired t-test analysis of mean ± SEM of CHC mass of *An. stephensi* for a few retention times indicated statistically significant differences. These differences were used to distinguish each age class. Mass of *n*-C_{25} between two day- and ten day old females was the first difference that appeared at RTs of 7.38 and 27.4 minutes (Fig. 2). Mass of *n*-C_{32} between 10 day- and 21 day old females was the second

differentiating criterion. That compound was eluted at 39.6 minutes (Fig. 3). Finally, masses of *n*-C_{12} and *n*-C_{24} were significantly different between two day- and 21 day old females. These two were eluted at 6.7 and 26.2 minutes, respectively (Fig. 4).

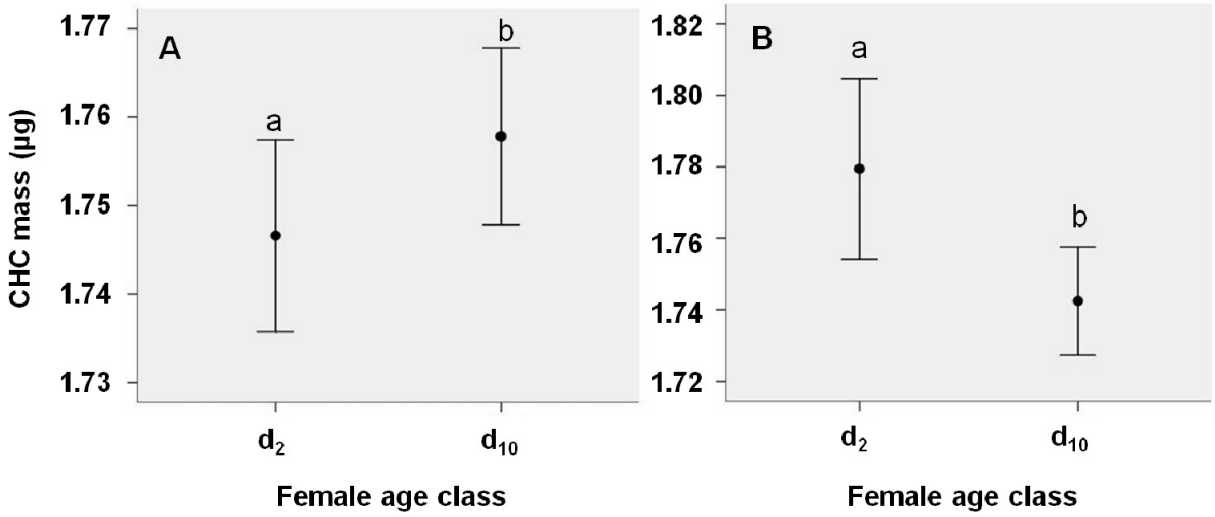

Figure 2. Mean ± SEM of CHC mass (µg) of *An. stephensi* (A) unidentified compound at RT 7.38 min, (B) *n*-C_{25} at RT 27.4 min. Paired t-test, n= 10, P< 0.05, d_2: young females (2 days old), d_{10}: mid-age females (10 days old). Different letters on bars indicate a statistically significant difference.

