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ORIGINAL ARTICLE

Factors affecting accuracy and precision of thermal summation models of insect development used to estimate post-mortem intervals

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Abstract This paper investigates the effects that different summary statistics (minimum, median, mean, or maximum), temporal sampling resolutions (duration between sampling events), and sample sizes (number of individuals sampled per sampling event) had on the accuracy and precision of the regression coefficients of a typical thermal summation model used to calculate minimum post-mortem interval (PMI). No significant differences were found in the values of the developmental constants calculated from different summary statistics of the duration of development. Sample size was found to affect the precision of measurement of the duration of development but had little overall influence on thermal summation constant (K) and developmental threshold (D_0) calculations (and therefore, subsequent PMI estimates), but temporal sampling resolution had a direct influence on the accuracy of K and D_0 calculations. These data suggest that when numbers of experimental maggots are limited, it is more important to sample more frequently using smaller sample sizes than to sample less frequently with large sample sizes. Furthermore, we suggest that the median is the most representative summary measure of the duration of development and should be used preferentially.

Keywords Forensic entomology · Statistics · Developmental threshold (D_0) · Thermal summation constant (K) · *Chrysomya chloropyga* · Calliphoridae

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Introduction

Insects are important forensic tools in estimating the minimum time between death and the discovery of a corpse or carcass, also known as the post-mortem interval (PMI) [14, 25]. The process of obtaining minimum PMI estimates is summarized in Fig. 1. These estimates are commonly made using a thermal summation model, such as the day-degree method, or from pre-calculated isomorphen diagrams [24, 26, 29]. If larval lengths are available, the isomorphen diagram estimate can be refined using an isomegalen diagram [40]. The thermal summation method relies on the analysis of development data from a minimum of six constant temperatures in the near-linear section of the temperature–growth response curve to make accurate estimates of the minimum PMI [30]. Few authors have published development data for more than five constant temperatures, and then only for a handful of forensically important blowfly species [29].

Furthermore, the inter-sample interval of published studies has ranged between 5 min [7] and 3 h [47] for egg development and between 2 h [3] and 24 h [18] for larval development. This represents a range of precisions in temporal sampling resolution. A related issue is the effect of changing relative error in temporal precision as the insects age: a 1-h error in measuring the duration of development 1 day after hatching is far more serious than a 1-h error after 2 weeks of development. The numbers of larvae or pupae in each sample also vary between studies, with Byrd and Butler [8, 9] sampling as few as two individuals per subsample per sampling event and Queiroz [39] sampling as many as 50 individuals per sampling event. This represents another aspect of precision and data quality that needs attention.

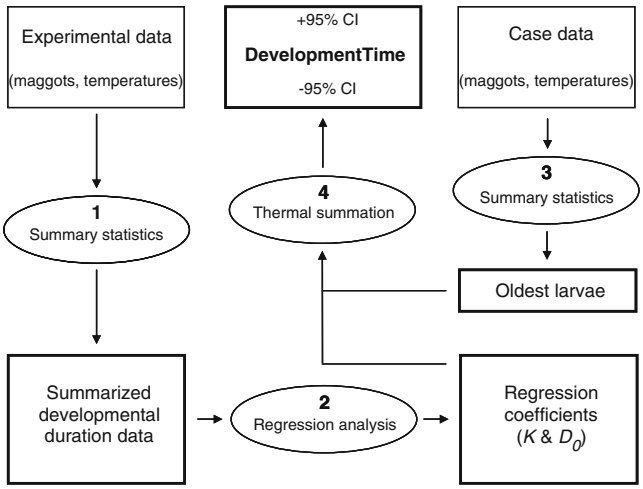

Fig. 1 Flow diagram summarizing the data sources and analytical process involved in estimating minimum post-mortem intervals (PMI) using a thermal summation model of insect development. The PMI is the duration of development (minimum PMI) plus the time between death and when the first insect laid eggs on the corpse or carcass. *Rectangles with light outlines* represent raw data, *rectangles with heavy outlines* represent processed data, and *ellipses* represent analytical steps. Analysis 2 is discussed by Ikemoto and Takai [30], and Analysis 4 by Higley and Haskell [29] and Greenberg and Kunich [26]

Other problems may lie in the different analytical approaches taken to characterize and summarize the duration of development, namely representing the durations of life stages using minima [17, 18, 25], medians [5, 7], modes [31], means [1, 3, 16], or maxima [4, 42, 45]. These summary measures differ in the accuracy with which they represent the true trend in development.

