Out-of-phase circadian biorhythms in gametocytes of *Plasmodium falciparum*

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Un biorythme peut en cacher un autre.
-- Dicton ferroviaire

Summary

The individual circadian periodicity in eight hosts of *Plasmodium falciparum* gametocytes is studied in Senegal. The average time series does not present any periodicity. As for filariasis (Pichon and Treuil, 2004), it could be considered that this lack of global periodicity is in fact due to the coexistence, in each host, of two completely periodic, out of phase rhythms. Here we propose a simple mathematical method to test this assumption, which is confirmed. There is a complete morning rhythm with peak at 10 a.m. and another evening rhythm with peak at 10 p.m., both rhythms being at equal proportions on average. A similar temporal pattern is suggested in Gambia. The same morning rhythm is observed in Tanzania, but the evening rhythm happens two hours earlier, explaining the occurrence of a global subperiodic fluctuation (Magesa et al., 2000). The paradoxical situation of gametocytes following a circadian morning rhythm in spite of the nocturnal activity of vectors is discussed.

Introduction

Gametocytes play a key role in the cycle of the plasmodia[1,2](1,2). Indeed, they constitute the stage that crosses the interface between the vertebrate host and the invertebrate vector. Once in the vector, these sexuated pre-gametes are transformed into gametes and carry out fertilisation. Among several plasmodia of monkeys[3], birds[3] and rodents[4,5], the multiplication of the asexual stages is synchronous and the mature, short-living gametocytes are able to infect the vector only during a few hours. This process, called the Hawking phenomenon, is timed so that the short period of parasite infectivity to occur at night, when the mosquitoes suck blood.

This phenomenon does not seem to occur in *P.falciparum,* but the studies on this subject, mainly dealing with infectivity, are somewhat contradictory[6,7,8,9] . Lack of periodicity in *P.falciparum* is conceivable because:

- the lifecycle of the asexual forms is 48 h. In an immune individual living in an area of stable transmission, these forms, generally coming from various broods, are not synchronous[10,11] .

- the maturation of the gametocytes, sequestered in the deep organs, requires 7 to 15 days, [12,13]. After such a time lag, even if there was a synchronous asexual cycle, it is unlikely that it is reflected at the unsequestered gametocyte stage. Once released in the blood, gametocytes still undergo an infecting maturation during two days.
- *P.falciparum* gametocyte longevity (several weeks with a period of half-life of 2-3 days[14]) is much longer than that of the majority of other plasmodia. Because of their extreme longevity, *P.falciparum* gametocytes do not have a timing requirement as rigorous as the other plasmodia to meet their vector.

However, Magesa et al. (2000) [15] recently highlighted in Tanzania a mean gametocyte circadian rhythm of low amplitude, culminating to 17 h, whereas the vectors have a nocturnal activity. This result relates to average gametocyte densities of eight subjects. Among lymphatic filariae, such a subperiodicity is explained by the coexistence in the same host of two completely periodic, out of phase rhythms, with peaks at 0 h and 14 h[16] . In a study in Senegal, by analysing the fluctuations of each host, in spite of their average, we test if this possibility also applies to *P.falciparum*.

Material and methods

2.1 Study area

The study was carried out in villages near Thiès, Senegal, where climate is Sahelian and malaria is hypoendemic. *Anopheles arabiensis* of the *An. gambiae* complex is the predominant malaria vector in this area[17].

2.2 Screening, blood sampling and microscopy

The study involved school children aged 8-13 years. Screening for *P. falciparum* gametocytes was obtained by making a thick smear from a finger prick. The blood smears were stained with 10% Giemsa and observed by microscope under high power objective (x100). After identifying individuals with medium to high gametocyte density by rapid detection method (at least 2 gametocytes every 15 microscope fields), a follow up was carried out on the next day. After staining, gametocyte counts against 1500 leucocytes were carried out by an experienced malariologist (C.B.). For each count, the density of gametocytes per mm^3 has been evaluated assuming a count of 7000 leucocytes per mm^3.

Eight healthy children, without clinical symptoms of disease and free from malaria treatment for a minimum of 2 weeks, were recruited. Taking into account the young age of the studied individuals, we only took four thick smears by finger-prick at 6-h intervals for the next 24 h. All samples were collected

with the informed verbal consent of the parents. The National Committee of Ethics approved the study.

2.3. Data analysis

2.3.1. Standardisation of data

Contrary to most papers on this subject that deal with average time series, our study was also conducted for each individual. In order to eliminate the effect of individual differences in the gametocyte density level, each observed count of a time series is divided by the mean of the series[16]. The transformed data are defined as Gametocyte Standard Densities (GSD). Their mean value is 1.

2.3.2.. Modelling a 24 h fluctuation.

The cosine function is regarded as a satisfactory approximation, and it is more convenient for the study of periodic phenomena. The classical method of Aikat and Das[18] fits, by maximum likelihood, a two-parameter (amplitude and peak hour) cosine function to a 24-h observed time series with 1-mean:

$$y=1+a\cos\omega(t-k) \qquad (1)$$

where y is the standardized density of gametocytes (GSD), t is the hour of blood sampling , k is the peak hour or acrophase , a is the (half) relative amplitude, and ω is a constant: $\omega= 2\pi/T$, with T=24 h.
t and k are expressed in decimal hours (3h30 pm : 15.5h).
Subperiodicity occurs when $a<1$, generally $a<0.5$.
There is complete periodicity when $a=1$: then the parasitic density can disappear ($y=0$) and culminate to 2 if $t=k$. It is the case of the nocturnal filariasis *Wuchereria bancrofti* and *Brugia malay*i, for which the peak hour $k=0$ h[16].

2.3.3. Mixture of two complete periodicities.

2.3.3.1. Justification

The majority of the studies on the parasitic periodicity only consider the average fluctuation of several observed biorhythms. In case of subperiodicity, this implies that all the studied individuals show, if sampling errors are neglected, the same rhythm. An equal peak hour for all is conceivable, but it is much more difficult to understand why the individual rhythms share the same amplitude. Pichon[19] has noticed in the filaria *Mansonella ozzardi*, regarded as typically non-periodical, that in fact certain hosts presented a marked periodicity, but in quasi-opposition of phase (‘crypto-periodicity’). In case of subperiodicity or

non-periodicity, it seems reasonable and parsimonious to suspect a composite periodicity.

