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Bionomics of *Anopheles fluviatilis* and *Anopheles culicifacies* (Diptera: Culicidae) in Relation to Malaria Transmission in East-Central India

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Abstract

The southern districts of Odisha State in east-central India have been highly endemic for *falciparum* malaria for many decades. However, there is no adequate information on the abundance of the vector species or their bionomics in relation to space and time in these districts. Therefore, a study was carried out on the entomological aspects of malaria transmission to generate such information. Collections of mosquitoes were made once during each of the three seasons in 128 villages selected from eight districts. Villages within the foot-hill ecotype had a significantly greater abundance of *Anopheles fluviatilis* James s. l., whereas the abundance of *Anopheles culicifacies* Giles s. l. was significantly greater in the plain ecotype. The abundance of *An. fluviatilis* was maximum during the cold season, whereas *An. culicifacies* abundance was highest during summer and rainy seasons. The maximum likelihood estimation of the malaria infection rate in *An. fluviatilis* was 1.78%, 6.05%, and 2.6% in Ganjam, Kalahandi, and Rayagada districts, respectively. The infection rate of *An. culicifacies* was 1.39% only in Kandhamal district; infected females were not detected elsewhere. Concurrently, the annual malaria parasite incidence (MPI) was significantly higher in hill-top (17.6) and foot-hill (14.4) villages compared to plain villages (4.1). The districts with more villages in hill-top and foot-hill ecotypes also had a greater abundance of *An. fluviatilis*, the major malaria vector, and exhibited a higher incidence of malaria than villages within the plain ecotype, where *An. culicifacies* was the most abundant vector.

Key words: : *Anopheles culicifacies*, *Anopheles fluviatilis*, bionomics, malaria

Malaria has been one of the most serious public health problems in India. Odisha State, a part of east-central India, has been afflicted with a high incidence of malaria for many years and contributed >48% of the total *Plasmodium falciparum* cases recorded in India during 2015 (National Vector Borne Disease Control Programme [NVBDCP] 2016). *Plasmodium falciparum* is the predominant malaria parasite (84.5%) in Odisha State (NVBDCP 2016). Of the total 30 districts of Odisha State, the eight southern districts (Rayagada, Nowrangpur, Kalahandi, Nuapada, Bolangir, Kandhamal, Gajapati, and Ganjam) have been most seriously affected by malaria, and control in these districts has become a formidable task. Having 25.8% of the 41.9 million population of the State, the eight southern districts contributed 40.7% of the total malaria cases ($n = 432{,}375$) and 33.3% of the total malaria deaths ($n = 78$) during 2015 (NVBDCP 2016).

Anopheles fluviatilis James and *Anopheles culicifacies* Giles are considered to be the primary malaria vectors in the malarious districts of Odisha State (Sharma et al. 2004, Gunasekaran et al. 2005, Sahu et al. 2014). Vector control is one of the essential components of any malaria control program; however, the control measures adopted by the National Program have not produced the desired level of control of these vector populations (Sharma et al. 2004). Detailed information on behavior and bionomics of the vectors is crucial for disease threat analysis and for the development and implementation of vector control strategies (Gunasekaran et al. 1989, Das et al. 2013, Sahu et al. 2014). Because of the continued persistence of malaria transmission, control activities in the southern districts of Odisha State recently were reviewed and reoriented with evidence-based approaches. For the direction of vector control, data on the bionomics of the malaria vectors were considered essential. However, adequate information on the prevalence of the vector species and their bionomics in relation to space and time were not available. Hence, a study was carried out to generate such information for the eight southern districts of Odisha State that have been highly endemic for *falciparum* malaria for many decades.

Materials and Methods

Study Site

The study was carried out in the eight southern districts (latitudes 18° 45′ and 21° 5′ N; longitudes 82° 2′ and 85° 05′ E) of Odisha State, east-central India. The area of the eight districts is 49, 313 km^2, which is 31.7% of the total area of the State. The districts have a population of 10,836,822 (2011 census), which is 25.8% of the State's total (41,947,358; Odisha-Wikipedia 2016). Most of these districts are hilly with forest cover. Dry summer (March–June), wet rainy (July–October), and dry winter (November–February) are the three prevailing seasons. The mean minimum and maximum monthly temperatures ranged from 5.0°C (December) to 26.8°C (June) and from 27.2°C (January) to 40.0°C (May), respectively. The average relative humidity ranged from 30.6% (March) to 85.6% (September). The average total annual rainfall was 1,171.9 mm (monthly range: 0 to 466 mm; Odisha-Wikipedia 2016).

Malaria Incidence and Vector Control Measures

The districts have been hyperendemic for malaria for many years (Sahu et al. 2014). *Plasmodium falciparum* is the predominant species, contributing to 84.5% of the total malaria cases (NVBDCP 2016). Incidence of malaria peaks during July to September and November to December (Sahu et al. 2014). The major vector control measure currently being carried out is indoor residual spraying (IRS) with DDT or synthetic pyrethroids (deltamethrin and alphacypermethrin; Sahu et al. 2015). The last indoor residual spray round was conducted during September–October 2010. In addition, long-lasting synthetic pyrethroid (deltamethrin)-treated mosquito nets (LLINs) were distributed among high malaria incidence villages during 2009 to 2012, covering half of the population in each district.

Selection of Study Site and Sampling

There are 94 community health centers (CHCs) in the eight study districts. Two CHCs from each district were selected based on population proportional sampling. Utilizing the data collected from the districts, a map showing district and CHC boundaries and altitude was prepared. A grid sampling design with 5-km grids was followed in each of the selected CHC and considering logistics issues it was decided to select 1% of the villages from each CHC. Accordingly, the required number of grids were selected randomly in each CHC in such a way that 1% of the villages was sampled. Following the same procedure in all the CHCs, a total of 128 villages from the eight districts were selected for the study. The selected villages were grouped into the three ecotypes viz., hill-top, foot hill, and plain based on topography. The elevation range of hill-top, foot hill, and plain villages are 231.2–942.1, 85.3–928.9, and 149–641.5 m, respectively. Villages that were situated either on hill tops or on slopes of the hills are grouped into the hill-top ecotope. Perennial streams are the only source of water in these villages. The streams and the terraced paddy fields were the major mosquito larval habitat. Villages which were located at or within half km from foot of the hills are classified as foot hill ecotope. Streams, rivulets, terraced paddy field, wells, and ponds are the major larval habitats in this ecotope. Villages located on flat, but undulating, land and at least 2 km away from foot hill or rivers are grouped as plain ecotope. Wells, ponds, and paddy fields are the major sources of anopheline larval habitats in this ecotope. The study protocol was approved by the Human Ethical Committee of Vector Control Research Centre (VCRC), Pondicherry, India.