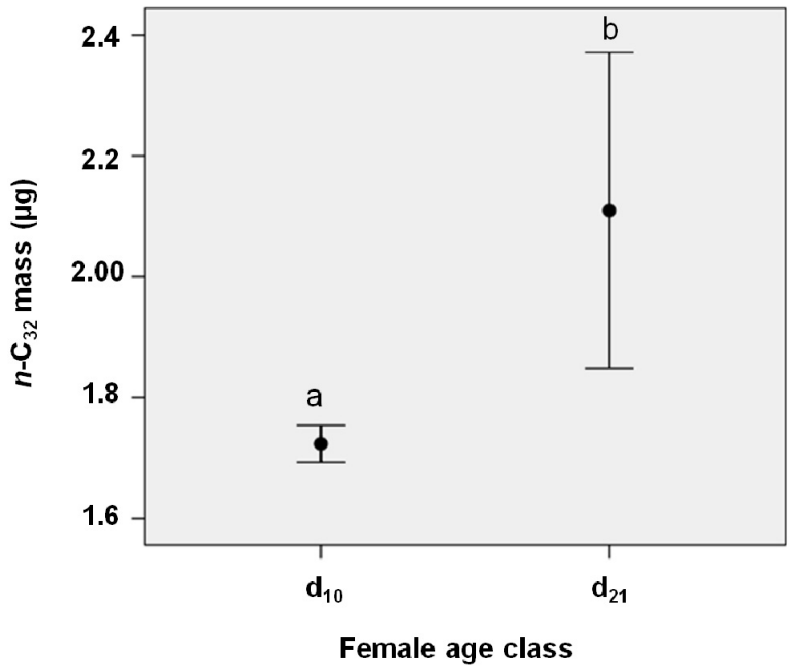

Figure 3. Mean ± SEM of *n*-C_{32} mass (µg) of *An. stephensi* at RT 39.6 min, Paired t-test, n= 10, P< 0.05, d_{10}: mid-age females (10 days old), d_{21}: old females (21 days old). Different letters on bars indicate a statistically significant difference.

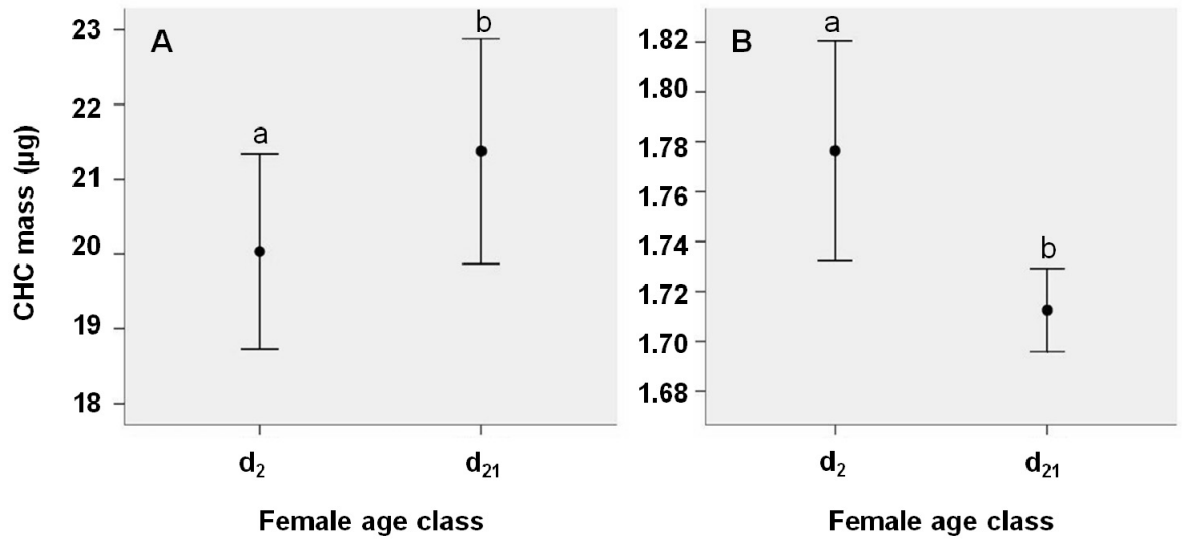

Figure 4. Mean ± SEM of CHC mass (µg) of *An. stephensi* (A) *n*-C_{12} at RT 6.7 min, (B) *n*-C_{24} at RT 26.2 min. Paired t-test, n= 10, P< 0.05, d_2: young females (2 days old), d_{21}: old females (21 days old). Different letters on bars indicate a statistically significant difference.

Trace analysis

A trace was considered to be a small peak for which the relevant mass could not be precisely calculated. A single CHC sometimes showed a trace amount at one age, while the mass of the same compound was much higher at a different age. Based on trace analysis, the mid-age group of mosquitoes (d_{10}) could be further distinguished from the young group (d_2) by the integratable peak of 3-MeC_{28} at RT 30.7, whereas d_2 only showed trace peaks (Fig. 5). Separation of d_{10} and d_{21} groups was also possible by a trace peak of 4-MeC_{28} at RT 30.6 for the former age class, but an integratable one for the latter (Fig. 5). Finally, the old females (d_{21}) could be easily differentiated from the young females (d_2) by two integratable peaks of 4-MeC_{28} and 3-MeC_{28} and the two corresponding trace peaks for d_2 (Fig. 5).

