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Age-grading and growth of *Wuchereria bancrofti* (Filariidea: Onchocercidae) larvae by growth measurements and its use for estimating blood-meal intervals of its Polynesian vector *Aedes polynesiensis* (Diptera: Culicidae)

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Abstract

Growth in length and width of *Wuchereria bancrofti* (Filariidea: Onchocercidae) larvae developing in its Polynesian vector *Aedes polynesiensis* (Diptera: Culicidae) was analysed using a mathematical approach to objectively extract patterns. L1 had a U-shaped growth in length, while widths followed an S-shaped function. L2 had an S-shaped growth in length and width. Growth in length of L3 was also S-shaped, while widths had an asymptotic size following a period of rapid shrinkage. The greatest difference between length and width was in stage 3 where the length was over 75 times greater than the width. The ratio of length to width was ≈ 50 for microfilariae and only 10 for the L1 ('sausage') stage. Characteristic mean length (and width) were $\approx 280(7)$ μm for microfilariae, ≈ 181 μm for L1 at their smallest, and $\approx 1584(22)$ μm for L3 infective larvae. There was a great increase in length during stage 2 from $\approx 322(27)$ to $\approx 982(31)$ μm. Stage duration decreased with increasing temperature while growth rate increased, giving steeper growth curves. There was no effect of temperature on size, except for L3, which were shorter when mosquitoes were reared at higher temperature. It appears that larval growth is a continuous process from microfilariae to the young L3 stage, and continuously modifies the larval parasite aspect, even within each stage. Thus, information on larval shape may be used as an age indicator and in some cases, may give an estimation on time elapsed since infection of the vector.

An important demographic parameter used in most mathematical models describing transmission of parasites by insect vectors is the length of the gonotrophic cycle of the vector, i.e. the time interval between two successive blood-meals. Usual methods for computing such a parameter are based on mark-recapture techniques. However, reliable estimates need substantial capture rates, which are not always possible. This paper presents another approach in which marked mosquitoes are those naturally infected by *W. bancrofti*. For one mosquito, the time since infection is simply the age of the developing larval parasite. Our method first expresses the age of larval parasite as a fraction of total development time (from microfilariae entering the vector to L3 larvae) using a regression model based on measurements of the parasite's length and width. This fraction of development is then converted to a chronological age since infection, using a back-calculation procedure involving ambient temperatures and growth rates of *W. bancrofti* larvae in the vector. The method is applied to wild caught *Ae. polynesiensis* in French Polynesia to compute the length of the gonotrophic cycle. This mosquito species comes to bite ≈ 3, 6–7 and 9 days after a first infectious blood-meal. Then the length of the gonotrophic cycle may be of 3–4 days. © 2002 Published by Elsevier Science Ltd. on behalf of Australian Society for Parasitology.

Keywords: *Wuchereria bancrofti*; Parasite growth patterns; Length; Width; Age indicator; *Aedes polynesiensis*; Gonotrophic cycle; Developmental rate; Age-grading; Growth shape

1. Introduction

Lymphatic filariasis due to sub-periodic *Wuchereria bancrofti* (Cobbold) affected humans in Oceania long before the arrival of European explorers during the 18th century (Iyengar, 1959; Pichon, 1981; Roux, 1993). In French Polynesia, *Aedes polynesiensis* Marks, 1951 has been recognised as the major vector of Bancroftian filariasis for a long time (Rosen, 1955). This mosquito species is widely distributed throughout the South Pacific region (Evenhuis and Gon, 1989) and is involved in disease transmission wherever it occurs (White, 1989).

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The cycle of *W. bancrofti* within a mosquito vector has been understood since Manson's work in 1878 (Manson-Bahr, 1959) and it was the first time that an insect had been revealed as the vector of any human or animal disease. Since then, others have described the morphological development of *W. bancrofti* in mosquitoes in detail, mainly in *Culex quinquefasciatus* Say (Lebredo, 1901; Abe, 1937; Kobayashi, 1940; Iyengar, 1956a,b) and in *Anopheles gambiae* Giles (Bain, 1972). Some typical measurements (total length, width and their ranges) for microfilariae and L3 were given by these authors. The morphology of the infective stage (i.e. end of the third stage) has been studied in particular detail (Nelson, 1959; Yen et al., 1982; Bain and Chabaud, 1986) as these 'mature' larvae are transmitted to man by the mosquito vector and should be differentiated from other close pathogens (see the review of Sasa (1976) on differential diagnosis).

Only preliminary studies were carried out on the development of *W. bancrofti* larvae in *Ae. polynesiensis* (Bahr, 1912; Manson-Bahr, 1941). The three stages of development of the filaria larvae within the *Aedes* mosquito were illustrated by photomicrographs by Byrd et al. (1945), while Iyengar (1957) outlined their life cycle. As before, some characteristic measurements were carried out, especially on L3, but no information was given on the growth patterns of the three stages. Moreover, none of the above studies attempted to describe the growth dynamics of the filaria in its vector.

Parasitic and infectious diseases control program may be enhanced by simulating transmission dynamics and control strategies with mathematical models (Scott and Smith, 1994; Ottesen and Ramachandran, 1995). These models require the quantification of many biological parameters, the time interval between two successive blood-meals often being one of them. This parameter, also called the gonotrophic cycle length of a vector, is essential to the understanding of the transmission dynamics of arthropod-borne diseases. It appears in many mathematical models as a variable itself (Saul et al., 1990) or is intensively used in models derived from Macdonald's (1957) approach to population dynamics of indirectly transmitted microparasites. For example, the gonotrophic cycle length is used in the computation of the rate of biting on humans as well as for the estimation of the basic reproductive rate (Anderson and May, 1991). For modelling indirectly transmitted helminth dynamics such as *W. bancrofti*, Rochet (1990) used the inverse of the duration of the gonotrophic cycle to estimate the frequency of blood-meals of the vector. In basic population dynamics studies, its value (n) is also used to estimate the daily survival rate of mosquitoes as the nth-root of the proportion of parous females (Coz et al., 1961).