Mosquito Collections

Indoor resting collections (IDRC) in human dwellings (HDs) and cattle sheds (CSs), outdoor resting collections (ODRC), and light trap catches indoors were performed once in each of the three seasons during September 2010 to August 2011. Owing to logistic problems, outdoor resting collections and light trap collections indoors could not be carried out in all the 128 selected villages. Therefore, among the villages selected in each district, about 50% (65 villages) was chosen randomly representing three ecotypes and designated as index villages where resting collections indoors (HDs and CSs) and outdoors and light trap collections indoors were carried out. In the remaining 50% (63 villages) of the villages, also representing three ecotypes, were designated as "random villages" and only resting collections indoors (HDs and CSs) were carried out. Diurnal resting collections indoors (0600 to 0730h) were made using an oral aspirator and flash light from nine catching stations (six HDs and three CSs) in each village. Each collector spent 10 min in each dwelling collecting resting mosquitoes from the eaves, walls, and roof. Diurnal resting catches also were made outdoors, with collectors spending one man hour (0800–0900 h) in each village from searching natural pit shelters (~0.3–0.5 m deep), culverts, and root interstices of trees, and newly dug pit shelters. Artificial pit shelters were pot (round) shaped with a small mouth (15–20 cm) and nearly 0.5 m deep and 0.3–0.5 m wide and were dug on the sides of well-shaded mounds of earth. These pit shelters were made at different fixed sites in all the directions around the village. Overall collection effort for day-time resting collections was 381 and 191.5 man-hours in HDs and CSs, respectively, in 128 villages, and 194 man-hours outdoors in 65 index villages. Light traps were used for mosquito collection indoors in the index villages. A modified version of the CDC miniature light trap with a 6-V DC motor and a 2.5-V bulb was operated from dusk to dawn in one index village from each terrain in each CHC. Four traps (two each in HDs and CSs) per night were used each season, yielding a total of 287 light traps in HDs and 288 in CSs.

Laboratory Processing

All mosquitoes were brought to the field laboratory, and identified morphologically to genus level. All the culicine mosquitoes were discarded and anopheline mosquitoes were identified to species and the number of females was recorded. Bloodmeals of the fully fed female mosquitoes of the known malaria vector species obtained from diurnal resting catches were analyzed to determine the bloodmeal source using the agar gel diffusion method (Crans 1969). The reagents were from MP Biomedicals, (Solon, OH). Before testing the bloodmeal, the reagents were tested with positive controls to confirm the specificity of the reagents.

The body parts of the individual specimens of *An. fluviatilis* and *An. culicifacies* were kept in eppendorf tubes, dried for 4–5 h at 90°C, and brought to the VCRC laboratory, in Pondicherry. Two legs were separated from the individual mosquito for DNA extraction and subsequent identification of sibling species using the molecular methods of Manonmani et al. (2001, 2007).

The individual mosquito after identification was cut in to two parts, one with head and thorax and the other with abdomen, and separately pooled for PCR assay to detect human *Plasmodium* infection. Each pool was composed of maximum five specimens of head-+thorax or abdomen. DNA was extracted from pools of mosquito body parts as per the manufacturers' protocol (QIAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany).

Nested PCR assay was performed using the primers and the methodology as described by Snounou et al. (1993) for amplification of the genus *Plasmodium*-specific fragment (PCR-1) and then the identification of *P. falciparum* and *P. vivax* (PCR-2). One negative (PCR mix without DNA) and one positive control (PCR mix with DNA known to work in amplification) were included in every PCR assay. *Plasmodium falciparum* and *P. vivax* DNA extracted from human blood samples were used for positive control. After electrophoresis of PCR products, the gel was photographed using UVP GelDoc-It Imaging System, UVP, LCC Upland, CA 91786 (Pradeep et al. 2012). PCR positives for *P. falciparum* (205 bp) and *P. vivax* (120 bp) were resolved by comparison with a standard 100 bp DNA ladder loaded alongside the samples. The parasite infection prevalence in *An. fluviatilis* and *An. culicifacies* was calculated using the CDC software PooledInfRate, Version 4.0 for pools of mosquitoes (Biggerstaff 2009).

Fever Surveillance

An active case detection fever survey was done during the day of mosquito collections in the 65 (29 foothill, 15 hill-top, and 21 plain villages) index villages in the eight districts, covering a population of 19,357. Blood smears (both thick and thin on the same slide) were collected from all fever patients and also from those who reported to have suffered from fever during the past 15 d prior to the team's visit. The blood slides were brought to the VCRC field laboratory, stained, and examined. The lists of the microscopically confirmed malaria patients were communicated to the respective CHCs, and they were treated with antimalaria drugs by Accredited Social Health Activist (ASHA) of the respective village.

Statistical Analysis

Considering only index villages (65), where both IDRC and fever surveillance were carried out, two-way analysis of variance (with 3 eco-types and 3 seasons as factors) was performed to test variation in the abundance of each vector species among eco-types and seasons followed by a pair-wise comparison using Bonferroni test. To normalize the man-hour abundance, log (y +1) transformation was carried out prior to analysis. Data were expressed back transformed or geometric means. Comparison of man-hour abundance between indoor and outdoor resting collections was done using a Student's "t" independent test on transformed man-hour abundance. Although developing a model with malaria indices and vector bionomics is complex, a simple linear correlation was done between vector abundance and slide positivity rate (SPR) or malaria parasite incidence (MPI). Because malaria transmission is perennial in the study districts, vector abundance for each village was calculated based on the number of vector mosquitoes collected and man-hour spent for all the three seasons. Significance of variation in SPR or MPI between eco-types and seasons in each district was ascertained using simple chi-square test. For all the statistical tests, a two-tailed probability of $P < 0.05$ was considered as statistical significance.

