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Autor: Gass, R.F.

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Swiss Tropical Institute, Socinstrasse 57, CH-4051 Basel, Switzerland

Influences of blood digestion on the development of *Plasmodium gallinaceum* (Brumpt) in the midgut of *Aedes aegypti* (L.)

R. F. Gass

Summary

Blood digestion of *Aedes aegypti* and development of *Plasmodium gallinaceum* were shifted against each other by giving the mosquitoes two consecutive blood meals. In this way the parasites were exposed to an environment where blood digestion was more advanced than in single blood meals. This procedure had an inhibiting effect on oocyst production when the two blood meals overlapped; an enhancing effect when they were well separated. The results are explained by the action of trypsin-like proteases on the parasites and indicate that plasmodia 0–10 h after blood meal are more sensitive to the enzymes than later stages of the parasites.

Key words: Host-parasite interrelationship; *Aedes aegypti;* multiple feeding; blood digestion; trypsin-like proteases; *Plasmodium gallinaceum;* ookinetes; oocyst production; adaptation.

Introduction

The susceptibility of mosquitoes to a given species of plasmodial parasites is known to be controlled by a great number of factors. Factor(s) responsible for innate immunity of the host have been demonstrated to be of systemic nature and most probably to be localized in the hosts' haemolymph (Weathersby, 1960; Weathersby and McCall, 1968). Their action upon the parasites is antiblastic and they are transferable to susceptible species (Weathersby et al., 1971).

Susceptible mosquitoes show great variations in their level of infection with a given species of plasmodia. The factors responsible for this intraspecific

Correspondence: Dr. Reto F. Gass, Swiss Tropical Institute, Socinstrasse 57, CH-4051 Basel, Switzerland

variation, ranging from good susceptibility to individual immunity, are only poorly understood. The grade of susceptibility, i.e. oocyst production, can be altered by feeding a variety of different substances to the mosquitoes such as salts, inorganic acids and bases, metabolites, vitamins, hormones, antibiotics, sulfonamides (Terzian, 1950, 1955; Terzian and Stahler, 1960; Terzian et al., 1952, 1953; Micks and Ferguson, 1961) and sugars (Weathersby and Noblet, 1973). As some of these diets lead to increased oocyst production we have to assume that the physiological conditions in the mosquito are suboptimal for plasmodial development; in other words that barriers are opposing plasmodial development.

Weathersby (1952) demonstrated that the passage through the gut is not essential for the sporogonic cycle. However under natural conditions this passage causes strong reduction of the parasite population; in the *Aedes aegypti/ Plasmodium gallinaceum* system 1000 gametocytes produce a mean number of 17.5 oocysts (Eyles, 1951). Degenerating ookinetes in the gut have been observed by Huff (1934) and Stohler (1957). Several barriers which could cause the loss of parasites have been suggested. Gass (1977a, b) suggested the hypothesis that prezygotic stages are destroyed if they do not develop to zygotes within 2 h after the blood meal. De Buck et al. (1932) and Howard (1962) considered the possibility that ookinetes are imprisoned in the blood clot. Stohler (1957) found that 30 h after the blood meal the peritrophic membrane forms a barrier which is impenetrable to ookinetes because of its thickness.

A further barrier has been supposed by several authors to be blood digestion and two possibilities for its mechanism have been considered. First Gooding (1966b, 1972) concluded that the developing ookinetes are dependent on nutrient provision by blood digestion because many procedures leading to delayed digestion were connected with decreased oocyst production. Second it was supposed that digestive enzymes could destroy the plasmodia (Gooding, 1966b, 1972; Briegel and Lea, 1975; Maier, 1976). An attempt to explain innate immunity of the mosquitoes by digestion of the parasites failed (Huff, 1927).

In this work it is attempted to find evidence for in vivo destruction of plasmodia by digestive enzymes by means of double feedings.

Materials and methods

Host-parasite system

For all experiments *Plasmodium gallinaceum* Brumpt, Liverpool strain, was used. This strain has been kept since 1969 in the Swiss Tropical Institute by two blood passages weekly in 10–20-day-old white leghorn chicks, and at least two cyclical passages annually. As susceptible host *Aedes aegypti* L., Rockefeller strain, was used. Its rearing was done according to usual methods and the adults were maintained at 27° C at a relative humidity of 75 ± 10%. Up to their experimental use at the age of 3–4 days they were fed exclusively on 10% sugar solution. For each experiment mosquitoes were chosen from the same date of emergence.

Double feedings

Usually blood digestion and plasmodial development start together and run parallel. The two processes can however be shifted relative to each other by giving the mosquitoes two blood meals, only the second of which is infective. At various time intervals (Δt_{BM} = 5, 10, 15, 20, 25, 30, 40, 45, 50, 70, 120 h) after the first, uninfective blood meal (BM I^-), a batch of females was allowed to feed on an infected chick (BM II^+). Under these conditions each plasmodial development stage in the midgut of the mosquitoes was exposed to an environment in which blood digestion was more advanced than under the condition of a single, infective blood meal, as was given to the control group (BM I^+). All blood meal sizes were estimated by weighing ether-anaesthetized mosquitoes in groups of 10–30 individuals 1½ h before and immediately after blood ingestion. To reduce deviations in the "empty-weights" of the mosquitoes, they were starved for one day prior to a blood meal. All experiments were started with such a number of females in each batch that after all loss due to unsufficient blood meals and early death, a number of about 50 individuals in both the test and control groups remained for final evaluation. The specific conditions for the three blood meal types were the following:

BM I^-: This first uninfective blood meal of the mosquito test group was interrupted for them to take a second one within a short time period. As proteolytic activity and blood meal size are correlated (Briegel and Lea, 1975), mosquitoes with a blood meal weight within a fixed range (50 ± 16% of the mosquito "empty-weight"; min. 0.9 mg, max. 1.7 mg) were chosen for all experiments.

