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Host selection by *Anopheles arabiensis* and *An. quadriannulatus* feeding on cattle in Zimbabwe

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Abstract. In the Zambezi valley, mosquito females of the *Anopheles gambiae* Giles complex (Diptera: Culicidae) were collected from a hut containing pairs of cattle distinguishable by known DNA markers. DNA was extracted from the blood-fed mosquito abdomens and primer sets for ungulate and mosquito DNA loci were used to identify the mosquito sibling species and individual host source(s) of their bloodmeals. The 67 mosquitoes comprised a mixture of *An. arabiensis* Patton (31%) and *An. quadriannulatus* Theobald (69%). DNA from one or both of the cattle present in the hut was detected in 91% of samples. When the hut contained an adult and a calf, the percentage of bloodmeals from the adult, the calf and adult + calf were 58%, 27% and 15%, respectively; the trend towards meals from the adult host was consistent but not always significant. When the pair of cattle comprised two adults of roughly equal size and age, then mosquitoes generally showed no significant bias towards feeding from one individual. There was no significant difference in the pattern of host selection made by *An. arabiensis* and *An. quadriannulatus* but the former had a significantly higher percentage (20%) of mixed meals than *An. quadriannulatus* (9%). These two members of the *An. gambiae* complex appear to be less selective in their choice of cattle hosts compared to day-active Diptera such as tsetse and *Stomoxys*, possibly because the hosts are generally asleep when *Anopheles* are active and there is therefore less selective pressure to adapt to host defensive behaviour. The slight bias of *Anopheles* towards older and/or larger cattle may be related to the host's larger surface area.

Key words. *Anopheles arabiensis*, *An. quadriannulatus*, bloodmeals, cattle, feeding behaviour, host defensive behaviour, host selection, microsatellite DNA, mosquito behaviour, Zambezi, Zimbabwe.

Introduction

Theoretical considerations suggest that heterogeneous biting patterns by insect vectors of disease will have an important impact on the epidemiology of vector-borne diseases. In particular, when vectors concentrate on certain hosts the basic reproductive rate of the disease, and the vectorial capacity of the vector, increase (Dye & Hasibeder, 1986). Experimental studies indicate that various aspects of host and mosquito behaviour could lead to heterogeneous biting. For instance, in various studies of *An. gambiae* Giles *sensu stricto* feeding on humans, pregnant mothers were bitten twice as frequently as their non-pregnant counterparts (Lindsay *et al.*, 2000), adults were more likely to be bitten than children (Port *et al.*, 1980), and even apparently similar human hosts attracted different numbers of vectors (Knols *et al.*, 1995). There are relatively few data on the patterns of host selection in wild populations of vectors – largely because, until recently, we lacked the tools to allow us to identify the individual host source(s) of bloodmeals. However, the development of molecular techniques for studying the feeding patterns of anthropophilic species of mosquito (Coulson *et al.*, 1990; Ansell *et al.*, 2000;

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Chow-Shaffer *et al*., 2000) has allowed field studies of the feeding behaviour of *An. gambiae* s.s. feeding on groups of humans (Gokool *et al*., 1993; Koella *et al*., 1998).

Less attention has been paid to the feeding biases of zoophilic species of *Anopheles*, because the more widespread and important mosquito-transmitted diseases such as malaria and filariasis are not zoonoses. Nonetheless, although wild and domestic animals are not hosts for these pathogens, they can be very important alternative hosts for the vectors, as for example in the cases of *An. arabiensis* Patton and *An. stephensi* Liston which transmit malaria in Africa and Asia, respectively. These two species feed on humans and cattle and thus the transmission of malaria by these vectors is dependent, amongst other things, on the frequency and proportion of feeds obtained from human and cattle hosts (Hadis *et al*., 1997). The balance between feeding on either cattle or human hosts for *An. arabiensis* almost certainly depends on the composition and feeding heterogeneities within the cattle and human populations. For instance, an increasing preponderance of, say, unattractive hosts in a cattle population might be expected to increase the proportion of meals taken from a sympatric human population.

The importance of cattle in the bionomics of some malaria vectors provides the exciting prospect of a novel means of controlling malaria, as illustrated recently in Pakistan where mosquitoes and malaria were controlled successfully by treating cattle with insecticide (Hewitt & Rowland, 1999; Rowland *et al*., 2001). This approach may also be appropriate for those parts of Africa where malaria is largely transmitted by *An. arabiensis* and where cattle are already treated with insecticide to control tick- and/or tsetse-borne diseases (Habtewold *et al*., 2001). Such areas include countries in the Greater Horn- and Southern regions of Africa and the more arid parts of East Africa. The rational development of this approach will require a better understanding of the responses of mosquitoes to cattle.