Results

Species Composition

A rich anopheline fauna was found in the eight southern districts of Odisha State, comprising 80.1% of the 18,643 female mosquitoes collected. Anophelines were represented by 18 *Anopheles* species, including *An. fluviatilis* and *An. culicifacies*, the recognized primary vectors, and *An. aconitus*, *An. annularis*, *An. jeyporiensis*, *An. maculatus*, and *An. varuna*, the reputed secondary vectors of malaria in India. Among the 18 species, *An. subpictus* (18.5%, n = 14,933 females) overall was the most abundant, followed by *An. nigerrimus* (16.9%), *An. culicifacies* (12.3%), and *An. jeyporiensis* (9.3%). District-wise summary showed presence of maximum number of species (17) in Kalahandi, Kandhamal, Nawarangpur, and Rayagada districts (Table 1).

Relative Abundance of the Vector Species

Among the eight southern districts, numerically the maximum number of female anophelines (3,821) were collected in Nuapada district followed by Bolangir district (2,920; Table 1). However, the proportion of the vector species (*An. fluviatilis* and *An. culicifacies* combined) was the highest in Gajapati district. *Anopheles culicifacies* was more abundant than *An. fluviatilis* in all the districts except in Rayagada, where *An. fluviatilis* was predominant. Distribution of species by district varied statistically ($\chi^2 = 11018$; df = 133, $P < 0.001$).

Efficiency of Collection and Habitat Occurrence by Vectors

Light traps set indoors (HDs and CSs) were more productive than hand catches, both indoors and outdoors, in terms of total number of *Anopheles* mosquitoes collected (Table 2). Comparison of man-hour abundance (log values) obtained by IDRC and ODRC among 65 index villages indicated that the abundance of *An. fluviatilis* was significantly ($t = 5.1$; df = 128; $P < 0.001$) higher in IDRC compared to ODRC. Similar result was obtained while comparing the corresponding man-hour abundance of *An. culicifacies* ($t = 6.78$; df = 128; $P < 0.05$). Even though, light traps were set indoors during night-hours, the number of *An. fluviatilis* and *An. culicifacies* collected by IDRC were markedly high compared to the light traps (Table 2). Comparison between the indoor habitats of all the study villages ($n = 128$) showed that while the abundance of *An. fluviatilis* was significantly higher in HDs than CSs ($t = 2.8$; df = 254; $P = 0.005$), the abundance of *An. culicifacies* showed the reverse trend, being greater ($t = 7.8$; df = 254; $P < 0.001$) in CSs than HDs in almost all the districts (Table 3). Overall, hand catch (day time) indoors was the most effective method for monitoring the abundance of the two primary vector species. Therefore, for further analysis as well as interpretation of results (discussion), only indoor resting catch (number collected per man-hour in both houses and CSs combined) of the vector species was considered.

Vector Abundance in Relation to Ecotypes

The abundance (mean ± SE) of *An. fluviatilis* was significantly higher in foot hill (1.02 ± 0.15/man-hour) and hill-top (0.78 ± 0.15/man-hour) villages than in the villages of plain ecotype (0.22 ± 0.06/man-hour) in all the districts ($F = 10.4$; df = 2,375; $P < 0.001$, two-way ANOVA).In contrast, *An. culicifacies* abundance (mean ± SE) was significantly ($F = 17.3$; df = 2,375; $P < 0.001$) greater in plain ecotype (7.31 ± 0.66/man-hour) compared to the other two ecotypes (foot hill:3.31 ± 0.43/man-hour, hill-top:2.91 ± 0.61/man-hour). In Rayagada, Ganjam, and Gajapati districts, a considerable number of *An. fluviatilis* also were collected from plain villages (Fig. 1). In Kandhamal and Rayagada, the abundance of *An. culicifacies* was relatively higher in hill-top and foot hill ecotypes (Fig. 2), which were predominant over plain ecotype.

Seasonal Abundance

Overall, *An.fluviatilis* abundance (mean ± SE)was significantly greater during winter (1.41 ± 0.19/man-hour) than rainy

Table 1. Mosquito species composition (%) observed in index and random villages with special reference to *Anopheles*

	Rayagada	Nowrangpur	Bolangir	Nuapada	Kandhamal	Ganjam	Gajapati	Kalahandi
Villages surveyed (index & random)	13	18	15	14	16	14	16	22
Man-hours spent (in IDRC & ODRC)	79.5	108	93.5	81	96	78.5	98.0	132
Trap Nights in index villages	72	72	72	72	71	68	72	76
No. of female *Anopheles*	2,447	2,845	2,920	3,821	1,334	2,580	618	2,078
Species								
An. fluviatilis James	3.02	0.49	0.14	0.21	2.10	1.82	11.49	3.08
An. culicifacies Giles	2.49	13.95	16.68	13.24	11.09	3.68	15.70	23.97
An. annularis Van der Wulp	0.69	0.18	1.40	0.10	0.45	0.47	10.36	0.34
An. aconitus Donitz	0.69	1.76	0.65	0.00	0.07	12.13	0.00	0.24
An. barbirostris Van der Wulp	9.77	7.94	14.97	0.97	0.52	7.02	0.32	1.06
An. jamesi Theobald	9.15	4.15	9.25	5.39	1.95	5.27	6.80	1.30
An. jeyporiensis James	17.94	1.34	0.27	10.34	36.06	0.39	8.90	14.44
An. karwari James	0.00	0.14	0.00	0.00	0.07	0.00	0.00	0.10
An. maculatus Theobald	2.45	1.86	0.27	2.12	4.27	2.33	9.87	2.84
An. nigerrimus Giles	10.42	18.35	15.21	37.74	6.52	11.20	0.97	5.39
An. pallidus Theobald	5.44	1.76	3.70	0.58	1.35	6.55	3.07	2.55
An. ramsayi Covell	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
An. splendidus Koidzumi	17.65	7.35	7.29	8.56	7.72	5.74	5.66	8.85
An. subpictus Grassi	5.52	32.16	18.29	5.84	21.14	25.12	20.71	28.10
An. tessellatus Theobald	3.06	3.13	1.10	3.01	4.35	3.99	0.65	0.82
An. theobaldi Giles	1.43	1.97	1.51	3.51	0.30	0.00	1.46	0.63
An. vagus Donitz	4.94	3.16	8.80	0.84	0.90	8.76	1.29	5.73
An. varuna Iyengar	5.23	0.32	0.48	7.56	1.12	5.54	2.75	0.58

