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Generalized Linear Modelling for Parasitologists

K. Wilson and B.T. Grenfell

Typically, the distribution of macroparasites over their host population is highly aggregated and empirically best described by the negative binomial distribution. For parasitologists, this poses a statistical problem, which is often tackled by log-transforming the parasite data prior to analysis by parametric tests. Here, Ken Wilson and Bryan Grenfell show that this method is particularly prone to type I errors, and highlight a much more powerful and flexible alternative: generalized linear modelling.

A major problem facing any parasitologist is how best to analyse his or her hard-earned data. What is the best way of determining, for example, whether human faecal egg counts decline with age, or whether male and female rabbits differ in their worm burdens? The problem of correctly identifying the best statistical test in parasitology is accentuated by the fact that parasites tend to be aggregated over their host population: most hosts have just a few parasites, or none at all, while others have many (for relevant discussions, see Refs 1–5). As a result, the parasite distribution is right-skewed with a long tail, and fails to conform to the normal or Gaussian distribution assumed by most of the commonly used statistical tests, ie. parametric tests, such as t-tests, analyses of variance, regression analyses, etc. This presents the parasitologist with a fundamental problem.

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Overcoming the problem of non-normality

The majority of parasitologists either ignore the fact that the non-normality of their data is a problem and use parametric tests regardless, or use non-parametric tests, such as Mann-Whitney U-tests, Wilcoxon signed ranks tests, Kruskal-Wallis tests, etc. (see Box 1). While

Box 1. Current Statistical Methods used by Parasitologists

We surveyed the statistical methods reported in 50 papers published in the past five years in the journal *Parasitology* (K. Wilson, unpublished; and see Table below). All of these papers contained data, such as egg counts and worm burdens, for which we would expect the untransformed distribution to be discrete (rather than continuous) and to conform to the negative binomial, or at least to the Poisson. Remarkably, in 20% of the papers surveyed, no statistical tests were applied at all; 46% used standard parametric tests (such as t-tests, analyses of variance and regression analyses), of which more than half failed to transform the data in any way prior to analysis; 28% used non-parametric tests (such as Mann–Whitney and Kruskal–Wallis tests); and just three of the 50 papers used sophisticated non-linear maximum-likelihood methods. Surprisingly, during this period, there was not one published paper that used generalized linear models.

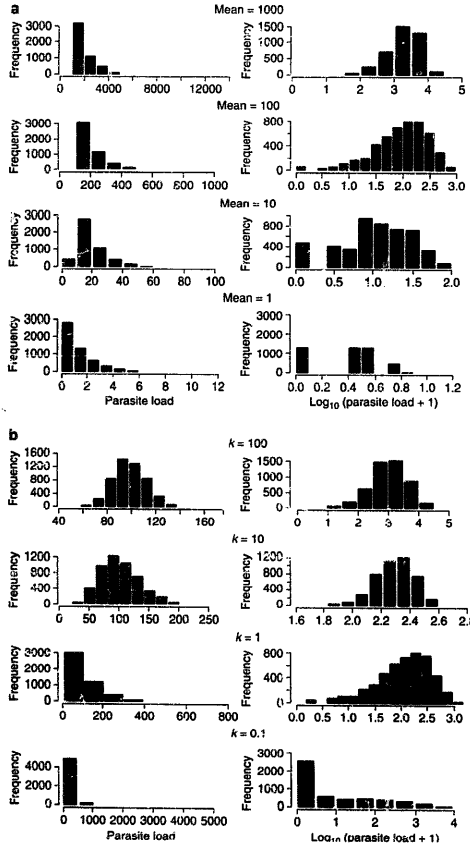
Table. Statistical tests used in 50 papers[a] published in *Parasitology* in 1991–1995

Statistical test	Mature parasites	Immature parasites	Both mature and immature parasites	Total No. (and %)
None	4	5	1	10 (20)
Parametric (*x*)	5	3	4	12 (24)
Parametic (log-*x*)	3	8	0	11 (22)
Non-parametric	5	5	4	14 (28)
Maximum-likelihood	1	2	0	3 (6)
GLM[b]	0	0	0	0 (0)
Total	**18**	**23**	**9**	**50**

[a] Papers are divided into those that discussed variation in the burdens of mature parasites (adult worms or ticks), immature parasites (eggs, oocysts, gametocytes, sporozoites, cercariae, microfilariae, etc.) and combined studies. Parametric tests are divided into those that analysed raw counts (*x*) and those that analysed log-transformed counts (log-*x*).
[b] GLM refers to generalized linear models and maximum-likelihood to non-linear maximum-likelihood models.

