The relationship between microfilarial load in the human host and uptake and development of *Wuchereria bancrofti* microfilariae by *Culex quinquefasciatus*: a study under natural conditions

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SUMMARY

The uptake of *Wuchereria bancrofti* microfilariae (Mf) by *Culex quinquefasciatus* and their development in relation to human Mf density were quantified by allowing a total of 1096 wild mosquitoes to feed on 13 volunteers sleeping under partially open bed-nets. For each volunteer, each hour between 18.00 and 06.00 h the Mf density in finger-prick blood was determined and engorged mosquitoes collected. Each hourly collection of mosquitoes was kept separately. Half of them was dissected within 18 h post-feeding for the presence of ingested Mf, the other half was reared for 12 days to allow for the development of L3 larvae. About 20% of the latter mosquitoes died during these 12 days and these harboured significantly more larvae than the surviving ones, which could be an indication of excess-mortality among heavily infected mosquitoes. Assuming that variability in Mf uptake and in the number of developed L3 larvae can be described by a negative binomial distribution, a maximum-likelihood procedure was applied to estimate the relationship between human Mf density and both the arithmetic mean Mf uptake and L3 development. Both were adequately described by a saturating hyperbolic function that significantly differed from linearity. The saturation level for Mf was estimated at 29 (CI: 20–54) and for L3 larvae at 6·6 (CI: 4·3–17·0). Next, the L3 yield was related to Mf uptake indicating that the *W. bancrofti–C. quinquefasciatus* complex shows 'limitation', i.e. a decreasing yield for an increasing uptake. Both the number of Mf ingested and the number of L3 larvae developing per mosquito were found to be highly aggregated, with the level of aggregation decreasing in a non-linear way with human Mf density.

Key words: *Wuchereria bancrofti*, *Culex quinquefasciatus*, mosquito feeding, microfilarial uptake, larval development, density dependence.

INTRODUCTION

The capability of vector mosquitoes to ingest microfilariae (Mf) of filarial parasites and to support their development after ingestion are important determinants of filariasis transmission (Bryan, McMahon & Barnes, 1990). Three processes, namely (1) uptake of Mf from the human host; (2) development of Mf to the infective-stage larvae (L3) and (3) transmission of L3 to human, determine the overall vector competence. Laboratory studies have demonstrated that the uptake of Mf by mosquitoes depends on the density and distribution of Mf in the human host (Bryan & Southgate, 1988*a*; Samarawickrema *et al*. 1985). For the number of L3 larvae developing from a particular number of Mf ingested, 3 possible relationships have been described (Pichon, 1974; Southgate & Bryan, 1992): *proportionality*, i.e. a constant ratio ($\leqslant 1$) of L3 to ingest Mf, *facilitation*, i.e. an increase in this ratio, and *limitation*, the converse of facilitation. Proportionality has been observed for the *Brugia malayi* (filarial parasite) – *Aedes togoi* (vector) combination in experimental cats and for *B. malayi* – *Mansonia bonneae*. Facilitation was found for *Wuchereria bancrofti* – *Anopheles gambiae*, *W. bancrofti* – *An. arabiensis* and *W. bancrofti* – *An. merus* and limitation for *W. bancrofti* – *Culex quinquefasciatus* (Southgate & Bryan, 1992). Although the epidemiological significance of such vector–parasite relationships has been widely discussed (Brengues & Bain, 1972; Pichon, 1974; Pichon, Perrault & Laigret, 1974; Southgate, 1992*a*,

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b), very little theoretical work has been done to demonstrate their effect under control programmes (Dye, 1992). The argument is that in the case of limitation it would be difficult to totally interrupt transmission even when control programmes reduce Mf prevalence and intensity to very low levels; whereas in the case of facilitation it would be relatively easier to block transmission and eradicate the parasite from the human population. However, as argued by Dye (1994) and Dye & Williams (1995) in speculating about the epidemiological impact of the different parasite–vector relationships it is also crucial to take into account the (often considerable) variation in larval uptake and development and not only consider mean values.

Unlike the *Anopheles* – *W. bancrofti* combination, quantification of the vector parasite relationship in *Culex* – *W. bancrofti* has received little attention (Southgate & Bryan, 1992). For a periodic strain of *W. bancrofti* it has been observed that the number of Mf ingested by *C. fatigans* (*C. quinuefasciatus*) is non-linearly related to the human Mf density (McGreevy *et al*. 1982). There are also reports indicating linearity in this vector parasite combination (Jordan & Goatly, 1962; Obiamiwe, 1977; Lowrie *et al*. 1989; Jayasekera, Kalpage & De Silva, 1991). It is further known that a large proportion of the ingested Mf is lost during the development to L3 both in the laboratory (Jordan & Goatly, 1962) and in nature (Subramanian *et al*. 1994).

All earlier reports are based on laboratory studies. Apart from the unnatural conditions, a drawback of such studies is that they often use a single cohort of vector mosquitoes which are allowed to feed on a restricted part of the body of a human Mf carrier. It has been suggested that, while studying the uptake and development of larvae in relation to human Mf density for anticipating the effects of proposed control programmes, it is essential to perform these studies under natural conditions, using local strains of mosquito and parasite (Southgate & Bryan, 1992). Therefore, such a study was carried out in Pondicherry, India, endemic for periodic *W. bancrofti* transmitted by *C. quinquefasciatus*. Wild mosquitoes, representing overlapping generations, were allowed to engorge on infected human volunteers under natural conditions and were collected throughout the night. Hourly collections were analysed in relation to the human parasite density. Using these data, attempts are made to quantify the relationship between human Mf density, with its periodicity in the host blood, and the uptake of Mf and output of L3 by mosquitoes. The Mf uptake and development of the parasites are considered as distinct processes in order to examine the evidence for density dependence in both processes separately. In analysing the data from the experiments, assumptions will be tested about the heterogeneity of uptake and development.

MATERIALS AND METHODS

Study design

From the Mf carriers, who were detected during a night blood survey in and around Pondicherry, a total of 13 carriers with varying Mf counts (1–394 Mf/20 μl of finger prick blood) and covering an age range of 10–50 years was selected for the present study. Only male patients were chosen in view of poor co-operation of female patients due to social and cultural factors. Informed oral consent of each patient was obtained before starting the collections. Each patient slept under a mosquito net ($1 \times 2{\cdot}5 \times 2$ m) in his own house, with one side of the net partly open for the entry of wild mosquitoes.