Molecular techniques to study the individual-specific feeding patterns of zoophilic Diptera such as tsetse have recently been developed (Torr *et al*., 2001). In the present study, we report the use of this technology to assess the feeding responses of members of the *Anopheles gambiae* complex to cattle in Zimbabwe.

Materials and methods

All field studies were carried out during April 2001 at Rekomitjie Research Station in the Zambezi Valley of Zimbabwe (16°9′ S, 29°26′ E), where sympatric populations of at least two members of the *An. gambiae* complex are present. The research station is in the Mana Pools National Park, where wild ungulate hosts such as buffalo (*Syncerus caffer* Sparrman), bushbuck (*Tragelaphus strepsiceros* Pallas) and warthog (*Phacochoerus aethiopicus* Pallas) are abundant. Apart from a herd of ~30 cattle held at the station for research purposes, no domestic livestock are found within 50 km of the station.

Specimens

Cattle. Mashona cattle, a breed of short-horned Sanga indigenous to Zimbabwe, were used in all studies. Blood samples from most of this herd had previously been collected and profiled (Torr *et al*., 2001). Animals introduced or born into the herd over the past 2 years had blood samples collected and were profiled as described below. All animals used in the experiment were weighed with a weigh crush.

Mosquitoes. Fed mosquitoes were collected from a hut containing a number of live cattle. The hut ($3 \times 4 \times 3$ m high) was fitted with an exit trap consisting of two netting funnels, 50 cm wide at their entrance and narrowing to a 10-cm wide exit. The funnels pointed in opposite directions and mosquitoes entered the chamber via one funnel and exited via the other. The exit funnel led to a gauze-covered box ($10 \times 10 \times 20$ cm long) where the mosquitoes were retained.

Cattle were placed in the hut at dusk. Each night different cattle were placed in the chamber. The treatments consisted of either: a single adult, a single calf, an adult and a calf, two calves, two adults or no animals. Treatments consisting of either a single animal or no animals were repeated once and the various pairs of cattle were repeated over four or five nights using different animals. The pairs of animals were selected so that they produced unique profiles using the OLADRB (Paterson *et al*., 1998) microsatellite marker (Torr *et al*., 2001). At 06.30 hours each morning, mosquitoes were collected from the exit trap and killed by placing them in a freezer (−20°C) for 30 min. A subsample of fed mosquitoes collected from the research chamber were held alive in an insectary (temperature 23–25°C, r.h. 60–70%) for 24 h before being killed to assess the effect of digestion on bloodmeal identification.

Field processing of mosquitoes

Blood-fed mosquitoes identified as being from the *An. gambiae* complex were selected from the catch and the bloodmeal was collected using the method of Torr *et al*. (2001). The remaining head, thorax and legs of the mosquitoes were placed in individual plastic tubes containing a crystal of silica gel for subsequent species identification.

DNA extraction and microsatellite analysis of cattle DNA

DNA extraction and analysis was undertaken at the Natural Resources Institute, U.K. DNA was extracted from the bloodmeal samples following Torr *et al*. (2001) and the concentration of extracted DNA was estimated by determining the absorbance at 260 nm.

All samples were then amplified using the OLADRB primer set (Paterson *et al*., 1998) and 10% of bloodmeals was also amplified using the BM4513 (Bishop *et al*., 1994) and IGF-1 (Kirkpatrick, 1992) primers. Samples were amplified using 30 ng of genomic DNA, 1 unit of Super

taq (HT Biotechnology Ltd, Cambridge, U.K.), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.01% (w/v) stabiliser, 1.5 mM $MgCl_2$, 150 μM dATP, dCTP, dGTP, dTTP and 6 μM of each primer, made up to a total volume of 25 μL using sterile distilled water. Samples were amplified under the following conditions: 94°C for 5 min, followed by 37 cycles comprising 30 s at 94°C, 30 s at 56°C and 1 min at 72°C, finally the mixture was incubated at 72°C for 10 min and stored at 4°C. The PCR product was then mixed with one volume of formamide loading buffer (0.3% bromophenol blue, 0.3% xylene cyanol FF, 10 mM EDTA, 97.5% de-ionized formamide, pH 7.5) and heated to > 95°C for 3 min before being loaded onto a 6% polyacrlamide gel containing 7 M urea, and run at 35 W for 2 h. The resulting bands were visualized using a silver staining kit (Promega, Southampton, U.K.). Samples that did not produce bands or produced patterns of bands that did not match any of the cattle in the area were re-analysed.