Box 2. Log-transformation often Fails to Normalize Parasite Distributions

A traditional method for normalizing right-skewed, overdispersed data is to $\log_{10}$- or $\log_e$-transform it, after first adding one to avoid zero counts. However, as illustrated below, this transformation fails when the mean of the distribution is small (a) or the distribution is highly aggregated, as indicated bysmall negative binomial k values (b).



Effect of the mean (a) and the skew (b) of the distribution (as determined by k of the negative binomial) on the efficacy of $\log_{10}$-transformation: both (a) and (b) show the frequency distribution of 5000 random samples taken from negative binomial distributions (using the *rnegbin* function in Splus), and the subsequent distribution after the samples were $\log_{10}$-transformed. In (a) the average k of the distribution is one and the average population mean varies between 1000 and 1, and in (b) the average population mean is 100 and average k varies between 100 and 0.1.

non-parametric tests make few assumptions about the underlying statistical distributions[6], and hence are preferable to parametric tests when the normality assumption is violated, they generally lack the power of equivalent parametric tests[6] and so are not an ideal solution.

A common alternative to using non-parametric tests is to use standard parametric tests after first transforming the data so that their distribution becomes approximately normal. Because most macroparasite distributions are empirically best described by the negative binomial distribution[5,7–9], an appropriate transformation is generally the $\log_{10}$- or $\log_e$-transformation (after first adding one to the parasite count to avoid zeros)[10,11]. However, this transformation often fails to normalize the distribution, especially when it is highly skewed (see Box 2).

Generalized linear models

A less common alternative to non-parametric tests is the family of generalized linear models (GLMs) (see Box 3). These are generalizations of classical linear models (such as linear regression, analyses of variance, etc.) in which the error distribution is explicitly defined (see Box 3). As emphasized above, classical linear models assume that the error distribution is normal. However, for macroparasite data, the appropriate error distribution is often the negative binomial, which is defined by its mean ($\bar{x}$) and the exponent k. The variance (s^2) of a negative binomial distribution is described as follows:

$$s^2 = \frac{\bar{x} - \bar{x}^2}{k} \qquad (1)$$

where $\bar{x}$ and s^2 are the mean and variance, respectively, of the sample and k is an inverse measure of the degree of aggregation, such that as aggregation declines so k increases until, as k approaches infinity (or in practice, above about 20), the distribution converges on the Poisson[8].

In order to fit the negative binomial distribution, we need to estimate the exponent. The most accurate estimate of k is obtained by maximum-likelihood methods[8], but a reasonably accurate moment-estimate can be calculated by rearrangement of Eqn 1:

$$\hat{k} = \frac{\bar{x}^2}{s^2 - \bar{x}} \qquad (2)$$

where $\hat{k}$ is the estimated value of k.

Box 3. Generalized Linear Models

Generalized linear models (GLMs), are generalizations of classical linear models (analyses of variance, t-tests, linear, regression analyses, etc.) that allow the error structure (the distribution of residuals about the fitted model) to be explicitly defined by one of a series of distributions, usually from the exponential family. GLMs also use a link function, which maps the expected values of the response variable (eg. faecal egg count) on to the explanatory variables (eg. age and sex). For classical linear models, the error structure is defined by the normal distribution and the link function is the identity link. For the negative binomial distribution (which is not in the exponential family), the GLM error structure is defined by Eqn 1 (in the text) and the link function is generally the log or square-root link[12,13]. For an accessible introduction to the uses of GLMs in ecological studies, see Ref. 12.

The significance of terms in GLMs are generally tested by comparing the deviances of models with and without those terms. Deviances are analogous to mean squares in classical linear models. For the Poisson and negative binomial distributions, deviances are distributed approximately as Chi-square (χ^2), with degrees of freedom equal to the difference in the number of parameters attributable to each of the models.