Table 2. Abundance (mean ± SD) of the malaria vectors by methods of collection

District	MHs		TNs	*An. fluviatilis*			*An. culicifacies*		
	IDRC	ODRC		IDRC	ODRC	LT	IDRC	ODRC	LT
Rayagada	58.5	21.0	72	1.40±1.0	0.14±0.26	0.21±0.18	1.05±1.11	0.00	0.03±0.20
Nowrangpur	81.0	27.0	72	0.12±0.2	0.00	0.13±0.20	5.41±2.02	0.00	0.01±0.03
Bolangir	69.5	24.0	72	0.05±0.1	0.00	0.01±0.03	7.73±5.85	0.00	0.14±0.20
Nuapada	60.0	21.0	72	0.19±0.2	0.00	0.00	9.62±7.72	0.00	0.07±0.17
Kandhamal	72.0	24.0	71	0.47±0.4	0.00	0.10±0.13	1.94±1.84	0.38±1.06	0.00
Ganjam	58.5	20.0	68	0.97±1.2	0.00	0.04±0.07	2.00±1.53	0.00	0.00
Gajapati	74.0	24.0	72	1.63±1.1	0.00	0.08±0.14	1.50±1.52	0.00	0.00
Kalahandi	99.0	33.0	76	0.85±0.7	0.00	0.05±0.13	6.06±4.70	0.00	0.00

IDRC—indoor (HD + CS) resting collection of females per man-hour (MH); ODRC—outdoor resting collection of females per MH; LT—light trap catch as females per trap-night; MHs—man-hours spent; TNs—trap nights.

Table 3. Abundance (mean ± SD) of *An. fluviatilis* and *An. culicifacies* in HDs and CSs

District	MHs		*An. fluviatilis*		*An. culicifacies*	
			MHD		MHD	
	HD	CS	HD	CS	HD	CS
Rayagada	39.0	19.5	1.43±1.62	1.33±1.34	0.48±0.57	2.19±2.61
Nowrangpur	54.0	27.0	0.15±0.24	0.07±0.16	1.04±1.24	14.15±5.93
Bolangir	45.0	24.5	0.08±0.23	0.00	3.83±4.69	14.92±8.53
Nuapada	40.0	20.0	0.29±0.34	0.00	2.71±1.82	23.43±20.83
Kandhamal	48.0	24.0	0.58±0.44	0.25±0.36	0.00	5.83±5.52
Ganjam	39.0	19.5	1.45±1.82	0.00	0.90±0.64	4.20±3.63
Gajapati	50.0	24.0	2.38±1.59	0.00	0.62±0.47	3.42±3.97
Kalahandi	66.0	33.0	0.82±0.75	0.91±0.78	1.06±1.56	16.06±11.46

MHD—man-hour abundance (number/man-hour); HD—human dwelling; CS—cattle shed.

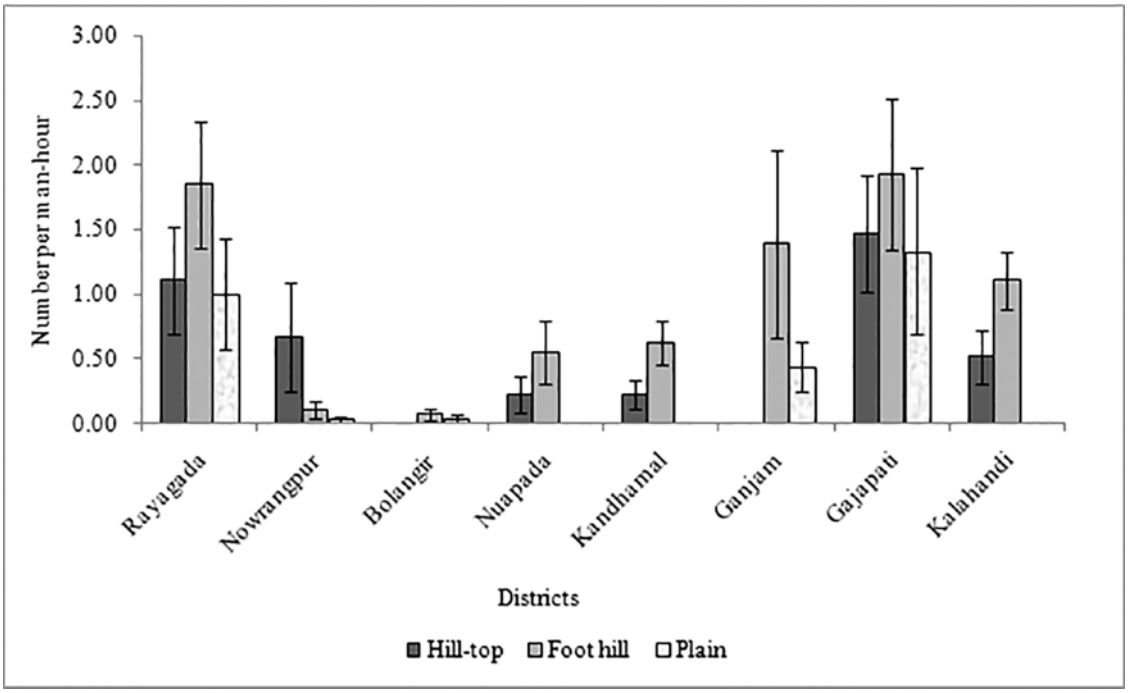

Fig. 1. Indoor resting abundance of *An. fluviatilis* in the three ecotypes in study districts.