2.3.3.2. Demonstration

The sum (or the average) of several circadian cosine functions is a circadian cosine function. What happens if two complete periodicities coexist, as it is the case for the subperiodic filariae?

The two curves y_1 and y_2, the peak hours of which are respectively k_1 and k_2 (it is supposed that $k_1 < k_2$) intersect in two points I and J separated by 12 h (Figure 1a). Point I is at equal distance from the peak hours k_1 and k_2:

$$t_I = (k_1 + k_2)/2.$$

In addition, I and J are equidistant from the horizontal line $y=1$. Any combination of these two fluctuations:

$$y_i = p\, y_1 + (1-p)\, y_2$$

where p is the proportion of the rhythm k_1 ($0<p<1$),

is a subperiodic cosine curve crossing both points I and J. The same is true for the average curve. Knowing k_1 and k_2, every individual rhythm is thus characterized by the proportion p: in a host, there is a proportion p of gametocytes k_1, and a proportion $(1-p)$ of parasites k_2. In practice, knowing the co-ordinates t_I and y_I of point I, one can calculate the peak hours k_1 and k_2:

$$k_{1/2} = t_I \pm (1/\omega) \cos^{-1} (y_I - 1) \quad (\text{mod } 24) \qquad (2)$$

The distance $d=| y_I - 1|$ depends on the relative position of the peak hours k_1 and k_2: $d=0$ indicates the opposition of phase ($k_2 = k_1+12$), and $d=1$ corresponds to synchrony ($k_1 = k_2$). If the simultaneous representation of several standardized individual time series reveals the existence of two nodes separated by 12 h, this should support the assumption of a double subjacent periodicity.

2.Results

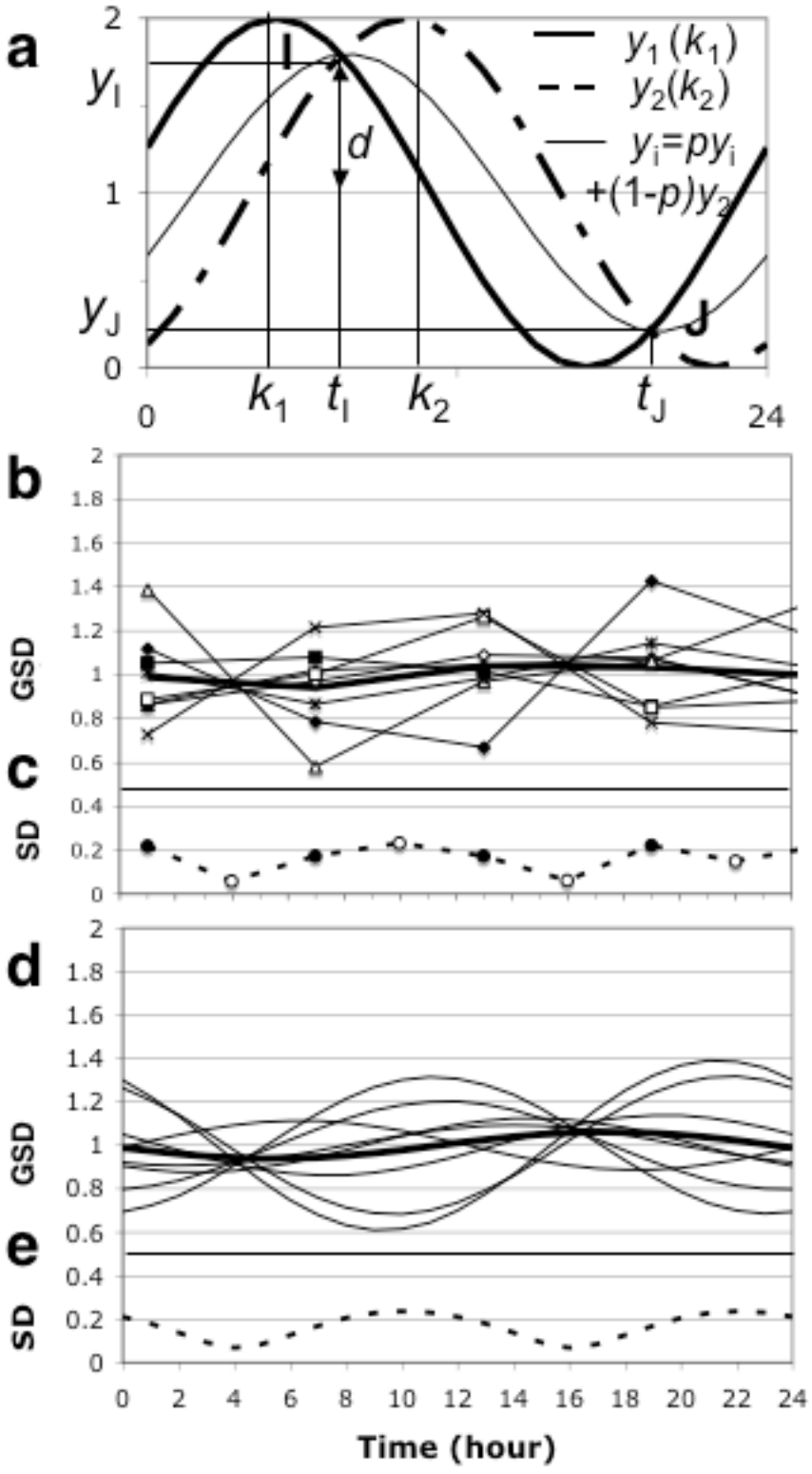

Figure 1. **a**. Coexistence of two completely periodic cosine functions y_1 and y_2, of amplitude 1 and respective peak hours k_1 and k_2. The thin curve y_i represents a mixture of these two functions in a host, p being the proportion of the first rhythm k_1. Observed (**b**) and fitted (**d**) 24-h fluctuations of gametocyte standard density (GSD) in eight individuals from Senegal. Corresponding standard

deviations (SD) of observed (solid circles) or interpolated (open circles) data (**c**) and of fitted cosine functions (**e**).