Usual field methods to estimate the gonotrophic cycle length belong to the general area of mark–release–recapture techniques. These techniques, which were first designed to estimate survival rates, are based on sophisticated mathematical models (Lebreton et al., 1992), some of which are specifically designed for haematophagous insects and the estimation of blood-meal intervals (Charlwood et al., 1986; Saul, 1987). However, if recapture rates are low, the estimation of the gonotrophic cycle length from field data may be inconsistent.

In French Polynesia, *Ae. polynesiensis* Marks is the main vector of sub-periodic lymphatic filaria *W. bancrofti*. It also transmits *Dirofilaria immitis* Leidy, the agent for dog heartworm and can transmit dengue virus (Rosen, 1955). Rivière (1988) used coloured powders as a marking technique of *Ae. polynesiensis* to estimate the length of the gonotrophic cycle. However, due to the (usually) small rate of recapture of this mosquito (4.3% in Rivière's experiments), only rough estimates of the gonodotrophic cycle length could be computed. Our study presents another approach by considering *Ae. polynesiensis* infected by *W. bancrofti* as 'naturally marked' by the parasite; the day 0 at which they were marked being the day when they took the infective blood-meal. The basic concept is that if a chronological age can be given to larval *W. bancrofti*, the time elapsed since infection of the vector and its capture can be determined. Because the morphology of *W. bancrofti* larvae, as summarized by length and width measurements, is continuously changing with time from microfilariae to L3 appearance, parasite larval aspect may be suggested as a statistically efficient marker of time elapsed since infection. Moreover, since $\approx$10% of wild caught *Ae. polynesiensis* females of endemic filariasis areas of French Polynesia are infected by the parasite (Lardeux et al., 1995), this 'natural' marking is much more efficient than usual marking–recapture techniques.

Here, we present experiments carried out at various temperatures, to analyse *W. bancrofti* growth *per se* in the vector *Ae. polynesiensis*, showing that *W. bancrofti* larval length and width may identify a time-characteristic shape of the parasite (i.e. an 'age indicator'). First the growth patterns of length and width of each *W. bancrofti* larval stage in relation to time and temperature are described, using a statistical model of growth description. Second, the effect of temperature on size (i.e. on age estimates) is analysed and characteristic measurements are given and the use of the shape of the larval parasite as an age indicator is discussed.

Laboratory experiments carried out to estimate the 'age' (expressed as a 'fraction of development') of *W. bancrofti* larvae using the filaria larvae growth rate function, which depends on the ambient temperature are presented. The aspect (shape) of larval *W. bancrofti*, estimated by length and width measurements, is correlated to the corresponding 'fraction of development' and then converted to a chronological age using a back-calculation procedure involving ambient temperatures of preceding days until microfilarial stage. As such, once an infected mosquito is captured, measurements of *W. bancrofti* larvae can be used to infer the time elapsed since the infectious blood-meal if ambient temperatures preceding the day of capture are known. The method is applied to field data to determine the time interval between two blood-meals of *Ae. polynesiensis* in French Polynesia.

2. Materials and methods

2.1. Infection of mosquitoes and filarial cycle

Aedes polynesiensis females (colonised strain) were blood fed on the forearm of a microfilaremic volunteer from the same locality as the mosquito strain (Tahiti-Paea), and who harboured a high microfilarial density (>5000 microfilariae per ml of blood as estimated from ten 10 μl blood drops). After the infective blood-meal, unengorged females were discarded, the remaining being kept in environmental chambers (MIR 250, Sanyo) set at 20, 22.5, 25, 27.5, 30, 32 and 34°C.

2.2. Larval length and width measurements

Each day, 15–20 mosquitoes from each environmental chamber were gently dilacerated in a drop of 1:1 water–glycerol to avoid excessive evaporation. *W. bancrofti* larvae were sought in the abdomen and thorax for microfilariae, in thorax for L1 and L2 and in the head, thorax and abdomen for L3. Overall lengths of *W. bancrofti* larvae were drawn on paper using a microscope equipped with a camera lucida (Laborlux K, Leitz) at the maximum possible magnification (i.e. ×625 for microfilariae and L1; ×625, ×390 or ×156 for L2 and ×156 for L3). Widths of larvae were measured approximately at the mid-length of each larva at ×625. With the camera lucida, larva images were projected on paper at a scale large enough for precise measurements. Measurements were carried out with a computerised digitiser (Summascketch II, Summagraphics).

2.3. Growth model and estimation of parameters

Larval growth was described as used by the growth model of Schnute (1981). It includes much of the 'traditional' models (Ricker, 1979) as sub-models which simply correspond to limiting parameter values. The parameters in the model always have stable statistical estimates and reasonable biological interpretation. The parametric properties of the model permit direct use of the data in selecting an appropriate model.

Schnute's model was fitted to *W. bancrofti* larval growth for each stage in each rearing temperature situation to test for a generalisation of growth patterns found. Because the model is non-linear in its parameters, the downhill simplex method in multidimensions (Nedler and Mead, 1965) was used to compute estimates of the parameters. Parameter estimates were then improved via a quasi-Newton method (Press et al., 1986). The loss function minimised was the weighted least squares of deviations of the observed values from those predicted by the model. Due to shrinkage with time, width measurements (Y) of stage 3 were transformed by 40-Y. The value of 40 μm was chosen as the closest integer above the maximum width observed in that stage. The transformation led to positive data giving a growth shape suitable for description by the model. Schnute's model cannot describe U patterns of growth as those observed for lengths of stage 1. For each growth curve (i.e. for each stage × temperature combination), the day of first appearance of a stage was denoted as day 1, so that all curves had the same initial time.

2.4. Statistical analysis of growth patterns

Sizes were compared by analysis of variance (ANOVA) with a 5% significance level. Before computations, measurements (x) were transformed by $\log(x)$ in order to normalise them and stabilise the variance. When ANOVA found significant differences, means were classified into homogenous groups using the Newman–Keuls procedure (Scherrer, 1984) which assesses the probability for each pair of means of obtaining greater differences under the null hypothesis (i.e. no difference among the means in the population).