BM II^+: The test group ingested its second blood meal on a infected chicken at a parasitaemia of 5–20%.

BM I^+: The control group taking only one single, complete blood meal was fed at the same time on the same infected chicken as the test group (BM II^+).

Evaluation of the infection of mosquitoes

The mosquitoes were killed 6 days after the infective blood meal and stored at −20° C. The success of the infection was recorded by removing the midguts and counting the number of oocysts using standard methods (Geigy and Herbig, 1955) and by calculating the infection rates. To evaluate the effect of double feedings on plasmodial development in the midgut, the oocyst production of the test and control groups was compared, a procedure which – as a consequence of the non comparable sizes of the infective blood meals – could not be done without introduction of a correction factor. Based on quantitative and statistical analyses of plasmodial infection in mosquitoes (Eyles, 1951) the hypothesis, that under our working conditions there is a linear, positive correlation between blood meal size and oocyst number, was tested and found to be useful (Gass, 1977a). As a consequence of these results, in the double feeding experiments the oocyst number of each single mosquito of the test group, which always ingested a smaller amount of infective blood compared to the control group, was corrected with a factor

$$k = \frac{\text{mean weight BM } I^+}{\text{mean weight BM } II^+}$$

The effects of the double feedings on the oocyst numbers could afterwards be evaluated by calculating the ratio of the median of the corrected oocyst numbers of the test group and the median of the control group.

The statistical comparison of the oocyst numbers was carried out with the U-test of Wilcoxon, Mann and Whitney using the formula of McNemar for the significancy test (Sachs, 1969). This comparison was exclusively calculated on the basis of oocyst carriers in both the test and the control group. The inaccuracy by unequal restriction of the two samples was neglected.

In addition to oocyst numbers, the infection rates of the mosquitoes were calculated. These were also dependent on the size of the infective blood meal, but in contrast to the oocyst numbers

they were not rendered comparable by the introduction of a correction factor, because the correlation between them and the blood meal size was found to be positive, but not linear (Eyles, 1951). Characteristics of the infection rates could therefore not be used as a significant measure of the effect of the double feeding experiments on oocyst production.

Assay of tryptic activity

Trypsin-like activity (MTLA = mosquito-trypsin-like-activity) of double fed mosquitoes was determined under conditions similar to those of the above described experiments. Deep frozen lots of 10 (exceptionally 8 or 9) blood fed females were thawed, mixed with 0.2 ml Tris-Cl buffer (0.1 M Tris, 0.02 M $CaCl_2$, pH 8.0), homogenized in a ice-cold glas-homogenizer and then centrifuged for 60 min at 26000 *g* in a refrigerated centrifuge. The clear supernatant was directly used for MTLA determination.

The assays, run in duplicate, followed the method of Hummel (1959). TAME (Nα-p-toluenesulphonyl-L-arginine methylester HCl, Calbiochem) was used as specific substrate for trypsin. The hydrolysis products were determined spectrophotometrically at 247 nm at a temperature of 30° C.