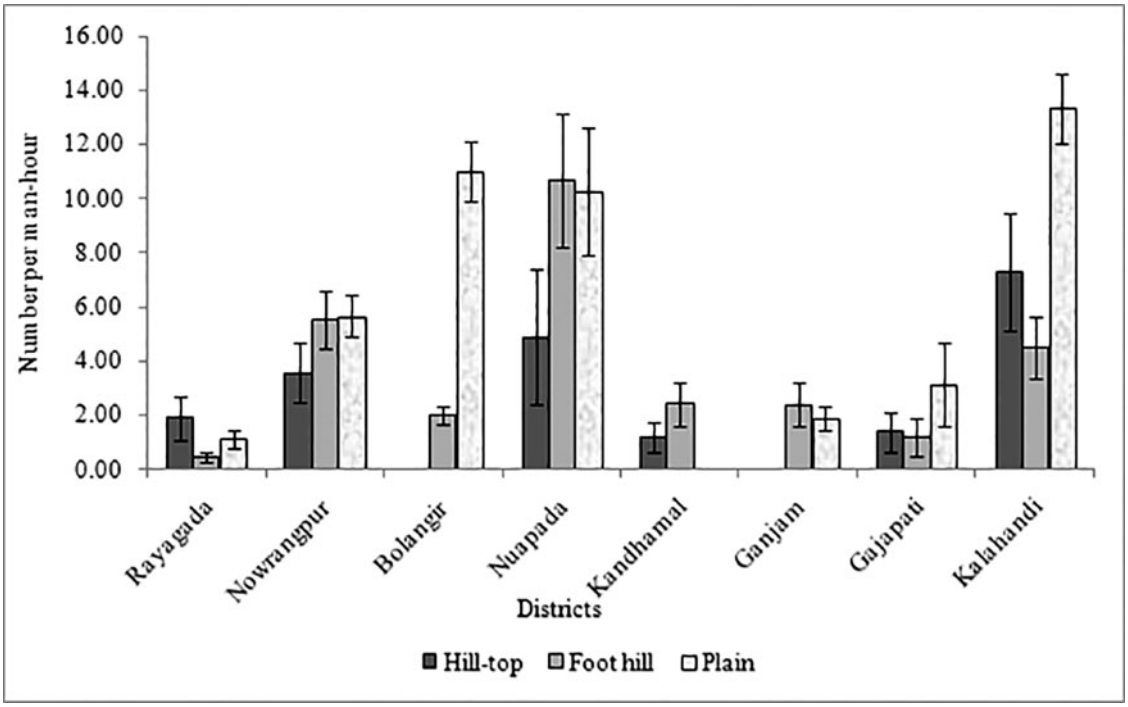

Fig. 2. Indoor resting abundance of *An. culicifacies* in the three ecotypes in study districts.

(0.62 ± 0.12/man-hour)and summer seasons (0.07 ± 0.24/man-hour; $F = 20.1$; df = 2,375; $P < 0.001$); summer recorded the lowest abundance. The same trend was seen in all districts. In contrast, the abundance of *An. culicifacies* was significantly ($F = 5.47$; df = 2,375; $P < 0.005$) greater during summer (5.56 ± 0.61/man-hour) than rainy (4.82 ± 0.57/man-hour) and winter (3.19 ± 0.53/man-hour) seasons. A similar seasonality was noticed in most of the districts except Nowrangpur, Bolangir, and Nuapada where the abundance was higher or almost equivalent during winter. Interaction of ecotype and season was found to be statistically significant for both *An. fluviatilis* ($F = 2.9$; df = 4,375; $P = 0.022$) and *An. culicifacies* ($F = 2.7$; df = 4,375; $P = 0.029$). Bivariate correlation analysis showed that overall, *An. fluviatilis* abundance showed a significant negative correlation with that of *An. culicifacies* ($r = -0.371$; $n = 384$; $P = 0.003$) during rainy ($r = -0.260$, $n = 128$; $P < 0.05$) and winter ($r = -0.278$, $n = 128$; $P < 0.05$) seasons.

Table 4. Percentage of *An. fluviatilis* fed on human or bovine blood

District	No. tested	% Fed on			No reaction (%)
		Human blood	Bovine blood	Mixed blood (H+B)	
Nowrangapur	35	57.1	40.0	0.0	2.9
Rayagada	51	52.9	31.4	2.0	13.7
Bolangir	17	41.2	52.9	0.0	5.9
Nuapada	15	93.3	0.0	6.7	0
Ganjam	37	86.5	13.5	0.0	0
Gajapathi	37	89.2	0.0	2.7	8.1
Kandhamal	32	65.6	28.1	3.1	3.2
Kalahandi	38	23.7	39.5	26.3	10.5
Total	262	62.2	26.0	5.3	6.5

H + B—human + bovine.

Table 5. Percentage of *An. culicifacies* fed on human or bovine blood

District	No. tested	% Fed on			No reaction (%)
		Human blood	Bovine blood	Mixed blood (H+B)	
Nowrangapur	50	0	100.0	0.0	0.0
Rayagada	50	2.0	98.0	0.0	0.0
Bolangir	50	0	86.0	4.0	10.0
Nuapada	50	2.0	92.0	2.0	4.0
Ganjam	50	0	94.0	2.0	4.0
Gajapati	50	2.0	98.0	0.0	0.0
Kandhamal	50	0	100.0	0.0	0.0
Kalahandi	50	0	100.0	0.0	0.0
Total	400	0.75	96.0	1.0	2.25

H + B—human + bovine.

Blood-Feeding Behavior

Of the 262 bloodmeals (cumulative of eight districts) from *An. fluviatilis* tested, 62.2% had fed on human and 26.0% bovine, 5.3% was mixed reaction to both human and bovine, and 6.5% was nonreactive, indicating important anthropophagic behavior. The percentage that fed on human varied from 41.2% (Bolangir) to 93.3% (Nuapada). In Kalahandi district, although the percentage that fed on human was 23.7%, when the percentage of *An. fluviatilis* that fed on mixed hosts (human + bovine; 26.3%) was included, the percent that fed on human increased to 50% (Table 4). In the case of *An. culicifacies*, of the 400 bloodmeals tested (50 samples from each district); only 0.75% were positive for human and 96% for bovine, 1.0% was mixed reaction to both human and bovine, and 2.25% was nonreactive, indicating a highly zoophagic behavior of this species. Human blood-positive samples were obtained from Bolangir and Nuapada (two from each district) and Gajapati and Rayagada (one from each district) districts (Table 5).

