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Laboratory colonization of the malaria vector *Anopheles* (*Cellia*) *superpictus* from Sanliurfa, Turkey

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Abstract

In this study, the first long-term colony of *Anopheles superpictus*, a malaria vector in Turkey, was established from a population from the endemic malaria region of Sanliurfa. Population parameters such as mean numbers of eggs per female, total number of eggs, egg hatching rate, pupation rate and sex ratio were determined from F_1 to F_{10} generations of the established colony.

Introduction

The establishment of laboratory colonies of malaria vectors is important in elucidating many aspects of their life cycle and in understanding the dynamics of malaria transmission under controlled conditions. Malaria, the World's most prevalent vector-borne disease, is endemic in 92 countries (Martens & Hall, 2000). It is endemic to the southeastern region of Turkey and is one of the most important health problems in the region. Şanlıurfa is important in the region because of its vector species and also its nuisance mosquitoes (Alten *et al.,* 2003). Despite years of attempted eradication, malaria remains a major public health threat in Şanlıurfa, with 1117 cases reported during 2001.

Anopheles superpictus Grassi is a widespread species in Syria, Israel, Palestine, Iraq, Iran, Algeria, Croatia, Armenian, Azerbaijan, Uzbekistan, Turkmenistan, Afghanistan, Pakistan, Spain, Greece and Italy (Knight & Stone, 1977; Merdivenci, 1984). *Anopheles superpictus* is also one of the most widely distributed species occurring from sea level to over 1700 metres and is considered to be an important vector, ranking next in importance to *Anopheles* (*Anopheles*) *sacharovi* Favre in Turkey (Parrish, 1959; Postiglione *et al.,* 1973; Merdivenci, 1984). Because of its exophilic and zoophilic tendencies, the vectorial role varies according to the social and biological characteristics of region (Postiglione *et al.,* 1973). On the other hand, importance of *An. superpictus* as a vector of malaria has not been thoroughly studied. Although no naturally infected or infective *An. superpictus* has been found in Turkey, the efficiency of *An. superpictus* as a vector of *Plasmodium vivax* has been demonstrated in the laboratory (Kasap *et al.,* 1987).

The purpose of this study was twofold: to gain preliminary information on laboratory colonisation of *An. superpictus* and to prepare the colony for use in future studies concerning the vectorial potential of the species.

Material and Methods

Field collection of females

In July 2001, naturally bloodfed, half gravid, and gravid females were collected from human dwellings and animal shelters using aspirators in Magralı, a village 10 km southeast of Birecik (37° 01' N and 37° 57' E) district of Sanlıurfa. Field collected females were held in 20 cm cubical cages placed in large freezers at 4° ± 2°C and transferred to the insectary of Hacettepe University Ecological Sciences Research Laboratories.

Insectary conditions

The insectary was maintained at 27° ± 2°C, 70% ± 5% RH with a 12:12 h light-dark cycle photoperiod. Dawn and dusk phases were supplemented with automatically dimmed fluorescent bulbs (40 W) activated between 06:00-07:00h and 18:00-19:00h.

Colonisation and maintenance

Field collected females were used to establish the colony. Individual bloodfed, half gravid, and gravid females were placed in netted paper cups (150 ml volume) lined with filter paper. One-third of the cups were filled with distilled water providing an oviposition medium for females. After eggs were laid on the water surface, they were counted using a binocular dissecting microscope. The eggs were allowed to incubate and hatching occurred within 2-3 days of oviposition.

After hatching, first-stage larvae were counted and were transferred into plastic rearing pans (20 x 10 x 15 cm) filled with 1 litre distilled water (25 ± 2°C). Five hundred larvae were placed in each pan. Larvae were not fed on the first day following hatching. On following days according to larval growth, larvae were fed twice daily with 0.01-0.04 g powdered larval food (TetraMin Fish Food®), which was spread evenly onto the water surface (Bangs *et al.*, 2002; Kuhn, 2002). Every two days all larvae were transferred to new plastic rearing pans with clean distilled water and were re-grouped according to size. This procedure was continued to pupation.

Pupae were transferred daily from rearing pans into netted cups (200 ml) containing clean distilled water using a Pasteur pipette (5 ml). Each adult that emerged from the cups was collected using an aspirator inserted through a hole in the net, and its sex identified. Afterwards males and females were transferred into mosquito cages (50 x 50 x 50 cm) containing cotton wicks soaked with 10% sucrose solution providing a carbohydrate meal. Each cage contained approximately 1000 adults. Sugar solution and wicks were replaced every two days in order to prevent bacterial growth.