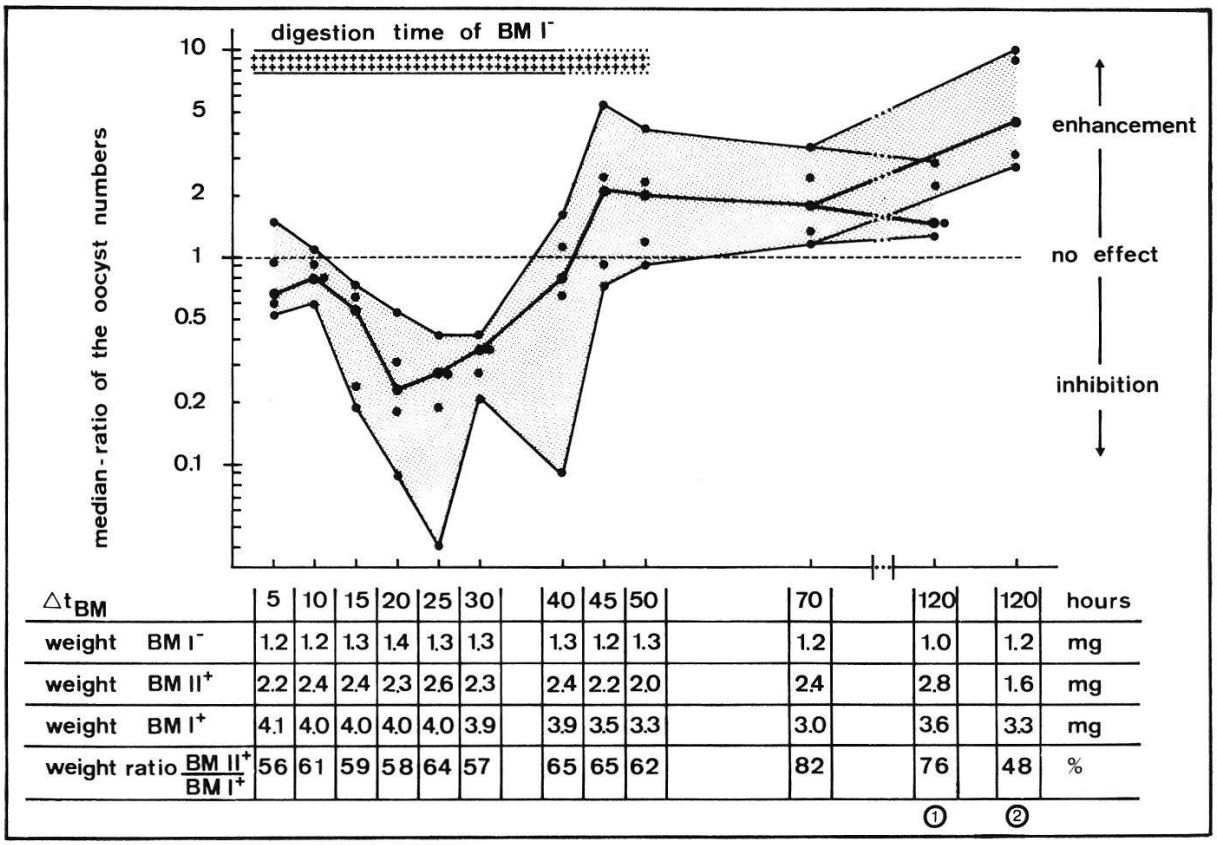
Results

Oocyst production in double fed mosquitoes

The effects of double feedings on oocyst production of *Plasmodium gallinaceum* in *Aedes aegypti* were measured in 5 independent experiments for each time interval Δt_{BM} between the uninfective BM I^- and the infective BM II^+. The results, representing only the effect upon oocyst numbers, are shown in Fig. 1 and demonstrate two different kinds of effects:

a) An inhibition of oocyst production is recorded at Δt_{BM} = 4–40 h, a time which corresponds approximately to the digestion time of the interrupted BM I^-. This indicates that the influence on plasmodial development is directly due to the previously ingested BM I^-, which is still present in the mosquitoes' midgut at the time of BM II^+. The inhibition curve shows first increasing, then decreasing inhibitory activity of BM I^- with a maximum at Δt_{BM} = 20–25 h. The extraordinary strong inhibition in one experiment at Δt_{BM} = 40 h has probably to be explained by a prolonged digestion time caused by unidentified factors.

b) If BM II^+ is ingested after complete digestion of the previous BM I^-, i.e. at $\Delta t_{BM} \geqslant$ 45–50 h, an enhancement of oocyst production occurs. This effect is strongest at Δt_{BM} = 45–50 h and tends to decrease at higher Δt_{BM}. The data about blood meal weights in Fig. 1 demonstrate that the mosquitoes of the test group compared to those of the control group always took smaller infective blood meals, even when they could ingest the blood into an empty midgut. The weight-ratios (weight of BM II^+ in percent of BM I^+) demonstrate that with growing Δt_{BM} the mosquitoes of the test group started to engorge higher blood amounts. It was therefore supposed that the decreasing tendency of enhancement and the increasing, relative weight of BM II^+ could be correlated. Such a hypothesis was supported by 5 additional experiments at Δt_{BM} = 120 h (Fig. 1: 120_2 h), where a reduction of the weight-ratio by interruption of BM II^+ produced a clear increase in oocyst numbers.



Δt_{BM}	5	10	15	20	25	30	40	45	50	70	120 ①	120 ②	hours
weight BM I⁻	1.2	1.2	1.3	1.4	1.3	1.3	1.3	1.2	1.3	1.2	1.0	1.2	mg
weight BM II⁺	2.2	2.4	2.4	2.3	2.6	2.3	2.4	2.2	2.0	2.4	2.8	1.6	mg
weight BM I⁺	4.1	4.0	4.0	4.0	4.0	3.9	3.9	3.5	3.3	3.0	3.6	3.3	mg
weight ratio $\frac{\text{BM II}^+}{\text{BM I}^+}$	56	61	59	58	64	57	65	65	62	82	76	48	%

Fig. 1. Oocyst numbers of *P. gallinaceum* after double feedings of *Aedes aegypti.* Each point represents the ratio: median of the corrected oocyst numbers (test group)/median of the oocyst numbers (control group) on a logarithmic scale and is plotted against the time Δt_{BM} between the two blood meals. The extreme values are connected with thin, the median values with thick lines. At Δt_{BM} = 120 h, the results after a complete BM II⁺ and an interrupted BM II⁺ are marked with ①, resp. ②. The weight ratios in the table below are calculated on the basis of all single experiments.

As the test groups always took smaller infective blood meals than the control groups, they were expected to develop smaller infection rates. Fig. 2 demonstrates that this is in fact so at Δt_{BM} = 5–50 h. At higher Δt_{BM} this clearly changes as the mosquitoes of the test groups had higher infection rates although they had engorged smaller amounts of infective blood. To get better information about the effects of double feedings upon the infection rates, the difference of the infection rates between test and control groups, expressed in percent of the infection rate of the test group, were plotted against Δt_{BM} (Fig. 3). The resulting curve is clearly similar to that in Fig. 1 and reflects in the same way the inhibiting and enhancing influences of the experimental procedure. In contrast to Fig. 1, the slight decrease of enhancement with growing Δt_{BM} is not manifested in the infection rates. However, the enhancing effect of a lowered relative weight of BM II⁺ ($\Delta t_{BM} = 120_2$ h) is clearly reflected.

The results of the statistical analysis for significance of the inhibition and enhancement of oocyst production, which is only based on the comparison of oocyst numbers, are shown in Fig. 4. They clearly demonstrate that at all tested