The detailed data are presented in Table A1 (supplementary information) and illustrated on Figure 1b. The smallest count was 79 gametocytes/µl. The average series, fitted by a cosine model (equation 1), does not present any significant periodicity (a=0.053, k=17.13 h, P = 0.26). A traditional analysis would thus conclude to the lack of periodicity in gametocytes from Senegal. One observes however that the individual series form between them a particular pattern of two nodes distant of 12 h. This is confirmed statistically in three ways.

-On both sides of each node, the counts of each individual are negatively correlated: r=-0.896 (P = 0.003) between 1 h and 7 h, and r=-0.897 (P = 0.003) between 13 h and 19 h.

-The standard deviations of the interpolated counts at the level of the nodes (Figure 1c) are significantly lower than the other standard deviations (Fmax test: P=0.002 and P=0.003).

-Direct calculation by simulation: on 100,000 random drawings, knowing the 8 lateral GSDs of each node, the respective probabilities that the first and second node occur at random are $1.31\ 10^{-3}$ and $1.00\ 10^{-5}$. Thus the probability of both events is $1.31\ 10^{-8}$.

The low value (0.066) of the minimum standard deviation suggests that the presence of another complete periodicity is unlikely.

The fit by a cosine function (equation 1) on each individual series is satisfactory (r>0.9). However, their amplitudes do not differ significantly from zero (a± SD=0.212 ± 0.114 h), what could come from the low number of measurements by 24 h. Indeed, the last volunteer was sampled every three hours and showed a significant amplitude (a=0.311; k=11.15; P=0.007). The calculation of the standard deviation as a function of time (Figure 1e) makes it possible to determine the points of intersection. The point I co-ordinates are t_I = 4.1 h, and y_I = 0.914, and point J co-ordinates are t_J = 16.1 h and y_J=1.086. These results are in conformity with the assumption of a composite periodicity. The distance d ± SE = 0.086±0.022 is very close to zero, which suggests that both subjacent biorhythms k_1 and k_2 are nearly in opposition of phase. The use of the formula (2) makes it possible to estimate them:

k_1±SE=10.5± 0.25 h
and
k_2±SE =21.9 ± 0.25 h.

Both biorhythms, shifted of 11.4 h, are indeed close to the opposition of phase (12 h), which accounts well for the average observed non-periodicity.

The individual proportion of k_1-rhythms is estimated to:

p ± SD = 0.50 ± 0.21

3. Discussion

Our study was designed to analyse periodicity in gametocyte densities from Senegal. A lack of periodicity was found for the average time series, but that is allotted to the mixture, in each host, of two completely periodic rhythms, nearly in opposition of phase.

Comparison with the Tanzanian study.

The protocol in Tanzania (-6) included eight subjects, 4-years old, sampled 12 times per 24 h. The majority of the subjects had a very low density of gametocytes, and these densities were only evaluated against 400 white blood cells, so the variance of the residuals (differences between the observed values and the values predicted from the cosine model) is 9.5 times larger (F test: P=0.02) for the Tanzania study that for the one from Senegal.

Magesa et al. (2000)(-6) find that the average of the time series follows a highly significant subperiodicity (P<0.001), of amplitude a=0.293 and peak hour k=17.23 h. Because of the important variability just noted, the identification of the points of intersection is less obvious than for the Senegal study (Figure A1a, supplementary information). Two low, 12-h distant standard deviations (F tests: P = 0.009 and P = 0.023) are detected respectively at 5.5 and 17.5 h, suggesting the existence of a composite periodicity. The fit of the cosine model (equation 1) to each individual series (Figure A1b, supplementary information) provides a satisfactory fit. (a± SD = 0.37 ± 0.16 h; k ± SD=16.82 ± 2.06 h). The calculated standard deviations between theoretical densities according to time allow locating the two nodes I (t_I =3.2 h, y_I = 0.74) and J (t_J = 15.2 h, y_J = 1.26). Their distance to the y=1 line d ± SE=0.26 ± 0.049 is significantly higher (P<0.001) than that of nodes of Senegal, which implies that peak hours are different. Starting from these points, one can estimate (equation 2) the peak hour of both basic biorhythms of Tanzania:

k_1±SE = 10.21 ± 0.19 h
and
k_2±SE = 20.19 ± 0.19 h

It is noticed that the k_1 of Senegal and that of Tanzania are not significantly different (Student's t: P=0.11). One thus admits that they have a common value k_1=10.3 h. In Tanzania, the proportion of k_1-rhythms is:

p ± SD = 0.43 ± 0.21

not significantly different from Senegal (Student's t: P=0.16).

On the other hand, the values of k_2 for both countries are very significantly different (P<0.001). We rename them k_S for Senegal and k_T for Tanzania.

Thus, for areas located on both sides of Africa, one notes that the gametocytes undergo a composite periodicity, due to the coexistence of two categories of basic periodicities, one in the morning (k_1 =10.3 h), and the other in the evening (k_S =21.9 h in Senegal; k_T =20.2 h in Tanzania). (Figure 2a). The average non-periodicity observed in Senegal is due to the fact that, not only both basic biorhythms are almost in opposition of phase, but also that their mean proportion is close to 50% ($p \pm SD = 0.50 \pm 0.21$). On the other hand, the average significant subperiodicity observed in Tanzania is due to the fact that the two peak hours being closer between them, the I and J intersection points are further from the line y=1.