Three days after adult emergence, females were offered a blood meal from live rabbits. Only the head of the rabbit was inserted into the cages and females were allowed to feed from behind the ears. This procedure was repeated daily. Half-gravid and gravid females sited on subsequent days were removed from the cages by an aspirator and inserted into individual netted cups for oviposition. Cups were checked daily and if females were still alive they were removed from the cups and placed into separate cages in order to distinguish between parous and nulliparous. After females were removed, the eggs in each cup were counted and cups were re-checked daily until hatching was complete.

Larvae emerging from these eggs were counted and recorded. The above procedure was repeated for four generations and the number of oviposited females, total number of eggs, mean number of eggs per female, egg hatching rate, pupation rate, male and female numbers and sex ratio were determined for each generation. After the 5th generation the number of oviposited females reached 500, and in the 6th generation was over 1000. Starting from the 5th generation, 150 bloodfed females were randomly selected from the cages for the parameters mentioned above and these parameters were checked up to the 10th generation.

Results and Discussion

Of the 143 females (54 bloodfed, 28 half gravid, and 61 gravid) collected from Mağaralı village in July, a total of 62 (43.4%) females (22 bloodfed, 11 half gravid, and 29 gravid) laid 6712 eggs. 81.7 % of these eggs hatched successfully and a total of 5486 first instar larvae were obtained. The fecundity (Table 1) and developmental attributes from immature to adult stages (Table 2) of the *An. superpictus* colony established from these larvae were evaluated from F_1-F_{10} generation.

Mean number of eggs from field-collected females of *An. superpictus* was 108.2 ± 2.73 and hatching rate was 81.7 %. Egg numbers decreased in the F_2 and F_3 generations. Mean number of eggs started to increase by the 4th generation and, except for a slight decrease in the F_8 generation, constantly increased, reaching peak numbers (111.6 ± 1.10) in the F_{10} generation over those of F_1. Even though there was a substantial decrease in egg hatching rate in the F_2 and F_3 generations relative to F_1, these numbers reached higher rates than the F_1 generation in the F_5, F_6, F_8 and F_{10} generations. The increase in mean egg number and hatching rate is important in order to show that *An. superpictus* can mate in the laboratory just as successfully as it can in the wild. However, mean number of eggs in the colony was relatively lower than other studies. Shannon & Hadjinicolao (1994) determined the mean number of eggs per batch as 162 (range 67-173) and 140 (range 93-163) for natural spring and summer population of *An. superpictus*, respectively. Rasnitsyn *et al.*, (1990) obtained 2700 eggs (mean 180) from 15 females under laboratory conditions.

Table 1. Fecundity of *An. superpictus* through F_1-F_{10} in the laboratory

Generation parent/progeny	Number of females ovipositing	Number of eggs per batch mean (±SE)	range	Number of eggs	Hatching %
P1/F1	62	108.2±2.73	45-142	6712	81.7
F1/F2	186	93.8±1.23	64-138	17441	64.5
F2/F3	161	89.4±1.02	59-122	14398	74.3
F3/F4	277	102.6±0.87	74-138	28429	80.2
F4/F5	138	104.5±1.28	79-137	14418	85.8
F5/F6	127	105.7±1.32	52-132	13421	76.1
F6/F7	142	107.3±1.19	61-129	15242	86.5
F7/F8	130	102.3±1.51	56-146	13298	82.5
F8/F9	121	107.3±1.63	67-145	12989	78.8
F9/F10	135	111.6±1.10	78-142	15070	83.7

Table 2. Developmental attributes of immature to adult of *An. superpictus* through F_1-F_{10} in the laboratory

