Tropical temperatures can inhibit development of the human malaria parasite *Plasmodium falciparum* in the mosquito

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In the poikilotherm mosquito the duration of the extrinsic cycle of the malaria parasite *i.e.*, development from gametocytes ingested with an infectious bloodmeal to sporozoites in the salivary glands is dependent on the ambient temperature and proceeds faster at higher temperatures. Completion of the extrinsic cycle is blocked at temperatures of 30°C and higher. It appears that exposure to 30°C during the first 30 h after ingestion of an infectious bloodmeal progressively prevents completion of the extrinsic cycle. During this period of 30 h differentiation of gametocytes develop into gametes, zygote formation and develop further into retort forms and ookinetes. Exposure to 30°C ambient temperature reduces, but does not prevent differentiation from gametocytes into ookinetes, although oocysts and sporozoites are not found. Once ookinetes have migrated to the surface of the midgut wall to form oocysts the parasite is no longer sensitive to exposure to 30°C ambient temperature and the extrinsic cycle is completed. These observations are relevant for experimental transmissions of the malaria parasite *Plasmodium falciparum* under field conditions.

*Keywords*: mosquito, malaria, parasite, temperature effect, life cycle, *Plasmodium falciparum*

The life cycle of the malaria parasites is complicated and species specific. Part of the life cycle of *Plasmodium falciparum* takes place in the human host and part in the mosquito vector, which is of the genus Anopheles. The parasite is transmitted with the saliva when infected female mosquitoes take a bloodmeal, being essential for egg-laying. Within minutes the injected parasites home in the liver and penetrate hepatocytes. The development and proliferation of the parasite inside hepatocytes takes approximately 5 days. Mature schizonts in hepatocytes rupture and release merozoites that infect red blood cells (rbc). The parasites in the rbc produce offspring that invade new rbc every 48 h. A small proportion of the blood stages become committed to development of sexual stages, the gametocytes. Development of mature, infectious gametocytes takes approximately 7-11 days. This part of the life cycle takes place in the human host with a body temperature of 37.4°C, or higher in case of disease. The second part of the life cycle, the sporogony takes place in the mosquito. The development from gametocytes in the ingested bloodmeal to sporozoites in the salivary glands is called the extrinsic cycle and takes place in the poikilotherm mosquito, implying that development is dependent on ambient temperature varying day and night, between seasons and in different geographical zones.

Under laboratory conditions an ambient temperature of 26°C is very useful for transmission studies. Gametocytes in the bloodmeal activate and release gametes that form zygotes in minutes. Formation of retort forms followed by ookinetes takes place between 15 and 30 h after the feed. The ookinetes migrate across the midgut wall and the oocysts on the surface of the midgut wall develop thousands of sporozoites that are released into the haemocoel approximately 9-12 days after the feed. The sporozoites migrate to and infect the salivary glands and are released from there into the saliva when the mosquito takes another bloodmeal. Aside from genetic factors the duration of the extrinsic cycle depends on environmental factors *e.g.*, the ambient temperature. The permissive temperatures for sporogonic development range from 16-32°C (Detinova, 1963). The effect of different temperatures on individual sporogonic stages is less well defined. This paper analyses the effect of different temperatures on the duration of the extrinsic cycle and development of different sporogonic stages.
MATERIALS AND METHODS

Mosquitoes
A colony of *Anopheles stephensi* mosquitoes obtained from a crossing of the Sind and Kasur strains derived from the corresponding places in Pakistan is maintained under laboratory conditions since 1987. The mosquitoes are grown at an ambient temperature of 30±1°C and a relative humidity (RH) of 80±10% or higher. Adult mosquitoes receive 5% glucose *ad libitum*. Female mosquitoes are separated from the males by exposure to a plastic bag with warm water which attracts the females that are removed with an aspirator. For the transmission experiments described in this paper 3- to 5-day-old mosquitoes were used.

*Plasmodium falciparum* parasites
The NF54 strain of the human malaria parasite *Plasmodium falciparum* (Ponnudurai *et al.*, 1989) is cultured in a semi-automated culture system (Ponnudurai *et al.*, 1982). Gametocytes that are used to infect the mosquitoes are collected from 14-day-old cultures. Transmission of the gametocytes to the mosquitoes is carried out using a feeder assay (Ponnudurai *et al.*, 1989).