Fully fed mosquitoes which were resting inside the net were collected at hourly intervals from 18.00 to 06.00 h. Following collection, mosquitoes were released into 1 cubic foot mosquito cages and transported to the laboratory. Hourly collections from each patient were kept separately. As the vector mosquitoes have the habit of resting on a nearby object after feeding, the chance of missing fully fed mosquitoes is negligible.

Half of each hourly collection was dissected on the following morning to assess the uptake of Mf. Each mosquito was teased into pieces in a few drops of saline to examine for the presence of Mf and other developmental stages of the parasite. The results of these immediate dissections, carried out within 18 h of the time of collection, were considered to represent the number of Mf ingested at the bloodmeal. This is justified because there exists no evidence of loss of Mf through dejecta in this mosquito species (Kartman, 1953; Jordan & Goatly, 1962). The remaining mosquitoes were reared for 12 days at 26–28 °C and 70–80 % relative humidity. They were maintained on raisin, and ovi-traps were provided for oviposition. Every day raisins were changed, ovi-traps were replaced and all dead mosquitoes were dissected for determining parity status and counting the stage-specific number of filarial larvae. Parity of the mosquitoes was determined by counting the number of dilatations following the method of Polovodova (Detinova, 1962). On the 13th day after collection, all surviving mosquitoes were dissected for infection and parity status. Since no subsequent bloodmeal was provided, mosquitoes laid eggs only once during the period of observation. Hence, parity of mosquitoes on the day of capturing was determined by subtracting 1 from the number of dilatations observed on the 13th day.

From 18.30 until 05.30 h, hourly samples comprising 2 or 3 smears of 20 μl were prepared from finger-prick blood. These moments coincide with the mid-point of each hour of mosquito collection. Paired observations of blood Mf counts (the arithmetic mean of the smears) and mosquito dis-

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sections will be henceforth referred to as 'patient hours' (theoretically 12 hours times 13 patients = 156 patient hours). All volunteers were treated with a standard course of DEC after the experiment.

Statistical methods

The hourly sampling of blood combined with the hourly catches of mosquitoes (patient hours) were used to quantify the relationship between the human Mf density (20 μl of blood) and Mf uptake by the mosquitoes or the number of L3 larvae developing in mosquitoes.

Since visual inspection of the (arithmetic) mean number of parasites W in the mosquitoes (Mf or L3) in relation to the human Mf density m suggested a saturation at high values for m, the following hyperbolic function was used to describe this relationship:

$$W(m) = a + \frac{bm}{1+cm}. \quad (1)$$

The interpretation of equation (1) is as follows. The parameter a (intercept), suggesting the possibility of infection of mosquitoes even when $m = 0$, is included to account for false-negative human Mf counts. At high human Mf counts, the relationship approaches a saturation level c', with $c' = a + (b/c)$. The initial steepness of the increase of W with m is given by parameter b. Equation (1) was used to explore 3 possible relationships for W with m: (i) constant (b is indistinguishable from zero); (ii) linear (c is indistinguishable from zero) and (iii) nonlinear (hyperbolic: all parameters > 0).

In estimating the parameters of this function, it is assumed that for a given human Mf density m, the variation in the number of parasites/mosquito (either Mf or L3) can be described by a negative binomial distribution with mean W and some unknown overdispersion parameter k (clumping factor). We will explicitly test whether and how (constant, linear or nonlinear) this k depends on the human Mf count m using the following power function:

$$k(m) = k_0 + \alpha m^{\beta}. \quad (2)$$

The parameters of equations (1) and (2) are estimated using the maximum likelihood procedure outlined in the Appendix. An important feature of this procedure is that it is based on the larval counts in individual mosquitoes rather than on the mean count or fraction positives/patient hour. The likelihood ratio statistic (which is approximately Chi-square distributed with D.F. equal to the difference between the number of parameters in the models being tested (Clayton & Hills, 1993)) was used to test different assumptions pertaining to W and k.

In order to assess whether the hour of the night is a confounding variable for the uptake of Mf, we carried out a logistic regression analysis (using SPSS) relating the success to engorge at least 1 Mf to the human Mf density and the hour of the night. Hour of the night was expressed as 1 for 18.00 to 19.00 h, 2 for 19.00 to 20.00 h, etc. In the regression equation we included hour itself, its logarithmic and quadratic transformation, and an interaction-term hour $\times m$ as independent variables. Non-significant variables were removed through backward elimination based on a likelihood-ratio test (Clayton & Hills, 1993).

The yield of L3 larvae, defined as ratio L3 output: Mf uptake (see Pichon, 1974; Southgate & Bryan, 1992), was determined for 9 classes of human Mf density (average of 2 or 3 smears, determined each hour of the night): 0–1, 1·3–4, 4·3–8, 8·3–25, 25·3–50, 50·3–100, 100·3–130, 130·3–200, and more than 200. These classes were chosen such that for each class, the numbers of mosquitoes immediately dissected and dissected after 12 days were at least 30. The observed yields will be compared with the expected yields based on a combination of the estimated relationships (equation (1)) for Mf uptake and L3 output. For a regular series of human Mf densities (0–300, steps of 1), both the expected Mf uptake (x) and the expected L3 output (y) were calculated, and for all these points the yield was expressed as y/x. This combination of the 2 estimated functions allows for all 3 Mf–L3 relationships mentioned in the Introduction section. If we disregard the intercept a, proportionality occurs if $c_{Mf} = c_{L3}$, limitation if $c_{Mf} < c_{L3}$, and facilitation if $c_{Mf} > c_{L3}$.

Throughout the manuscript mean values refer to (either or not weighted) arithmetic means.