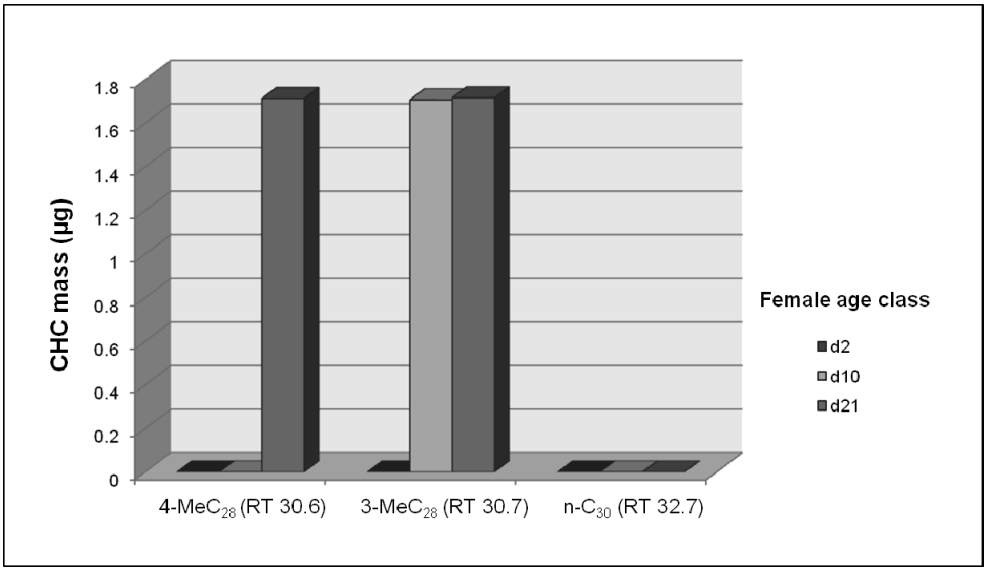

Figure 5. The changing trend of CHCs mean values from young (d_2) to old females (d_{21}) at RTs 30.6, 30.7 and 32.7 min. Numerical values close to 0.00 µg are trace.

Discussion

The life span of a female *Anopheles*, along with the survival rate of a population, influence the probability of encountering infectious hosts, surviving the extrinsic incubation period and transmitting the *Plasmodium* infection (McDonald, 1957). The period necessary for the parasite to reach its infective stage within the vector is often an appreciable portion of the vector's life-span and, therefore, only a small proportion actually survive long enough in nature to transmit the infection (Dawes et al., 2009). As a result, the basic reproduction number (R_0) of vector-borne infections is critically dependent on the life-span of the vector, and in particular on the infective life expectancy (Garrett-Jones, 1964; Dye, 1992). Clements & Patterson (1981) concluded that many species exhibit age-dependent mortality. More recently, Styer et al. (2007) showed that mortality was highly age-dependent in both sexes of *Aedes aegypti*, and that the age at which a mosquito first bites an infectious host is an important indicator of the probability of transmitting a pathogen.

A female *Anopheles* is potentially dangerous when she reaches a specific physiological age, i.e., at the completion of a sporogonic cycle right after feeding on an infected host. In order to calculate the potentially dangerous age (PDA), we must know the duration of sporogonic and gonotrophic cycles (Warrel & Gilles, 2002). McDonald (1957) proposed his famous formula for precise calculation of sporogonic cycle in different *Plasmodium* species. The sporogonic cycle requires a minimum temperature, so it occurs more quickly if the temperature consistently stands on the higher end of the preferred temperature frame.

It has been shown that while the length of sporogonic cycle is highly dependent on the parasite species, *Anopheles* species has an insignificant impact on the cycle duration (Zahirnia et al., 2001).

$$n = \frac{T}{(t - t_{\min})}$$

Therefore, the sporogonic cycle (n) for *P. vivax* under our rearing conditions (T_{vivax}= 105, t= 28°C, t_{min}= 14.5°C) was calculated as:

$$n = \frac{105}{(28 - 14.5)} = 7.77 \text{ degree-days}$$

To lay its first batch of eggs, a female mosquito usually requires at least one blood meal. The duration of the gonotrophic cycle, defined as the period between the blood meal and the subsequent oviposition, is temperature-dependent (Knell, 1991). When the average day-night temperature is high, the gonotrophic cycle is shorter, which means that *Anopheles* females seek a suitable source of blood more frequently. In areas where the proportion of *Plasmodium* gametocyte carriers is high, the mosquitoes have a high likelihood of becoming infected sooner. Provided that the average mosquito's life span is long enough for the sporogonic cycle of the parasite to be completed, high rates of human infection can occur.