2.5. Computing the 'fraction of development' of W. bancrofti larvae reared at constant temperature

The developmental rate of *W. bancrofti* is temperature-dependent and has been modelled by Lardeux and Cheffort (1997) using Lactin et al. (1995) function (see Appendix A).

For complete development of *W. bancrofti* larvae, i.e. for a microfilariae entering a mosquito and developing up to the L3 stage, by definition

$$r(T)\Gamma_T = 1 \tag{1}$$

where $r(T)$ is the developmental rate at temperature T and Γ_T is the total development time at temperature T.

If development is incomplete then Eq. (1) can be rewritten as

$$r(T)D_T = A \qquad (0 < A < 1) \tag{2}$$

where D_T is the time elapsed since the beginning of development at temperature $T(D_T < \Gamma_T)$ and A measures the 'fraction of development' of *W. bancrofti* larvae.

When dissecting a wild caught mosquito, if larval *W. bancrofti* are found and if an estimate of their 'fraction of development' A can be given, the development time D of these larvae can simply be computed using Eq. (2) as:

$$D = A/r(T) \tag{3}$$

D is also the time elapsed since mosquito infection and as such, D may also estimate the time between two blood-meals if the mosquito has been caught while attempting to blood-feed. However, since $r(T)$ is temperature-dependent and T varies in field conditions, computations are not as straightforward as in Eq. (3) but should be carried out using the following protocol.

2.6. Estimation of W bancrofti 'fraction of development' using larval shape

Laboratory experiments consisted in rearing infected

mosquitoes in environmental chambers at various constant temperatures (20, 22.5, 25, 27.5, 30 and 32°C) and dissecting each day a batch of 15–20 mosquitoes and measuring length and width of each *W. bancrofti* found (see Section 2.2 for complete details). In these experiments, the values D_T were simply the times (in days) elapsed between infection and the dissection of mosquitoes. Values of the function $r(T)$ were computed using function parameter estimates from Lardeux and Cheffort (1997) (see also Appendix A), and A values were computed using Eq. (2).

In total, our data set consisted of 1174 triplets (lengths, widths, A) obtained for various rearing temperatures and for various stages of *W. bancrofti* larval development. This data set was used to analyse the relationship among the 'fraction of development' A and the measurements of length (L) and width (W) by use of multiple linear regression of the form:

$$A = \alpha + \beta L + \gamma W \tag{4}$$

where α, β and γ were regression coefficients.

As lengths and widths may be correlated, a stepwise procedure was used to build the regression, and test whether only one or both measurements should be taken into account in the model. Multicolinearity was assessed by tolerance computation, assuming that a tolerance value >0.2 indicates no intercorrelation between the variables. This was also checked by inspection of the correlation matrix (values >0.9 revealing bivariate multicolinearity). The relative importance of 'length' and 'width' in the equation was indicated by the magnitude of their beta-coefficients. The overall significance test of the multiple regression was carried out by ANOVA, and residuals were checked for normality (Shapiro–Wilk test) and random dispersion (Sokal and Rohlf, 1995).

2.7. Estimation of the time since infection D (i.e. development time of W. bancrofti larvae) in varying temperature environment

Parameters of the function $r(T)$ describing the developmental rate of *W. bancrofti* larvae were estimated under laboratory conditions at constant rearing temperatures. However, for certain animals, such a type of function may not be adequate under variable temperature conditions (Stinner et al., 1974). To test the accuracy of the developmental rate function $r(T)$ of *W. bancrofti* in varying temperature environments, experiments were carried out in environmental chambers where temperatures were allowed to vary on a daily basis between 22.5 and 30°C, following a sinusoid pattern: infected mosquitoes were reared at 22.5°C just after infection (day 0), at 25°C during day 1, at 27.5°C during day 2, at 30°C during day 3, at 27.5°C during day 4 etc. Each day, a batch of mosquitoes were killed and *W. bancrofti* larvae identified by stages and counted. The procedure of Pontius et al. (1989) was used to estimate the mean time of appearance of L3 stages as described in Lardeux and Cheffort (1997). This value, which corresponds to the observed real time to L3 stage, was compared with the development time computed from Eq. (6) (see below) to validate the accuracy of the function estimates under varying temperature conditions.

To compute the development time D from field data, records of temperatures during the days preceding the capture of the infected mosquito are needed. The least information required is the daily minimum and maximum temperatures, and D can be computed as follows:

During a day, the ambient temperature behaves according to a sine function oscillating between a minimum ($T_{\min}$) and a maximum ($T_{\max}$) (Baskerville and Emin, 1969). The function is:

$$T = a\ \sin(t) + b \tag{5}$$

where T is the temperature, a the amplitude of the sine curve ($a = T_{\max} - T_{\min})/2$), t the time of day in radians (1 day $= 2\pi$) and b the mean of the sine curve ($b = (T_{\max} + T_{\min})/2$).

A day may be divided into 24 h and for each h ($h = 0 \cdots 23$), a corresponding temperature may be computed by replacing t by $2\pi h/24 - \pi/2$ in Eq. (5).

Since temperature T is a function of time t (i.e. $T = f(t)$, where f is the sine function (Eq. (5)), Eq. (1) for complete development may be rewritten as

$$\int_0^D r(T)\mathrm{d}t = \int_0^D r(f(t))\mathrm{d}t = 1 \tag{6}$$

where D is the developmental time in h, $f(t)$ the sine function and $r(\cdot)$ the developmental rate function or

$$\sum_{t=0}^{D} r(f(t))\Delta t \cong 1 \tag{7}$$

where Δt are periods of constant temperatures ($\Delta t = 1$ h in our computations).

For incomplete development (for example, when an immature larvae are found in a wild caught dissected mosquito), the right side of Eq. (7) is not 1, but the value A ($0 < A < 1$) measuring the 'fraction of development' of larvae.

Using Eq. (5) for T and the equation given in Lardeux and Cheffort (1997) for $r(T)$ (see also Appendix A for parameter values used in equation $r(T)$), Eq. (7) can be easily solved iteratively for D.