RESULTS

Blood smear counts and mosquito collections

Paired observations of human blood Mf density and number of fully engorged *C. quinquefasciatus* are available for 119 (immediate dissection) and 62 (dissected after 12 days) patient hours. Both numbers

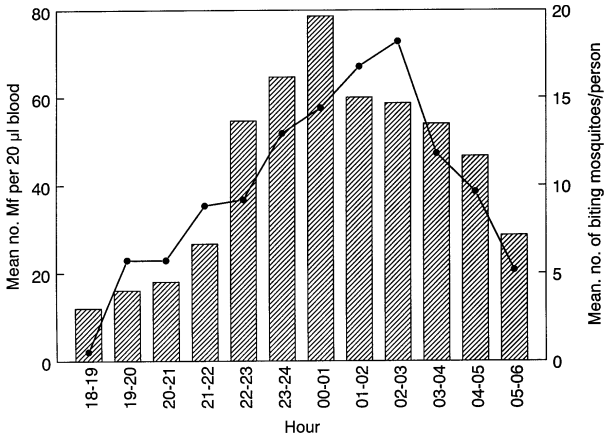

Fig. 1. Comparison of periodicity in vector biting (solid line) and microfilaria appearance in the peripheral blood of human (bars) in relation to hour of the night.

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Table 1. Summary of mosquito dissection results

	Immediate dissection	Died before 12 days of follow-up	Dissected after 12 days
Number of patient hours	119	29	62
Number of mosquitoes dissected			
All	592	104*	400
Nulliparous	314 (53 %)	53 (54 %)	114 (29 %)
1-parous	207 (35 %)	41 (41 %)	210 (53 %)
2- or 3-parous	71 (12 %)	5 (5 %)	76 (19 %)
Number (and %) of infected mosquitoes			
All	267 (45 %)	66 (63 %)	166 (42 %)
Nulliparous	140 (45 %)	28 (53 %)	42 (37 %)
1-parous	93 (45 %)	33 (80 %)	84 (40 %)
2- or 3-parous	34 (48 %)	2 (40 %)	40 (53 %)
Number with Mf	267 (45 %)	15 (14 %)	—
Number with L1	—†	15 (14 %)	—
Number with L2/L3	—	42 (40 %)	166 (42 %)
Number with L3	—	11 (11 %)	149 (37 %)
Mean‡ number of parasites (S.D.)/mosquito			
Mf	9·3 (26·2)	10·5 (45·7)	—
L1	—	1·6 (8·3)	—
L2/L3	—	6·4 (13·5)	4·2 (9·8)
L3	—	1·2 (5·8)	2·4 (5·7)
Mean‡ number of parasites (S.D.)/positive mosquito			
Mf	20·0 (35·6)	73·1 (99·4)	—
L1	—	11·3 (19·3)	—
L2/L3	—	15·9 (17·4)	10·0 (16·4)
L3	—	11·1 (14·5)	6·4 (7·8)

* Parity of 5 mosquitoes could not be determined since they dried up.
† Not applicable/none found.
‡ Arithmetic means.

are considerably smaller than the theoretically expected 156. There were 28 patient-hours, particularly during dusk and dawn, without biting mosquitoes and 9 patient-hours in which volunteers were reluctant for repeated finger pricking. For another 28 patient-hours only a few mosquitoes (2 or 3) were collected and all of them were dissected immediately for assessing the Mf uptake, leaving no mosquito for further observation on L3 development. Finally, in 29 patient-hours, all the mosquitoes died and were dissected before reaching 12 days post-feeding. A plot of human Mf load and the number of mosquitoes biting per volunteer against the hourly interval indicates that these variables coincide and both peak between 22.00 and 05.00 h (Fig. 1).

Dissection results

A summary of the dissection results is given in Table 1. None of the mosquitoes for examining Mf uptake died within the 18 h interval needed for dissection. Other developmental stages of the parasite together with Mf were observed in only 3·3 % of the immediately dissected mosquitoes, suggesting a previous infective bloodmeal. As a consequence, it is fairly unlikely that L3 larvae found after 12 days do not originate from the volunteers. Since shortly after feeding, Mf-positive mosquitoes harbour a considerable number of Mf (20 on average), the loss of infection after 12 days is much more apparent from the decline in larval load – from 9·3 to 4·2, i.e. about 55 % reduction – than the reduction of the percentage infected mosquitoes (which only declines from 45 to 42 %). Of the 504 mosquitoes kept for further observation on parasite development, 20 % died before reaching 12 days post-feeding. As many as 64 % of these dead mosquitoes harboured a parasite of any stage. This proportion is significantly higher than that observed in mosquitoes dissected after 12 days (42 %; $P < 0{\cdot}05$). Also the mean number of developing larvae (L2 or L3) per infected dead mosquito ($15{\cdot}9 \pm 17{\cdot}4$) was significantly higher ($P < 0{\cdot}001$) than in those dissected after 12 days ($10{\cdot}0 \pm 16{\cdot}4$). This could be an indication of parasite-induced mortality among heavily infected mosquitoes.

Mosquito parity

Table 1 also provides details of the parity status of the 3 groups of mosquitoes. The fraction (1-, 2-, and 3-) parous mosquitoes among those dissected immediately and those dying before 12 days follow-up is about equal ($\sim 0{\cdot}46$) and this makes it not very likely that mortality of the latter group is a

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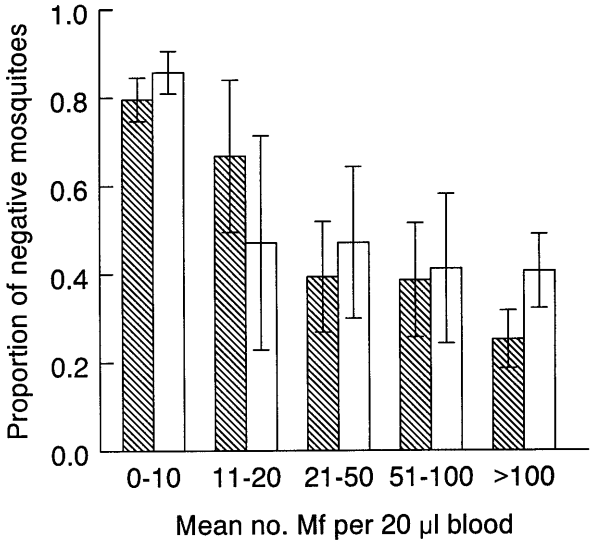

Fig. 2. Relationship between observed human microfilarial density and the number of mosquitoes failing to ingest Mf (hatched bars) and to develop infective L3 larvae (empty bars). Error bars are 95% confidence intervals.