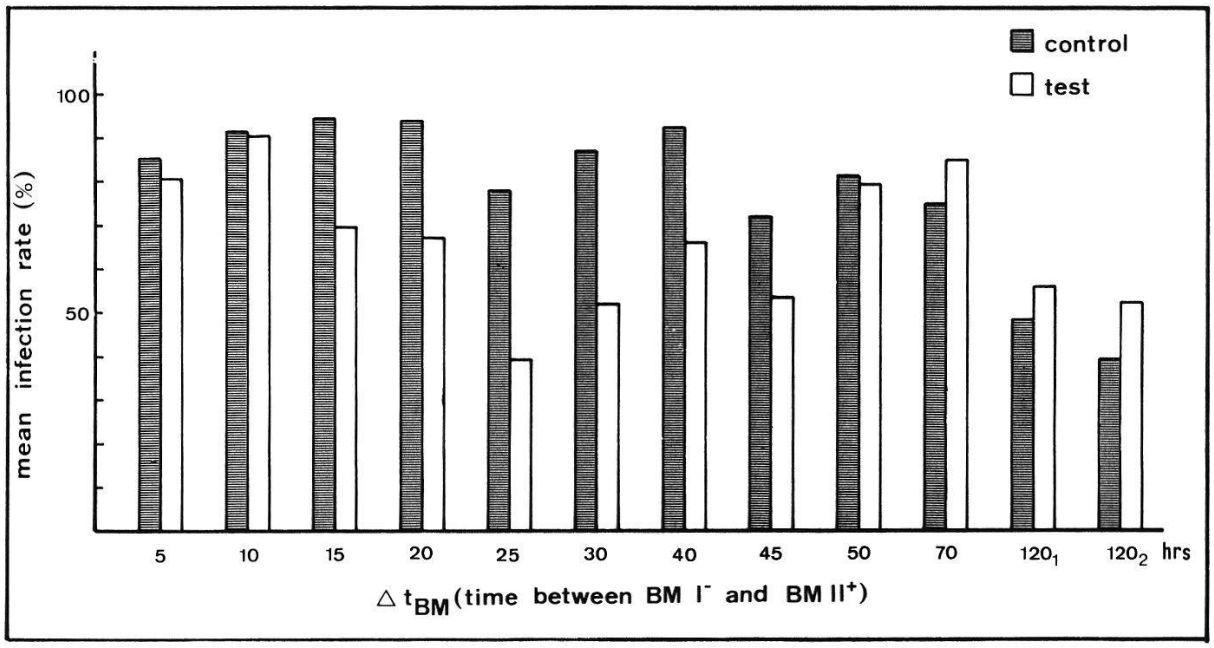


Fig. 2. Infection rates (*P. gallinaceum*) of *Aedes aegypti* after double feedings.

$\Delta t_{BM} = 120_1$: complete BM II$^+$

$\Delta t_{BM} = 120_2$: interrupted BM II$^+$

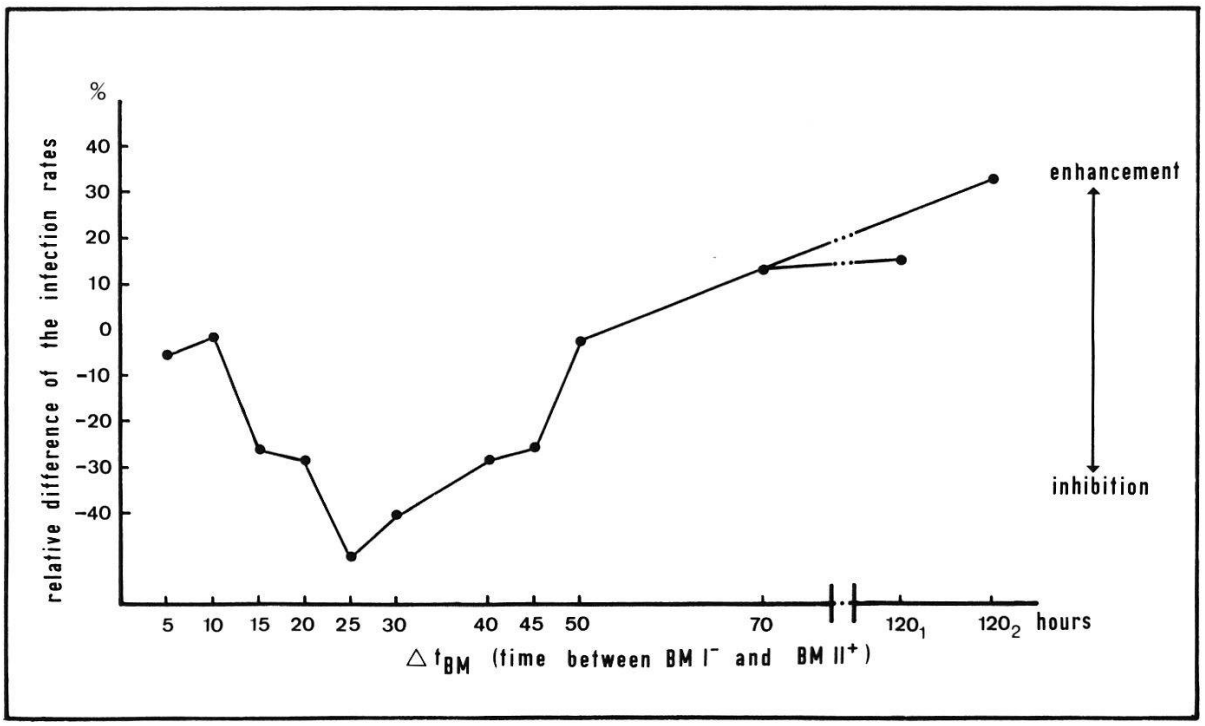


Fig. 3. Differences of the infection rates (*P. gallinaceum*) between test and control groups of *Aedes aegypti* in percent of the test groups after double feedings.