Identification of mosquito species

The species of each mosquito was identified following the method of Scott *et al*. (1993) using 2 μL of the DNA solution extracted from each bloodmeal. If identification was not possible from this sample, DNA was extracted from the mosquito bodies as follows. A single mosquito specimen was homogenized in 100 μL of STE buffer (10 mM Tris-HCl, 1 mM EDTA, 10 mM NaCl). The resulting solution was then incubated at 95°C for 12 min and then centrifuged at 11 000 **g** for 4 min; 20 μL of this solution was then placed in a fresh Eppendorf tube and stored at 4°C.

Results

Identification of bloodmeals

Of the 183 bloodmeals collected, 167 (91%) contained DNA from at least one of the animals inside the experimental chamber that night (Table 1). Of the remaining 16 mosquitoes, seven produced allelic bands that did not correspond with any of the cattle at Rekomitjie and nine did not produce any bands when amplified using the OLADRB primers. As all the cattle in the area had been profiled, and all the analysed mosquitoes contained blood, then samples that produced bands that did not match any of the cattle presumably represented meals taken from wild ungulates prior to entering the chamber. None of the samples contained blood from cattle outside the chamber only, but 4% contained blood from one animal outside the chamber as well as one inside.

As expected, when a single animal was in the hut, all (14/14) the identified meals were from the animal in the hut. On one night, no cattle were placed in the hut and the single mosquito found in the exit trap the following morning did not produce allelic patterns that corresponded with the cattle at Rekomitjie.

Effects of digestion on bloodmeal identification

The gut contents of 10 mosquitoes that had been captured from the experimental hut and kept alive for 24 h before being killed were analysed. Only 50% of these samples could be identified, compared to 94% (17/18) for samples that were collected the same day and processed immediately. None of the samples collected after 24 h of digestion contained DNA from more than one host.

Probability of identity

All the cattle at Rekomitjie had been profiled and only animals with unique profiles were used in this experiment. However, the OLADRB primer is not species-specific and it is therefore possible that wild ungulates could produce an identical allelic pattern. To assess the probability of such a misidentification, approximately 10% ($n = 20$) of the samples were also profiled using the BM4513 and IgF-1 markers. In all cases, the profiles from these markers confirmed the identification obtained using OLADRB.

Host selection

The data for the four nights where a calf and an adult were in the hut show that the adults were consistently fed upon more frequently than the calves (Table 2). There was no significant difference ($\chi^2 = 7$, d.f. = 6) in the pattern of hosts for the four different pairs and, overall, the number of meals from the adults (58%) was significantly ($\chi^2 = 5.2$, d.f. = 1, $P < 0.05$) greater than that from the calf (27%).

In general, when similar animals (i.e. two adults or two calves) were in the hut there was no significant difference in the numbers of meals from each host. Indeed, there was only one case where there was a significant bias towards one particular animal (Table 2).

Differences in the mass or surface area of a host might be expected to be important determinants of the probability of being bitten (Port *et al*., 1980). The mean weights of the calves and adults were 74 kg and 385 kg, respectively, and thus if a host's mass determined the probability of it being bitten, then the expected ratio of adult : calf meals would be 1 : 5.2 compared to the observed ratio of 1 : 2.1. Moulton's

Table 1. The percentage of *Anopheles* bloodmeals identified as containing DNA from at least one of the test animals. The test chamber contained: a single adult (A), a single calf (C), two adults (A + A), two calves (C + C), an adult and a calf (A + C) or no animals (0).

	Host(s)						
	A	C	A + A	C + C	A + C	0	Total
Percentage identified	100	100	95	71	91	0	91
Replicates (nights)	1	1	5	4	4	1	16
n	12	2	74	14	80	1	183

Table 2. Bloodmeal identities for *Anopheles arabiensis* and *An. quadriannulatus* females attracted to various pairs of cattle: comprising two adults, or two calves or an adult + calf. †No mosquitoes captured. The number of meals from animals A, B and A + B and the respective liveweights of each host are shown separately for each pair. *P* indicates the probability that the numbers from each animal are significantly different at the $P < 0.05$ (*) or $P < 0.01$ (**) level of probability. Samples not producing allelic bands for test animals are not shown.