The gonotrophic cycle includes three phases of host seeking, blood digestion and oviposition site selection. The duration of host and oviposition site seeking takes about 12 hours in Baluchistan conditions (Zahirnia et al., 2001). The length of the gonotrophic cycle is mainly determined by the blood digestion and ovulation periods (Faghih, 1969). The length of blood digestion was first calculated by Blunk for laboratory reared *An. maculipennis* and was also found to be applicable to *An. stephensi* in Baluchistan (Faghih, 1969).

$$S = \frac{K}{C - N} = \frac{36.5}{28 - 9.9} = 2.01 \text{ degree-days}$$

In the above formula, S is the blood digestion period; K is a constant; which varies according to temperature and relative humidity; C is the average daily temperature; and N, which is the second constant, indicates the temperature thresholds. The whole gonotrophic cycle is accordingly calculated as 2.01 + 0.5= 2.51 degree-days. The probability of completion of the sporogonic cycle will increase in *Anopheles*, according to the time elapsed after reaching the potentially dangerous age (PDA). Potentially dangerous age under our laboratory conditions was reached in approximately three days. That indicates that *An. stephensi* females are potentially able to transmit malaria to humans after three days, if they have been exposed to gametocytes in any of their previous blood feedings.

Considering the three age classes of our studied mosquitoes, two day old *Anopheles* were the only ones which had not reached the dangerous age. The two other classes certainly passed PDA under our rearing conditions, and could be infectious after feeding on any gametocyte carrier.

Our experiments indicated a qualitative difference of cuticular hydrocarbons among different age classes of female *An. stephensi*. We interpret a qualitative difference to mean that a hydrocarbon is present at one age but not present at another one, which includes a trace amount as well. Once the abundance of a hydrocarbon falls below the limit of detection, there is no way to determine whether the hydrocarbon is truly absent or present in trace amounts.

Quantitative differences among our three age classes were observed at RTs 30.6 (4-MeC_{28}) and 30.7 (3-MeC_{28}). Although 4-MeC_{28} may be used to recognize the relatively old *A. stephensi mysorensis*, it is 3-MeC_{28}, which may ultimately give the high precision for recognizing potentially dangerous *A. stephensi* in Baluchistan. Field studies have shown that the average life span of *A. stephensi mysorensis* in Baluchistan is about a month (Zahirnia et al., 2001). Since older female *Anopheles* (d_{21}) have a considerably lower daily survival probability; the middle-age class (d_{10}), which is designated by a combination of trace 4-MeC_{28} and quantifiable 3-MeC_{28}, should be considered the most potent group of females. The not-yet-dangerous young females can be differentiated from the most dangerous age group (d_{10}) by their lower mass of a yet-unknown hydrocarbon at RT 7.38, and a higher amount of *n*-C_{25} at RT 27.4. Similarly, d_2 is differentiated from d_{21} by a lower mass of *n*-C_{12} (RT 6.7) and higher mass of *n*-C_{24} (RT 26.2).

Brei et al. (2004) stated that the abundance ratio of two CHCs influenced the dangerous age of *An. stephensi*. Desena et al. (1999) similarly analyzed the CHCs of *Aedes aegypti* and found a correlation between pentacosane and nonacosane as the mosquito gets older. Hugo et al. (2006), who tried to estimate the age of Australasian mosquitoes using CHCs, detected a relationship between the age and CHC relative abundance in laboratory reared *An. farauti* and *A. aegypti*, which are vectors of malaria and dengue, respectively, while no correlation was observed in *Ochlerotatus vigilax*.

