

Ecology of malaria vector *Anopheles culicifacies* in a malarious area of Sistan va Baluchestan province, south-east Islamic Republic of Iran

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السمات الإيكولوجية للأنوفيلة البعوضية الوجه الناقل للملاريا في مناطق متأثرة بالملاريا في ولاية سيستان وبلوشستان، جنوب شرق جمهورية إيران الإسلامية

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الخلاصة: لاتزال الملاريا تمثل إحدى المشاغل الرئيسية للصحة العمومية في الجزء الجنوبي الشرقي من جمهورية إيران الإسلامية. وقد أجرى الباحثون دراسة حول السمات البيولوجية، والحساسية لمبيدات الحشرات والتهيجية للأنوفيلة البعوضية الوجه في ولاية سيستان وبلوشستان، وجمعت العينات لتعيين كل من النوع والنشاط الفصلي، واختبارات الحساسية لكل من اليرقات والكاهالات، واختبارات التهيجية ومنسب الولوج بالبشر، ومعدل الحيوانات البوغية. ووجد الباحثون أن الأنوفيلة البعوضية الوجه البالغة هي حساسة لجميع مبيدات الحشرات من المركبات البيروثرويدية، وهي تبدأ بالظهور داخل المباني في أواخر شهر أيار/مايو، مع ذروتين إحداهما في حزيران/يونيو والثانية في أيلول/سبتمبر. وفي حين يتمتع الـ د.د.ت بأعلى تأثير مهيج فإن الفينيتروثيون والسيفلوثرين والبيرمثرين هي الأقل إهاجة. وكان بعوضتان من أصل 860 بعوضة أنثى مُعدَّيتين بأنواع المتصورات (معدل الحيوانات البوغية 0.25%)، وأجرى الباحثون اختبار مقياسه المتمز المناعي المرتبط بالإنزيم على 250 وجبة دموية مأخوذة من مجموعات من البعوضات الإناث تناولت وجبتها المسائية من الناس ومن الأبقار واتضح أن 12.5% منها قد تغذت على البشر.

ABSTRACT A study was done of the bionomics, insecticide susceptibility and irritability status of *Anopheles culicifacies* in Sistan va Baluchestan province. Sampling was performed to determine the following parameters: species identification, seasonal activity, adult and larval susceptibility tests, irritability tests, anthropophily index and sporozoite rate. *An. culicifacies* adults were susceptible to all tested pyrethroid insecticides. *An. culicifacies* started to appear indoors in late May, showing 2 peaks in June and September. Fenitrothion, cyfluthrin and permethrin had the least irritancy effect and DDT the highest. Only 2/860 females tested were infected with *Plasmodium* spp. (sporozoite rate: 0.25%). ELISA testing of 250 blood meals derived from night-biting collections of female mosquitoes from humans and cows revealed that only 12.5% were human-fed.

Écologie du vecteur du paludisme *Anopheles culicifacies* dans une région impaludée, de la province du Sistan-Balouchistan, au sud-est de la République islamique d'Iran

RÉSUMÉ Une étude de la bionomie d'*Anopheles culicifacies*, de sa sensibilité et de son irritabilité aux insecticides a été conduite dans la province du Sistan-Baloutchistan. Des échantillons ont été prélevés pour déterminer les paramètres suivants : identification des espèces, activité saisonnière, test de sensibilité des adultes et des larves, tests d'irritabilité, indice d'anthropophilie et indice sporozoïtique. Les *An. culicifacies* adultes étaient sensibles à tous les insecticides pyréthroïdes testés. Des individus de l'espèce *An. culicifacies* commençaient à être observés dans les bâtiments vers la fin du mois de mai avec deux pics, un en juin et un en septembre. Le fénitrothion, la cyfluthrine et la perméthrine avaient le pouvoir irritant le plus faible, à l'opposé du dichlorodiphényltrichloroéthane (DDT) qui était le plus irritant. Seules deux femelles sur les 860 analysées étaient infectées par *Plasmodium* spp. (indice sporozoïtique : 0,25 %). L'analyse par la technique ELISA de 250 repas de sang provenant de piqûres nocturnes par des moustiques femelles sur des humains et des vaches a révélé que seuls 12,5 % des repas étaient composés de sang humain.