Amendt et al. [2] provide a detailed account of internationally accepted standard procedures for collecting, packaging, transmitting and processing of entomological evidence. This follows numerous other publications detailing the same objective [12, 13, 25, 26], but little attention has focused on best methods for experimental data collection and summary. This paper investigates how the collection and summary of experimental data (Fig. 1: Analysis 1) affects the accuracy and precision of developmental models for *Chrysomya chloropyga* (Wiedemann) (Diptera: Calliphoridae), a blowfly found in most parts of Africa [51], where it feeds prolifically on carrion, giving it forensic importance [6, 35, 45].

Materials and methods

Data collection

Adults of *C. chloropyga* were collected with Red Top® fly traps (Miller Methods, Pretoria) in Grahamstown (33°19′ S, 26°32′ E) to start a laboratory culture. The culture was maintained within a degree or two of 23°C under a lighting cycle of 12:12 h (light/dark). Flies were fed milk powder, sugar, and water ad libitum for 1 day and then provided with a 200-g pork chop as an oviposition medium. Initially, flies would feed on the chop but on the morning of the third or fourth day, numerous eggs would be found on the chop and were monitored frequently until they hatched. The duration between oviposition and hatching was not recorded because of uncertainty about the timing of oviposition and about the occurrence of precocious egg development in the mothers' oviducts [48].

Groups of ten approximately 1-h-old larvae were placed on 20 g of fresh chicken liver in separate tapered, 250 ml polystyrene cups The low density of maggots prevented measurable accumulation of maggot-generated heat [23] that might have stimulated growth. Each cup was steadied in 3.0–3.5 cm of river sand in its own 8×8×5 cm plastic tub. This provided sufficient sand for pupariation once larvae left the cups (presumed wandering or post-feeding). Once larvae began to wander, the remaining larvae still feeding and larvae that had began pupariation were not sampled. This allowed feeding larvae to reach wandering phase and pupariating larvae to reach eclosion. Eight to 20 cups were placed in Labcon 3104U incubators set at eight constant temperatures of 15°C, 17.5°C, 20°C, 22.5°C, 25°C, 27.5°C, 30°C, and 32.5°C. To avoid stunted growth, additional chicken liver was added when the original food dried out or was consumed rapidly.

Every 4 h for the first 48 h and every 8 h from then to pupariation, one maggot was removed from each of the five random cups at each temperature and its instar was recorded.

Analytical methods

First, to compare the effects of using different summary statistics (Fig. 1: Analysis 1), all of the raw data were used to calculate the minimum, median, mean, and maximum durations between hatching and either first ecdysis, second ecdysis, wandering, pupariation, and eclosion at each temperature. The mode was not calculated because the samples were capped at predetermined numbers of maggots and therefore could not represent the distribution of development times.

Next, to gauge the effects of precision and relative error of temporal sampling (Fig. 1: Experimental data), only the first sample of maggots of each day was used to calculate the same summary measures. This maintained the sample size at five larvae per event but decreased the temporal precision from 4 or 8 h to 24 h.

Finally, to examine the effect of sample size (Fig. 1: Experimental data), all of the larvae sampled within a day were pooled as though they were all collected at the end of the day, and the summary statistics were recalculated. This increased the sample size to 30 larvae per sampling event in

the first 2 days and to 15 larvae per sampling event thereafter, but decreased the temporal precision of sampling from 4 or 8 h, respectively, to 24 h. It also slightly increased the variability in the size of the larvae because they were not sampled in a single brief event, but since we used only the measures of central tendency, this did not affect the analysis in significant ways.