$\Delta t_{BM} = 120_1$: complete BM II$^+$

$\Delta t_{BM} = 120_2$: interrupted BM II$^+$

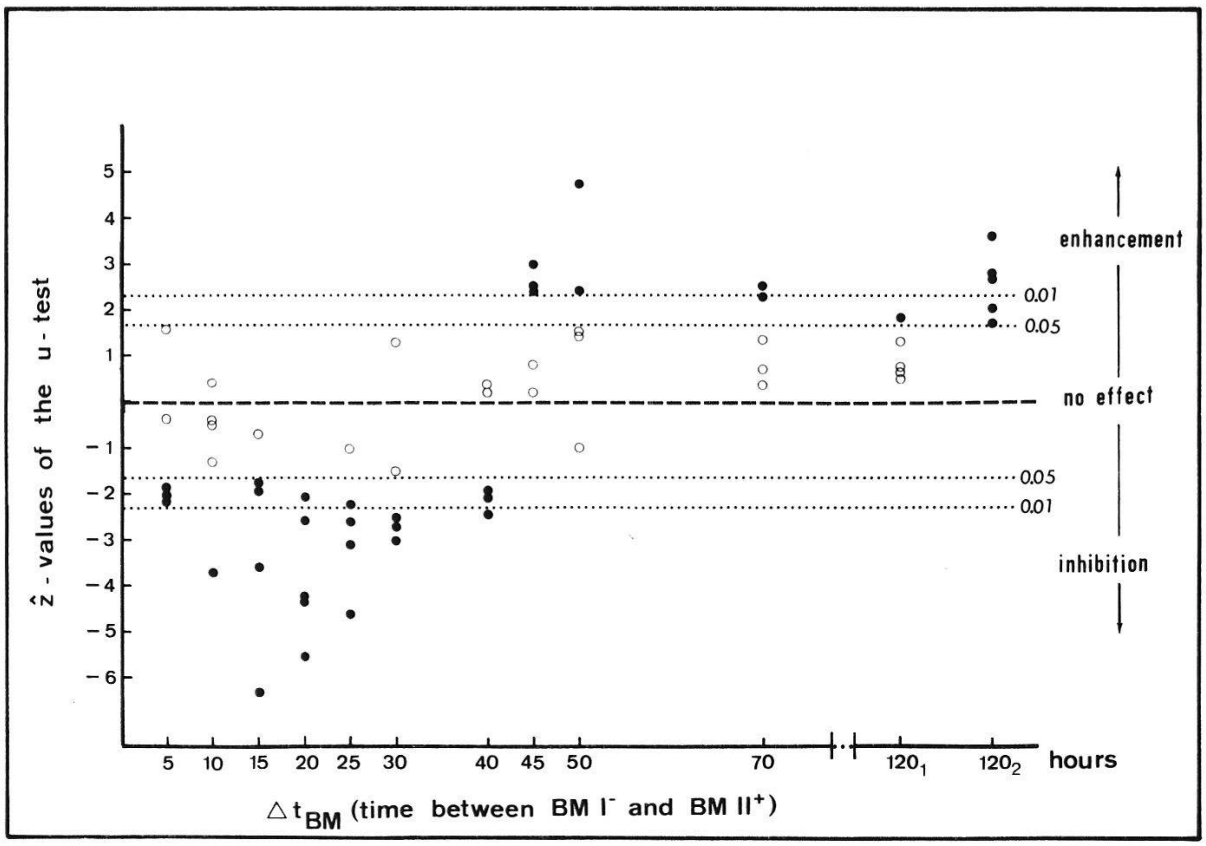
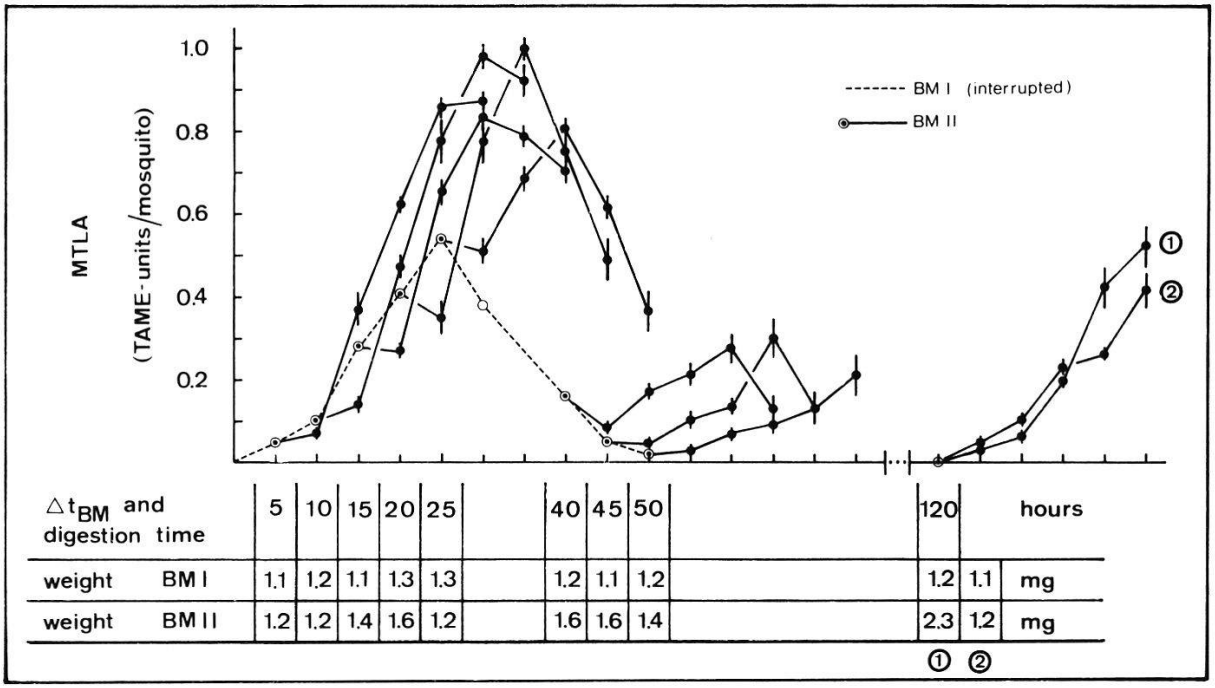


Fig. 4. Statistical analysis of the differences in oocyst numbers between control and double fed mosquitoes (*Aedes aegypti/P. gallinaceum*). The 0.05 and 0.01 levels of significance are marked with dotted lines. Negative ẑ-values mean smaller oocyst numbers in the test group.