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Introduction

Malaria remains a major public health concern in the south-east of the Islamic Republic of Iran in the provinces of Sistan va Baluchistan, Hormozgan and the tropical areas of Kerman provinces, where it is characterized as "refractory malaria". About 80% of all malaria cases in the country occur here [1,2]. The pattern of malaria is unstable, with 2 seasonal peaks, primarily in the spring and autumn, with outbreaks usually occurring after the rainy season. Although 6 anopheline mosquitoes are known to be the malaria vectors in the south-east Islamic Republic of Iran (*Anopheles culicifacies*, *An. stephensi*, *An. dthali*, *An. fluviatilis*, *An. superpictus* and *An. pulcherrimus*) [3–18], *An. culicifacies* is the major vector. Its relative density is high even at altitudes up to 3000 m. Studies of its feeding behaviour show that *An. culicifacies* is predominantly zoophilic, feeding mainly on cattle (anthropophilic index less than 10%), but where cattle are scarce, its anthropophilic index can reach in excess of 20% [19].

Traditionally, the sporozoite rate of mosquitoes was assessed by dissection of the salivary glands of individual mosquitoes; however, this is often a laborious process [20]. In recent years more efficient sporozoite detection methods have been developed, including serological methods, e.g. enzyme linked immunosorbent assay (ELISA) or immunochromatographic assay [21] and molecular techniques, e.g. polymerase chain reaction (PCR) [22]. PCR methods have been used to amplify specific DNA sequences of *P. falciparum* for highly sensitive detection of mosquito resident parasites [22]. The PCR procedure can detect as few as 10 sporozoites in the salivary glands (0.2 pg of parasite DNA) and is therefore a powerful tool for screening small numbers of anophelines [23].

There are important differences in the bionomics of *An. culicifacies* in different regions, including differences in

seasonal relative density, diurnal activity and human-biting behaviour [24]. Knowledge of the profile of *An. culicifacies* in different areas of the Islamic Republic of Iran is crucial for understanding the impact of mosquito control strategies that rely principally on indoor spraying of houses with residual insecticide. The objective of this study therefore was to determine the bionomics, insecticide susceptibility and irritability status of *An. culicifacies* in Sistan va Baluchestan province, south-east Islamic Republic of Iran.

Methods

Study area

The investigation was carried out over a period of 12 months in Sistan va Baluchestan province (25°3' to 28°31' N and 58°48' to 63°19' E) during 2005. This province has a subtropical climate and is prone to seasonal malaria transmission. The province comprises 3 regions of differing geography: the coastal region in the south, a mountainous region in the west and the desert region in the east and north. It is bordered by the Oman Sea in the south.

Iranshahr district is located in the centre of Sistan va Baluchestan province with a surface area of 30 230 km², between long 58°59' to 61°15' E and lat 26°55' to 28°40' N. The area is subtropical and suitable for malaria maintenance and transmission. Iranshahr is a city with a population of 25 9700. The total number of malaria cases was about 7228 in 2005 [personal communication, Iranian Health Ministry disease management centre]. It is an agricultural region irrigated by rivers, deep wells and cement pools, which are the main breeding sites for mosquitoes.

Based on these criteria and from a statistical point of view, several localities in each region were randomly selected for the survey: Bandan, Machkor and Seraydan from the mountainous region and Abchekan, Nokjoob and

Malekabad from the plateau. These sites have been the principal malarious zones in the province in the last 3 years. During the fieldwork, Machkor from the mountainous region and Malekabad from the plateau were omitted from the study due to transport problems and natural disasters such as flooding.

Adult and larval collection methods

Pyrethrum space spray catches were performed monthly in 8 shelters (4 human and 4 animal shelters) located in different parts of the villages in each region by standard methods using 0.2% pyrethrum spray [25]. The density was calculated as the number of mosquitoes per shelter. Attempts were also made to catch anophelines outdoors using shelter pits, night catch and window traps.

Larvae were sampled using standard dippers (500 mL) from breeding places close to adult collection sites. The mean number of larvae was calculated per 10 dippers.