Thus, if an estimate of the 'fraction of development' A can be given, using the multiple regression equation of length and width measurements, the corresponding time (D days) elapsed between a mosquito's infection and its capture may be easily computed in a back-calculation way, starting at the time of capture ($t = 0$), using the minima and maxima of temperatures of the preceding days and stopping the summations when

$$\sum_{t=0}^{D} r(f(t))\Delta t \cong A \tag{8}$$

2.8. Estimation of Ae. polynesiensis blood-meal intervals with field data

Wild *Ae. polynesiensis* females were caught in Opoa village (Raiatea Island), an endemic area of Bancroftian filariasis transmission (Lardeux et al., 1995). A house-to-house survey was carried out and mosquitoes were collected on human baits (i.e. females seeking for blood-meal) using Bonnet et al. (1956). Mosquitoes were dissected and *W. bancrofti* larvae measured as described above. Measurements of length and width of *W. bancrofti* larvae were recorded for 59 infected mosquitoes. In all, 266 larvae were measured in length and width, and aged using Eqs. (5) and (8). The 'ages' refer to the time since infection. The statistical analysis of histogram of 'ages' was carried out using a modal analysis procedure described in Lardeux and Tetuanui (1995), involving algorithms of Bhattacharya (1967) and Hasselblad (1966). This modal analysis enabled the decomposition of histograms into their Gaussian components, giving estimates of their $\bar{x}$ and standard deviation (SD). Because several larvae were found per mosquito, a mean 'age' for each insect was computed and once again, the modal analysis of the histogram of ages was carried out using the above procedure. Because mosquitoes were captured on human baits while attempting to bite, the computed modes of histograms were interpreted as blood-meal intervals.

Daily minima and maxima for temperatures were obtained from a nearby meteorological station (Meteo France, Raiatea).

3. Results

3.1. Infection of mosquitoes and filarial cycles

At 34°C, most mosquitoes died within a few days, before the larval cycle of *W. bancrofti* was completed. At 32°C, morphological alterations of L3 indicated that the evolution of the parasite cycle was not normal. L3 lengths were considerably shortened and widths larger than expected. The parasite cycle was completed but considered as not natural (Lardeux and Cheffort, 1997) and this experiment was not analysed for growth patterns.

Only larvae which could be clearly delineated among mosquito fragments were drawn. As such, from each daily batch of 15–20 mosquitoes, the minimum number of measurements carried out was about five per parasite larval stage per day. Usually, for each mosquito batch, a mean number of 15–20 measurements were carried out per stage per day. With the camera lucida and depending on the larval stage, lengths were between 10 and >25 cm while widths were >0.5–2 cm, enabling a correct measurement. Basic measurements for lengths and widths of *W. bancrofti* larvae are given in Table 1.

3.2. Growth patterns

3.2.1. Growth in length of first stage

The growth pattern for length of L1 exhibited at each temperature is U-shaped. Lengths decreased in time from a size about that of microfilariae, reached a minimum and increased until recovery of the initial size. The maximum shrinkage in length was 0.1807 mm (SD = 0.0204) and was not different among rearing temperatures ($F = 1.35$; d.f. $= 4, 91$; $P = 0.25$). Mean age of L1 at that minimum length was 6 days at 20°C, 4 days at 22.5 and 25°C, 2 days at 27.5 and 30°C. This minimum was approximately situated at half stage duration. The smallest individual length measured was 75.2 μm (associated with a width of 19 μm) which made clear the denomination of 'sausage' stage usually given for stage 1. Stage 1 lasted 13 days at 20°C, 6 days at 22.5 and 25°C and 4 days at 27.5 and 30°C.

3.2.2. Growth in width of first stage

Unlike length, width in the L1 stage exhibited strictly increasing growth. Schnute's model indicated that curves were almost S-shaped, of the Richards (1959) type. However, parameter estimates indicated that the S form was not well pronounced and that growth in width may be

Table 1
Mean and standard deviation for length (μm) and width (μm) of *W. bancrofti* larvae at the beginning and end of different developmental stages in the vector *Ae. polynesiensis* (note that differences in sizes due to temperature occur only at the end of third stage)

	Beginning of stage					End of stage				
	No.	Length		Width		No.	Length		Width	
		$\bar{x}$	SD	$\bar{x}$	SD		$\bar{x}$	SD	$\bar{x}$	SD
Microfilariae	80	278.6	025.2	7.1	0.8	88	267.7	19.8	8.1	1.0
Stage 1	80	217.7	024.3	9.8	1.7	84	223.7	33.6	24.9	3.4
Stage 2	94	322.4	094.9	27.3	2.4	67	098.2	28.1	31.2	4.1
Stage 3 (average)	128	1470.9	233.4	26.5	3.4	–	–	–	–	–
Stage 3 (20°C)	–	–	–	–	–	18	1714.4	183.3	22.9	1.4
Stage 3 (22.5°C)	–	–	–	–	–	26	1792.6	154.4	23.3	1.8
Stage 3 (25°C)	–	–	–	–	–	42	1608.3	130.6	22.3	1.3
Stage 3 (27.5°C)	–	–	–	–	–	39	1616.2	88.5	22.0	1.1
Stage 3 (30°C)	–	–	–	–	–	58	1411.2	153.3	23.2	2.4
Stage 3 (32°C)	–	–	–	–	–	21	816.0	93.0	35.0	5.0

approximately linear, beginning and ending plateaus of the S function being very short.

3.2.3. Growth of second stage

At the five different rearing temperatures, length and width of L2 could be modelled by an S-shaped curve of Richards type. Sizes of L2 were not significantly different among rearing temperatures. As before, the shape of curves became straighter as temperature increased. During stage 2, lengths were increased by a factor of three (Table 1). Stage 2 lasted 12 days at 20°C, 11 days at 22.5°C, 8 days at 25°C, 7 days at 27.5°C and 6 days at 30°C.

3.2.4. Growth in length of third stage

Growth in length of L3 could be modelled by an S-shaped function of Richards type. L3 had an asymptotic growth in length, which was smaller at higher temperature. This asymptotic size was rapidly reached and, depending on rearing temperature, occurred between 2 and 4 days after L3 appearance. The first L3 appeared 26 days after infection at 20°C, 17 days at 22.5°C, 12 days at 25°C, 11 days at 27.5°C and 9 days at 30°C.

