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Errors in instar determination of mayflies (Ephemeroptera) and stoneflies (Plecoptera) using the simple frequency, Janetschek, Cassie and Dyar's law methods

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SUMMARY. 1. The reliability of the simple frequency, Janetschek, Cassie and Dyar's law methods for determining or corroborating instars of mayflies and stoneflies was evaluated using data from published studies, a population of *Baetisca rogersi* and populations simulated through use of random numbers and generated normal distributions.

2. The Janetschek and Cassie methods are variations of the simple frequency method that offer no significant advantage. Modes of the Cassie method, thought to represent instars, are much more difficult or impossible to detect than are the corresponding peaks of the other two methods.

3. Overlap in size between adjacent instars can lead to false instar peaks or modes in frequency plots. The potential for overlap in mayflies and stoneflies is greatly increased, compared to other insects, because of their large number of instars and known developmental variability. The normal distribution simulations demonstrated that instar size variability as low as 5–7.5% COV (coefficient of variability) may lead to false instar peaks when the number of instars is in the typical range. These simulations also indicated that even simple frequency plots with distinct peaks may result in inaccurate instar determinations.

4. The number of size classes used in an analysis was correlated with the number of peaks or modes revealed. The number of peaks greater than zero in the Janetschek plots for the *Baetisca rogersi* population varied from 5 to 53 as the number of size classes was varied from 20 to 188. Similarly for the random number simulations, the number of peaks varied from 6 to 41 as the number of size classes varied from 22 to 127.

5. Dyar's law semi-logarithmic plots do not corroborate instars determined through frequency methods, because the uniform spacing of 'instar' data points is the direct result of the uniform spacing of peaks in frequency plots of most data sources (including random numbers), whether or not peaks actually indicate instars. Also Dyar's law plots will

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'corroborate' different numbers of instars depending on the peak selection criteria used. The potential for corroborating instars through supplemental rearing and best-fit analysis is discussed.

6. The future of mayfly–stonefly instar determination lies in the increased and more rigorous application of the rearing and Palmen body (mayflies only) methods.

Introduction

Insect development may be defined as the progressive changes in size, morphology and physiology of the insect throughout the insect's life cycle. Moulting and the related processes of apolysis and new cuticle formation are vital parts of insect development that lead to the formation of a series of instars. Our understanding of the biology of many insects will be greatly improved when the number of instars and degree of development per instar are correlated with environmental factors. Unfortunately, methods of instar determination suffer from several severe problems.

Rearing and the simple frequency method are the two most widely used methods. Rearing directly determines instars through observations of exuviae; however, logistical problems in culturing insects preclude the universal application of rearing. By contrast, the frequency method is simple to use and easily applied to field populations. Instars are indirectly determined by this method through a plot of the number of individuals (collected throughout at least one complete life-cycle) per size class, where each distinct peak in the plot infers one instar (Figs. 1–3).

Apparently, the simple frequency method has successfully been used to determine instars for many species, but has been inadequate for others (see references in Fink, 1980, page 371). Gaines & Campbell (1935) and Schmidt, Campbell & Trotter (1977) have demonstrated for lepidopteran larvae that the simple frequency method will only yield clear results for populations with a fairly homogeneous rate of development and number of instars.

In the last decade workers on mayflies and stoneflies have used increasingly the simple frequency, Janetschek and Cassie methods to determine instars (Table 1). The Janetschek method (Janetschek, 1967) is currently the most popular and requires the calculation of a gliding mean (running mean or moving average) for each simple frequency size class; for the xth size class the gliding mean ($\bar{Y}_x$) may be calculated as the quantity

$$\bar{Y}_x=[(Y_{x-2}+Y_{x-1}+Y_x+Y_{x+1}+Y_{x+2})/5]$$

The gliding mean values are then subtracted from the respective simple frequency values to yield positive and negative values. Plotting these values results in a graph, the periodic maxima–minima or Janetschek plot, in which each distinctive peak is presumed to indicate an instar (Fig. 1).

The Cassie method (Harding, 1949; Cassie, 1950, 1954, 1963) requires the calculation of cumulative size frequency percentages from the original size frequency data. These values are then plotted on the probability axis of probability paper versus size classes on the arithmetic axis. The component (e.g. instar, age class) distributions of a polymodal distribution are presumably indicated by modes and inflections in the plot. A mode, analogous to a peak in the simple frequency and Janetschek methods, indicates a 'relatively rapid' increase in cumulative frequency percentage due to an accumulation of individuals in a restricted size range (Figs. 4 and 5). The mode is thought to represent one component of the total distribution, while an inflection indicates little or no accumulation of individuals and represents the gap between two successive components. The range, mean and standard deviation can be estimated for each component by plotting adjusted values on probability paper; however, aquatic entomologists have not been concerned with this aspect of the Cassie method.