Entomological survey

Entomological evaluations were carried out in the villages in which malaria transmission occurs and where the presence of *An. culicifacies* had been reported previously. Space-spray collections were carried out monthly in 8 fixed shelters: 4 animal and 4 human shelters in each village, according to World Health Organization (WHO) procedures [26]. In addition, larval collections, night biting and shelter pit collections of adults were carried out monthly and continued for a period of just over 5 months.

Adult susceptibility tests

The insecticide susceptibility tests on field adults and larvae were performed on the *An. culicifacies* collected from mountainous area. For the adult tests, female *Anopheles* spp. reared from collected larvae were exposed to discriminating doses of etofenprox 0.50%, permethrin 0.75%, deltamethrin 0.05%, lambda-cyhalothrin 0.05% and cyfluthrin 0.15%

for 60 min. at 25–29 °C and 65%–80% relative humidity [27]. The effects of these insecticides were studied in accordance with WHO standards, using mortality rates after 1-hour exposure followed by monitoring over a 24-hour recovery period [28]. Silicon oil impregnated papers were used for control exposures for pyrethroid insecticides.

Larval susceptibility test

For the larval insecticide susceptibility tests fenitrothion was used at the discriminating dose. Butanone 2% in absolute ethanol was used as a control. Late third and early fourth instar larvae were tested with larvicides according to the method described by WHO [29]. The larvae were exposed to discriminative dose of larvicide. A total of 100 larvae representing 4 replicates of 25 larvae were tested. The larvae were fed with Bemax and fish food, and mortality counts were made after a 24-hour exposure period.

Irritability tests

The irritability levels of *An. culicifacies* against diagnostic dose of dichlorodiphenyltrichloroethane (DDT) and pyrethroids (lambda-cyhalothrin, permethrin, cyfluthrin and deltamethrin) insecticides were measured in an exposure chamber according to WHO methods using light intensity of approximately 8 foot candles was used [30]. The field mosquitoes were collected from different larval breeding places of Iranshahr county. In each test, 30 sugar-fed 2–3-day-old adult females were exposed and the number of take-offs at 1 min. intervals was recorded. for 15 min. The Abbott formula was used to correct the observed mortalities [31]. The results were subjected to statistical analysis using SPSS, version 13 and the collected data were analysed by Student *t*-test and the chi-squared test.

Host preference pattern

To determine the host preference pattern of *An. culicifacies*, blood-fed

mosquitoes were collected by suction tube inside human dwellings, stables, storerooms and outdoor resting places around villages. The blood meals of the identified anophelines were smeared on circles of Whatman no.1 filter paper. They were interleaved with non-absorbent onionskin paper and sent to the Department of Parasitology in the Pasteur Institute of Iran for ELISA testing [32].

Detection of malaria parasites

Investigation of malaria parasites were performed on 860 fed female *An. culicifacies* mosquitoes (head and thorax) that were collected during 2 malaria transmission peaks: June/July and September/October 2005. Parasite DNA was extracted by phenol–chloroform solution (1:1). Following DNA extraction, nested PCR was conducted due to the high sensitivity of this method for detecting the human malaria parasite [33]. Extracted DNA was processed by the genus-specific primers corresponding to small subunit ribosomal ribonucleic acid (ssr RNA) from malaria parasites such as *P. vivax* and *P. falciparum* [33]. The amplified DNA products were detected in ethidium bromide stained 2.5% agarose gels using electrophoresis [33].

Results

Entomological survey

An. culicifacies were found in all rural districts inspected. Larvae were found in man-made water collections (including wells, cisterns, fountains, ornamental ponds and in the water stored in drums and building construction sites); from pools, stream margins, catch basins and seepage canals; sewage water, wells and the drainage containers of air conditioners in houses. In rural areas, *An. culicifacies* were found in pools, stream beds, palm irrigation canals, at the margins of stream and rivers, in seepage and in marshy areas with a gentle flow of water.