Sibling Species Composition of Vectors

Analysis of 365 specimens of *An. fluviatilis* collected from the eight districts showed that species S (reported to be more efficient vector, Nanda et al. 1996) formed 72.1% (range:58.0% [Kandhamal] to 95.5% [Ganjam]) and species T (relatively lesser efficient vector) comprised 24.9% (range: 33.9% [Kandhamal] to 38.5% [Nowarangapur]). Among the districts, the proportion of species S was maximum in Ganjam (95.5%), followed by Gajapati (87.5%), Kalahandi (66.6%), and Rayagada (62.7%). In Bolangir and Nuapada, the sample size tested was too small to comment on the sibling species composition (Table 6). In the case of *An. culicifacies* ($n = 567$), 52.9% was species B (non/poor vector, Subbarao 1998), 43.9% species E (relatively more efficient vector), and 0.9% species C (poor vector with low anthropophagy, Subbarao 1998). The highest proportion of species E (56.7%) was recorded in Nowrangpur district followed by Gajapati (51.5%), Nuapada (46.6%), and Kalahandi (45%; Table 7).

Table 6. Sibling species composition of *An. fluviatilis*

District	Females analyzed	Sibling species (%)	
		S	T
Rayagada	75	47 (62.7)	28 (37.3)
Nowrangapur	13	8 (61.5)	5 (38.5)
Bolangir	4	2 (50.0)	2 (50.0)
Nuapada	6	3 (50.0)	3 (50.0)
Kandhamal	62	36 (58.0)	21 (33.9)
Ganjam	67	64 (95.5)	3 (4.5)
Gajapati	72	63 (87.5)	3 (4.2)
Kalahandi	66	40 (66.6)	26 (36.4)
Total	365	263 (72.1)	91 (24.9)

Infection Rate

Anopheles fluviatilis collected from five districts ($n = 183$) were grouped into 35 pools, of which 3 pools were positive (one *P. falciparum* and two *P. vivax*) by PCR assay. The MLE infection with sporozoites and oocysts was 1.78% (56 mosquitoes, 11 pools), 6.05% (16 mosquitoes, 3 pools), and 2.6% (38 mosquitoes, 7 pools) in Ganjam, Kalahandi, and Rayagada districts, respectively. Positive

pools were not found in *An. fluviatilis* in Kandhamal and Gajapati districts. Since the number of *An. fluviatilis* collected from Bolangir, Nowrangpur, and Nuapada districts was very low; PCR assays could not be done (Table 8). A total of 438 *An. culicifacies* collected from the eight districts were grouped into 87 pools and tested by PCR for malaria parasite DNA. Positive results were obtained only for Kandhamal district, with an infection rate of 1.39% (72 mosquitoes, 14 pools; Table 9).

Malaria Incidence

Overall, the malaria parasite incidence per 1,000 population (MPI) in the southern districts of Odisha was 10.7 (aggregate active case detection data from all villages for the three seasons). There was a wide variation in MPI recorded in the districts. Malaria parasite incidence was the highest in Gajapati district (42.0) followed by Ganjam (23.9). Next to these two districts, Rayagada, Kalahandi, and Kandhamal recorded an MPI of 16.4, 12.3, and 8.4, respectively. In the remaining three districts (Nowrangpur, Bolangir, and Nuapada), the MPI was relatively lower and was in the range of 1.2–6.9. The overall SPR recorded in the southern districts was 19.7%.

The MPI was significantly higher in hill-top (17.6) and foot hill (14.4) villages compared to plain villages (4.1; $P < 0.05$ by χ^2). In Rayagada, Kandhamal, and Nuapada districts, the MPI was greater in foot hill villages (22.1, 15.6, and 9.49, respectively) than hill-top villages (10.7, 16.9, and 2.5, respectively). The lower MPI recorded in the hill-top villages could be due to distribution of LLINs, an additional intervention. In all the eight southern districts, *P. falciparum* was the predominant malaria species constituting 86.6% (including the mixed infections of *P. vivax* and *P. falciparum*) and the proportion of *P. vivax* was 13.4% (*P. falciparum*—169, *P. vivax*—28, and *P. falciparum* + *P. vivax*—12).

The SPR and the MPI recorded in the eight southern districts during the three seasons are given in Table 10. The overall MPI was significantly ($P < 0.05$ by χ^2-test) higher during rainy (4.1) and winter (3.7) seasons than summer season (2.9; Fig. 3). Correspondingly, the abundance of *An. fluviatilis* was also higher during rainy and winter seasons compared to summer. The indoor resting abundance of *An. fluviatilis* showed a significant positive association with SPR ($r = 0.412$; $P = 0.001$) as well as MPI ($r = 0.438$; $P = 0.001$), whereas the abundance of *An. culicifacies* did not indicate any association with incidence of malaria ($P > 0.05$; Fig. 3).

Table 7. Sibling species composition of *An. culicifacies*

District	Females analyzed	Sibling species (%)		
		B	C	E
Rayagada	40	26 (65.0)	0	7 (17.5)
Nowrangapur	171	74 (43.3)	0	97 (56.7)
Bolangir	61	46 (75.4)	0	15 (24.6)
Nuapada	103	54 (52.4)	0	48 (46.6)
Kandhamal	37	17 (46.0)	2 (5.4)	14 (37.8)
Ganjam	53	33 (63.3)	0	20 (37.7)
Gajapati	33	14 (42.4)	2 (6.1)	17 (51.5)
Kalahandi	69	36 (52.2)	1 (1.4)	31 (45.0)
Total	567	300 (52.9)	5 (0.9)	249 (43.9)

Table 8. Maximum likelihood estimation (MLE) of the malaria infection rate in pools of *An. fluviatilis* mosquitoes tested by PCR assay

Districts	*An. fluviatilis*			
	No. ofmosquitoes	No. ofpools	No. +ve	Infectionrate (95%CI)
Rayagada	38	7	1	2.6(CI:0.16–12.3)
Nowrangpur	0	0	0	0
Bolangir	0	0	0	0
Nuapada	0	0	0	0
Kandhamal	15	3	0	0
Ganjam	56	11	1	1.78(CI:0.01–8.47)
Gajapati	58	11	0	0
Kalahandi	16	3	1	6.05(CI:0.04–29.1)
Total	183	35	3	

CI—confidence interval.