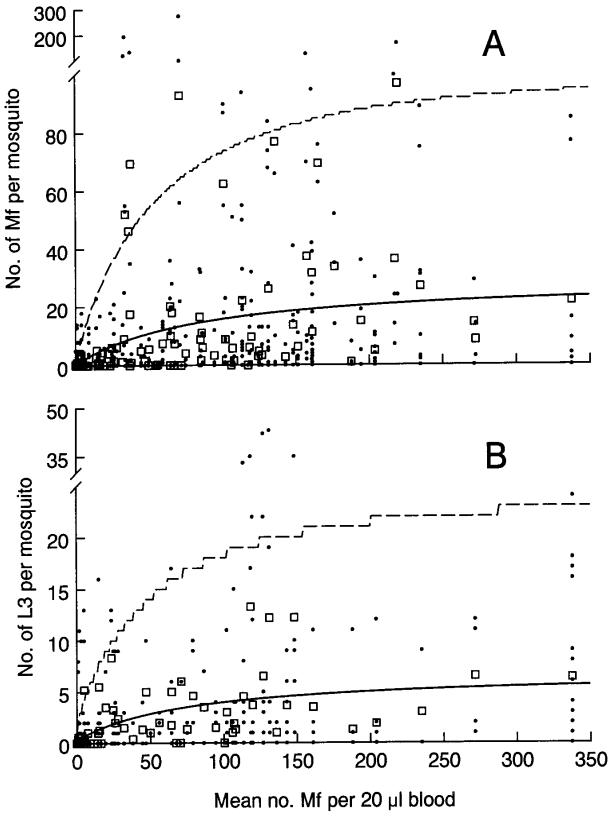

Fig. 3. Comparison of observed (dots) and predicted (solid line) number of microfilariae (A) and L3 (B) per mosquito in relation to human Mf density. The dashed line is the estimated 95th percentile of the negative binomial distribution of Mf or L3 in mosquitoes as a function of the human Mf density. The empty squares represent the arithmetic mean Mf uptake or L3 output for a particular human Mf density in a particular patient hour.

consequence of relatively older ages. Somewhat counter-intuitive (and difficult to explain) is the large proportion of parous mosquitoes among those dissected after 12 days (0·71; statistically significantly different from the other 2 groups, $P < 0{\cdot}05$). The opposite is to be expected should age be an important determinant for survival within the considered interval. None of the dissection results for the 3 groups pointed to statistically significant differences between nulliparous and parous mosquitoes. This applies to both the fraction infected ($P > 0{\cdot}05$ in all comparisons; see Table 1) and the mean number of parasites (Mann–Whitney U test for independent samples, $P > 0{\cdot}05$; data not shown).

Uptake and development of Mf in relation to human Mf density

As stated earlier, both Mf uptake and the number of developing larvae varied considerably among mosquitoes. From Fig. 2, showing the proportion of mosquitoes failing to engorge Mf or develop larvae, we can learn that this can only partially be explained from the differences in blood Mf density between patients and patient hours (see Discussion section). Although the failure rate declines with blood Mf density, even at high densities of more than 100 Mf/20 μl a considerable fraction of mosquitoes remains uninfected. The variability is also clearly shown in Fig. 3, where for all dissected mosquitoes the larval load (Mf or L3) is plotted against the human Mf density in a particular patient hour.

Results of fitting relationships

Fig. 3 also shows the estimated hyperbolic relationships for the Mf uptake and L3 output as a function of the human Mf density (equation (1)). The dashed line in these graphs is based on the estimated trend in the mean W and clumping factor k (equation (2)) and represents the 95% upper limit of the corresponding negative binomial distributions. Parameter estimates, including 95% confidence interval (CI), for the relationships are provided in Tables 2 (for Mf uptake) and 3 (for L3 output). In these Tables, estimates and log-likelihoods for the 'full models' (i.e. comprising all parameters) are compared with those for simpler hypotheses about how W and k vary with the human Mf density m. Both for Mf uptake and L3 output the full model results in a significantly better fit to the data than the simpler alternatives (likelihood ratio test, $P < 0{\cdot}05$ for all comparisons). We have also tested a more complicated relationship, in which the factor bm (see equation (1)) was replaced by bm^d (resulting in a sigmoid function if $d > 1$), but this did not improve the fit (d indistinguishable from 1), neither for Mf nor for L3. The intercept a of the hyperbolic functions for Mf and L3 is small when compared to the theoretical saturation level c'. Furthermore, the

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Table 2. Parameter estimates and associated log-likelihoods (LL) for the relations describing the Mf uptake, $W(m)$ (equation (1)), and the clumping factor of the negative binomial distribution, $k(m)$ (equation (2)), as a function of the human Mf density

(Values in parentheses are 95% confidence intervals for the full model.)

	Parameter estimates equation (1)				Parameter estimates equation (2)			
Hypothesis	a	b	c	$c' = a + b/c$	k_0	α	β	LL
Constant W	14·4	—*	—*	—	0·00786	0·0258	0·515	−1325·8
Linear $W(m)$	0·411	0·164	—*	—	0·0325	0·0521	0·334	−1300·6
Constant k	0·216	0·294	0·00865	34·2	0·231	—*	—†	−1310·3
Linear $k(m)$	0·206	0·317	0·0108	29·6	0·121	0·00143	—†	−1296·1
Full model	0·168	0·342	0·0119	28·9	0·0304	0·0514	0·355	−1292·2
	(0·028–0·56)	(0·22–0·55)	(0·0045–0·026)	(20–54)	(0·004–0·11)	(0·008–0·105)	(0·20–0·68)	

* Not estimated, fixed to zero.
† Not estimated, fixed to 1.

Table 3. Parameter estimates and associated log-likelihoods (LL) for the relations describing the L3 output, $W(m)$ (equation (1)), and the clumping factor of the negative binomial distribution, $k(m)$ (equation (2)), as a function of the human Mf density

(Values in parentheses are 95% confidence intervals for the full model.)

	Parameter estimates equation (1)				Parameter estimates equation (2)			
Hypothesis	a	b	c	$c' = a + b/c$	k_0	α	β	LL
Constant W	3·62	—*	—*	—	< 0·0001	0·0401	0·435	−674·4
Linear $W(m)$	0·535	0·0288	—*	—	< 0·0001	0·0862	0·276	−658·6
Constant k	0·216	0·106	0·0178	6·17	0·260	—*	—†	−665·9
Linear $k(m)$	0·219	0·104	0·0170	6·34	0·135	0·00142	—†	−658·3
Full model	0·276	0·0904	0·0143	6·60	< 0·0001	0·0887	0·281	−654·4
	(0·010–0·65)	(0·041–0·24)	(0·0025–0·051)	(4·3–17·0)	(∼ 0–0·091)	(0·051–0·150)	(0·15–0·41)	

* Not estimated, fixed to zero.
† Not estimated, fixed to 1.