Table 1 Relative density of *Anopheles culicifacies* s.l. females sampled by 5 techniques at Iranshahr, Baluchestan, over a 5-month period of 2005

Month	Window trap inlet		Window trap outlet		Pit shelter		Pyrethrum space-spray		Human bait		Animal bait	
	N	Mean (n = 2)	N	Mean (n = 2)	N	Mean (n = 2)	N	Mean (n = 2)	N	Mean (n = 1)	N	Mean (n = 1)
June	37	18.5	16	8.0	297	148.5	707	353.5		93.0		123.0
July	0	0.0	0	0.0	298	149.0	60	30.0		3.0		183.0
August	0	0.0	1	0.5	27	13.5	73	36.5		22.0		74.0
September	1	0.5	4	2.0	4	2.0	132	66.0		21.0		143.0
October	0	0.0	9	4.5	14	7.0	423	211.5		43.0		417.0
Total	38	19.0	30	15.0	640	320.0	1395	697.5		182.0		940.0

N = total number of mosquitoes; n = number of collections.

They were also found in water with a high salinity.

In the mountainous area, *An. culicifacies* were found together with *An. fluviatilis*, *An. dthali*, *An. hyrcanus*, *An. superpictus*, *An. turkhudi* and *An. stephensi*. In the plain regions, this species was found with *An. dthali*, *An. turkhudi*, *An. stephensi*, *An. fluviatilis*, *An. superpictus* and *An. sergentii*.

Table 1 shows the relative densities of *An. culicifacies* s.l. females ($n = 3325$) sampled by the 5 techniques. Pyrethrum indoor space-spray was the most productive sampling method, collecting 52.7% of the total number of mosquitoes analysed. The window-trap method was not the most suitable method. The results demonstrate the 2 peaks of *An. culicifacies* mosquito activities in June/July and September/October.

The abdominal conditions of *An. culicifacies* ($n = 2103$) using different methods of collection are shown in Table 2. From this table it can be concluded that most females were freshly fed (42.4%), gravid or semi-gravid.

Seasonal pattern

This species remained reproductively active throughout 5 months in the province (Figure 1). Its activity started in June, reached a peak in July and then gradually decreased. Subsequently, its relative density again increased from September in the mountainous and plain regions, with a second peak in October. The density of *An. culicifacies* in mountainous areas was higher in the autumn. There was low activity during the cold winter and hot summer periods.

Host preference pattern

The host preference pattern of *An. culicifacies* is presented in Figure 2. Night biting collections resulted in more than 940 specimens from animal bait (cows) and 182 from human bait. This showed that *An. culicifacies* was highly zoophilic in the rural mountainous areas and in the rural plains.

In ELISA testing of 550 blood meals of this species, the proportion giving positive reactions from (+) to (++++), with alkaline phosphatase anti-human conjugate varied between collection sites. Of those collected 12.5% had fed on humans alone. All of the blood meals gave a detectable reaction to one or other of the antisera.

Susceptibility tests

The results of larval susceptibility tests showed that diagnostic doses of fenitrothion caused 100% mortality of larvae of *An. culicifacies* after a 24-hour recovery period.

The adult susceptibility tests using WHO criteria (98%–100% mortality indicating susceptibility and < 98% mortality indicating resistance), showed that *An. culicifacies* was susceptible to all the insecticides tested: etofenprox, permethrin, deltamethrin, lambda-cyhalothrin and cyfluthrin.

Irritability tests

The results of irritability tests showed that DDT had the most irritancy effect against *An. culicifacies* whereas permethrin and cyfluthrin displayed the lowest irritancy effect. The mean number of take-offs/minute/adult with

permethrin was 0.58 (SD 0.08). The figures for DDT, deltamethrin, lambda-cyhalothrin, cyfluthrin and etofenprox were: 4.47 (SD 0.33), 0.77 (SD 0.09), 0.92 (SD 0.11), 0.73 (SD 0.10) and 1.54 (SD 0.15) respectively. Using one-way analysis of variance and the Games–Howell *post-hoc* test, the results showed a statistically significant difference in the irritability level among pyrethroids, with permethrin showing a higher irritancy in *An. culicifacies* compared with DDT, lambda-cyhalothrin, cyfluthrin and deltamethrin ($P < 0.05$).

Detection of malaria parasites

The analysis of malaria parasites revealed that out of 860 *An. culicifacies* females tested, 2 samples were infected by *Plasmodium* spp., a sporozoite rate of 0.25%.