Treatment	Host pairs			Host A	Host B	Hosts A + B	Total	*P*
Ox vs. calf	1	Fed	*n*	8	6	4	18	ns
		Weight	kg	400	55			
	2	Fed	*n*	9	4	4	17	ns
		Weight	kg	350	80			
	3	Fed	*n*	15	3	2	20	**
		Weight	kg	395	80			
	4	Fed	*n*	10	7	1	18	ns
		Weight	kg	395	80			
Ox vs. Ox	5	Fed	*n*	4	2	0	6	ns
		Weight	kg	400	350			
	6	Fed	*n*	9	6	2	17	ns
		Weight	kg	395	395			
	7	Fed	*n*	10	4	5	19	ns
		Weight	kg	400	395			
	8	Fed	*n*	2	6	0	8	ns
		Weight	kg	350	295			
	9	Fed	*n*	14	5	1	20	*
		Weight	kg	400	350			
Calf vs. Calf	10	Fed	n	1	2	0	3	ns
		Weight	kg	80	55			
	11†	Fed	n	0	0	0	0	–
		Weight	kg	80	80			
	12†	Fed	n	0	0	0	0	–
		Weight	kg	80	80			
	13	Fed	n	4	3	0	7	ns
		Weight	kg	80	55			

formula for the relationship between the weight and surface area of cattle (Hogan & Skouby, 1923) suggests that the mean surface areas of the calves and adults were ~1.7 and 4.9 m^2, respectively. If this parameter determined the probability of being bitten, then the ratio of would be 1 : 2.8, which is not that dissimilar from the observed ratio, especially given the crude estimate of the host's surface area.

Specific feeding behaviour

Of the 183 mosquitoes collected, 91% (167/183) produced allelic bands for either *An. arabiensis* or *An. quadriannulatus*. In 95% (159/167) of cases, identification of the mosquito species was made using mosquito DNA co-extracted with ungulate DNA from the bloodmeal. The remaining 5% (8/167) of samples required the extraction of DNA from the body of the mosquito.

The mosquitoes collected comprised a mixture of *An. quadriannulatus* (69%) and *An. arabiensis* (31%). The preponderance of *An. quadriannulatus* may reflect the stronger zoophilic tendencies of this species rather than the relative abundance of the two species.

Analysis of the bloodmeal sources for the two species (Table 3) indicates that there was no clear or significant difference in the proportion of meals taken from specific

Table 3. Bloodmeal identities for *An. quadriannulatus* (QUAD) and *An. arabiensis* (ARAB) attracted to pairs of cattle. The pairs comprised: two adults, two calves or an adult and a calf and the identities indicate the number of meals from the most popular animal of the pair (A), the least popular (B) and unidentified ungulate hosts (X) not present in the experimental hut. For the adult + calf pair, host A is the adult. Results include only samples that produced allelic bands for both mosquitoes and ungulate DNA.

	Adult + calf		Two Adults		Two calves	
Host	QUAD	ARAB	QUAD	ARAB	QUAD	ARAB
A	26	7	29	7	3	1
B	10	5	15	0	1	0
X	0	2	1	1	1	0
A + B	5	6	4	3	0	0
A + X	2	9	1	1	0	0
B + X	0	2	1	1	1	0
Sample size	43	31	51	13	6	1

animals. For instance, where mosquitoes were presented with the choice of feeding on an adult or a calf, 71% of *An. arabiensis* were found to have fed on the adult compared to 77% of *An. quadriannulatus*. There was, however, a significant difference in the proportion of mixed meals in two respects. First, the percentage of meals that contained blood from both test animals was 9% for *An. quadriannulatus* compared to 20% for *An. arabiensis*. Second, the percentage of mixed meals comprising blood from one test animal and an unidentified host outside the chamber showed similar trends, with 5% *An. quadriannulatus* samples containing DNA from an unidentified host compared to 29% for *An. arabiensis*.

Discussion

Use of microsatellite markers

The present study made two improvements on the technique used by Torr *et al.* (2001) to identify ungulate bloodmeals taken by tsetse. First, Torr *et al.* (2001) like other studies (Gokool *et al.*, 1993; Ansell *et al.*, 2000; Chow-Shaffer *et al.*, 2000) used several microsatellite markers to distinguish reliably between individuals. In the present study, however, the entire local cattle population had been profiled and by using animals with unique combinations of alleles only a single marker was required. This reduced the time and reagents required to identify the sample and hence the cost of the procedure. However, a single marker could not be used for unidentified populations. Second, in contrast to Torr *et al.* (2001), a silver-staining procedure, rather than radiolabelled primers, was used to visualize the PCR products. This technique is not only cheaper but does not necessitate the specialist facilities required for handling radioactive materials.