We believe that the changing proportions of two hydrocarbons can be used as a discriminatory criterion among young, mid- and old age classes of *An. stephensi*. 3-MeC_{28}, which was eluted at RT 30.7 appears to be specific for those females that have reached the dangerous age; however, its potential in age grading needs to be verified in the field under differing climatic and nutritional conditions. A sufficient number of field-collected females can provide critical information on the age structure of natural populations of *An. stephensi* and, secondly, how it may vary over time or in concert with malaria transmission. Individual cuticular analysis of a higher number of field mosquitoes and studying their ovarian tracheal systems, as the standard method of age grading, would help determine the accuracy of our hydrocarbon biomarkers for age grading of *An. stephensi mysorensis*.

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References

Anyanwu, G. I., D. H. Davies, D. H. Molyneux, A. Phillips, & P. J. Milligan, 1993. Cuticular-hydrocarbon discrimination/variation among strain of mosquito, *Anopheles* (Cellia) *stephensi* liston. Annals of Tropical Medicine and Parasitology, 87: 269-275.

Anyanwu, G. I., D. H. Molyneux, & A. Phillips, 2000. Variation in cuticular hydrocarbons among strains of *Anopheles gambiae sensu stricto* by analysis cuticular hydrocarbons using gas liquid chromatography of larvae. Memórias do Instituto Oswaldo Cruz, 95: 295-300.

Anyanwu, G. I., D. H. Davies, D. H. Molyneux & A. Priestman, 2001. Cuticular-hydrocarbon discrimination between *Anopheles gambiae* s.s and *An. arabiensis* larval karyotypes Annals of Tropical Medicine and Parasitology, 95: 843-852.

Basseri, H. R., N. Safari, S. H. Mousakazemi & K. Akbarzadeh, 2004. Comparison of midgut hemagglutination activity in three different geographical populations of *An. stephensi*. Iranian Journal of Public Health, 33: 60-67.

Basseri, H. A., S. Doosti, K. Akbarzadeh, M. Nateghpour, M. M. A. Whitten & H. Ladoni, 2008. Competency of *Anopheles stephensi* mysorensis strain for *Plasmodium vivax* and the role of inhibitory carbohydrates to block its sporogonic cycle. Malaria Journal, 7: DOI: 10.1186/1475-2875-7-131.

Blomquist, G. J., D. R. Nelson & M. Renobales, 1987. Chemistry, biochemistry and physiology of insects' cuticular lipids. Archives of Insect Biochemistry and Physiology, 6: 227-265.

Brei, B., J. D. Edman, B. Gerade & J. M. Clark, 2004. Relative abundance of two cuticular hydrocarbons indicates whether a mosquito is old enough to transmit malaria parasites. Journal of Medical Entomology, 41: 807-809.

Carlson, D. A. & M. W. Service, 1979. Differentiation between species of the *Anopheles gambiae* Giles complex (Diptera: Culicidae) by analysis of cuticular hydrocarbons. Annals of Tropical Medicine and Parasitology, 73: 589-592.

Carlson, D. A. & M. W. Service, 1980. Identification of mosquitoes of *Anopheles gambiae* species complex A and B by analysis of cuticular compoundes. Science, 207: 1089-1091.

Chapman, R. F., 1998. The Insects: Structure and Function. Cambridge University Press, Cambridge, 771 pp.

Chen, C. S., M. S. Mulla, R. B. March & J. D. Chaney, 1990. Cuticular hydrocarbon patterns in *Culex quinquefasciatus* as influenced by age, sex and geography. Bulletin of the Society for Vector Ecology, 15: 129-139.

Clements, A. N. & G. D. Paterson, 1981. The analysis of mortality and survival rates in wild populations of mosquitoes. Journal of Applied Ecology, 18: 373-399.

Dawes, E. J., E. S. Churcher, S. Zhuang, R. E. Sinden & M. G. Basáñez, 2009. *Anopheles* mortality is both age- and *Plasmodium*-density dependent: implications for malaria transmission. Malaria Journal, 8: doi: 10.1186/1475-2875-8-228.

Desena, M. L., J. D. Edman, J. M. Clark, S. B. Symington & T. W. Scott, 1999. *Aedes aegypti* (Diptera: Culicidae) Age determination by cuticular hydrocarbon analysis of female legs. Journal of Medical Entomology, 36: 824-30.

Detinova, T.S., 1962. Age-Grouping Methods in Diptera of Medical Importance. WHO, Geneva, 216 pp.