3.2.5. Growth in width of third stage

Parameter estimates of Schnute's model for L3 growth in width indicated that widths shortened with age and rapidly reached an asymptotic value (i.e. 6 days after L3 appearance at 22°C; 2.5 days at 25°C and 1 day at 27.5 and 30°C).

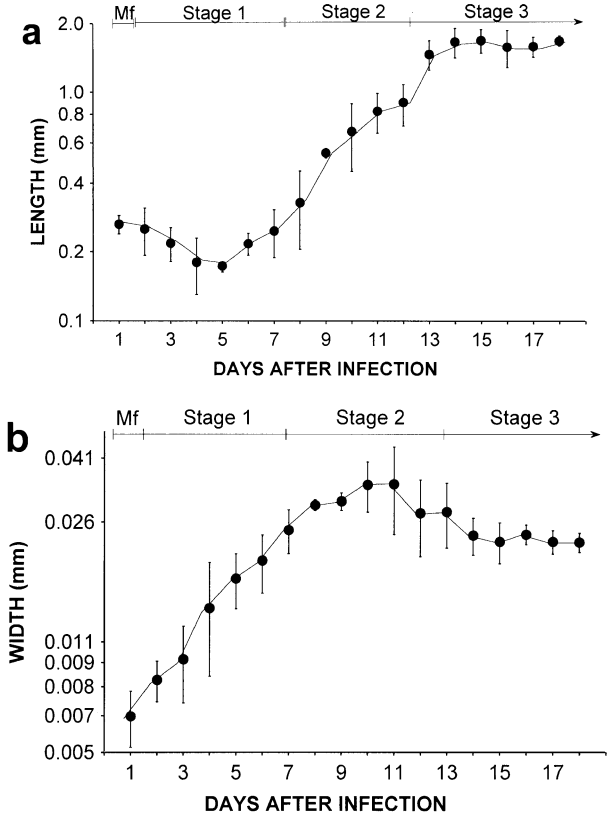

Fig. 1. Overall growth curves of daily mean sizes (log scale), with 95% confidence intervals, of *W. bancrofti* larvae (microfilariae, Mf and 3 larval stages, stages 1, 2, and 3) in *Ae. polynesiensis* at 25°C. (a) Growth in length. (b) Growth in width.

3.2.6. Overall pattern of growth and relationship between larval shape and age

Because of the constant rearing temperature in each experiment, the development of larvae was well synchronised among mosquitoes from the same batch and adjacent larval stages did not widely overlap. As such, it was valuable to sum up larval growth from microfilariae to stage 3 in one curve. Curves constructed from measurements from different temperatures had the same 'typical' shape, even if higher temperatures induced steeper forms. The temperature of 25°C, which is the mean annual temperature in Tahiti, was chosen for illustration (Fig. 1a,b). The worm aspect of all larval stages of *W. bancofti* could be summarised by the ratio of length to width. This ratio is a measure of the shape of the parasite and can be plotted against time since infection of the vector. For example, mean ratios measured at 25°C during 18 days are given in Fig. 2. The curve shows that the shape of larval *W. bancrofti* is continuously changing with time in the mosquito vector. As such, the shape can be used as an age indicator. Ratios ranged from ≈ 40 for microfilariae to <10 for L1 larvae and up to 75 for L3. These values were consistent within all temperatures studied.

3.3. Effect of temperature on sizes of third stage

Neither lengths nor widths were significantly different at the beginning of stage 3 among rearing temperatures ($F = 1.82$; d.f. $= 4, 123$; $P = 0.13$ for lengths, $F = 1.65$; d.f. $= 4, 123$; $P = 0.16$ for widths). On the other hand, at the end of the stage, both lengths and widths differed significantly $F = 41.06$; d.f. $= 4, 178$; $P < 0.0001$ for lengths and $F = 3.74$; d.f. $= 4, 178$; $P = 0.006$ for widths). Newman–Keuls comparisons made three homogenous groups for lengths. The first one was constituted by L3

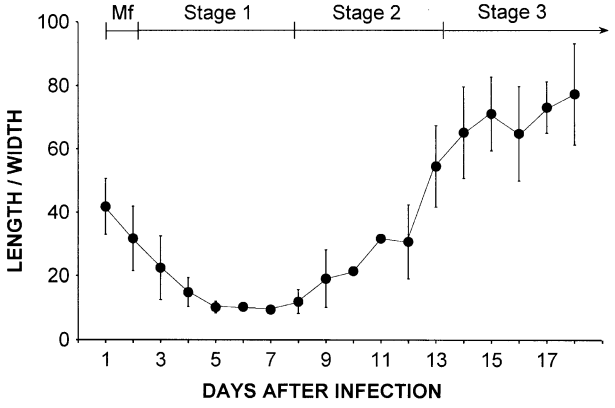

Fig. 2. Daily mean ratio of length to width, with 95% confidence intervals, of *W. bancrofti* larvae (microfilariae, Mf and 3 larval stages, stages 1, 2, and 3) in *Ae. polynesiensis* at 25°C.