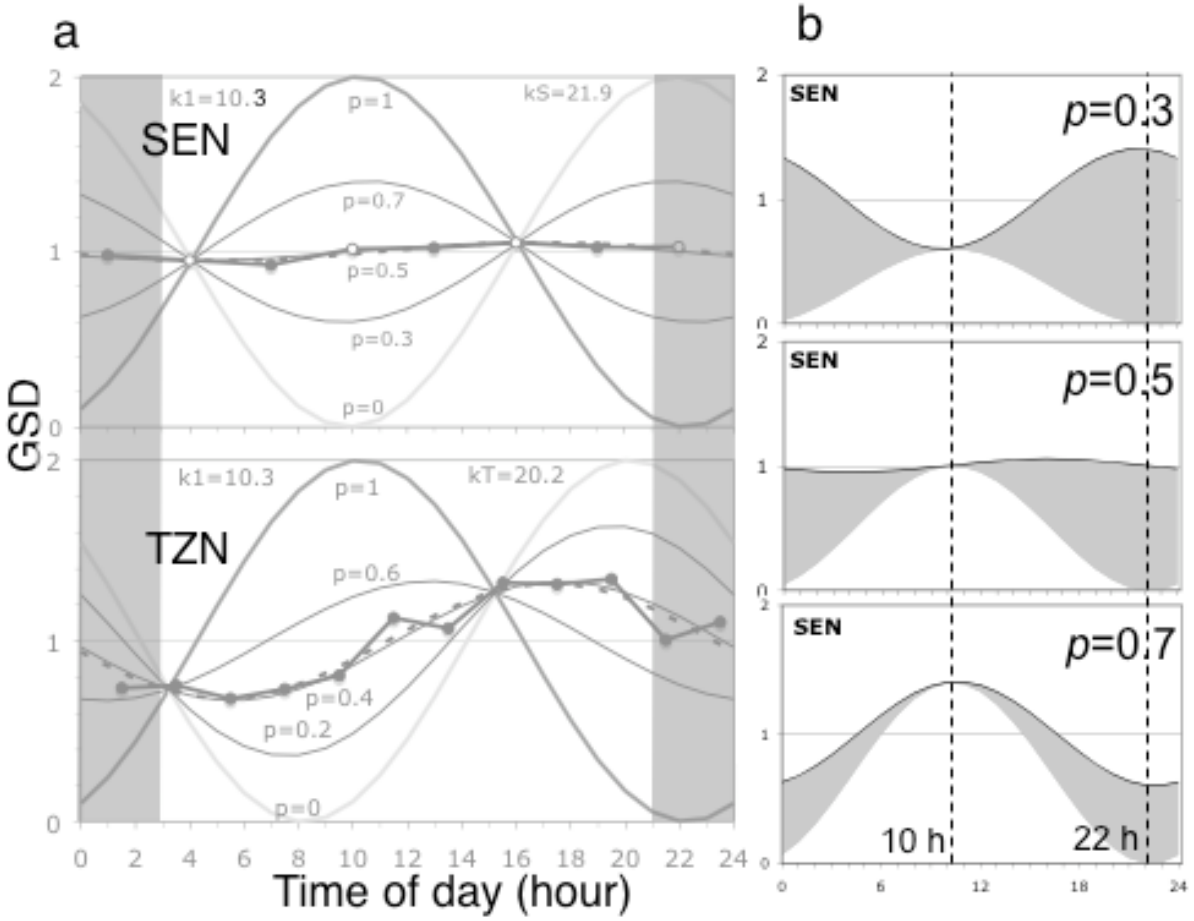

Figure 2. **a**. Basic curves of periodicity (with peak hours), curves of averages calculated (interrupted line) and observed (dots and thick line), curves (thin lines) corresponding to mean ± standard deviation for Senegal (top) and Tanzania (bottom). Proportions p of the k_1-rhythm are indicated. The grey areas represent the activity period of the mosquito vectors. **b**. Senegal- Gambia temporal patterns and infectivity to vectors at 10 h and 22 h. The white area corresponds to the k_1-rhythm, and the grey area corresponds to the k_2-rhythm.

When the proportion p of the k_1-rhythm in a host deviates from 0.5, periodicity appears.

Assumption of a genetic determinism of the two types of biorhythms

In microfilarial parasites, which are diploid, three types of genotypes can arise from two alleles of the same gene (15). Gametocyte haploidy makes the situation simpler: each allele is supposed to determine a circadian behaviour, an individual gametocyte being assumed to follow a k_1-rhythm *or* a k_2-rhythm. The vectors being strictly nocturnal, the night k_2-rhythm plays a prevailing role in the transmission and the continuation of the biological cycle, while the diurnal k_1-rhythm plays a negligible role, at least directly. The nocturnal density of k_1-gametocytes at midnight being lower than 5%, they should disappear in some generations. However not only the k_1-rhythm does not seem to vary in spite of the geographical distance, suggesting that it does not depend on natural selection, but its frequency reaches approximately 50% of the gametocytes. This could be explained simply if the k_1-allele conferred a considerable selective advantage on the gametocytes that carry it, significant enough in order to compensate for its scarcity during the nocturnal transmission period. One may also explain the persistence of k_1 if the only diploid stage of the parasitic cycle, the zygote, was clearly favoured at the heterozygous state.

Biorhythms and infectivity.

The work of Bray et al[20] (1976)(19) may allow testing the validity of these assumptions. They study the ability of gametocytes to infect anopheline vectors. They fed batches of mosquitoes on twelve gametocyte carriers only twice, at 10 h and 22 h, and dissect the midguts of vectors to count oocysts five to seven days later. This study was carried out in Gambia, a country bordering Senegal. It is noted that the 12 pairs of gametocyte standard densities corresponding to 10 h and 22 h are negatively correlated (r=-0.764; P=0.004). By reiterated linear interpolations, the minimum standard deviation was observed at 17.12 h and the corresponding GSD estimated $y \pm SE = 1.046 \pm 0.024$, not significantly differing from the node J in Senegal (P=0.082). These data are in conformity with the null hypothesis of the same biperiodicity as Senegal. From the observed GSD at 10 h and 22 h, one estimates the proportions p of the k_1-rhythm for each subject $p \pm SD = 0.52 \pm 0.10$. They are similar to those of Senegal (P= 0.353). Thus the samples of Gambia and Senegal seem qualitatively ($k_1$and k_S) and quantitatively (p) similar.
Bray et al. conclude that there is no difference of infectivity between 10 h and 22 h. Quite oddly, both times correspond almost exactly to both theoretical peak hours k_1 and k_S. Whatever the proportion p of k_1-gametocytes in a host, every circulating gametocyte at 10 h has a k_1- rhythm, and no circulating gametocyte at

22 h has any k_1- rhythm (Figure 2b). We conclude that, even if they take a slight part in transmission, k_1-gametocytes are potentially as infective as k_2-gametocytes.
Lack of periodicity in infectivity was also confirmed in Kenya (Gytheko (8)). In Burkina Faso, Boudin et al.[21](20) could get high infections of laboratory-bred mosquitoes feeding them during daytime.