Dye, C., 1992. The analysis of parasite transmission by bloodsucking insects. Annual Review of Entomology, 37: 1-19.

Faghih, M., 1969. Malarialogy and Malaria Eradication. Tehran University Press, Tehran, 726 pp. (in Persian).

Garrett-Jones, C., 1964. Prognosis for interruption of malaria transmission through assessment of the mosquito's vectorial capacity. Nature, 204: 1173-1175.

Gillies, M. T., 1956. A new character for the recognition of nulliparous females of *Anopheles gambiae*. Bull WHO, 15: 451–459.

Howard, W. R. & G. J. Blomquist, 2005. Ecological, behavioral and biochemical aspects of insect hydrocarbons. Annual Review of Entomology, 50: 371-93.

Hugo, L. E., B. H. Kay, G. K. Eaglesham, N. Holling & P. A. Ryan, 2006. Investigation of cuticular hydrocarbons for determining the age and survivorship of Australasian mosquitoes. American Journal of Tropical Medicine and Hygiene, 74: 462-474.

Knell, A. J., 1991. Malaria. Oxford University Press, London, 92 pp.

Krugher, E. L. & C. D. Pappas, 1993. Geographic variation of cuticular hydrocarbons among fourteen populations of *Aedes albopictus* (Diptera: Culicidae). Journal of Medical Entomology, 30: 544-548.

Manouchehri, A. V., E. Javadian, N. Eshghy & M. Motabar, 1976. Ecology of *Anopheles stephensi* Liston in southern Iran. Tropical and Geographical Medicine, 28: 228-232.

Manouchehri, A. V., M. Zaim & A. M. Emadi, 1992. A review of malaria in Iran (1975-90). Journal of the American Mosquito Control Association, 8: 381-385.

McDonald, G., 1957. The Epidemiology and Control of Malaria. Oxford University Press, London, 201 pp.

Montooth, K. L. & A. G. Gibbs, 2003. Cuticular pheromones and water balance in the house fly *Musca domestica*. Comp Biochem Physiol A: Molecular and Integrative Physiology, 135: 457-465.

Motabar, M., 1972. Malaria and its Control. Tehran University Press, Tehran, 97 pp. (in Persian).

Nasirian, H. & H. Ladonni, 2006. Artificial blood feeding of *Anopheles stephensi* on a membrane apparatus with human whole blood. Journal of the American Mosquito Control Association, 22: 54-56.

Rasoolian, M., J. Sadrai & M. R. Nikbakhtzadeh, 2008. Identification of the *Anopheles* mosquitoes (Diptera: Culicidae) of southern Iran using cuticular hydrocarbons. Animal Cells and Systems, 12: 165-170.

Rasoolian, M. & M. R. Nikbakhtzadeh, 2009. Identification of Iranian vectors of malaria by analysis of cuticular hydrocarbons. Animal Cells and Systems, 13: 331-337.

Schal, C., V. L. Sevala, H. P. Young & J. A. S. Bachman, 1998. Sites of synthesis and transport pathways of insect hydrocarbons: cuticle and ovary as target tissues. American Zoologist, 38: 382-393.

Styer, L. M., J. R. Carey, J. L. Wang & T. W. Scott, 2007. Mosquitoes do senesce: departure from the paradigm of constant mortality. American Journal of Tropical Medicine and Hygiene, 76: 111-117.

Warrel, D. A. & H. M. Gilles, 2002. Essential Malariology. Hodder Arnold, Oxford, 348 pp.

WHO, 1959. A course in advanced entomological techniques applied to malaria eradication. working document WHO/ Mal/ 238.

Yaghobi, F., 2005. Morphologic and genetic variety of *Anopheles stephensi* (Diptera Culicidae) populations in Bluchistan and Hormozgan, Iran. MSc thesis in Medical Entomology. Tehran School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. (in Persian).

Zahar, A. R., 1985. Vector Bionomics in the Epidemiology and Control of Malaria. VBC/85.2. World Health Organization, Geneva, VBC/85.2, 136 pp.

Zahirnia A. H., H. Taherkhani & H. Vatandoost, 2001. Observation of sporozoite in *Anopheles culicifacies* S.l. (Culicidae: Diptera) in Sistan and Baluchistan province, Iran. Hakim, 4: 149-153. (in Persian).