Using reduced major axis regression [30], the developmental threshold (D_0; slope) and thermal summation constant (K; y-intercept) of each developmental event (and the associated 95% confidence intervals) were estimated for each summary statistic for each sampling design.

Results

The mean coefficient of variation (R^2) for the regressions (Fig. 2) did not vary significantly between the four summary statistics (Main effects ANOVA, $F_{3,\ 50}=2.53$, $p=0.067$), the two temporal precisions ($F_{1,\ 50}=1.17$, $p=0.285$) or the two sample sizes ($F_{1,\ 50}=0.75$, $p=0.390$), but the mean R^2 for first ecdysis (0.926) was significantly lower than those of the other developmental events ($F_{4,\ 50}=16.77$, $p=0.000$), which all lay between 0.98 and 0.99. This last effect was due to the large relative error in measuring the timing of the first ecdysis. Thus, the overall quality of the models was generally excellent (as can be expected from the way that the linear part of the developmental curve is identified), with little to choose between most of them.

Effects of different summary statistics

Estimates of D_0 of each developmental event did not differ significantly between any of the four measures of duration within each sampling design, since all of their 95%

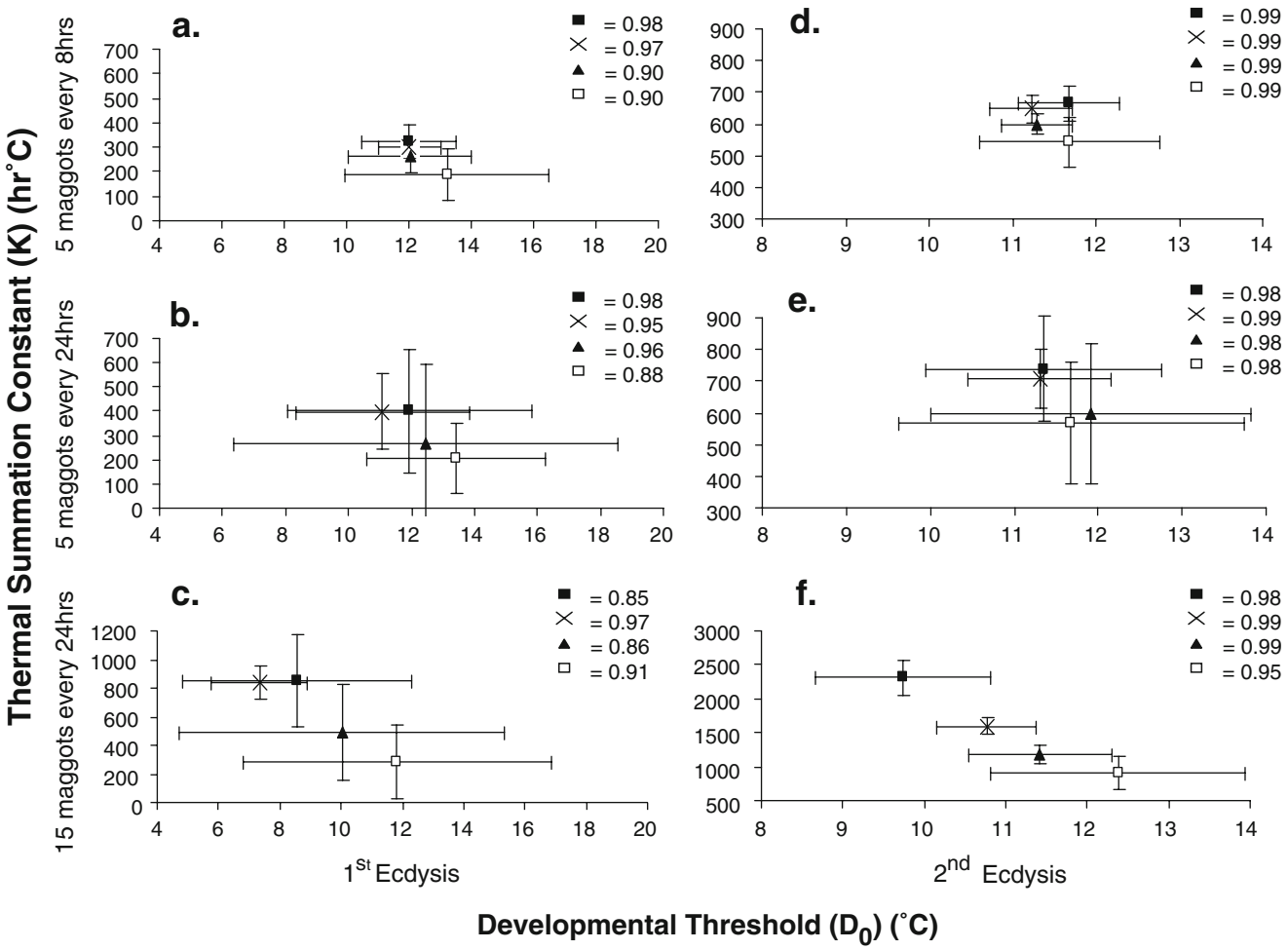

Fig. 2 **a**–**o** Scatterplots of D_0 and K estimated for first ecdysis, second ecdysis, onset of wandering, pupariation, and eclosion, from the minimum (*open square*), median (*closed triangle*), mean (*cross*) and maximum (*closed square*) time taken to reach those developmental events, using data of differing temporal precision and sampling density. *Error bars* represent 95% confidence intervals. (Note: the y-axis for (**c**), (**f**) and (**i**) are at different scales to those of the graphs above in the same column). Coefficients of determination (R^2) of the regressions used to calculate D_0 and K values for each measure of tendency are given in each figure