Research for a composite periodicity should be undertaken in other geographic areas and in the few 'non-periodic' malaria parasites such as *P.gallinaceum* and *P.lophurae*, that Hawking suspects to be in fact more highly evolved plasmodia, not more primitive ones.
For Hawking[22],(21) periodicity is regarded as a compromise between the short-term and long-term survival of the microfilarial parasite. During 12 h, the microfilaria actively stops in the precapillary arterioles of the lungs in order to avoid an abrupt increase in oxygen concentration. Then, during 12 h, it is passively transported by the blood circulation, allowing continuation of the biological cycle in the vector. This process makes it possible the parasite to survive a long time (more than one year for *W.bancrofti*[23]). It is likely that the circadian sequestration of the mature gametocytes, which was not suspected before this study, has the same purpose: avoiding too frequent crossings of the lungs and excessive exposures to oxygen[24] (23). This could help to explain the extreme survival of *P.falciparum* gametocytes.
One may conclude with Hawking et al. [25] (1971)(24-10): '…it might seem that it would have been simpler and more effective if plasmodia had produced gametocytes which continued to be infective to mosquitoes … Then there would have been no need for the complicated mechanisms of synchronized cycles to make gametocytes match the mosquitoes.' However, this complex strategy seems to have been adopted by *P.falciparum,* undoubtedly the most effective of human parasites. In particular, the persistence during evolution of a diurnal rhythm is probably due to mechanisms much more complex than those considered here.

References

1. Boudin, C., Robert, V., 2003- *Plasmodium falciparum*: épidémiométrie de la transmission homme-moustique et de l'infection chez le vecteur. Bull Soc Pathol Exot, 2003, 96, 4, 335-340

2. Talman, A.M, Domarle, O., McKenzie, F.E. , Ariey F., Robert, V., 2004- Gametocytogenesis : the puberty of *Plasmodium falciparum. Malaria Journal* 2004, 3:24

3. Hawking F., Worms M.J., Gammage K.,1968. Twenty-four- and 48-h cycles of malaria parasites in the blood; their purpose, production and control. Trans.Roy.Soc.Trop.Med.Hyg., 62, 731-760;

4. Gautret P., Chabaud A.G., Landau I., 1995- *Plamodium vinckei vinckei* and *P.yoelii nigeriensis*: pattern of gametocyte production and development. Parasitologia, 37, 17-24.

5. Gautret P., Gantier J.C., Baccam D., Miltgen F., Saulai M., Chabaud A.G., Landau I., 1996 – The gametocytes of *Plamodium vinckei petteri*, their morphological stages, periodicity and infectivity. Int.J.Parasitol., 26 , 1095-1101.

6. Hawking F., Margaret E., Wilson E., Gammage K., 1971- Evidence for cyclic development and short-lived maturity in the gametocytes of *Plasmodium falciparum*. Trans.Roy.Soc.Trop.Med.Hyg., 65, 549-559.

7. Bray R.S., McCrae A.W.R., Smalley M.E., 1976- Lack of circadian rhythm in the ability of the gametocytes of *Plasmodium falciparum* to infect *Anopheles gambiae*. Int.J.Parasitol, 6, 399-401.

8. Githeko AK, Brandling-Bennett AD, Beier M, Mbogo CM, Atieli FK, Owaga ML, Juma F, Collins FH., 1993 - Confirmation that *Plasmodium falciparum* has aperiodic infectivity to *Anopheles gambiae*. Med Vet Entomol., 7, 373-376.

9. Gautret, P., 2000- Letter to the Editor: *Plasmodium falciparum* gametocyte periodicity. Acta Tropica, 78, 1-2

10. Magesa, S.M., Mdira Y.K., Akida, J.A., Bygbjerg I.C., Jakobsen P.H., 2000- Observations on the periodicity of *Plasmodium falciparum* gametocytes in natural human infections. Acta tropica, 76, 239-246. http://www.elsevier.com/locate/actatropica

11. Delley, V., Bouvier, P., Breslow, N., Doumbo, O., Sagara, I., Diakite M., Mauris A., Dolo A., Rougemont A., 2000- What does a single determination of malaria parasite density mean? A longitudinal survey in Mali. Trop.Med.Int.Hlth., 5, 404-412.

12. Eichner M, Diebner HH, Molineaux L, Collins WE, Jeffery GM, Dietz K, 2001- Genesis, sequestration and survival of *Plasmodium falciparum* gametocytes: parameter estimates from fitting a model to malariatherapy data. Trans R Soc Trop Med Hyg, 95:497-501.

13. Day KP, Hayward RE, Dyer M: The biology of *Plasmodium falciparum* transmission stages. Parasitology 1998, 116:S95-S109

14. Smalley ME, Sinden RE, 1977- Plasmodium falciparum gametocytes their longevity and infectivity. Parasitology, 74:1-8.

15. Pichon G., Treuil J.-P., 2004- Genetic determinism of parasitic circadian periodicity and subperiodicity in human lymphatic filariasis. C. R. Biologies, 327, 1087–1094. http://www.sciencedirect.com

16. Robert, V., Dieng, H., Lochouarn, L., Traoré, S.F., Trape, J.F., Simondon, F., Fontenille, D., 1998- La transmission du paludisme dans la région de Niakhar, Sénégal. Trop.med.Intl.Hlth, 3, 667-677.

17. Aikat, T.K., Das M.,1977- A modified statistical method for analysis of periodicity of microfilaria. Indian J. Med. Res., 65, 58-64

18. Pichon, G., 1983- Crypto-periodicity in *Mansonella ozzardi*, Trans.R. Soc. Trop. Med. Hyg. 77, 331–333

19. Bray R.S., McCrae A.W.R., Smalley M.E., 1976- Lack of circadian rhythm in the ability of the gametocytes of *Plasmodium falciparum* to infect *Anopheles gambiae*. Int.J.Parasitol, 6, 399-401.

20. Boudin, C., Olivier, M., Molez, J.-F., Chiron J.P., Ambroise-Thomas, P., 1993- High human malarial infectivity to laboratory-bred *Anopheles gambiae* in a village in Burkina Faso. Am.J.Trop.Med.Hyg., 48, 700-706

21. Hawking, F., 1967- The 24-hour periodicity of microfilariae: biological mechanisms responsible for its production and control, Proc. R. Soc. Ser. B 169, 59–76.