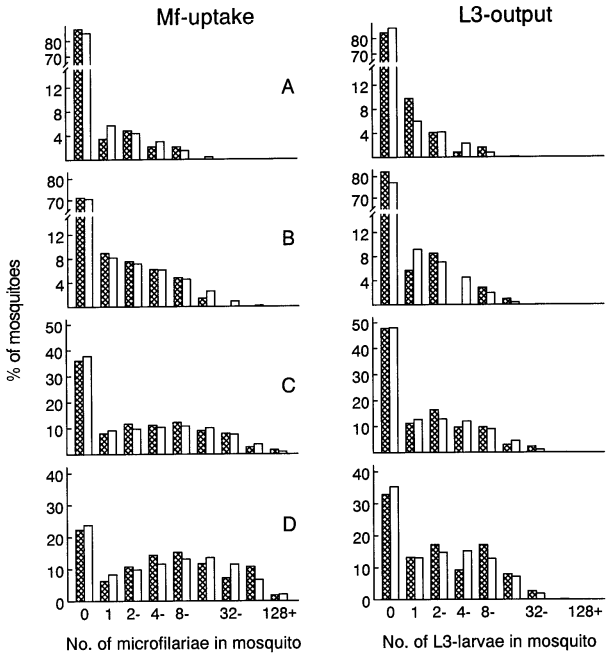

Fig. 4. Observed (hatched bars) and expected (empty bars) frequency distributions of larval counts (left series: Mf uptake; right series: L3 output) in relation to the human Mf density: (A) 0–2 Mf/smear, (B) 2·33–16, (C) 16·33–128, (D) more than 128 Mf/smear. Larval count classes represent a geometric series with doubling class width's (classes are 16–31 and 64–127 are not printed).

CIs almost comprise zero, indicating that false negative blood Mf counts do not constitute an important bias of the experiment. For L3 both the initial slope b (i.e. efficiency of developing larvae at low human Mf counts) and the theoretical saturation level c' are about a factor of 4 times lower than those for Mf (no overlapping CIs).

If we express the results of the estimated relationships for $k(m)$ in terms of k values at 3 human Mf densities of 1, 10 and 100 Mf/smear, then we found for the Mf uptake $k = 0{\cdot}08$, 0·15 and 0·29, respectively and for the L3-output $k = 0{\cdot}09$, 0·17 and 0·32, respectively. These values indicate that larval counts are always highly over-dispersed, but that the degree of aggregation decreases with increasing human Mf counts. Furthermore, the values for Mf and L3 are strikingly similar.

A detailed representation of the estimated relationships is provided in Fig. 4. Observed and expected frequency distributions of Mf uptake and L3 output are given for 4 ranges of human Mf densities: 0–2, 2–16, 16–128 and > 128 Mf/blood smear. The procedure for obtaining the expected distributions is provided in the Appendix. The agreement with the observations is satisfactory: for Mf, $\chi^2_{\text{D.F.}=16} = 11{\cdot}7$, $P = 0{\cdot}75$; for L3, $\chi^2_{\text{D.F.}=9} = 12{\cdot}4$, $P = 0{\cdot}20$ (in both cases subtracting the number of estimated parameters – i.e. 6 – from D.F.). The distributions again underline the large variability in Mf uptake and L3 output.

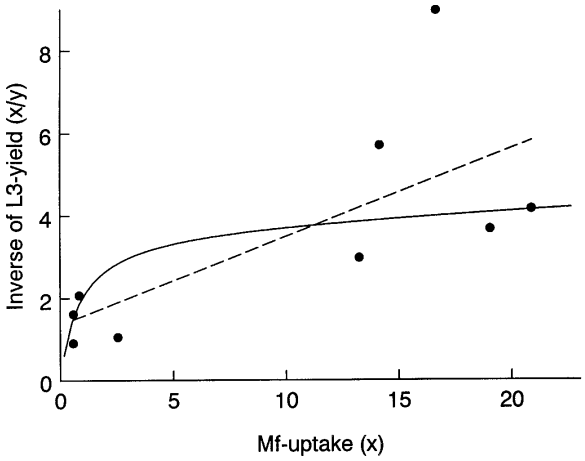

Fig. 5. Relationship between the arithmetic mean number of microfilaria ingested (x) and the inverse of L3 yield (x/y; see text for details). The observations (dots) are obtained by determining the mean Mf uptake and mean L3 output for 9 categories of human Mf density (see Materials and Methods section). The dashed line is the result of a simple linear regression on the observations. The curve (solid line) is based on estimated relationships between human Mf density and the number of Mf ingested and the number of infective L3 larvae developed/mosquito.

L3 yield

The yield of L3 larvae (ratio L3 output (y): Mf uptake (x)), was calculated both from the observations (defining 9 classes of human Mf counts; see Materials and Methods section) and on the basis of the estimated relationships. Following the procedure of Southgate & Brian (1992), the inverse of L3 yield (so: x/y) was plotted against the Mf uptake (x); see Fig. 5. Linear regression was performed on the observed points and this showed a significantly better fit than a constant relationship (which signifies 'proportionality'; $P < 0{\cdot}05$). The positive slope (0·21; 95% CI: 0·023–0·40) suggests 'limitation', i.e. an L3 yield which declines with an increasing Mf uptake. As a result of the considerable variability of larval counts, one of the observations is below the theoretical lower limit of 1·0 (parasites do not multiply in the vector). The solid line of Fig. 5 shows the results of combining the estimated relationships for Mf uptake and L3 output. The initial sharp increase suggests that the reduction in L3 yield is most prominent over the lower range of Mf uptake values.