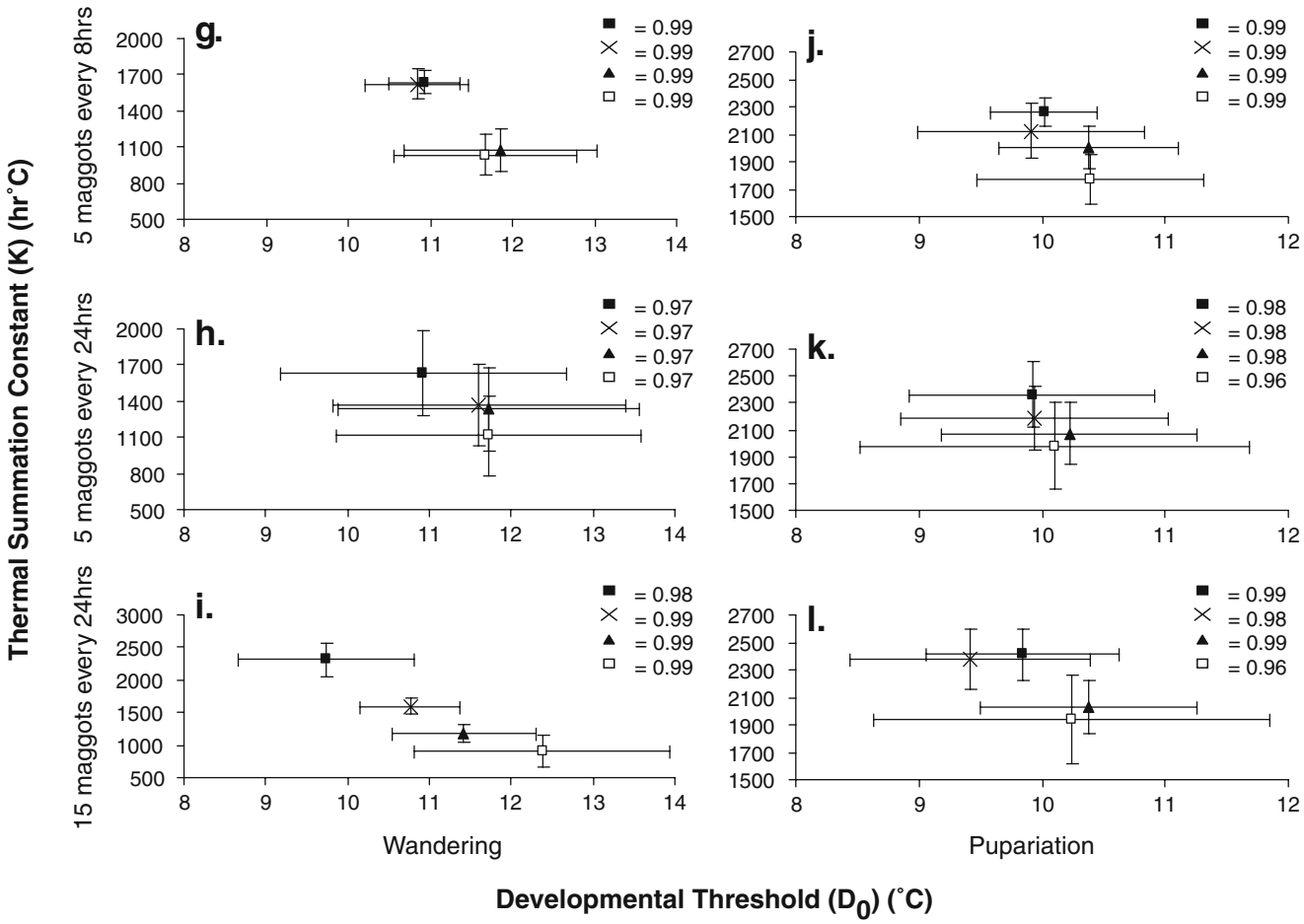

Fig. 2 (continued)

confidence intervals overlapped (i.e., within graphs in Fig. 2), but showed wider variation between measures when the relative error in duration was large, i.e., for early developmental events. The minima always produced the lowest estimates of K, and the maxima the highest, but this pattern was not seen in the estimates of D_0 (Fig. 2).

K differed significantly at first ecdysis between maximum data and minimum data (Fig. 2c); at wandering phase between maximum data and minimum, median, and mean data (Fig. 2i), and between mean data and minimum, median, and maximum data (Fig. 2i); and at pupariation between maximum data and minimum data (Fig. 2j). K did not differ significantly within any of the durations of development between any of the four measures of duration for the 24-h precision samples with the smaller sample size (within the middle row in Fig. 2).

Effects of temporal precision

With a few minor exceptions that did not form a consistent pattern, the estimates of K and D_0 also did not differ significantly between each sampling design, i.e., between the top and middle graphs in the same column of Fig. 2.

However, K increased and D_0 decreased between developmental events (i.e., between graphs within rows in Fig. 2), and the associated 95% confidence intervals were consistently narrower, for both K and D_0, when the relative error of the sampling was larger, i.e., for earlier developmental events, namely first ecdysis, second ecdysis, and wandering. This is most easily seen by comparing the estimates for first ecdysis (with relative errors of up to 24 h in a day, i.e., 100%) with those for eclosion (with relative errors never more than 24 h in 6 days, i.e., 17%).

Effects of sample size

Without focusing on the measures of duration, increased sample size failed to improve the precision of the K and D_0 calculations as both sample sizes produced equally large 95% confidence intervals.