22. Eberhard, M.L., 1986-Longevity of microfilariae following removal of adult worms, Trop. Med. Parasitol., 37, 361–363.

23. Krieg, N. R., Hoffman, P. S., 1986- Microaerophily and oxygen toxicity. Ann. Rev. Microbiol., 40,107-30. http://www.annualreviews.org/aronline

---

[1] Boudin, C., Robert, V., 2003- *Plasmodium falciparum*: épidémiométrie de la transmission homme-moustique et de l'infection chez le vecteur. Bull Soc Pathol Exot, 2003, 96, 4, 335-340

[2] Talman, A.M, Domarle, O., McKenzie, F.E. , Ariey F., Robert, V., 2004- Gametocytogenesis : the puberty of *Plasmodium falciparum. Malaria Journal* 2004, 3:24

[3] Hawking F., Worms M.J., Gammage K.,1968. Twenty-four- and 48-h cycles of malaria parasites in the blood; their purpose, production and control. Trans.Roy.Soc.Trop.Med.Hyg., 62, 731-760;

[4] Gautret P., Chabaud A.G., Landau I., 1995- *Plamodium vinckei vinckei* and *P.yoelii nigeriensis*: pattern of gametocyte production and development. Parasitologia, 37, 17-24.

[5] Gautret P., Gantier J.C., Baccam D., Miltgen F., Saulai M., Chabaud A.G., Landau I., 1996 – The gametocytes of *Plamodium vinckei petteri*, their

morphological stages, periodicity and infectivity. Int.J.Parasitol., 26 (10), 1095-1101.

[6] Hawking F., Margaret E., Wilson E., Gammage K., 1971- Evidence for cyclic development and short-lived maturity in the gametocytes of *Plasmodium falciparum*. Trans.Roy.Soc.Trop.Med.Hyg., 65, 549-559.

[7] Bray R.S., McCrae A.W.R., Smalley M.E., 1976- Lack of circadian rhythm in the ability of the gametocytes of *Plasmodium falciparum* to infect *Anopheles gambiae*. Int.J.Parasitol, 6, 399-401.

[8] Githeko AK, Brandling-Bennett AD, Beier M, Mbogo CM, Atieli FK, Owaga ML, Juma F, Collins FH., 1993 - Confirmation that *Plasmodium falciparum* has aperiodic infectivity to *Anopheles gambiae*. Med Vet Entomol., 7(4):373-376.

[9] Gautret, P., 2000- Letter to the Editor: *Plasmodium falciparum* gametocyte periodicity. Acta Tropica, 78, 1-2

[10] Magesa, S.M., Mdira Y.K., Akida, J.A., Bygbjerg I.C., Jakobsen P.H., 2000- Observations on the periodicity of *Plasmodium falciparum* gametocytes in natural human infections. Acta tropica, 76, 239-246. http://www.elsevier.com/locate/actatropica

[11] Delley, V., Bouvier, P., Breslow, N., Doumbo, O., Sagara, I., Diakite M., Mauris A., Dolo A., Rougemont A., 2000- What does a single determination of malaria parasite density mean? A longitudinal survey in Mali. Trop.Med.Int.Hlth., 5 (6), 404-412.

[12] Eichner M, Diebner HH, Molineaux L, Collins WE, Jeffery GM, Dietz K, 2001- Genesis, sequestration and survival of Plasmodium falciparum gametocytes: parameter estimates from fitting a model to malariatherapy data. Trans R Soc Trop Med Hyg, 95:497-501.

[13] Day KP, Hayward RE, Dyer M: The biology of Plasmodium falciparum transmission stages. Parasitology 1998, 116:S95-S109

[14] Smalley ME, Sinden RE, 1977- Plasmodium falciparum gametocytes their longevity and infectivity. Parasitology, 74:1-8.

[15] Magesa cf 6

[16] Pichon G., Treuil J.-P., 2004- Genetic determinism of parasitic circadian periodicity and subperiodicity in human lymphatic filariasis. C. R. Biologies, 327, 1087–1094. http://www.sciencedirect.com

[17] ref sur vecteurs au Sénégal

[18] Aikat, T.K., Das M.,1977- A modified statistical method for analysis of periodicity of microfilaria. Indian J. Med. Res., 65, 58-64

[19] Pichon, G., 1983- Crypto-periodicity in *Mansonella ozzardi*, Trans.R. Soc. Trop. Med. Hyg. 77, 331–333

[20] Bray R.S., McCrae A.W.R., Smalley M.E., 1976- Lack of circadian rhythm in the ability of the gametocytes of *Plasmodium falciparum* to infect *Anopheles gambiae*. Int.J.Parasitol, 6, 399-401.

[21] Boudin, C., Olivier, M., Molez, J.-F., Chiron J.P., Ambroise-Thomas, P., 1993- High human malarial infectivity to laboratory-bred *Anopheles gambiae* in a village in Burkina Faso. Am.J.Trop.Med.Hyg., 48, 700-706

[22] F. Hawking, The 24-hour periodicity of microfilariae: biological mechanisms responsible for its production and control, Proc. R. Soc. Ser. B 169 (1967) 59–76.

[23] Eberhard, M.L., 1986-Longevity of microfilariae following removal of adult worms, Trop. Med. Parasitol., 37, 361–363.

[24] Krieg, N. R., Hoffman, P. S., 1986- Microaerophily and oxygen toxicity. Ann. Rev. Microbiol., 40,107-30. http://www.annualreviews.org/aronline

[25] Hawking cf (10)

SUPPLEMENTARY INFORMATION

Hidden circadian periodicity in gametocytes of *Plasmodium falciparum* in natural human infections.

Table I. Observed and *standardised* (*GSD*) individual gametocyte densities at 6-h intervals for the eight individuals of the Thiès area (Senegal).