● differences, significant on the level 0.05
○ differences, not significant
$\Delta t_{BM} = 120_1$: complete BM II$^+$
$\Delta t_{BM} = 120_2$: interrupted BM II$^+$

Δt_{BM} significant differences between the oocyst numbers of the test and control group occur, which are unlikely to be due to the introduction of a correction factor (see "Materials and methods" and Gass, 1977a). Inhibition is shown to be most pronounced at $\Delta t_{BM} = 20$ h with 5 significant results. The enhancement of oocyst production is shown to be less effective than the inhibition. This is expressed in lower numerical values $|\hat{z}|$ and lower numbers of significant results. However, the ẑ-values were positive in all experiments (except one at $\Delta t_{BM} = 50$ h), indicating that this enhancement is real. Furthermore Fig. 4 also reflects the above stated decreasing enhancement from $\Delta t_{BM} = 45$–120 h and the importance of the relative weight of the infective blood meal BM II$^+$ ($\Delta t_{BM} = 120_2$ h).

The shape of the inhibition curve showing a maximum at $\Delta t_{BM} = 20$–25 h is very similar to that of proteolytic activity in a single blood meal as it has been demonstrated by several authors (Gooding, 1972, review). We therefore postulated that the proteolytic activity (MTLA) in BM I$^-$ at the time of the intake of



Δt_{BM} and digestion time	5	10	15	20	25	40	45	50	120		hours
weight BM I	1.1	1.2	1.1	1.3	1.3	1.2	1.1	1.2	1.2	1.1	mg
weight BM II	1.2	1.2	1.4	1.6	1.2	1.6	1.6	1.4	2.3	1.2	mg
									①	②	

Fig. 5. MTLA after double feedings ± standard errors in *Aedes aegypti.* Each point represents the mean of 8–10 experiments, each experiment carried out with a pool of 8–10 mosquitoes. The mean blood meal weights for each Δt_{BM} are listed in the table below. At Δt_{BM} = 120 h, the results after a complete and an interrupted second blood meal are marked with ①, resp.②.

the parasites might be an important factor for plasmodial development and decided to investigate the course of MTLA in double fed mosquitoes.

Trypsin-like activity in the midgut of double fed mosquitoes

A further series of experiments was carried out, designed to investigate the possibility that plasmodial development is influenced by the level of trypsin-like activity (MTLA) in the gut. The mean weight of the first blood meal was the same as in the earlier experiments (1.2 mg) but the second one was lower (1.5 opposed to 2.4 mg). For all MTLA determinations uninfective blood meals were given to the mosquitoes. The MTLA was only measured up to 25 h from the ingestion of the second blood meal, i.e. during a digestion time which is of real importance for the sporogonic gut-stages of the plasmodia.

The results (Fig. 5) demonstrate that BM II produces first a 5-hour-period with reduced MTLA, which could be due to the inhibitory action of the serum (Gooding, 1966a; Huang, 1971). When BM II is taken at a time which is characterized by increasing or maximum MTLA during the digestion of the previous BM I, i.e. at Δt_{BM} = 5–25 h, strong stimulation of enzyme secretion is recorded. Maximum MTLA reaches thereby twice the amount that occurs in an interrupted blood meal and is also clearly higher than after a complete blood meal (Fig. 6).

In clear contrast to this are the results at Δt_{BM} = 40–50 h. BM II taken immediately after the completed digestion of BM I stimulates only low MTLA,

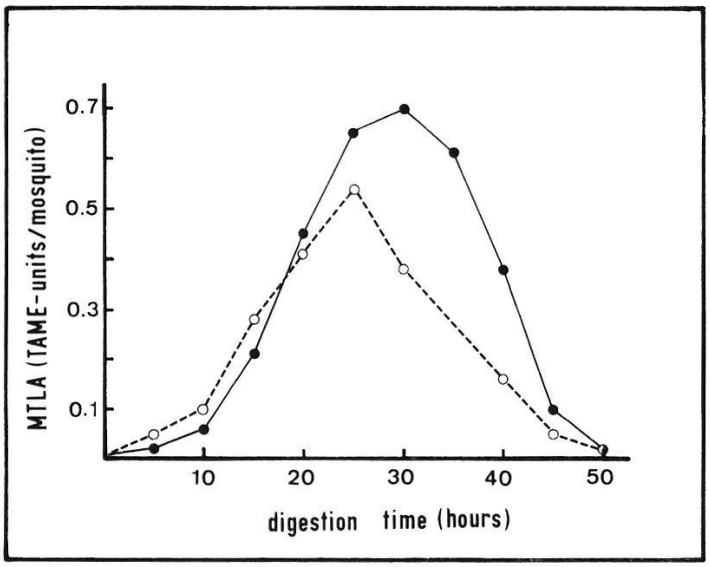


Fig. 6. MTLA after a complete blood meal (●——●/ mean weight 2.3 mg) and a interrupted blood meal (○----○/ mean weight 1.2 mg) in *Aedes aegypti.*

reaching a maximum of only 50% of that in BM I. These findings point to a post-digestive exhaustion period. This idea is supported by quantitative, ultrastructural examinations of the midgut cells (Hecker and Rudin, in prep.). At Δt_{BM} = 120 h, i.e. 3 days after the completed digestion of the previous blood meal, the midgut seems to have recovered and the second blood meal is digested with a similar MTLA pattern to the first one. The MTLA is somewhat lower, noticeably after digestion times of 20 and 25 h (Fig. 6 and Fig. 5 at Δt_{BM} = 120 h). However significant differences are only recorded for interrupted blood meals (Student t-test).

Discussion and conclusions

Double feedings of *Aedes aegypti* are demonstrated to have inhibiting and enhancing effects on oocyst production of *P. gallinaceum.*

Inhibition of oocyst production

Inhibition occurs when the second, infective blood meal is ingested during the degestion of a previous, uninfective one, i.e. when the two blood meals overlap. As under these conditions blood digestion follows a modified course (Fig. 5), the living environment of the developing plasmodial parasites is altered. The recorded inhibition of their development points to an inadequate adaptation to conditions in the midgut.