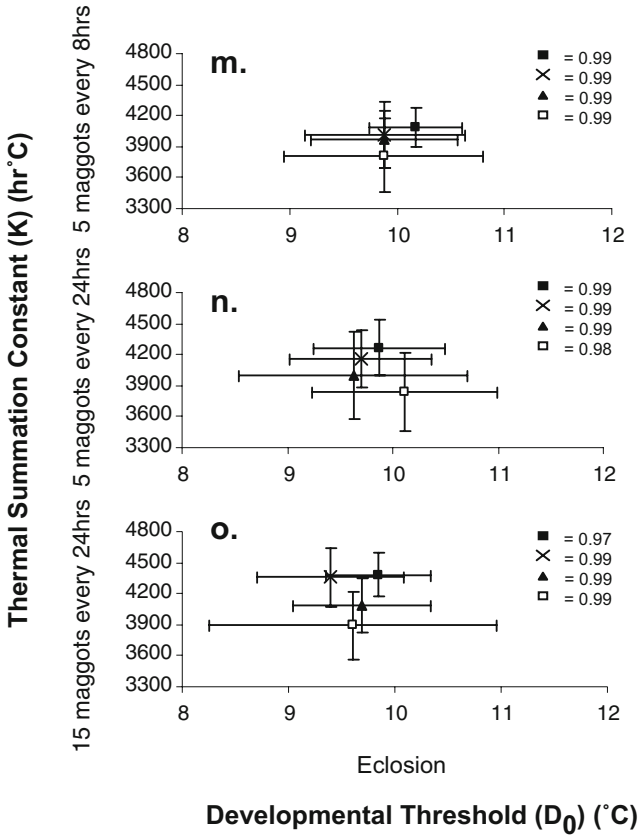

Fig. 2 (continued)

Discussion

Effects of different summary statistics

No significant differences were found in the coefficients of determination or the values of the D_0 calculated from different summary measures of the duration of development (Fig. 2). This could be attributed to a false negative (type II) error, a failure to detect actual differences through lack of statistical power because sample sizes were too small. Overcoming this would require using more temperatures in the regression models. In the case of estimating D_0, a larger sample is unlikely to have much effect because there was no consistent pattern of variation in the estimates made from the various summary measures (Fig. 2), and this source of inaccuracy in the estimation of minimum PMIs is of little concern.

On the other hand, minima and maxima always produced the lowest and highest estimates of K, respectively (Fig. 2), which is a logical relationship that might indicate a false negative error. For that reason, it also indicates that some discrimination needs to be shown in choosing a summary measure of duration of development, especially if the number of temperatures used in a particular regression model does provide adequate statistical power. Unfortunately, only a handful of publications meet the minimum number of temperatures for adequate statistical power [30]. These include: hatching [7, 18, 24, 34]; first ecdysis [24, 41]; second ecdysis [24, 41]; wandering (Hanski 1976) [18, 20, 24, 40, 41]; pupariation (Hanski 1976, Marchenko 1988, 2001) [11, 18, 24, 41, 49]; and eclosion (Marchenko 1988, 2001) [16, 24, 38]. This is an area of concern in forensic entomology and has been discussed briefly in Richards et al. [41].

Any particular investigation will need to estimate a window for development time [2] and perhaps for this reason, authors have published their results using various summary measures of duration (listed in the Introduction). This means that there is currently no single standard measure of tendency accepted in the forensic entomology literature. Accuracy is defined as "the closeness of a measured or computed value to its true value" [43] and, unfortunately, the measure of tendency chosen to represent each sampling population can have a significant effect on the accuracy of the K value (e.g., Fig. 2c, f, g, and i), to represent the true duration of development and thus the accuracy of the minimum PMI estimate. For this reason, it is important to choose the most representative measure of tendency of the true duration of development.

It is commonly recommended to estimate development times using a large sample (i.e., at least 30 individuals) of the oldest immature blowflies from a case [2], as they are considered to be the most representative indicator of the first oviposition event. These would include the largest feeding larvae, first larvae to enter wandering phase, first puparia to form, etc. Unfortunately, these recommendations have been practiced during collection of experimental data, and several studies have sampled the largest feeding blowfly larvae as the most representative sample for that population [8–10, 24, 26]. Such samples would represent maxima. Minimum and maximum data represent outlying values [43] of the lower and upper extremes of the duration of development. Because the largest immature blowfly is an outlier, it is not the most representative measure of the duration of development and should not be used to calculate thermal summation constants. It is imperative that authors either sample randomly (all sizes of a single cohort) [15] or sample all individuals [19] during experimental data collection to better understand the variation in size with respect to actual age.