Generation parent/progeny	Number of first instar larvae	Number of pupae	Pupation (%)	Adults ♂	Adults ♀	Sex ratio ♂ : ♀	% emergence from eggs
P1/F1	5486	3753	68.4	1091	1362	0.80 : 1	36.5
F1/F2	11258	5113	45.4	1218	1387	0.88 : 1	14.9
F2/F3	10701	6712	62.7	1721	1870	0.92 : 1	24.9
F3/F4	22789	15826	69.4	3876	4793	0.81 : 1	30.5
F4/F5	12377	8861	71.6	2321	2692	0.86 : 1	34.8
F5/F6	10213	8321	81.5	2611	2486	1.05 : 1	38.0
F6/F7	13181	10257	77.8	3217	2912	1.10 : 1	40.2
F7/F8	10976	8113	73.9	2436	2591	0.94 : 1	37.8
F8/F9	10236	8017	78.3	2379	2737	0.87 : 1	39.4
F9/F10	12621	9698	76.8	2761	2587	1.07 : 1	35.5

Pupation and adult emergence rate from eggs of *An. superpictus* decreased substantially in the F_2 and F_3 generations when compared with those of the F_1 generation (Table 2). However pupation rates reached higher levels than those of the F_1 generation starting from the F_4 generation up to the F_{10} generation while rate of adult emergence reached higher levels than the F_1 generation between F_6-F_9 generations.

In general, after the critical F_2 and F_3 generations, all parameters reached a certain stability. The sex ratio can be accepted as one of the most important indicators of a stable laboratory colony. Many research papers (Gomez *et al.*, 1977; Alptekin & Kasap, 1988; Maharaj, 2003) reporting on the sex ratio of mosquitoes point out that the sex ratio is generally very close to 1:1. Similarly the sex ratio of our colony was generally close to 1:1, though the bias towards females and males changed from generation to generation.

Commonly, colonisation of anophelines from field material is frustrating, as many species do not adapt readily to laboratory culture, often involving difficulties in mating, ovipositing, feeding and survival (Shannon & Hadjinicolao, 1994). Especially, free mating between the individuals constitutes one of the most important problems.

Successful mating for *An. superpictus* (Rasnitsyn *et al.*, 1990), *Anopheles campestris* (Loong & Cheong, 1986) and *Anopheles litoralis* (Darsie & Cagampang Ramos, 1970) was only possible when using artificial mating techniques. Additionally, Kasap (1983) had to use extra large cages (50 x 100 x 150 cm) for the first five generations in order to achieve free mating between *Anopheles sacharovi* individuals. In the present study no such problem were encountered in natural mating among *An. superpictus* individuals and mating was conducted readily with mated females laying batches of eggs successfully in relatively small cages (50 x 50 x 50 cm).

This factor undoubtedly played a very important role in the successful colonisation of the species but some difficulties nevertheless were encountered. The most important of these were at the larval development stage. The species prefers clean and gently running waters as a natural habitat (Jetten & Takken, 1994) and therefore the property of the water used in the laboratory during the larval stage becomes extremely important.

Highest pupation rates occurred when clean distilled water was used as larval medium compared with other alternatives such as natural lake water and tap water. Furthermore by itself distilled water is not enough and it is necessary to change the water at least every two days. It was possible only in this fashion to keep the water clean enough to meet the requirements of the larvae. Otherwise bacterial growth, which results in larval mortality, is unavoidable. Another important factor is the quality and quantity of the nutrition provided. Nutrition quantity above the consumption capacity of the larvae resulted in increased contamination of the water.

TetraMin Fish Food gave very good results due to properties such as low fat content, high protein values and its ability to float on top of the water long enough for the larvae to consume it, resulting in over 70% larval survival between the F_5-F_{10} generations. In addition the daily consumption rate of larvae must be observed very carefully and nutrition quantity must be adjusted accordingly. High quantity of nutrition will result in bacterial contamination and low quantities of nutrition will result in cannibalism. Yasyukevich (1999) obtained 90% larval survival by feeding larvae with a mixture containing ground combined fodder, *Daphnia* and wheat bran in a ratio of 2:1:4 but the use of this mixture might cause problems in long term studies as the availability of the ingredients might change through time.

Colonisation of mosquitoes from the field becomes paramount for many investigations concerning the biology and behaviour of this species (Bangs *et al.*, 2002). Colonisation is also the first step in developing and maintaining standardised and specific reference strains for numerous genetic and non-genetic research purposes (WHO, 1966).

In our study, the first long-term colony of *An. superpictus* from Turkey was established and an enormous quantity of information on colony rearing was obtained. In conclusion, this colony is presently in the 40th generation, is free mating and feeds on both rabbits and humans. It is now ready for additional studies concerning all aspects of its life history, the mechanism of development of resistance to insecticides and host preference behaviour.

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