A common alternative to explicitly defining a negative binomial error distribution is to assume a Poisson distribution and to adjust the scale parameter (or dispersion parameter) so that the ratio of the residual deviance and its degrees of freedom is approximately equal to one (Refs 12, 17). Thus, instead of asssuming that the variance of the parasite distribution is equal to its mean ($s^2 = \bar{x}^2$, for the Poisson distribution), we assume that it is proportional to it, ie. $s^2 = \Phi\bar{x}$, where Φ is referred to as the empirical scale or dispersion parameter. When empirical scale parameters are used, model parameter estimates are not affected but the standard errors are higher[12] and, in a manner similar to standard analyses of variance and regression models, the scaled deviances for terms in the model are compared using F-tests instead of χ^2-tests[12,17].

A number of statistical packages (eg. GLIM, Genstat, SAS and Splus) include GLMs and have a range of error structures already defined (including Gaussian, gamma, binomial and Poisson). Negative binomial errors are not usually included in the available set, but they can be defined by the user or obtained from sources within the public domain (for example, Ref. 12 provides GLIM macros on disk both for estimating k by maximum-likelihood methods and defining negative binomial errors, and equivalent Splus functions are available by anonymous ftp from StatLib; see also Refs 13, 14).

An alternative to defining a negative binomial error distribution explicitly is to generate an empirical estimate based on Poisson errors. Equation 1 can be simplified to:

$$s^2 = \frac{\bar{x}(1-\bar{x})}{k} = \bar{x}\Phi \qquad (3)$$

Thus, the negative binomial distribution can be approximated by assuming that Φ is approximately constant over all values of $\bar{x}$. In practice, we define a Poisson error distribution and estimate the value of Φ empirically (Φ is then defined as the empirical dispersion or scale parameter; see Box 2).

A comparison of methods

Wilson, Grenfell and Shaw[14] have recently compared the log-transformation method described earlier with these two GLM methods, using (1) simulated data sets (Box 4), and (2) real parasite data from an unmanaged population of Soay sheep on the Scottish island group of St Kilda (Box 5).

From the simulated data sets, they conclude that, when sample sizes are small, the frequency of type II errors, ie. incorrectly accepting the null hypothesis, H_0, is generally slightly higher when using standard parametric tests on log-transformed data than when using either of the GLM methods. However, much more importantly, they also conclude that, almost regardless of the sample sizes, when the distributions being compared differ in their degree of aggregation (as indicated by their negative binomial k estimates), type I errors, ie. incorrectly rejecting H_0, are likely to be extremely common when using the log-transformation method, but negligible when using either of the GLM methods (Box 4). This strongly suggests that when using the log-transformation method, parasitologists are much more likely to report spurious differences in parasite burdens than when using either of the GLM methods. Fortunately, it appears to matter little which of the two GLM methods is used. This means that standard statistical packages such as GLIM, Genstat, SAS and Splus, can be used without recourse to writing specific macros or functions to define the appropriate error structure.

Analyses using real parasite data also indicate that the choice of statistical method used is also important here[14]. For example, an analysis of the worm burdens of Soay sheep dying during one winter on St Kilda strongly suggests that the log-transformation method is capable of generating type II errors, and an analysis of August faecal egg counts of the same population of sheep suggests that this method may also generate type I errors (see Box 5 for details). In both cases, these results were confirmed by more sophisticated non-linear maximum-likelihood models. This latter method is also needed when analysing patterns in the degree of aggregation, such as changes in k with age[4,15].

The use of explicit maximum-likelihood error structures is not restricted to field data. For example, Box 6 shows an analysis of the results of experimental infections of cats with filarial worms. In this case, the method also allows us to estimate the death rate of adult parasites (Box 6).

Conclusions

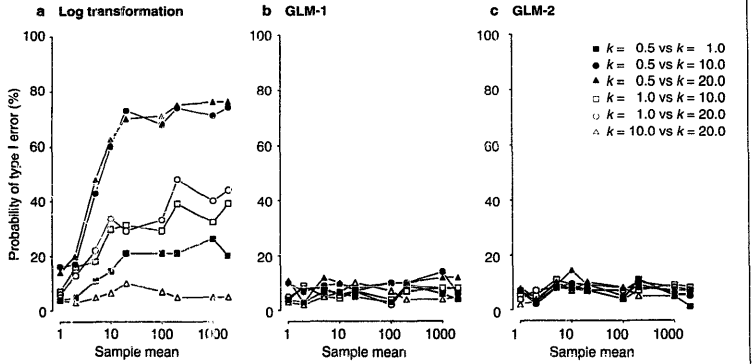
The analyses summarized here clearly indicate that classical linear regression models using log-transformed data are usually much more likely to generate both type I and type II errors than are generalized linear models. GLMs are becomingly increasingly incorporated into modern statistical packages and being used by ecologists and social scientists. With familiarity, they are only marginally more difficult to use and understand than the statistical models currently being employed by parasitologists. Obviously, they are not the be-all-and-end-all in statistical