Role of periodicity in Mf-uptake

The bars in Fig. 6 (with 95% CI) show the percentage of mosquitoes engorging Mf (Mf prevalence; Fig. 6A) and the mean Mf uptake/mosquito (Fig. 6B) for the different hours of the night. Also the expected values based on the fitted relationships are shown (see Appendix for their calculation). It can

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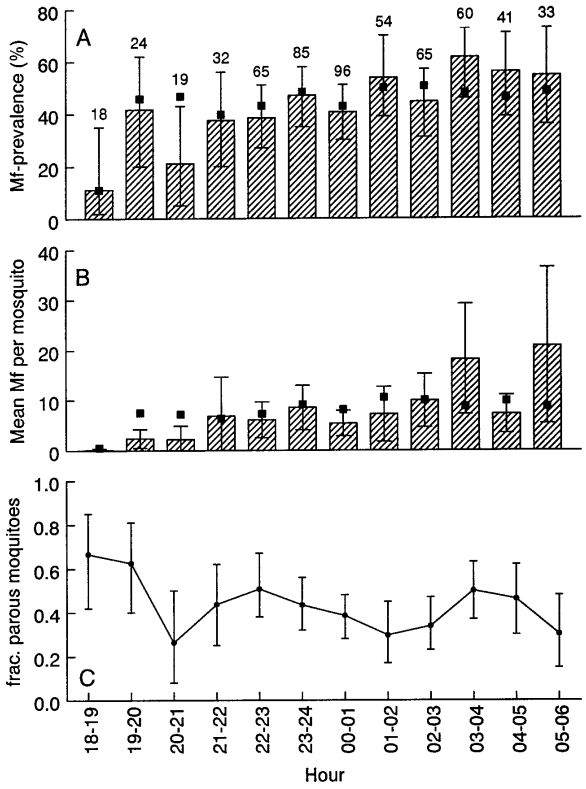

Fig. 6. Comparison of observed (bars) and predicted (filled squares) prevalence (A) and intensity (B; arithmetic means) of microfilariae in mosquitoes throughout different hours of the night. Error bars are the 95% confidence intervals for the observed prevalence and intensity of microfilariae. The number of dissected mosquitoes is given above the prevalence bars (A). (C) The fraction of parous mosquitoes (with 95% confidence intervals).

be seen that most of the expected values are within the CIs of the observed Mf prevalence and Mf uptake, suggesting satisfactory fit of the model to the data. However, the over-estimation is rather systematic. A possible reason is that the model (equations (1) and (2)) over-estimates the fraction of mosquitoes with high Mf uptake at intermediate (2–16 Mf/smear) human Mf densities (see the upper tail of the predicted distribution for Mf in Fig. 4B).

Figure 6A shows that the Mf prevalence increases during the night and then stabilizes around 50% in spite of the decline in the human Mf density towards the end of the night (Fig. 1). However, the mean human Mf density during 05–06 h is still twice as high as during 18–19 h and since Mf uptake relates in a non-linear way to blood Mf density one expects an Mf uptake of more than twice the level during the early evening. This is still no explanation for the apparent increase of the observed mean Mf uptake (Fig. 6B), which suggests that the efficiency of the *Culex* vector to engorge Mf increases during the night. However, in a logistic regression analysis of the success of engorging Mf as a function of the human Mf density and the hour of the night, this latter variable appeared to be non-significant (likelihood-ratio test, $P \gg 0{\cdot}05$). This is also suggested by the wide CIs, which are mainly the result of a few very high counts. In the interval 05–06 h, the exclusion of 2 excessively high uptakes (170 and 196 Mf) would bring the mean uptake down to 10·4 Mf. A plot of the fraction parous mosquitoes (Fig. 6C) demonstrates that a possible increase in uptake efficiency cannot be explained from a trend in mosquito age during the night.

DISCUSSION

This paper reports the analysis of *W. bancrofti* larval counts in wild *C. quinquefasciatus* mosquitoes fed under natural conditions on volunteers sleeping under a partially opened mosquito net. The results thus obtained are of great value for gaining quantitative insight into the transmission of the parasite. Other distinguishing features of the present study are that the Mf density and intake were studied throughout the night, accounting for the periodicity of Mf in the peripheral blood, and that Mf uptake and larval development were studied in parallel permitting the study of possible density regulation during both of these processes.

Uptake of Mf

The results of fitting relationships through a maximum likelihood procedure show that the mean uptake of Mf depends on the human Mf density in a nonlinear saturating way (Fig. 3A). The assumption of a straight line had to be rejected in favour of a hyperbolic relationship. It is important to note that a proportional relationship would not have been rejected if our analysis had been based on a simple least-square regression of mean Mf uptake to mean human Mf density, mainly because these mean uptakes are extremely scattered (in some patient hours, only a few mosquitoes could be collected) and sometimes largely dominated by extremely high uptakes (up to 200–300 Mf). Our finding corroborates several publications for different vectors and *W. bancrofti* combinations (*C. quinquefasciatus*, *A. aegypti*, *An. gambiae*: McGreevy *et al.* (1982); *An. gambiae*, *An. arabiensis*, *An. melas*, *An. funestus*; Bryan & Southgate (1988*a*); Bryan & Southgate (1988*b*); *A. polynesiensis*; Failloux *et al.* (1995)), in which a nonlinear saturating relationship for the uptake of Mf of periodic *W. bancrofti* was found. Though the experimental settings will not always be comparable the results disagree with the linear relationship concluded in various other studies (Jordan & Goatly, 1962; Obiamiwe, 1977; Lowrie *et al.* 1989; Jayasekera *et al.* 1991).

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Loss of larvae

Irrespective of the human Mf density or Mf uptake, the overall loss of larvae during 12 days, is estimated at an average of 80 %. This estimate includes the 104 mosquitoes that died during development and, hence, do not carry larvae at all. If these latter mosquitoes are disregarded, the loss is estimated at 74 %. These figures are well in agreement with observations by Jordan & Goatly (1962) who found losses of 55–99 %. Higher losses were found by McGreevy *et al*. (1982): 87–96 %, and lower by Jayasekara *et al*. (1991) who observed a loss of 24–67 % of Mf during their development to the L3 stage. The loss of larvae in the *Mansonia dives – B. malayi* complex was reported to be minimal (Wharton, 1960). While the loss of larvae is high, the percentage of mosquitoes losing all larvae is estimated much lower: 17 % (when only considering the survivors after 12 days) or 37 % (when treating dead mosquitoes as if they lost their infection). The latter percentage is well in agreement with observations on wild mosquitoes caught in Pondicherry where the reduction in the number of infected mosquitoes during development from Mf to L3 was estimated at 25–33 % (Subramanian *et al*. 1994).