Individual village,age and sex	Gametocyte densities per ml of blood and *GSD* Blood sampling time 1:00	7:00	13:00	19:00	Mean density	Standard deviation
PD11F	368	258	220	469	328.75	112.610
	1.1194	*0.7848*	*0.6692*	*1.4266*	*1.0000*	*0.343*
PD12M	1184	1330	1492	1475	1370.25	143.880
	0.8641	*0.9706*	*1.0889*	*1.0764*	*1.0000*	*0.105*
NF10F	156	184	188	193	180.25	16.581
	0.8655	*1.0208*	*1.0430*	*1.0707*	*1.0000*	*0.092*
NF12M	196	82	137	150	141.25	46.914
	1.3876	*0.5805*	*0.9699*	*1.0619*	*1.0000*	*0.332*
NF13M	567	486	551	641	561.25	63.668
	1.0102	*0.8659*	*0.9817*	*1.1421*	*1.0000*	*0.113*
NF11F	518	532	500	421	492.75	49.594
	1.0512	*1.0797*	*1.0147*	*0.8544*	*1.0000*	*0.101*
PD11M	1829	2070	2605	1757	2065.25	383.920
	0.8856	*1.0023*	*1.2613*	*0.8507*	*1.0000*	*0.186*
KM13M	79	132	139	85	108.75	31.117
	0.7264	*1.2138*	*1.2782*	*0.7816*	*1.0000*	*0.286*
Mean	612.13	634.25	729.00	648.88		
SD	602.82	705.02	881.23	628.99		
GSD mean	*0.9888*	*0.9398*	*1.0384*	*1.0331*		
GSD SD	*0.2038*	*0.1944*	*0.1907*	*0.2068*		

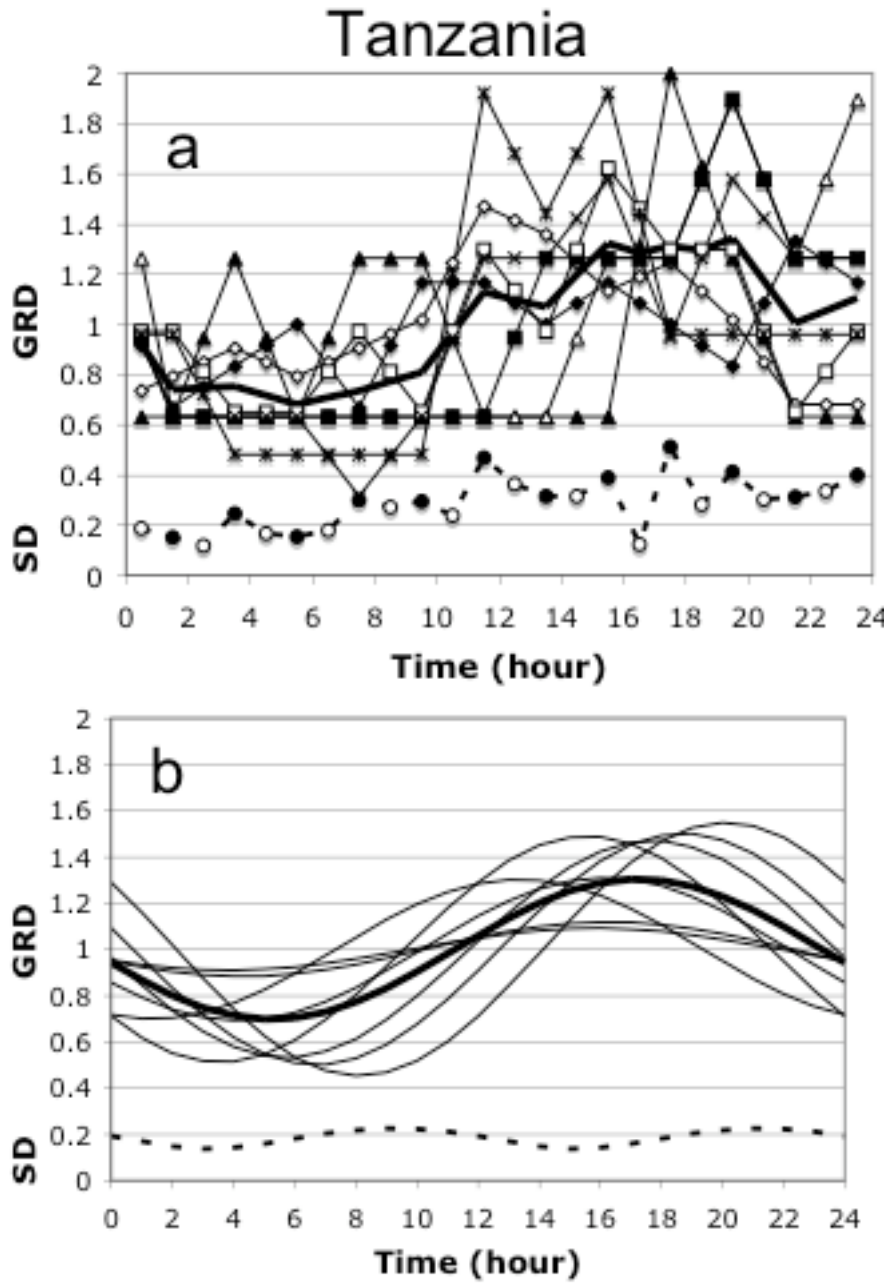

Figure A1- Tanzania : observed 24-h fluctuations (B) and fitted curves (D). Observed and interpolated standard deviations (B) and calculate standard deviation.(interrupted line (D). (data: Magesa et al., 2000).