Box 4. Simulated Data Sets

In order to compare the log-transformation method with the GLM approach, Wilson, Grenfell and Shaw[14] used the two methods to analyse a series of randomly generated data sets from the negative binomial distribution. The data sets comprised 20, 100 or 500 samples from distributions with means ranging between one and 2000, and with k values ranging between 0.5 and 20. These ranges cover those of most parasite burdens and faecal egg counts. For each of a pair of data sets, with either identical means or means differing from each other by 100% (1 vs 2, 5 vs 10, 10 vs 20, etc.), the statistical significance of the difference between the means was assessed over 100 trials using three models of increasing sophistication: a classical linear model using $\log_{10}$-transformed data (a, ir. Fig. below); a GLM with Poisson errors and an empirical scale parameter, using untransformed data (b); and a GLM with negative binomial errors, again using untransformed data (c).

The explanatory variable of the three models comprised a single factor, which coded for each of the pair of distributions. The number of times that the different models detected significant differences between distributions was scored over 100 trials using F-tests (a and b) or Chi-square tests (c). Thus, by comparing the output of the three models it was possible to assess the probabilities of each performing type I errors, ie. incorrectly rejecting the null hypothesis of no difference between the means; and type II errors, ie. incorrectly accepting the null hypothesis.

The biggest differences among the three models came when comparing data sets that had the same means, but different k values (for details of the other comparisons, see Ref. 14). In this series of comparisons, the probability of the log-transformation model producing type I errors ranged between between 0 and 75% and increased with sample size (n), sample mean $\bar{x}$ and difference between the component k values. By comparison, both the GLMs produced many fewer type I errors over all values of n, $\bar{x}$ and k; and both models failed with a probability ranging between about 0 and 15% (see Fig.). Thus, the log-transformation method is much more likely than the GLM method to indicate spurious differences between data sets.



A comparison of the rate of type I error production when the component distributions have the same mean but different k values: (a), (b) and (c) each shows the probability of making a type I error when using each of the three statistical methods (see main text and Ref. 14, for a description of the models and the simulations). Each point refers to the probability of incorrectly rejecting the null hypothesis of no difference between the means, and is based on 100 simulations comprising 20 data points taken from two negative binomial distributions with identical population means but different population k values. The six symbols refer to different combinations of k values, as indicated in the figure. Simulations based on larger samples (100 or 500 data points) produce qualitatively similar trends (see Ref. 14). The results for the log-transformation method, shown in (a), indicate that this has a high probability of incorrectly rejecting the null hypothesis over a range of sample sizes, sample means and component k values. The results for the GLM method with an empirical scale parameter is shown in (b), and for the GLM with negative binomial errors in (c). For both (b) and (c), the probability of type I error is small.

analyses[14], and more sophisticated methods, such as non-linear maximum-likelihood analysis and bootstrapping, may be appropriate when sample sizes are large or the statistical models simple (eg. Refs 4, 11, 16). However, GLMs are a significant improvement on most current methods of analysis, and their use by parasitologists must be encouraged.

Acknowledgements

We thank Steve Albon, Tim Coulson, Mick Crawley, Brian Ripley, Darren Shaw, Bill Venables and An Verbyla for positive discussions about negative binomials, Jill Pilkington for collecting most of the Soay sheep parasite data, and David Denham and Edwin Michael for the data on filarial worms. This work was supported by the NERC and the Wellcome Trust.

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Box 5. Soay Sheep Parasite Data

The log-transformation method was compared with the two GLM methods using post-mortem worm counts and faecal egg counts of an unmanaged population of Soay sheep on the Scottish island group of St Kilda (see Refs 18, 19). This population exhibits severe 'crashes' every 3–4 years, when up to 70% of the sheep die, due mainly to the population over-exploiting its winter food supply[20,21], although parasites have also been implicated (Refs 18, 22, 23; and K. Wilson *et al.* unpublished).