Regulation of larval density

More interesting than an over-all percentage loss is to know whether and how this depends on the number of Mf engorged: is there any evidence of density regulation during larval development in addition to the density regulation we concluded for the Mf-uptake? The data presented in Fig. 5 suggest such a regulation by showing a statistically significant decrease in the L3 yield (or an increase in the inverse) for increasing Mf uptakes. Furthermore, given the sharp initial increase in the curve based on the estimated relationships, this 'limitation' is probably not a phenomenon that only occurs at (extremely) high Mf intakes. This latter phenomenon should, however, be considered carefully, because it also arises from the fact that both estimated relationships of Fig. 3 have a positive intercept *a* (to take account of the few, false-negative blood smears). Since *a* for L3 output is even slightly higher than for Mf uptake (see Tables 2 and 3), at 'zero' human Mf densities the L3 yield is even higher than the theoretical value of 1. Hence, the sharp rise of the curve in Fig. 5 could in part be due to the (lack of) sensitivity of the blood smear for detecting Mf. Another comment to be made is that Fig. 5 only applies to mean Mf uptakes while Fig. 3A and Fig. 4 clearly show that, for a given human Mf density, there is a large individual variation in the uptake of Mf. An important, but as yet unsolvable, question is what happens with the (extremely) high Mf uptakes (the upper tail of the distribution). To answer this question one should be able to examine mosquitoes at a moment that the engorged Mf can still be counted while it is already clear which of these Mf will develop to the L3-stage. For *Simulium damnosum*, this can be done by distinguishing between the Mf encapsulated in the peritrophic membrane and those outside the membrane and/or entering the thoracic muscles (Basáñez *et al*. 1995). In this approach, one should be aware that excess mortality of mosquitoes at later stages of larval development, a potential mechanism of density regulation, is not taken into account.

The density regulation of both Mf uptake and L3 yield can be due to increased mortality of the larvae at high densities or to parasite-induced mortality of the vector (Dye, 1992; Subramanian *et al*. 1994; Das *et al*. 1995). The experimental design of our study does not permit far-reaching conclusions about mosquito-survival because during the 12 days period no record was kept of the day on which a mosquito died and, more important, because no parallel dissections of live mosquitoes were carried out during this period. However, the results as presented in Table 1 suggest that the excess mortality of highly infected mosquitoes may play a role. Both the percentage infected and the intensity of infection in the positives among the 104 mosquitoes which died before 12 days were significantly higher than those for the survivors (64 % *vs*. 42 % and 16 *vs*. 10 L2/L3 larvae, respectively). Parasite-induced vector mortality was also reported by Crans (1973), who observed a mortality rate twice as high as in *C. quinquefasciatus* females harbouring *W. bancrofti* larvae when compared to mosquitoes without infection. Failloux *et al*. (1995) observed a mortality rate of 20 to 60 % in *A. polynesiensis* populations infected by *W. bancrofti* and concluded that the level of mortality was associated with parasite load.

Heterogeneity in Mf uptake and larval yield

Both the number of Mf ingested and the number of L3 larvae/mosquito show marked variability. Fig. 2 shows that, even for high human Mf densities of more than 100 Mf/20 μl blood still 25 % of the mosquitoes fail to ingest Mf and 40 % do not carry L3 larvae. This variation can in part be explained from variation in the human Mf density, both in time and across different sites of the body. While mosquitoes were collected continuously throughout a (patient) hour, the Mf density was only determined at the mid-point and only from finger-prick samples. Though a clear periodicity was observed for all carriers together, considerable hour-to-hour variations were observed for each individual, and this will also imply variation within an hour. Further, while human Mf density is determined in the capillary blood of a finger, mosquitoes bite all over the body. This spatial variability of Mf in the host as one

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explanation of variation in Mf uptake was suggested earlier by Jordan & Goatly (1962) and Ramachandran & Zaini (1968). However, variation in human Mf density can neither be the only nor the most important reason for the large differences in Mf uptake by and larval development within the vector. In our experiment, this is suggested by the virtual absence of 'false positives', i.e. mosquitoes which engorge Mf or develop larvae from patient-hours with a zero Mf density. This number would be larger if the blood smears were not representative of the blood engorged by the mosquitoes. Most likely, factors related to the feeding itself are responsible for the heterogeneity, such as the presumed ability of mosquitoes to concentrate Mf in the blood close to the feeding site (Bryan & Southgate, 1988*a*), differences in pool and capillary feeding habits of the mosquitoes (Gordon & Lumsden, 1939; Gubler *et al*. 1973), or perhaps the non-homogeneous (clustered) distribution of Mf in the blood (Hairston & Jachowski, 1968), which becomes more important as the amount of blood declines.

In our analysis, the variability of the Mf uptake and the L3 output is represented by a negative binomial distribution. Both the mean and the clumping factor k of this distribution were found to vary with the human Mf density m. Among the tested relationships, the assumption of a power-function for describing k as a function of the human Mf density resulted in the best fit to the observations (maximum-likelihood). The low values of this k (ranging from close to zero to 0·3) indicate a high level of aggregation of the numbers of Mf or L3/mosquito (Anderson & May, 1985; Wenk, 1991; Basáñez *et al*. 1994, 1995; Das *et al*. 1995). The increase in k, and hence a decrease in the degree of over-dispersion with human Mf density, could imply that the levelling-off of the mean uptake or larval output is partially due to the absence of excessively high Mf uptakes: the upper tail of the distribution is lopped off, reducing the estimates for W and increasing the estimates for k (see also Anderson & Gordon, 1982; Pacala & Dobson, 1988).

Methodological issues

Though the relationships in Fig. 3 are based on many data-points (592 for Mf and 400 for L3), the number of patients involved in the study is only 13. From these 13 patients we derived 119 (Mf) and 62 (L3) 'patient-hours' by treating the hourly collection of blood together with the hourly catches of mosquitoes as independent samples. Independent, of course, not in the sense that for a patient the successive blood smears are not correlated (depends on the worm load of a person), but in the sense that it is exclusively the Mf density in the blood which determines the Mf uptake and not the hour of the night or any (unknown) patient factor. By means of logistic regression analysis we have excluded the hour of the night as a confounder. However, both the considerable within-patient (hour-to-hour) and the between-patient variation in blood smear counts, together with the highly variable Mf uptake make it very difficult to resolve the problem of systematic differences between volunteers in their ability to infect mosquitoes. But even if such patient factors should exist, the implications for our findings are likely to be limited, mainly because the ranges of Mf densities shown during the night by each of the volunteers are considerably overlapping. This implies that it is, for example, not just 1 volunteer who delivers the data points at the higher end of human Mf densities or just 1 with the zero and low counts. In order to obtain a second data set for verification of our results, another experiment, similar to the one here presented but with more volunteers, is now being carried out at VCRC.