Table 9. Maximum likelihood estimation (MLE) of the malaria infection rate in pools of *An. culicifacies* mosquitoes tested by PCR assay

Districts	*An. culicifacies*			
	No. ofmosquitoes	No. ofpools	No. +ve	Infectionrate (95%CI)
Rayagada	62	12	0	0
Nowrangpur	71	14	0	0
Bolangir	34	7	0	0
Nuapada	20	4	0	0
Kandhamal	72	14	1	1.39(CI:0.08–6.6)
Ganjam	46	9	0	0
Gajapati	87	18	0	0
Kalahandi	46	9	0	0
Total	438	87	1	

CI—confidence interval.

Table 10. Seasonal status of malaria by district and population

District	Total population	Surveyed population	Rainy					Summer					Winter				
			BSE	+ve	*Pf*	SPR	MPI	BSE	+ve	*Pf*	SPR	MPI	BSE	+ve	*Pf*	SPR	MPI
Rayagada	961959	2,681	45	27	26	60.00	10.07	40	11	5	27.50	4.10	40	6	6	15.00	2.24
Nowrangpur	1218762	3,079	45	4	4	8.89	1.30	50	6	6	12.00	1.95	58	11	9	18.97	3.57
Bolangir	1648574	3,425	73	4	0	0.00	0.88	48	0	0	0.00	0.00	39	0	0	5.48	0.47
Nuapada	606490	3,072	30	1	1	3.33	0.33	14	2	2	14.29	0.80	17	4	4	23.53	1.30
Kandhamal	731952	2,014	47	7	1	14.89	3.08	26	3	3	11.54	1.32	69	9	6	13.04	3.96
Ganjam	3520151	1,089	53	15	14	28.30	13.77	32	8	7	25.00	7.35	38	3	1	7.89	2.75
Gajapati	575880	1,309	43	11	10	25.58	8.40	44	16	15	36.36	12.22	49	28	25	57.14	21.39
Kalahandi	1573054	2,688	76	15	14	19.74	5.58	47	10	7	21.28	3.72	40	8	3	20.00	2.98

BSE—blood smears examined.
+ve—positives.
Pf—*Plasmodium falciparum*.
SPR—slide positivity rate (%).
MPI—malaria parasite incidence/1,000 population.

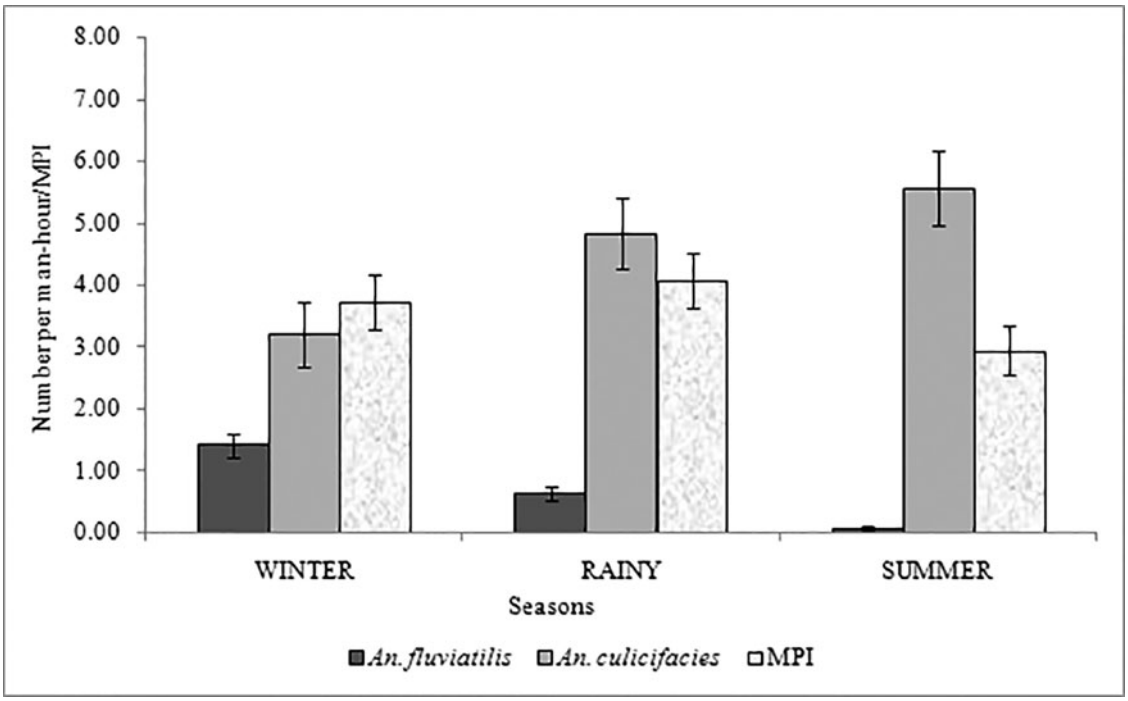

Fig. 3. Malaria parasite incidence (MPI) and indoor resting abundance of the vectors by seasons.