Two plausible mechanisms have been suggested for the influence of blood digestion in mosquitoes on plasmodial development. Firstly, Gooding (1966b, 1972) has proposed that a change in mosquito digestion could lead to a change in nutrient levels and hence death of plasmodia by starvation. This hypothesis

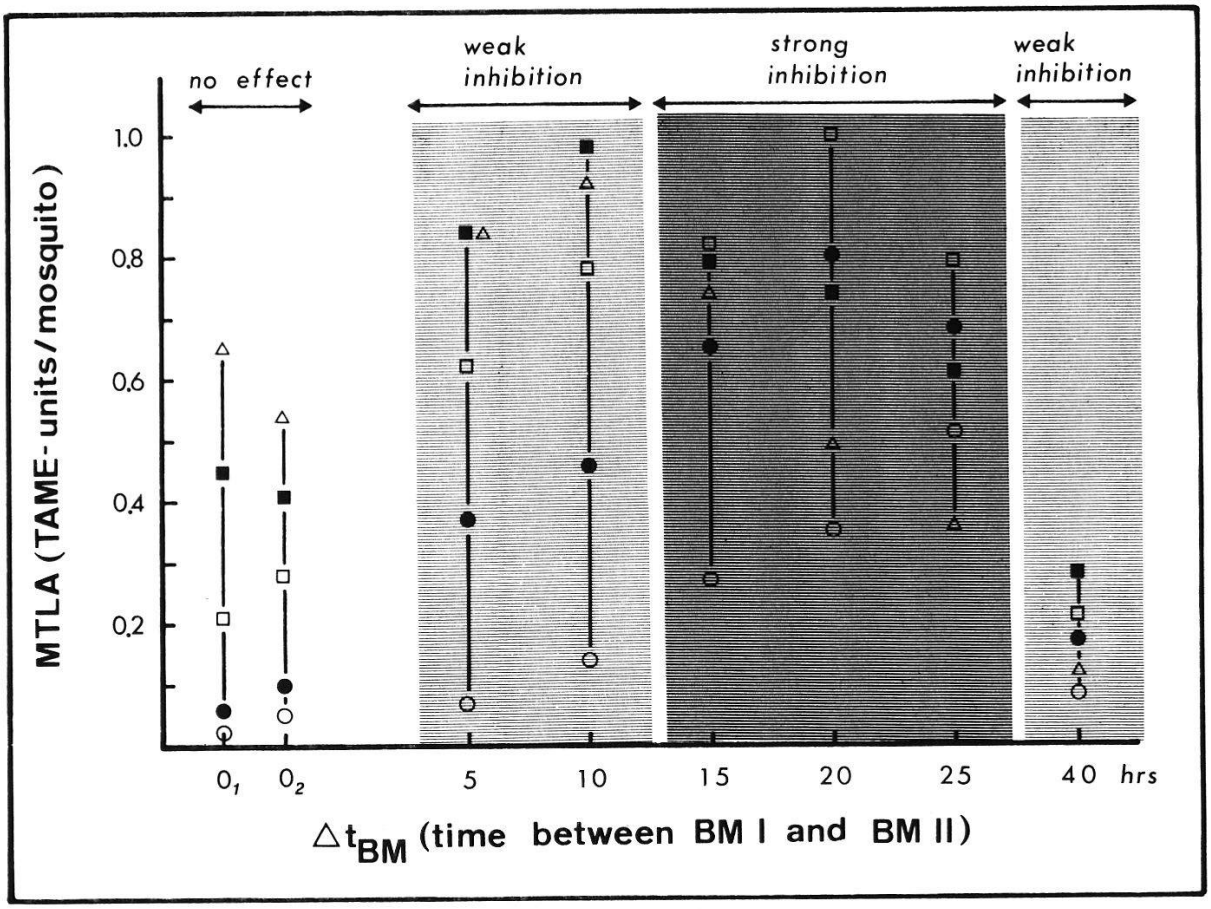


Fig. 7. Ambient MTLA-levels for various ookinete stages after double feedings producing inhibition of oocyst production (*Aedes aegypti*/*P. gallinaceum*).
0_1: complete, single blood meal
0_2: interrupted, single blood meal
Plasmodial development stages (hours after blood meal):
○ 5 h □ 15 h Δ 25 h ● 10 h ■ 20 h

cannot explain our inhibition experiments. It rather seems that after double feedings the nutrient of the parasites is improved, because they enter an environment in which nutrients, provided by the digestion of the previous blood meal, are already available.

The second mechanism whereby mosquito digestion could alter plasmodial development is through changes in the level of destructive digestive enzymes of the host (Gooding, 1966b, 1972; Briegel and Lea, 1975; Maier 1976). The digestive enzymes of *Aedes aegypti* which have been examined are trypsin-like protease, invertase, amylase, non-specific esterase and triglyceride lipase (Gooding, 1972, 1975, reviews; Briegel and Lea, 1975; Geering and Freyvogel, 1974, 1975; Kunz, 1977; Yang and Davis, 1968). Of these we consider the trypsin-like protease the most probable cause of plasmodial destruction – the invertase rises and falls too quickly to explain our results; the triglyceride lipase and amylase are only present at very low levels; non-specific esterase is implausible on chemical grounds as an agent of cell destruction. We therefore confine the follow-

ing discussion to the possible role of MTLA. It should be borne in mind that the measured enzyme levels are an average over different compartments in the gut – compartments which are built up firstly by the digestion proceeding inwards from the periphery of the blood clot and secondly by the formation of two peritrophic membranes after double feedings (Freyvogel and Stäubli, 1965).