In contrast to many other studies (Jordan & Goatly, 1962; Obiamiwe, 1977; McGreevy *et al*. 1982; Basáñez *et al*. 1994, 1995; Southgate & Bryan, 1992), we have based our conclusions on the analysis of larval counts in individual mosquitoes and not on the mean uptake of a batch of vectors or the fraction of vectors infected. We believe that, if possible, utilization of the basic unit of measurement (the mosquito) results in the most powerful estimation of relationships and of parameters for over-dispersion.

Implications for control

The conclusion that the *W. bancrofti-C. quinquefasciatus* complex is of the 'limitation' type, which we draw on the basis of an experiment under natural conditions, could have important consequences for the effectiveness of control measures (Southgate & Bryan, 1992). The estimated relationship shown in Fig. 3B (which is the consequence of the saturated uptake of Mf plus the 'limitation' phenomenon) makes it clear that low human Mf densities show a relatively high capability to generate infectious mosquitoes. Hence, control should bring down and maintain Mf densities at low levels in order to sufficiently break transmission by mosquitoes. This could be one of the reasons for the difficulties to bring about a major decline in the parasite population through 5 years of vector control in Pondicherry (1981–85; see Subramanian *et al*. 1989). It was found that, although in this region the trend in prevalence and intensities of Mf continued to decline, the annual transmission index (number of infective larvae/person/year estimated from entomological biting collections) has considerably increased from 1986 onwards (Das *et al*. 1992). Similarly eradication appears to be difficult in areas where control programmes are solely aimed at reducing the parasite reservoir in the human host (Biswas *et al*. 1989; Jayasekera *et al*. 1991; Southgate & Bryan, 1992).

On the other hand, our findings do not justify firm conclusions about the implications for control. The series of observations obtained from the 13 volunteers selected for the study do not constitute a representative sample from the population of Pondicherry. During a survey in 1981 (before starting vector control; see Rajagopalan *et al*. 1989) about 10 % was found to be Mf positive on the basis of a single smear of 20 μl. Among those positive, only a small fraction (< 3 %) showed high counts of more than 50 Mf/smear. A re-analysis of the data for Mf uptake and L3 output after excluding patient hours with more than 50 Mf/smear resulted in estimates of c which did not significantly differ from 0; i.e. relationships which do not differ from linearity. Though this could only be concluded when assuming a positive intercept a (and again higher for L3 than for Mf), which complicates reasoning about density regulation during development from Mf to L3, this stresses that one should like to do this kind of experiment with a (large) number of patients which together reflect the distribution of Mf density in the population (see also Dye (1994) and Dye & Williams (1995)).

However, probably more important than density regulation of mean larval counts, is the occurrence of very high Mf uptakes and L3 outputs even for relatively low human Mf densities. Though the present experiment does not provide information on the survival chances of mosquitoes with large numbers of L3 larvae (say $\geqslant 8$) in the field, they might play a disproportionate large role in transmission and considerably hamper the elimination of *W. bancrofti*.

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APPENDIX

Let $W(m)$ be the hyperbolic function describing the mean Mf intake or L3 output as a function of the human Mf density m and $k(m)$ the function describing the clumping factor of the negative binomial distribution for the number of larvae in the vector as a function of m (see eqns (1) and (2); Materials and Methods section), then the parameters a, b, c, k_0, α, and β of these functions are estimated by maximizing the likelihood function:

$$L = \prod_{i=1}^{13} \prod_{h=1}^{12} \prod_{j=1}^{J_{hi}} P_{NB}(x = l_{hij} \,|\, W(m_{hi}), k(m_{hi})), \qquad \text{(A 1)}$$

with:

$P_{NB}(x \,|\, W, k)$ probability to find x parasites (Mf or L3) given a mean Mf uptake or L3 output of W and a clumping factor k of the negative binomial distribution:

$$P_{NB}(x \,|\, W, k) = \frac{\Gamma(k+x)}{\Gamma(x+1)\,\Gamma(k)} \left(\frac{W}{W+k}\right)^{x} \left(1+\frac{W}{k}\right)^{-k}, \qquad \text{(A 2)}$$

where ($\Gamma(.)$ is the gamma function); $\mathcal{J}_{hi}$ number of mosquitoes collected in hour h (totally 12 h) from patient i (totally 13 patients); l_{hij} number of parasites found in mosquito j caught from patient i during hour h; m_{hi} Mf density of patient i during hour h. Maximizing this likelihood function is achieved with a downhill-simplex method (Nelder & Mead, 1965) implemented in a computer program written in C.

The expected prevalences of Fig. 6A are, for each hour h, calculated as follows:

$$p_h = \frac{\sum_{i=1}^{13} \mathcal{J}_{hi} \times P_{NB}(x > 0 \,|\, W(m_{hi}), k(m_{hi}))}{\sum_{i=1}^{13} \mathcal{J}_{hi}} \qquad \text{(A 3)}$$

with:

$$P_{NB}(x > 0 \,|\, W, k) = 1 - \left(1+\frac{W}{k}\right)^{-k}. \qquad \text{(A 4)}$$

The expected mean Mf uptake within each hour h (Fig. 6B) is calculated as:

$$w_h = \frac{\sum_{i=1}^{13} \mathcal{J}_{hi} \times W(m_{hi})}{\sum_{i=1}^{13} \mathcal{J}_{hi}}. \qquad \text{(A 5)}$$

The predicted distributions shown in Fig. 4 are, for each of the considered ranges of values of human Mf densities m_{hi} (0–2, 2–16, 16–128, > 128), calculated as:

$$Pr(x = l) = \frac{\sum_{i=1}^{13} \sum_{h=1}^{12} \mathcal{J}_{hi} \times P_{NB}(x = l \,|\, W(m_{hi}), k(m_{hi}))}{\sum_{i=1}^{13} \sum_{h=1}^{12} \mathcal{J}_{hi}}, \qquad \text{(A 6)}$$

with $Pr(x = l)$ expected probability to engorge l Mf or to produce l L3 larvae.

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