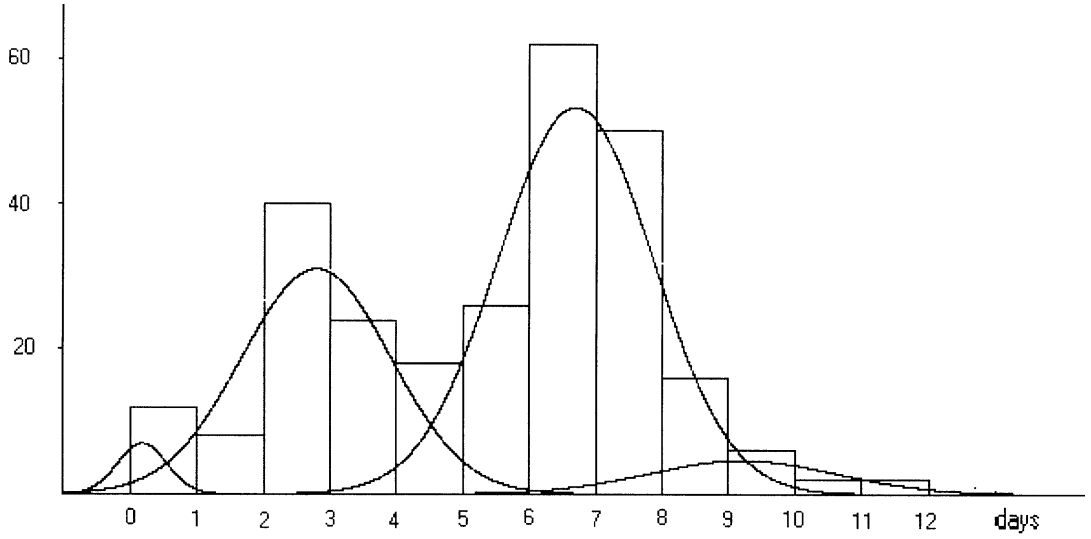

Fig. 3. Modal decomposition of histogram of *W. bancrofti* larval 'ages' into Gaussian components. The total number of ages, computed by our model that use length and width of the parasite larvae as 'age indicator',and ambient temperature, is 266. The 'age' of the parasite larvae represents the time elapsed since ingestion of the microfilariae by the vector. Four Gaussian components are identified by the modal decomposition method, which means (in days units) are mean time between two mosquito bites.

reared at 20 and 22.5°C, the second one by larvae reared at 25 and 27.5°C and the third one by larvae maintained at 30°C. These groups were ranked from largest to smallest lengths as temperature increased. In the same manner, Newmann–Keuls tests grouped widths in two homogenous groups: widths measured from larvae reared at 20 and 22.5°C were slightly larger than those reared at 25, 27.5 and 30°C. The particular case of lengths at 32°C confirmed that length decreases with temperature. The mean length at 32°C recorded on 21 observations was 816 μm (SD = 93), half the size recorded at lower temperatures, while widths were 35 μm (5). These L3 were banana-shaped, unlike the usual long snake-like form (Lardeux and Cheffort, 1997).

3.4. Effects of varying temperatures on the parasite development time

The laboratory experiment carried out at variable temperature lasted 18 days (i.e. until the last batch of mosquitoes was dissected). The first L3 appeared at day 12 and the procedure of Pontius et al. (1989) gave a mean time of appearance of the L3 stage of 12.58 days (variance = 0.21). The computation of development time D using Eq. (7) gave 12.04 days. The two values were similar and as such, the developmental function $r(T)$ used to compute the 'fraction of development' of *W. bancrofti* larvae can also be used in varying temperature conditions (i.e. field conditions).

3.5. Estimation of W. bancrofti 'fraction of development' using larval shape

Beta-coefficients of multiple regression were 0.78 for 'width' and 0.26 for 'length' indicating that most of A can be explained by width measurements. However, the stepwise procedure included both variables 'width' and 'length' in the model, though a light correlation existed between the two (correlation = 0.6, but <0.9). Moreover, the tolerance value was 0.64 (i.e. >0.2), indicating no large inter-correlation of the two variables. The multiple regression equation was (in brackets: SE of coefficients):

$$A = -0.0913(0.005) + 0.238(0.008)\text{Length} + 23.359(0.284)\text{Width}$$

with a correlation coefficient $R = 0.96$.

ANOVA indicated a good overall significance test for regression ($F = 7968$; d.f. $= 2, 1171$; $P \approx 0.000$). Plots of residuals vs. predicted values showed no particular structure and as such, residuals were randomly dispersed. The Shapiro–Wilk test ($W = 0.97$; $P = 0.09$) indicated that residuals were normally distributed.

So, the above regression equation could be used to predict the 'fraction of development' A of a *W. bancrofti* larvae, knowing its length and width.

3.5.1. Estimation of Ae. polynesiensis blood-meal intervals with field data

The histogram of computed ages of the 266 *W. bancrofti* larvae measured amongst the 59 dissected mosquitoes is given in Fig. 3. This histogram was decomposed into four Gaussian components by modal analysis. Means and standard deviations of these components are given in Table 1. The histogram of the 59 mean ages for mosquitoes (computed as the mean of ages of *W. bancrofti* found in each mosquito), and its decomposition into three Gaussian components is given in Fig. 4. Values of $\bar{x}$ and SD are given in Table 1. Mark–recapture data of Suzuki (1977) and Rivière (1988) were re-analysed using modal analysis, in

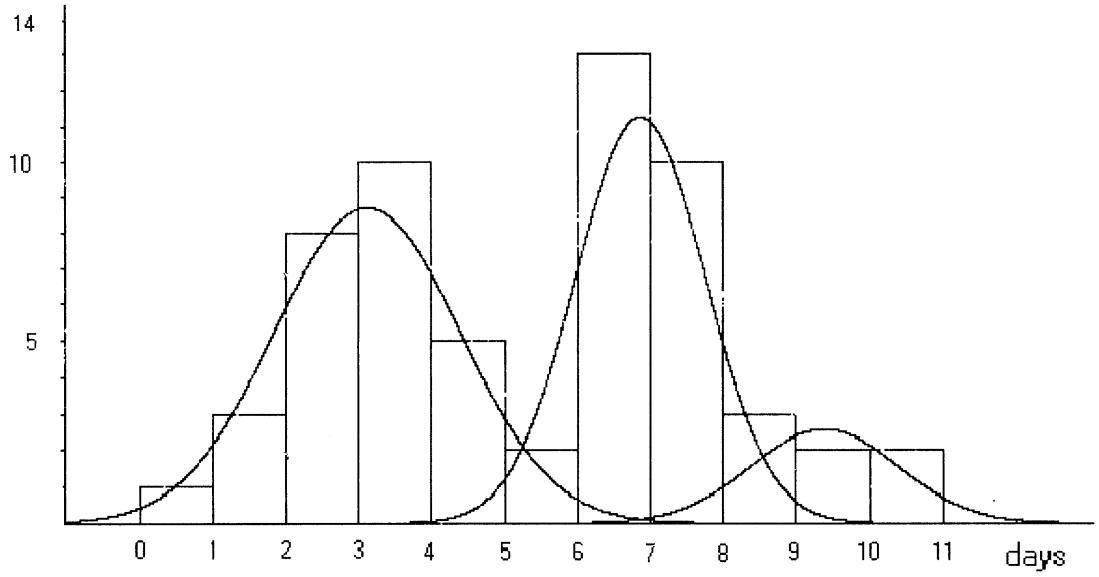

Fig. 4. Modal decomposition of histogram of the 53 mean 'ages' of *Ae. polynesiensis* computed by our model. Ages are grouped in several modes that are identified by the modal decomposition method. Values of means of the Gaussian components identified are the mean time (in days) since the infectious blood-meal.

order to better compare our results with theirs (Table 2). A quick look at the results indicates that the mean time between histogram modes are $\approx$ 3–4 days, representing the length of the gonotrophic cycle.