Discussion

Among the eight southern districts of Odisha State, the indoor resting abundance of *An. fluviatilis*, the recognized primary vector of malaria, was highest in five districts viz., Rayagada, Kandhamal, Ganjam, Gajapati, and Kalahandi, where more of the villages sampled were positioned within hill-top and foot hill ecotypes having perennial streams, the preferred larval habitat of *An. fluviatilis* (Sahu et al. 1990). In these five districts, the malaria parasite incidence was also highest. In the remaining three districts viz., Nuapada, Bolangir, and Nowrangpur, although the abundance of *An. fluviatilis* was lower, *An. culicifacies* was present in high densities even when compared to the other five districts. The malaria parasite incidence was lower in these three districts, indicating that *An. culicifacies* supported only low level of transmission of malaria even though it was highly abundant. In agreement, this species had a low infection rate (1.39%, recorded in only one district) and low frequency of human bloodmeals. By comparison, the infection rate of *An. fluviatilis* (1.78 to 6.05 recorded in three out of five districts) was higher than that of *An. culicifacies*. Therefore, the prevalence and abundance of *An. fluviatilis* was associated with malaria transmission and its incidence. The abundance of *An. fluviatilis* was greatest during winter and rainy seasons when the malaria incidence was also highest. This trend was seen in all the eight districts. In agreement with an earlier study in Keonjhar district of Odisha State, *An. fluviatilis* showed a higher indoor resting abundance in HDs than CSs and the abundance peaked during rainy and winter seasons when malaria incidence was higher (Sahu et al. 2011). In the present study, the abundance of *An. culicifacies* was maximum during summer, but the incidence of malaria was lowest during this season. Prasad et al. (2015) reviewed the resting behavior of *An. culicifacies* in India and reported its preference to rest indoors, mainly in CSs. In addition, the abundance of *An. fluviatilis* was positively correlated

with the MPI through all the seasons ($P < 0.05$), whereas there was no such correlation between the abundance of *An. culicifacies* and the MPI ($P > 0.05$).

Greater frequency of feeding on humans than animals increases the vectorial capacity of an anopheline species. In the southern districts of Odisha State, *An. fluviatilis* frequently fed on humans, with a human blood index (HBI) of 0.67. In contrast, *An. culicifacies* infrequently fed on humans with an overall HBI equal to 0.02; most bloodmeals were of bovine origin, indicating low man-vector contact as reported earlier (Subbarao 1998).

Anopheles fluviatilis in southern India is a complex of three sibling species S, T, and U (Subbarao et al. 1994). Among them, species S has been reported to be an efficient malaria vector, T a poor vector, and U a nonvector (Nanda et al. 1996, Subbarao 1998). Species S reportedly was primarily anthropophagic, whereas species T and U were zoophagic (Subbarao 1998). In the current study, species S was collected most frequently (72.1% of total identified), in seven of the eight southern districts. Where the abundance of *An. fluviatilis* and malaria incidence were highest, species S was predominant. In Gajapati and Ganjam districts, where a very high incidence of malaria was reported, the proportion of species S was near 90%. A similar proportion of species S (90.9%) and T (9.1%) were reported previously in East-Central India (Sahu et al. 2009).

In India, *An. culicifacies* is a complex of five sibling species viz. A, B, C, D, and E and among them, species A, C, D, and E have been reported to be vectors of malaria (Subbarao 1998). In the southern districts of Odisha State, species B, C, and E were collected, with species B and C considered poor vectors. Therefore, the presence of species E assumes importance with reference to malaria transmission. Although, the proportion of species E was 43.9%, the overall human blood index of *An. culicifacies* was only 0.8% (range 0% to 2% in different districts) with a low infection rate (1.39) recorded only in one district. These data indicated a low potential for this species to transmit malaria compared to *An. fluviatilis*. However, it has been reported that despite its low HBI, *An. culicifacies* could play a major role in the transmission of malaria in India, because it is generally found in high numbers (Dash et al. 2007). Therefore, a low infection rate (1.39%) and a poor anthropophagy might be compensated by high abundance and the prevalence of sibling species E (44%). Considering these facts, the role of *An. culicifacies* in malaria transmission cannot be overlooked.

The abundance of *An. fluviatilis* varied between ecotypes, being higher in foot hill and hill-top villages, where the incidence of malaria was also higher. The abundance of the major vector, *An. fluviatilis*, showed a significant positive correlation with malaria incidence through all the three seasons. The districts with more villages on hills and foot hills recorded a higher abundance of *An. fluviatilis* and a higher incidence of malaria. Therefore, the proportion of villages in these two ecotypes defined the level of endemicity of malaria with in the districts. A similar study carried out in two distinct tribal areas—BaigaChak (thick forested) in Dindori district and Bichhia block (forest fringe area) in Mandla district, Madhya Pradesh, India, showed that the abundance of *An. culicifacies* was significantly higher in forest fringe area, whereas *An. fluviatilis* abundance was higher in thick forested area. While *An. culicifacies* was incriminated as a vector from both the areas, *An. fluviatilis* was incriminated only from the thick forested area (Chand et al. 2015). Study concluded that regions should be prioritized for spraying and supplying long-lasting insecticidal nets according to the current information on vectors and malaria incidence (Chand et al. 2015).

The infection rate of the individual sibling species of both vectors and their insecticide resistance status was not determined in the current study. Another limitation was that the infection rate of *An. fluviatilis* was estimated only for five districts because its abundance was too low in the other three districts. The strength of our study was that a systematic approach was used in conducting the entomological and epidemiological surveys covering a vast geographical area and in all the three seasons of a year.

Currently, indoor residual spraying using DDT or synthetic pyrethroids (deltamethrin and alphacypermethrin) is the major vector control intervention measure being implemented in the southern districts of Odisha State. In addition, LLINs have been distributed in these districts. In spite of implementing these intervention measures, there is no sign of malaria abating in these areas. Although *An. fluviatilis* was susceptible to both DDT and synthetic pyrethroids, *An. culicifacies* has developed resistance to these insecticides (Sahu et al. 2014, 2015). Considering the biological differences in the vector species and their susceptibility status to the common insecticides, the ongoing vector control strategy may need to be modified. DDT could be the choice of insecticide for indoor residual spraying in hill-top and foot hill villages where *An. fluviatilis* is predominant, and synthetic pyrethroids for IRS in plain villages, where *An. culicifacies* is predominant and showing the low resistance to synthetic pyrethroids (Sahu et al. 2015).

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