Worm burdens

In the first comparison, Wilson, Grenfell and Shaw[14] analysed the worm burdens of sheep that died during the population crash of 1991–1992. The models included two factors potentially affecting the burdens of six species or genera of adult helminth worms: AGECLASS (two levels: lambs and adults) and SEX (two levels; males and females). This analysis identified three qualitative discrepancies between the results of the three models. In two of these (*Trichuris ovis* and *Dictyocaulus filaria*), the two GLMs identified SEX as a significant heterogeneity in worm burdens, when the log-transformation method failed to do so. In the third (*Teladorsagia* spp), the two GLMs identified a significant AGECLASS–SEX interaction, whereas the log-transformation method again failed to do so. Thus, it appears that the log-transformation method committed type II errors in these analyses and this was confirmed by non-linear maximum-likelihood methods.

Faecal egg counts

In the second comparison, Wilson, Grenfell and Shaw[14] examined the factors influencing the faecal egg counts of sheep in the month of August between 1988 and 1993. When male egg counts were considered in isolation, the models again had two factors: AGECLASS (four levels: lambs, yearlings, two-year-olds and adults) and YEAR (six levels).

While all three methods indicated important between-year variation in male faecal egg counts, only the log-transformation method identified significant differences between the four age-classes ($P<0.01$). Thus, the standard log-transformation method appears to have made a type I error that is not made by the two GLMs, and this assertion was further supported using a non-linear maximum-likelihood model.

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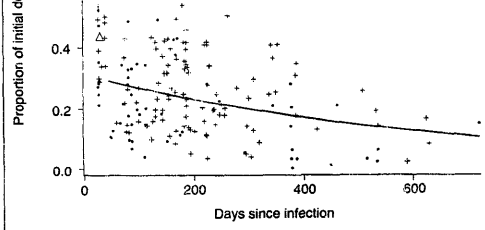
Box 6. Dealing with Aggregation in Experimental Infections

Estimating parasite death rates in cats infected with *Brugia pahangi*. The Fig. (below) shows a famous parasitological data set[24,25]: the number of adult worms recovered after infection of cats with a single dose of the filarial nematode *Brugia pahangi* [initial dose = 100 larvae (dots) or 200 larvae (crosses)]. After a sharp initial decline in recoveries (reflecting the initial establishment of worms[26]), the proportion of parasites declines gradually, though variably, with time. Given a Poisson-distributed infection rate, we might expect a Poisson distribution for parasite numbers[23]; however, these data are much more aggregated than that (estimated $k \pm SE = 3.29 \pm 0.41$), probably reflecting heterogeneities in parasite demographic parameters between hosts[24,26]. This aggregation is confirmed by a good fit of the negative binomial model (residual deviance = 163, degrees of freedom = 150, $P = 0.222$), which also indicates no significant difference in proportional recovery between infection doses.



The fitted line in the Fig. (above) is for the regression of worm burden on time for recoveries after 40 days, using a log link function. Comparison with earlier burdens (mean indicated by the triangle) illustrates the initial drop in parasite load. The regression also allows us to estimate the adult parasite's average death rate and lifespan (E. Michael, B.T. Grenfell, D.A. Denham and D. Bundy, unpublished). The death rate is equal to the slope of the regression line (= 0.538 per year) and the lifespan equal to 1/slope (= 1/0.538 = 1.86 years).

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Letters

Plasmodium cdc2-related Kinases: Do they Regulate Stage Differentiation? Reply

I can only add my support to Kinnaird and Mottram's comments (this issue): currently available data on *Plasmodium* cdc2-related kinases are indeed insufficient to define a role for these enzymes in the parasite's life cycle (as was clearly stated in our original paper on *Pfcrk-1*)[1]. Nevertheless, a touch of cautious speculation is useful to define working hypotheses whose value can be tested experimentally.

Our hypothesis that *Pfcrk-1* may be involved in establishment or maintenance of the differentiated, nondividing state of gametocytes was based on the facts: (1) that it is most homologous to a known downregulator of cell proliferation ($p58^{GTA}$); and (2) that its mRNA can be detected only in gametocytes. Furthermore, expression of $p58^{GTA}$ during mouse embryonic development coincides with the cessation of cell division that accompanies differentiation[2] – a pattern similar to that exhibited by *Pfcrk-1*. However, as mentioned by Kinnaird and Mottram, $p58^{GTA}$ is just one member (namely PITSLRE-β1) of the PITSLRE family of kinases, of which several very closely related isoforms exist; the function of these isoforms is unknown, but most (the exception being PITSLRE-β1 and -α2) appear not to have deleterious effects on cell viability when expressed ectopically suggesting that they do not act as downregulators of cell division[3]. The possibility exists that *Pfcrk-1* is a gametocyte-specific functional homologue of one of these rather than of PITSLRE-β1 and plays no role in the regulation of gametocytogenesis. The search for the function of $p58^{GTA}$ homologues in Apicomplexans will benefit from the finding that *Theileria annulata* appears to possess such a gene, as indicated by Southern blot experiments using *Pfcrk-1* as a probe; this putative homologue has been cloned and is currently being characterized (G. Langsley, pers. commun.).