Fig. 7 contains a further analysis of our inhibition experiments. The ambient MTLA-level is plotted against the time between the two blood meals for plasmodia at different stages of development. This shows:

a) Compared to a single blood meal plasmodia 5 and 10 h after the infection are always exposed to increased MTLA; this reaches its maximum at Δt_{BM} = 15–25 h, when strongest inhibition of oocyst production is recorded.

b) Compared to a single blood meal plasmodia 15 h and more after the infection are also exposed to increased MTLA. However for these forms the highest ambient MTLA-levels are observed when inhibition of oocyst production is low (Δt_{BM} = 5–10 h).

A further important point is shown in Fig. 5: Compared to a single blood meal prezygotic stages (gametocytes and gametes) are always exposed to increased MTLA. However it is remarkable that at Δt_{BM} = 5 h significant inhibition occurs (Fig. 4) though these forms are exposed only to very low MTLA, while plasmodia 5 and 10 h after the infective blood meal are exposed to very high enzyme activity (Fig. 7). For this reason it seems probable that the inhibition of oocyst production which we observed at Δt_{BM} = 5 h was caused by destruction of young ookinetes rather than of prezygotic stages.

It follows that our results can only be explained by the sensitivity to MTLA of those forms of plasmodia existing 0–10 h after the infection of the mosquito.

Enhancement of oocyst production

If the infective blood meal is ingested after the completed digestion of a previous, uninfective one, on enhancement of oocyst production is recorded. Terzian et al. (1956) obtained similar results with *Aedes aegypti* and *P. gallinaceum* with a interval of 9 days between the two meals. In this work it is shown that the enhancement occurs as soon as the parasites enter a gut in which the digestion of the first blood meal is finished and is demonstrated up to Δt_{BM} = 120 h, the degree of enhancement probably being negatively correlated with the size of the infective blood meal.

Akov (1966) has reported that *Aedes aegypti* digests a second blood meal more slowly than the first, even with a interval between the two blood meals of several days. This seems plausible after our findings that MTLA is decreased in the second blood meal. We therefore are again unable to explain the enhanced oocyst production by improved nutrition for the parasites.

The analysis of the ambient MTLA values for different development stages of plasmodia (Fig. 8) shows:

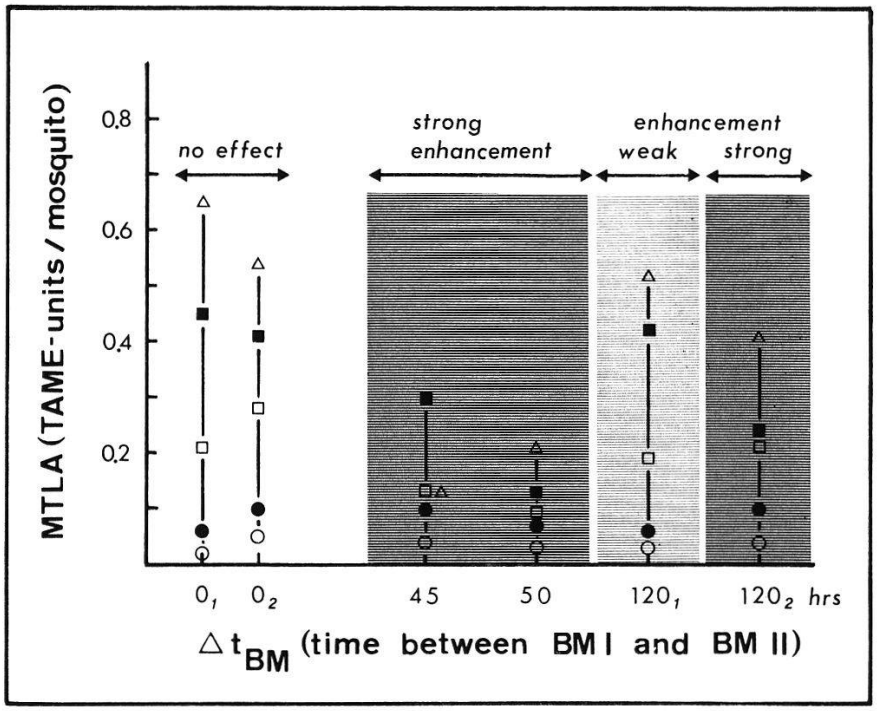


Fig. 8. Ambient MTLA-levels for various ookinete stages after double feedings producing enhancement of oocyst production (*Aedes aegypti/P. gallinaceum*).
0_1: complete, single blood meal
0_2: interrupted, single blood meal
Plasmodial development stages (hours after blood meal):
○ 5 h □ 15 h Δ 25 h ● 10 h ■ 20 h

a) Compared to a single blood meal plasmodia 5 and 10 h after infection are not exposed to altered MTLA.

b) Compared to a single blood meal plasmodia 20 and 25 h after infection at Δt_{BM} values of 45, 50 and 120_2 h are always exposed to lower MTLA. Under these conditions a strong enhancement of oocyst production is recorded.

c) At $\Delta t_{BM} = 120_1$ h the plasmodia 20 and 25 h after infection are exposed to MTLA similar to that in a single, interrupted blood meal. Under these conditions a weak enhancement is recorded.

This data lead to the suggestion that at least two factors cause the observed enhancement. The first is proposed to be the improved environment for plasmodia 20 and 25 h after the infective blood meal, caused by decreased levels of trypsin-like enzymes. The other factor(s) are yet undetermined. Without the positive effect of decreased MTLA on plasmodial development they still produce a weak enhancement of oocyst production.

Conclusions

Our data provide strong evidence that in double fed mosquitoes the survival of plasmodia is critically influenced by the levels of the surrounding digestive enzymes of the host. Almost all our data can be explained in terms of the

levels of trypsin-like protease in the mosquito gut, suggesting that this enzyme is a major agent of plasmodial destruction. Analysis of our inhibition data indicates that the plasmodial stages between 0 and 10 h after infection of the mosquito are most sensitive to destruction by the host protease. This suggests some sort of developmental adaptation of the parasite to the host's digestive processes; a hypothesis which is being investigated further.

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