4. Discussion

Measurements made in our study are in agreement with previous studies. The studied range of temperatures corresponds to the mean minimum of 21.5°C (August) and the mean maximum of 29.5°C (March) recorded in Tahiti. Above 31–32°C, the development of *W. bancrofti* larvae is abnormal (Basu and Rao, 1939; Nakamura, 1964; Rozeboom et al., 1968; Bruhnes, 1969a,b; El-Dine and Habib, 1969). Our experiments carried out at 32°C and exhibiting alterations in the shape of larvae, confirmed these results. Moreover, a temperature of 34°C seems to be a limiting rearing factor for *Ae. polynesiensis*. Thus, our data are representative of mean developing conditions for *W. bancrofti*. The duration of the parasite's cycle in its vector is reduced as temperature increases, passing from 26 days at 20°C to 9 days at 30°C (time of first appearance of L3 after blood-feeding).

Most growth patterns were objectively identified as S-shaped functions by Schnute's model, with the exception of L1 growth in length (U-shaped) and L3 growth in width (negative exponential function). The general growth curve of Fig. 2 has the same form as those given for Fiji strains of *W. bancrofti/Ae. polynesiensis* by Suzuki (1977) or for close filaria species such as the dog heartworm *D. immitis* Leidy (Kartman, 1954), which is also transmitted by *Ae. polynesiensis* in French Polynesia, or for *Brugia patei* (Laurence and Pester, 1961). The shape of these growth curves may be a characteristic of such filarial worms. Here, the greatest difference between length and width was found in the L3 stage in which the length was over 75 times greater than the width. For *D. immitis*, Kartman (1954) found a ratio of about 40, indicating that infective larvae of this species have a less thin and elongate aspect than *W. bancrofti*. On the other hand, microfilariae in both species exhibited the same ratio of about 50. In the L1 ('sausage') stage, the difference between length and width has reduced to its smallest value since the length was only ten times the width. Thus, the overall increase in length was very great, whereas the change in width was quite limited, especially after the L3 stage had been reached. During the L1, width increases 2.5 times and during the L2, 1.5 times. The increase in length is relatively greater in L2, as the size of larvae is multiplied by three from the beginning to end of stage.

Temperature does not seem to modify the general pattern of growth in length and width. At different temperatures, the shape of curves for each larval stage are the same, but are of different time scales and 'slopes': because temperature has no effect on final L1 and L2 stage measurements, growth rates have to increase as the duration of stages shorten with increasing temperature. This is also the case for L3 growth, except that final lengths are approximately 18% smaller at 30°C than at 20°C. One important result for filaria transmission to a vertebrate host is that L3 become rapidly infective (i.e. their shape is rapidly stabilised) and at 25°C, this stage may be attained a few days after moulting from stage 2 to stage 3.

Wuchereria bancrofti larvae are growing continuously in *Ae. polynesiensis* from microfilariae to L3 stage. Larval

Table 2
Number of days ($\bar{x}$) and SD since infection of *Ae. polynesiensis*, as computed by extraction of Gaussian components in three data sets from wild mosquitoes captured on human baits and attempting to blood-feed: individual larvae measurements, mean age of larvae in each mosquito, Suzuki (1977) and Rivière (1988) data of recapture experiments

	Larvae measurements ($n = 266$)		Mean age per mosquito ($n = 59$)		Suzuki's data of recapture ($n = 40$)		Rivière's data of recapture on Tereia Island ($n = 360$)		Rivière's data of recapture on Tahiti Island ($n = 132$)		Rivière's data of recapture on Scilly Island ($n = 37$)	
Gaussian component	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
1	0.16	0.34	–	–	–	–	1.31	0.67	–	–	–	–
2	2.79	1.14	3.11	1.26	3.01	0.91	3.78	1.89	3.16	0.81	3.85	0.95
3	6.71	1.16	6.86	0.88	6.44	1.08	7.12	1.1	6.43	1.49	6.16	0.62
4	9.31	1.38	9.38	1.01	9.97	0.72	14.45	0.79	11.3	0.59	–	–

length and width are continuously changing with time and therefore, the shape of larvae may be used as a time indicator. Precision in 'size at age' measurements is achieved if sizes rapidly change with time, so that each 'size' may correspond to a unique 'time'. This may not always be the case in S-shaped growth patterns where plateaus at the beginning and end of the curve denote a slowing growth. However, for *W. bancrofti*, these plateaus do not last long enough to indicate a stop in shape changes except for infective larvae. Young L3 may still be used to correlate sizes and time, but not infective larvae whose shape stay the same and can be retained in the vector during several days until blood-feeding. *Wuchereria bancrofti* larval sizes do not depend on temperature, except for excessively high ones (i.e. >30°C). However, growth rates are temperature-dependent: the lower the temperature, the slower the growth. The time unit used to descibe a 'size at age' relationship should therefore take into account this temperature effect. If an accurate time unit can be found, the shape of larvae may be correlated with its 'age'. Chronological time elapsed since vector infection may, therefore, be inferred. If vectors are captured just before feeding, time between two blood-meals (i.e. the gonotrophic cycle length) may be determined.