Kinnaird and Mottram suggest that cell cycle arrest during gametocytogenesis is more likely to be achieved through the action of a CDK inhibitor (CDI) than through that of an additional kinase activity. While there are cases in which a CDI appears to be sufficient to arrest the cell cycle (eg. in yeast mating-pheromone response[4]), the fact that, in higher eukaryotes, additional elements (such as $p58^{GTA}$ for example) appear, in some instances, to be required to stop cell proliferation indicates that inhibiting a cell cycle kinase with a CDI may not, by itself, be enough to induce or maintain cell cycle arrest; this may reflect the relatively higher complexity of the cell cycle machinery in such organisms. The complexity of *Plasmodium*'s life cycle is presumably mirrored by an underlying complexity at the level of molecular regulation of the progression of this cycle. The large number of CRKs already identified in other protozoan parasites with complex life cycles[5] (versus the apparently much simpler situation of yeast for example) is consistent with this view. Likewise, the harvest of *Plasmodium* CDKs has probably only just begun, and one can reasonably expect more enzymes to be added to the as yet short list (indeed, we are presently characterizing a novel Pfcrk homologue). Lack of success in heterologue mutant complementation system (eg. yeast *cdc-2*) does not necessarily mean that the genes under investigation do not have a similar function to that lacking in the mutant; absence of functional complementation may well be due to poor expression of the parasite's genes in the heterologous host, or to the inability of the gene product to establish the required interactions with the host's machinery. In other words, the best way to resolve this question is to study the function of these genes in the parasites themselves. Stable transfection protocols for both trypanosomatids and *Plasmodium*[6] are now available, which should make this goal attainable. It would, of course, also be of great interest to identify CDIs in *Plasmodium*.

Kinnaird and Mottram rightly point out that there are several steps in the parasite's life cycle that require cell cycle arrest. Although there is no reason *a priori* to expect that the same gene products are involved at different stages (after all, if *Plasmodium* uses different sets of ribosomes at different developmental stages[7], it may well use different enzymes to fulfil similar functions at different stages), it is certainly worth looking at Pfcrk-1 expression during the entire life cycle, especially in nondividing stages (eg. sporozoites). In this respect, preliminary data suggest that a *Pfcrk-1* gene product peaks in late gametocytogenesis (Day 15) and is still detectable (but decreasing rapidly) after gametocyte activation has been initiated (M. Kariuki, C. Doerig and S. Martin, unpublished). If confirmed, such data would be consistent with Pfcrk-1 expression being correlated with the cell-division status of the parasite. The absence of detectable *Pfcrk-1* in asexual parasites argues against the idea that this enzyme is involved in the development commitment to gametocytogenesis, since this commitment appears to occur in the preceding schizont[8] (the possibility cannot be excluded that *Pfcrk-1* is indeed involved in the developmental decision, but is expressed at subdetectable levels or only in a small subpopulation of asexual parasites). Demonstrating a link between *Pfcrk-1* and cell surface components (as is the case for mammalian $p58^{GTA}$) would lend support to the hypothesis that it may function in sensing and/or transducing environmental changes that are likely to trigger developmental processes such as gametocyte activation; clearly, much more work is required before a picture of *Pfcrk-1* function and mode of action emerges.

All colleagues working on the regulation of growth and development in *Plasmodium* would presumably agree that functional characterization of candidate regulatory genes is the obligate next step towards an understanding of this phenomenon. This endeavour will be greatly facilitated by the recent availability of reverse genetics procedures to *Plasmodium*. In the meantime, we will still favour the working hypothesis that *Pfcrk-1* is an important regulator of differentiation: if empirical evidence shows it to be the case, so much the better, we will have gained some insight into the mechanisms of *Plasmodium* development; if not, we will nevertheless have delighted for a while in the excitement of perhaps uncovering a fundamental aspect of the parasite's biology.