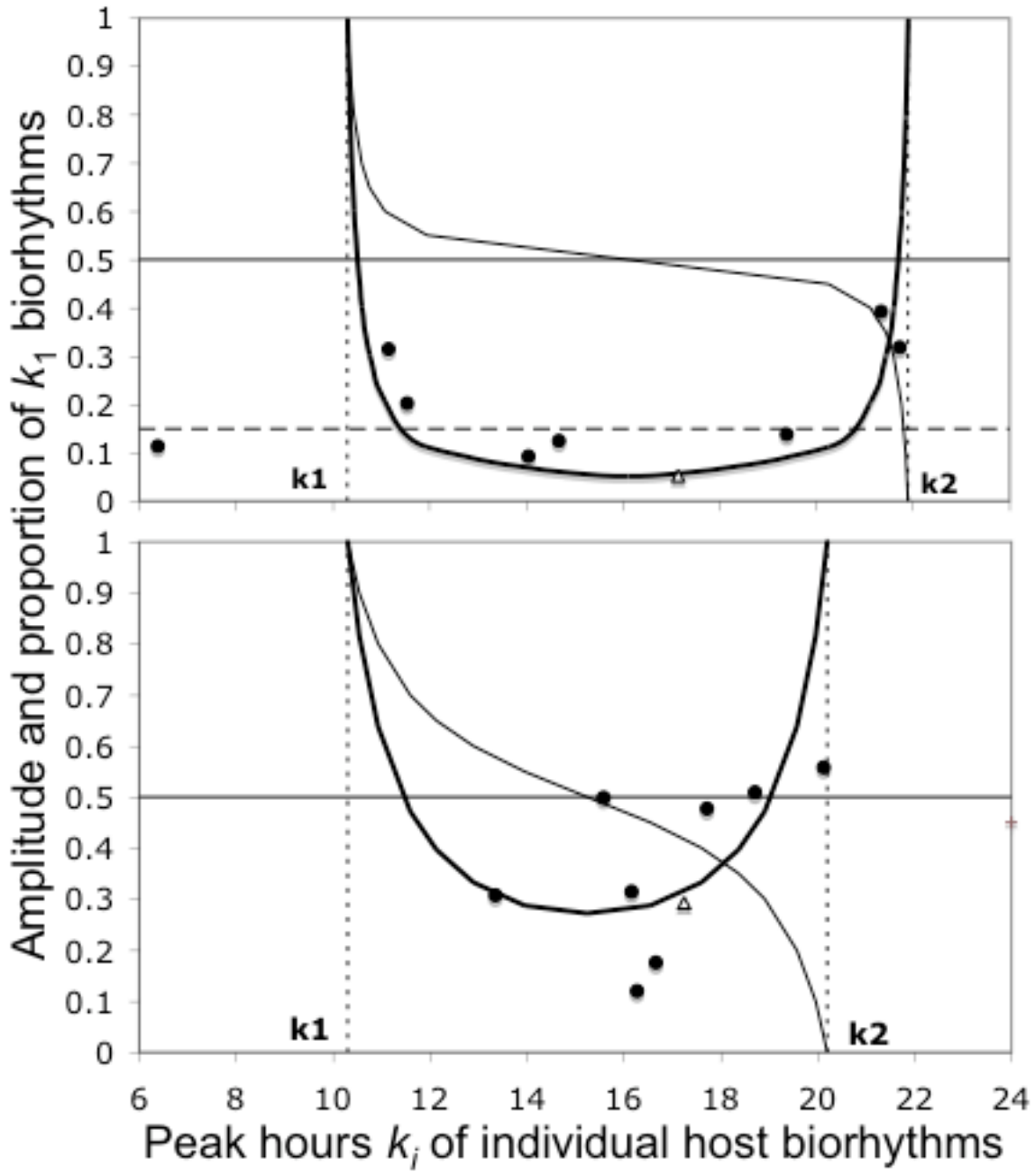

Figure A2. Relation of amplitude and p(k_1) vs individual peak hours k_i.
Top: Senegal; bottom: Tanzania. (data: Magesa et al., 2000)

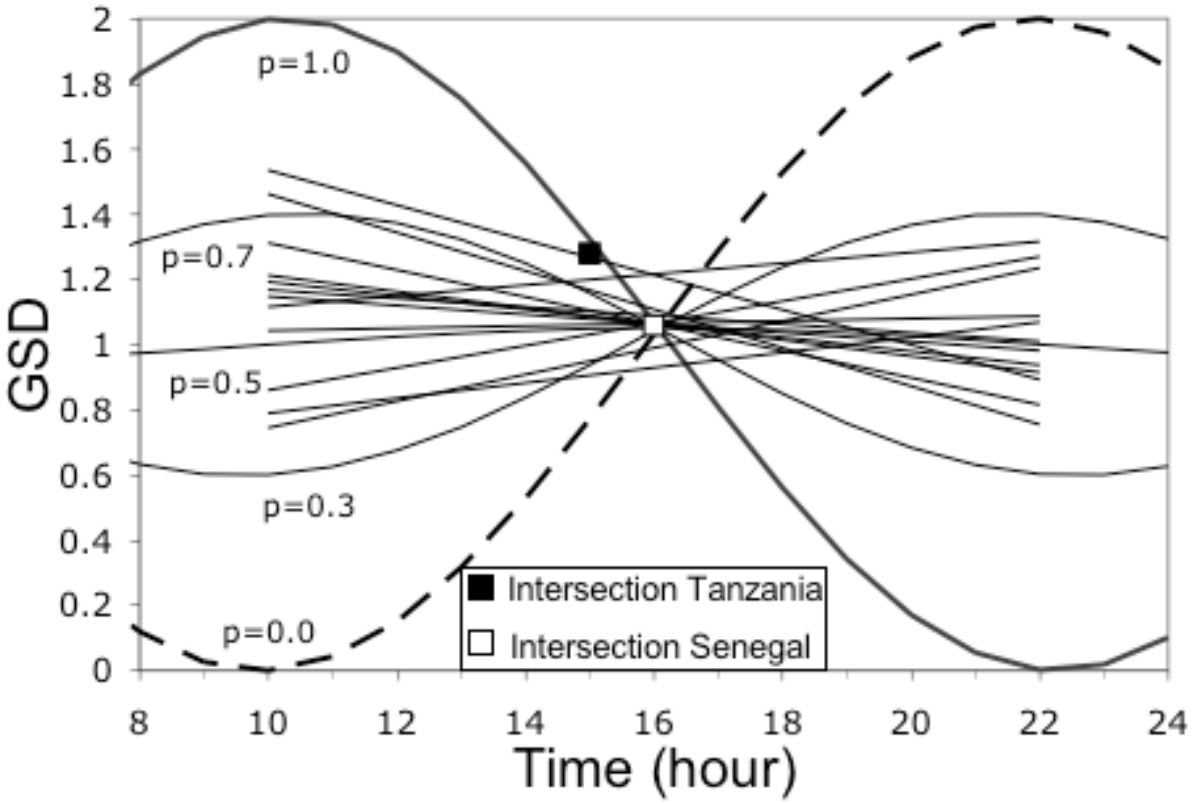

Figure A3- Basic biorhythms of Senegal and observed GSD at 10 h and 22 h in Gambia. (data Bray et al).