For these same reasons, development times should not be estimated using minimum and maximum data, especially if any window of precision (e.g., a standard error) is incorporated into the estimate. We recommend that an alternative measure of tendency (discussed below), from a large sample size (i.e., at least 30 maggots where possible) of the oldest immature blowflies from a case be used to estimate a development time [2], and that the appropriate lower and upper bounds (e.g., 95% confidence intervals) of

the regression equation should be used to calculate the relevant window for development.

A mean developmental duration is the most frequently used measure of tendency within the forensic entomology literature [36]. It represents the average time at which individuals are observed to experience developmental change [27], i.e., the average duration of pupariation or the average duration of wandering. Sokal and Rohlf [43] advocate the use of the mean in biological statistics as it is easy to calculate, and it has a smaller standard error (i.e., higher precision) than other measures of tendency. However, means are sensitive to outlying minimum and maximum values because of the way means are calculated. When the data are symmetrical, the mean gives an accurate summary of the duration of development (Fig. 3a), but if the data are skewed the mean is affected by the outliers and becomes a particularly skewed estimate of the general trend of development (Fig. 3b) [43]. Nishida et al. [37] showed that such skewing is not unusual in blowfly development. For this reason, mean developmental durations are not ideal for calculating K and D_0.

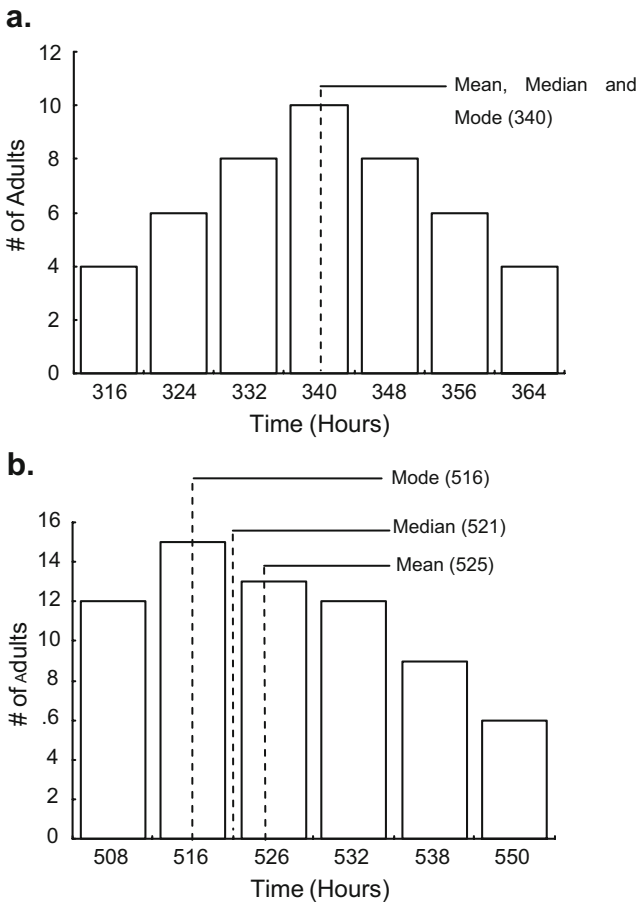

Fig. 3 Relative positions of the mean, median, and mode of hypothetical samples with distributions that are **a** symmetrical and **b** positively skewed

The mode is a measure of tendency that is statistically robust to the effects of outlying values [21, 43]. Kamal [31] is the only author to summarize development data using the mode. It refers to the most common duration of developmental within the experimental population [27], i.e., the most common duration to pupariation or the most common duration to wandering. But, to obtain a reliable mode for K and D_0, the sample size at each sampling event must be at least 30 individuals if it is to represent the underlying frequency distribution [43]. This radically increases the total number of individuals needed for an experiment, which is often not feasible when conducting development studies at more than two temperatures [11]. Another problem when using the mode is that it is not uncommon to have more than one mode (bimodal or multimodal) at any experimental event, possibly due to sexual dimorphism or circadian rhythms, resulting in two different estimates of development time with identical statistical support [43]. For these reason, modal developmental data are not ideal for the routine calculation of K and D_0.