Detinova (1963), computed the length of the gonotrophic cycle of *Anopheles maculipennis* using morphological changes of ovarioles and the duration of digestion of the mosquito's blood-meal (as an hyperbolic function depending on ambient temperature and relative humidity). However, Detinova's method is not always possible as morphological changes in mosquitoes' ovarioles with time are not always as easy to interpret. This is true for *Ae. polynesiensis* (Rivière, 1988). Duke (1968) and Philippon (1977) computed the length of the gonotrophic cycle of *Onchocerca volvulus* (Leuckart) developing in *Simulium damnosum* Theobald, using an approach similar to ours, but with less precision because they did not take into account temperature variations. However, they pointed out the usefulness of using the parasite growth as an age indicator. Describing ambient temperature variations by a sine function is an imperfect approximation of the day's temperature pattern since it is symmetric, whereas temperature pattern generally is not. However, in French Polynesia, the minimum temperature occurs at 2 AM, while the maximum occurs at 1 PM (Pasturel, 1993). As such, the pattern is nearly symmetrical on a 12:12 h basis and the sine approximation is accurate. Nonetheless, the function provides a mechanism to incorporate the fluctuations in temperatures during the day rather than relying on a mean temperature (with possible adjustments). Temperatures used for computations are those measured in meteorological stations, i.e. at 1 m above the ground level, under shelter. Mosquitoes may choose resting places where the ambient temperature may be slightly different from that measured at meteo stations. These temperatures may not be the actual temperature conditions during the maturation of the larvae in the mosquitoes. However, the behavioural patterns and the resting places of *Ae. polynesiensis* may be close to these artificial conditions (Rivière, 1988). As such, the bias introduced should be minimum, and 'age' estimates computed more precise than if not taking into account ambient temperature fluctuations.

Using L3 measurements may not be accurate to compute larval 'age'. Their shape is rapidly that of L3 infective, not changing with time, and can survive several days in the mosquito, waiting for their release during a blood-meal (Lardeux and Cheffort, 1996). So, our method cannot be used to compute the time between an infectious blood-meal and the following infective one. In our data, young L3 measurements formed the tail of the histogram in Fig. 3. These L3 were not infective and proved that *Ae. polynesiensis* can take a blood-meal a few days before being able to transmit the parasite. We measured microfilariae in one individual, represented by the first bar in histogram of Fig. 3 or Fig. 4 and Rivière (1988) recaptured mosquitoes just the day after marking and releasing partially engorged females. Depending on the state of repletion from the first blood-meal, *Ae. polynesiensis* can visit hosts again as soon as a few hours after the partial blood-meal. Observations of young L3 or microfilariae in biting mosquitoes indicate that blood-meals may be more frequent than usually believed for this mosquito species. The shape of the histograms, with peaks not as well separated as expected, also corroborates this hypothesis. Nevertheless, mean times since infection can be computed, which interval between them designates the mean length of the gonotrophic cycle. Our data suggest that most times since infection for *Ae. polynesiensis* occur at ≈3, ≈6–7 and ≈9.5 days. As such, the length of the gonotrophic cycle may be of 3–4 days for *Ae. polynesiensis*. Our results are in agreement with Suzuki (1977) and Rivière (1988) who found the same value. Depending on the temperature, the incubation period of *W. bancrofti* in *Ae. polynesiensis* in the Society Islands (French Polynesia) varies between 10 and 16 days (Lardeux and Cheffort, 2001). As such, *Ae. polynesiensis* coming to bite humans with infective larvae should have finished at least three blood-meals.

However, Ingram (1954) and Jachowski (1954) reported the interval between two blood-meals of *Ae. polynesiensis* by individual rearing in the laboratory. In both studies, the interval was estimated to be ≈7 days. This value is much longer (about double) than our estimate in the field. It is presumed that, under artificial and restricted laboratory conditions, it sometimes takes a long time from oviposition to blood-meal, even if a blood source is made available continuously, as was the case of the above studies; while in the field, mosquitoes would take a blood-meal almost immediately after oviposition.

Usual marking–recapture techniques need several consecutive days of significant recaptures of marked animals. It is time consuming and subject to biased estimates for computation of gonotrophic cycle length (Rivière, 1988). The

method presented here bypasses these disadvantages, and in areas where mosquito prevalence of infection is high (such as in French Polynesia), enough infected mosquitoes can be captured in 1 day to carry out an analysis.

The method presented here is well adapted to a statistical determination of time since infection; i.e. a statistical analysis of histogram distribution of ages. Estimating time since infection for one single mosquito with a good precision is still possible if several larvae are found within the vector, and if these larvae exhibit small length and width measurement variances.

Our method also has the advantage that with only one mosquito sample, other population dynamic parameters (such as daily survival rates using the ratio of the number of mosquitoes with infective larvae to the total number of infected mosquitoes (Laurence, 1963), or from the number of mosquitoes with first, second and third stages larvae (Van Dijk, 1966), or methods outlined in Burkot and Graves (1995)), can be derived following mosquito dissection and parasite larval counts.

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Appendix A. Developmental rate function for *W. bancrofti* larvae (Lardeux and Cheffort, 1997)

Larval *W. bancrofti* developmental rate $r(T)$ at temperature T can be modelled by means of Lactin et al. (1995) function:

$$r(T) = \exp(\rho T) - \exp\left[\rho T_{\mathrm{m}} - \frac{(T_{\mathrm{m}} - T)}{A}\right] + \lambda$$

where T_{m} is a thermal maximum, i.e. the 'lethal' temperature at which life processes can no longer be maintained for prolonged periods of time, A the temperature range over which 'thermal breakdown' becomes the overriding influence, and ρ can be interpreted as a composite value for critical enzyme-catalysed biochemical reactions. The parameter λ is the value of $r(T_{\mathrm{m}})$ (i.e. when $T = T_{\mathrm{m}}$) and allows the curve to intersect the abscissa at sub-optimal temperatures, permitting estimation of the base temperature (i.e. the temperature below which development stops) by allowing $r(T) = 0$ to be solved for particular parameter values. For the combination *W. bancrofti*/*Ae. polynesiensis*, parameters (and their SE) were $\lambda = -1.179$ (0.189), $A = 10.98$ (1.28), $\rho = 0.0126$ (0.0005) and $T_{\mathrm{m}} = 56.72$ (0.51).

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