Transmission of *Plasmodium falciparum* by a standard membrane feeder assay
In the standard membrane feeder assay cultured parasites are suspended in normal human serum, mixed with human red blood cells (50% v/v) and introduced into a pre-warmed (37°C) glass feeder covered with parafilm at an ambient temperature of 26°C (Ponnudurai *et al.*, 1989). Mosquitoes are allowed to take a bloodmeal for 10 minutes. Unfed and partially fed mosquitoes are eliminated. In the standard membrane feeder assay mosquitoes are housed at an ambient temperature of 26°C and 80±10% RH until dissected to determine sporogonic stages. For the experiments at different ambient temperatures mosquitoes fed using the standard membrane feeder assay were transferred to separate rooms with the desired temperature and 80% relative humidity for the specified periods of time.

Detection of sporogonic stages
Analysis of development of all sporogonic stages in the mosquito midgut after the bloodmeal is dependent on recognition of these stages. There are no markers for the recognition of zygotes. For the detection of retort forms and ookinetes midguts of 5 mosquitoes were pooled in phosphate buffered saline, triturated and stained with a FITC-labelled anti-Pfs25 antibody in 0.05% Evans blue in PBS (Ponnudurai *et al.*, 1989). Pfs 25 is expressed on the surface of retorts and ookinetes. The pooled midguts were suspended in 25 µL and the number of fully developed retort forms and ookinetes was determined in 64 fields of a Bürker-Türk counting chamber.

Oocysts can be counted on dissected midguts stained with merbromide The oocysts were determined in each of 20 midguts dissected in a drop of 1% merbromide in distilled water on a glass slide to stain the oocysts, covered with a coverslip and counted under the microscope. The average number of oocysts is depicted in the figures.

RESULTS
An increase in ambient temperature from 21°C to 28°C resulted in a decrease of the period needed to complete the extrinsic cycle in mosquitoes from 15 to 9 days (Fig. 1).

Ambient temperature and development of retort forms
Formation of retort forms is observed at all ambient temperatures in the range of 21-30°C and all time points from 18-26 h after the feed (Fig. 2). Infected mosquitoes kept at 21°C ambient temperature exhibited a peak number of retorts 22 h after the feed. At higher temperatures the overall maximum number of retorts is lower, with highest numbers already 18 h after the feed and decreasing over time.
Mosquitoes were allowed to take an infectious bloodmeal and groups of fully fed mosquitoes were housed respectively at 23, 26 and 28°C. Mosquitoes were dissected 7 days after the bloodmeal and oocysts were counted. The average number of oocysts of 20 dissected mosquitoes is depicted.

The number of ookinetes 18 h after an infection the feed increases with the ambient temperature (Fig. 2). At 21°C and 26°C the number of ookinetes increases from 18-26 h after the feed. At 30°C the highest number of ookinetes is observed at 18 h after the feed and decreases thereafter. It is remarkable that the number of retorts counted 18-26 h after the feed in mosquitoes housed at an ambient temperature of 21°C is considerably higher than the number of ookinetes, the next developmental stage after the retorts, over the same period. At 26°C and 30°C the data of the retort and ookinete counts are compatible with a shift of retorts to ookinetes during this period.

The number of oocysts observed 7 days after the feed in comparison to the number of ookinetes observed 21 h after the feed and in relation to housing of mosquitoes at different temperatures is depicted in Fig. 3. The oocyst number decreases with increasing temperature and no oocysts are formed when infected mosquitoes are housed at 30°C. The ookinete numbers peak at 26°C and are produced at 30°C in contrast to oocysts.
Figure 3. Mosquitoes were allowed to take an infectious bloodmeal and groups of fully fed mosquitoes were housed at 21°C, 26°C and 30°C. Groups of 5 mosquitoes were dissected 21 h after the feed and their midguts were pooled to determine the number of ookinetes present. Duplicate counts were performed and the average number depicted. From the same groups 20 mosquitoes were dissected 7 days after the infectious feed and the oocysts were counted of 20 mosquitoes and the average depicted.

Figure 4. Mosquitoes were allowed to take an infectious bloodmeal. A control group was housed at 26°C for the whole period of 7 days. In addition, groups were housed at 30°C for increasing periods of time and then returned to 26°C and 20 mosquitoes were dissected 7 days after the feed and their oocysts counted. The oocyst count of the controls housed at 26°C for the whole period of 7 days after the feed was taken as 100% transmission.