The median is a measure of tendency that is also robust to the effects of outlying values [27], and numerous authors have used the median to summarize development data in the forensic entomology literature [5, 7, 15, 38, 41, 44]. It refers to the time at which 50% of all individuals experience developmental change [27], i.e., the time taken for 50% of the population to reach first ecdysis or pupariation. Although medians have a larger standard error (i.e., less precision) than means for purely mathematical reasons, this has only minor implications for calculating thermal summation models.

Logarithmic transformation of right-skewed data can help to make their distribution more symmetrical and thus bring the median and the mean closer together, especially for population variables like biological rates [50]. However, it is not guaranteed to eliminate skewness and will not alleviate severe outliers, and the mean has to be transformed back before it can be used for further analysis, with implications for precision. Related exponential transformations, like the square-root transformation, could also be tried under the same caveats, but have less theoretical justifiability and must be applied empirically [22]. For these reasons, we propose that the median is the most representative measure of the duration of development when analyzing both symmetrical and skewed data and would provide the most reliable routine summary data for estimating K and D_0.

Effects of temporal and sampling precision

Precision is defined as "the closeness of repeated measurements of the same quantity to each other" [43] and can be represented by measures such as standard errors. There are

three sampling strategies for developmental studies that affect the precision of the duration data and the precision of K and D_0 calculations, namely, sample size (number of individuals sampled per sampling event), temporal sampling resolution (duration between sampling events), and the number of constant temperatures used to calculate K and D_0 [30]. If experimental sampling methods rely on killing maggots to measure them, and sample size and temporal sampling resolution are constrained by the number of eggs laid by females, then scientists are faced with an experimental trade-off between more sampling events and smaller sample size, or larger sample sizes and fewer sampling events.

The K and D_0 values of the four summary measures were more similar for smaller sample sizes than those calculated from the greater sample size (compare the middle and bottom graphs in the same column of Fig. 2), particularly when the relative error of the sampling was larger, i.e., for earlier developmental events. Furthermore, 95% confidence intervals of K and D_0 were generally narrowest when the temporal sampling resolution was high (in the top row of Fig. 2). Therefore, an increase in the sample size affects the precision of measurement of the duration of development, but because it is not itself a direct measure of time, it will not influence the K and D_0 values (and subsequent estimates of minimum PMI) as much as temporal sampling resolution. Therefore, to increase the precision of the K and D_0 values, and subsequent PMI estimates, it is more important to sample more frequently using fewer samples than to sample less frequently using more samples. This is empirically evident from comparing the top and middle rows of each column of Fig. 2.

It has been shown elsewhere that the precision of the K and D_0 values can be further increased by increasing the number of temperatures along the regression line used to calculate them [30]. Both their precision and their accuracy can be improved by covering as much as possible of the range of temperatures on the linear section of the temperature–growth response curve [46].

For the most accurate K and D_0 estimates, sampling frequencies should be substantially shorter than the duration of each developmental event to counteract the effects of relative error. Any remaining uncertainty in K and D_0 is then inherent biological variation that is outside the control of experimental design. A relative error of about 10% in the resolution of temporal sampling of the total development time for each event is an optimal trade-off between effort and accuracy [41]. This translates to about 2–6 h for first ecdysis, 4–8 h for second ecdysis, 8–12 h for wandering, and 12–24 h for pupariation and eclosion, depending on temperature.

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