Discussion

Mosquitoes of the *An. culicifacies* complex are widespread in tropical Asia and are the principle vectors of malaria across the Indian sub-continent and Arabian peninsula, including parts of the Islamic Republic of Iran. *An. culicifacies* occurs in scattered foci along the Gulf area as far west as Bushehr, in the south of the Islamic Republic of Iran. *An. culicifacies* Giles with *An. stephensi* is regarded as the chief vector of malaria in the south-eastern corner of Islamic Republic of Iran [34]. In most areas the *An. culicifacies* s.l. population have 2 main seasonal peaks of relative density, during the spring and autumn [35,36]. We too found the main peak in June and a

Table 2 Abdominal condition of *Anopheles culicifacies* s.l. females captured by different collection methods at Iranshahr, Baluchestan, over a 5-month period of 2005

Source of collection	No. tested	Unfed		Freshly fed		Semi-gravid		Gravid	
		No.	%	No.	%	No.	%	No.	%
Window trap	68	31	45.5	37	54.4	14	21.1	13	19.1
Pit shelter	640	305	47.6	335	52.3	86	13.5	102	15.9
Pyrethrum space-spray	1395	877	62.9	520	37.3	423	30.3	342	24.5
Total	2103	1213	57.6	892	42.4	523	24.9	457	21.7

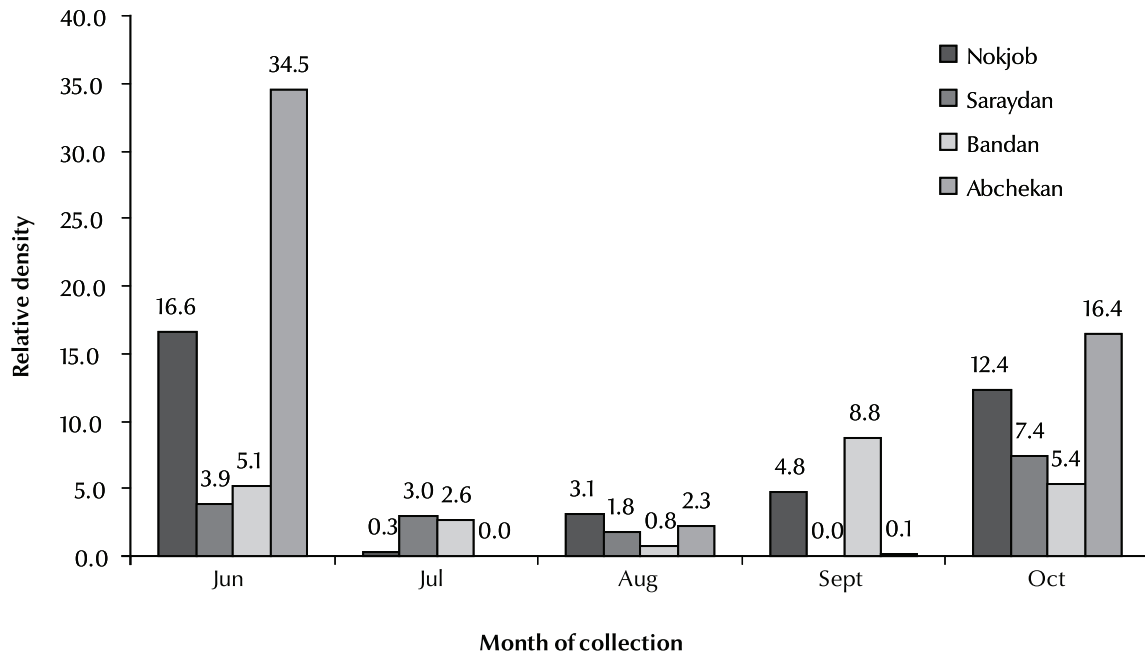


Figure 1 Relative density of *Anopheles culicifacies* in different rural areas of Sistan va Baluchestan province using the total catch method

secondary peak in October. Iranshahr district, including our unsprayed study villages, is a major rice-growing area of Baluchistan where, during April to September, irrigated rice is the main breeding source of *An. culicifacies* s.l., along with irrigated palms and stream pools. During the winter months, from

October to April, stream pools serve as the predominant breeding sites of *An. culicifacies* s.l. in the majority of areas [34]. Further studies are needed to determine the aestivation and hibernation habits of this species in winter conditions.