Figure 5. Mosquitoes were allowed to take an infectious bloodmeal. The controls were housed at 26°C for the whole period of 7 days. Additional groups were housed either directly at 30°C or first for increasing periods at 26°C followed by housing at 30°C until they were dissected 7 days after the feed and their oocysts were counted. The oocyst count of the controls housed at 26°C for the whole period of 7 days after the feed was taken as 100% transmission.
To determine sensitivity of different sporogonic stages to exposure to 30°C mosquitoes were kept at 30°C for increasing periods of time after the infectious feed and returned to 26°C until dissected to determine oocyst numbers. The results depicted in Fig. 4 show that increasing periods of exposure to 30°C immediately after the infectious feed increasingly decreases transmission as measured by the oocyst count at day 7 until it is completely blocked after an exposure period of 22 h.

When fed mosquitoes are housed at 26°C and groups are transferred to 30°C for selected periods of time (Fig. 5) it is clear that exposure to 30°C during the first 30 h after the infectious feed blocks transmission in comparison to mosquitoes kept at 26°C for the whole period. In addition, infected mosquitoes exposed to 26°C for 30 h followed by housing at 30°C even for long periods of time produce normal numbers of oocysts. Mosquitoes kept at 30°C for periods of 2 or 3 weeks also develop sporozoites (results not shown).

DISCUSSION

Sporogony of *Plasmodium falciparum* in *Anopheles stephensi* is temperature dependent and takes longer at lower temperatures (Boyd, 1949; Noden *et al.*, 1995; this paper). Detinova (1962) indicated that the permissive temperatures to complete the extrinsic cycle range from 16-32°C. We have not determined the minimum permissive temperature, but the data show that infected mosquitoes exposed after the infectious feed to 30°C ambient temperature do not complete the extrinsic cycle. A remarkable point is that only part of the extrinsic cycle is sensitive to the blocking effect of exposure to 30°C. The period of 30 h between ingestion of gametocytes in an infectious bloodmeal and migration of ookinetes across the midgut wall is sensitive to the transmission reducing effect of an ambient temperature of 30°C. Development of oocysts, formation of sporozoites and invasion of salivary glands is not blocked at 30°C ambient temperature.

It is also interesting that at 30°C ambient temperature zygote formation and further development into retort forms and ookinetes is reduced but not completely blocked. However, these ookinete stages are unable to form oocysts and possibly are unable to migrate across the midgut wall.

At lower ambient temperatures it not only takes longer to complete the extrinsic cycle but the number of oocysts produced significantly increase. Whereas the number of ookinetes present 21 h after the infectious feed in mosquitoes kept at 26°C correlates with the number of oocysts found 7 days later (data not shown), this is not obvious at other temperatures.

The number of retort forms observed during 26 h after the feed is highest at an ambient temperature of 21°C and much higher than the number of ookinetes counted during this period. Since the ookocyst counts are highest when infected mosquitoes are housed at an ambient temperature of 21°C, probably the development of retorts into ookinetes is delayed and takes more than the observation period of 26 h. Another possibility is that at 21°C ookinetes are able to cross the midgut wall more effectively and over a longer period of time. Our data support and extent the observations made by Noden *et al.* (1995).

The speed of digestion of the infectious bloodmeal is considered of importance in relation to completion of the sporogonic cycle (Ponnudurai *et al.*, 1987; Feldman *et al.*, 1990). A more rapid digestion of the infectious meal was not only related to lower oocyst loads but could also be used to select for mosquitoes refractory to sporogonic development of this parasite. The digestive process may either limit the possibility of ookinetes to migrate across the midgut wall, or ookinetes may be digested before they can migrate across the wall. Thus, delay of induction or inhibition of the digestive process in infected mosquitoes housed at an ambient temperature of 21°C may permit more ookinetes to cross the midgut.

Under field conditions mosquitoes take their bloodmeal preferentially during the night when temperatures are lowest, not only while taking the bloodmeal but also during the following hours. This feature contributes to a more effective transmission of the parasite and completion of its sporogonic development. Analysis of infection in captured mosquitoes as well as experimental transmissions under field conditions, which are usually performed during daytime, should take into account this temperature effect on sporogonic development and parasite load.
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REFERENCES