Previous studies have shown that human microsatellite markers can be used to identify the individual specific source(s) of human bloodmeals taken by mosquitoes (Gokool *et al.*, 1993; Ansell *et al.*, 2000; Chow-Shaffer *et al.*, 2000). The present study demonstrates that ungulate primers can also be used to study the responses of mosquitoes feeding on cattle. The present results showed that digestion rapidly reduced the ability of microsatellite markers to identify a bloodmeal; 24 h after feeding, only 50% of the samples collected could still be identified. This is markedly less than the 100% success achieved with bloodmeals from tsetse analysed 24 h after feeding using identical markers (Torr *et al.*, 2001). The difference is presumably due largely to the smaller size of bloodmeals taken by mosquitoes (Takken *et al.*, 1998; Torr & Hargrove, 1998).

DNA extracted from a bloodmeal comprises a mixture of DNA from the host's blood and the insect. Ansell *et al.* (2000) estimated that ~20% of the DNA extracted from a mosquito bloodmeal comprised DNA from the mosquito rather than the host species. The present study showed that in most cases, the mosquito DNA extracted with the bloodmeal is sufficient to allow the identification of the mosquito species. Thus, a single DNA extraction can be used to identify both the species of mosquito and the source of any bloodmeals it may have taken.

Host selection

When presented with the choice of feeding on a calf or an adult, both *An. arabiensis* and *An. quadriannulatus* fed more frequently from the adult. Similar preferences have been observed with other haematophagous Diptera including horn flies (Steelman *et al.*, 1993), tsetse (Torr *et al.*, 2001) and stable flies (A. Prior, unpublished data). However, the bias towards adult cattle shown by mosquitoes is much less marked than that seen with tsetse, where 100% of flies fed from the adult when given a choice between feeding from a calf or an adult (Torr *et al.*, 2001).

Host defensive behaviour is an important determinant of host choice for mosquitoes (Edman & Scott, 1987). Previous studies of the behaviour of Mashona cattle indicate that younger hosts exhibit higher rates of defensive behaviour, such as stamping their legs and rippling their skin (Torr & Mangwiro, 2000), and thus it might be expected that mosquitoes will feed more successfully on adult cattle. However, if hosts within a herd were completely quiescent, then differences in an individual's mass or surface area might be expected to be important determinants of the probability of being bitten. The ratio of calf : adult bloodmeals was very different to the respective ratio of liveweights but only slightly less than the ratio of surface areas. It may therefore be that simple differences in the surface area of different animals within a herd determines the probability of being bitten.

Mixed bloodmeals

The proportion of mixed meals (10–20%) was similar to that observed for *An. gambiae* s.s. collected from houses in a Tanzanian village (Koella *et al.*, 1998), but much higher than the rate observed for tsetse feeding on pairs of cattle (1.5%; Torr *et al.*, 2001). The latter comparison is particularly interesting because the results pertain to the same herd of Mashona cattle used in this experiment.

Burkot *et al.* (1988) suggested that, in principle, the frequency of multiple feeding should be greater where there is little difference in the probability of feeding on different hosts. The present results, showing a higher rate of multiple feeds in members of *An. gambiae* complex compared to tsetse, fit with this suggestion in two respects. First, young cattle have a far higher inherent rate of defensive behaviour than adult cattle and consequently day-active Diptera, such as tsetse, are much less successful at feeding on calves than adults (Torr & Mangwiro, 2000; Torr *et al.*, 2001). Second, for *An. gambiae* complex feeding on cattle at night, hosts will generally be asleep. Thus, age-related differences in both defensive behaviour and feeding success will be less. We might therefore expect a higher rate of mixed meals in

mosquitoes attracted to a heterogeneous group of young and adult cattle.

On the other hand, there are also published data that do not fit with the prediction of Burkot *et al.* (1988). Port *et al.* (1980), for instance, found that the incidence of multiple meals for *An. gambiae* s.s. feeding on pairs of humans was 3–6%, which is more similar to the results for tsetse than *Anopheles*. Similarly, Torr *et al.* (2001) found that the incidence of mixed meals in tsetse was low even when they were presented with similar hosts. Inter-specific differences in the incidence of multiple feeding must be related to a species' propensity to persist with feeding when disturbed by a host. This propensity may be a fundamental behavioural characteristic of biting Diptera that varies between species according to differential selective pressures governed by their contrasted life histories (Anderson & Roitberg, 1999; Schofield & Torr, 2002).

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