An. culicifacies s.l. has been regarded as mainly endophilic [37], with outdoor-

resting behaviour reported recently in some parts of India [38]. In this study, pyrethrum space-spray indoors was the most productive sampling method for *An. culicifacies* s.l. The results are consistent with those obtained in a previous study in this part of Islamic Republic of Iran [34]. Nevertheless,

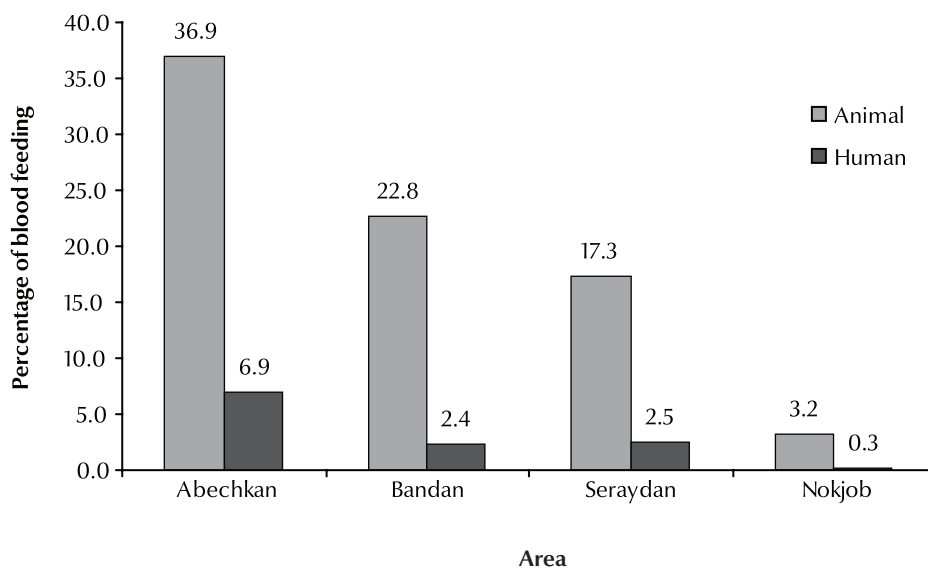


Figure 2 Host preferences of *Anopheles culicifacies* in 4 different rural areas of Sistan va Baluchestan province ($n = 940$ from animal bait; $n = 182$ from human bait)

our study found considerable numbers of *An. culicifacies* s.l. resting outdoors in caves and pit shelters. Our analysis of abdominal conditions from females caught from pyrethrum space-spray, window trap and pit shelters showed that most were freshly-fed gravids or semi-gravids.

In field populations of the malaria vectors *An. culicifacies* and *An. subpictus* at a village in the low country of Sri Lanka during 1994–96, analysis of blood meals by ELISA showed that 8.3% of 242 *An. culicifacies* were human-fed and 80% of these (i.e. 6.6%) were concurrently bovid-fed [39]. These figures are much lower than the usual human blood index for afrotropical malaria vectors [40].

In our study the host preference pattern of *An. culicifacies* showed that 12.5% had fed on humans alone.

Irritability tests showed that *An. culicifacies* adults were susceptible to all the insecticides tested and that irritability to permethrin was significantly lower than for DDT, lambda-cyhalothrin, cyfluthrin and deltamethrin. There are different sibling species of *An. culicifacies* in the world, for instance occurrence of sibling species A,B,C,D and E from India [41] A and B from Islamic Republic of Iran [42], B and E from Sri Lanka [43]. This species has several sibling species in the south-eastern part of the Islamic Republic of Iran and each sibling seems to have a different behavioural response.

The PCR procedure can detect as few as 10 sporozoites in the salivary glands (0.2 pg of parasite DNA) and is therefore a useful tool for screening small numbers of anophelines [23]. The infectivity rate among *An. culicifacies* females was very low (0.25%) in the season in which our samples were collected. This method has proved to be more sensitive than other diagnostic methods and is recommended for epidemiological studies in different malarious regions of the country.

We aim to establish a national laboratory for the detection of parasites in malaria vector mosquitoes using the molecular method described in our study.

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