Only two workers have expressed concern about the reliability of the above methods for determining instars of mayflies and stoneflies. Winterbourn (1966) could not reliably determine the number of instars for two New Zealand stonefly species using the Cassie method because of considerable size variability resulting

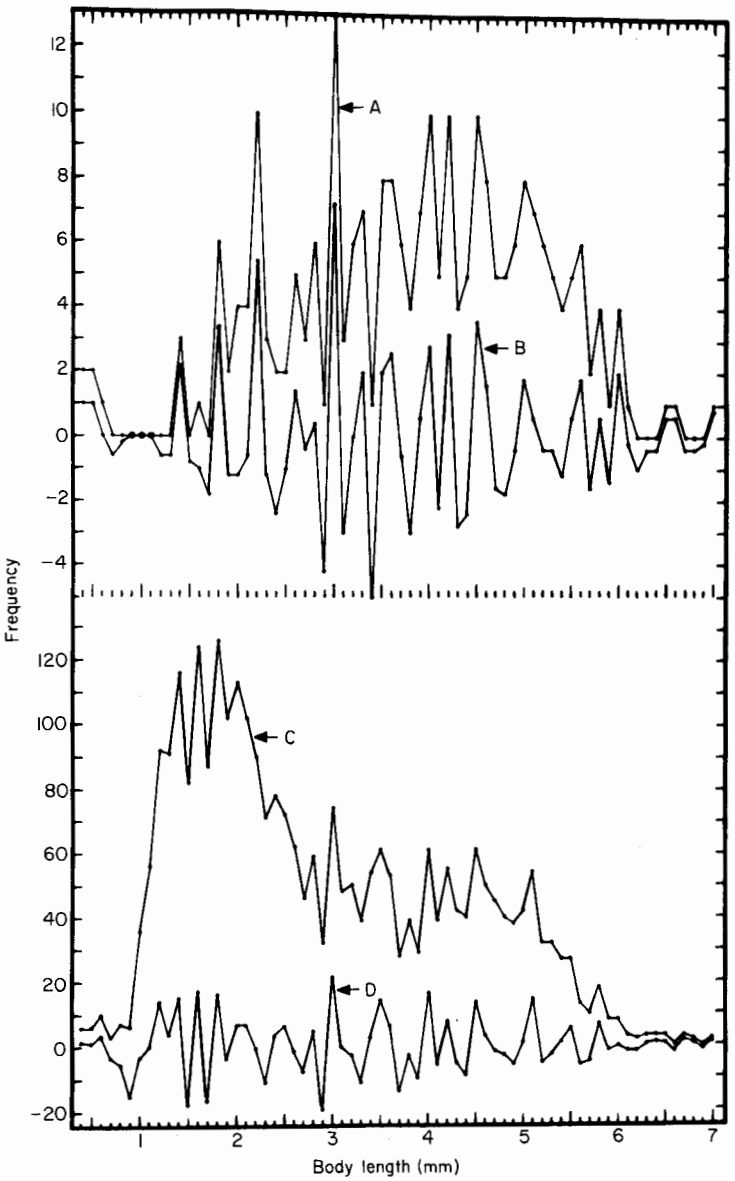

FIG. 1. Simple frequency and Janetschek periodic maxima–minima plots of size–frequency data from *Tricorythodes minutus* nymphs (both sexes): A–B, nymphal exuviae (N=244); C–D, whole nymphs (N=2913). A and C are simple frequency plots. B and D are Janetschek periodic maxima–minima plots using the JN gliding mean. Data taken from simple frequency plots of Newell (1976) Figs. 21 and 23 (corresponding to Fig. 10 of Newell & Minshall, 1978).

from extensive delayed egg hatching. Fink (1980) in a general review of mayfly instar determination methods and later (1982) in a critical analysis of the number of instars of *Stenonema modestum* noted the great similarity between the simply frequency, Janetschek and Cassie methods, and that these methods could only be used reliably with homogeneously developing mayfly populations, thereby excluding most species. The purpose of this paper is to show in detail why the simple frequency, Janetschek, Cassie and even Dyar's law methods are not suitable for determining or corroborating instars of these insects. An outline of the

TABLE 1. Summary of studies that used the simple frequency, Janetschek, and Cassie methods of instar determination.*

Study	Order – Species	Instar method	No. of size classes	No. of nymphal instars reported	No. of JN per. max. peaks>0†	
					Found	Predicted
(1) Janetschek (1967)	Col. – *Gomphiocephalus hodgsoni* Carpenter	S, JN	79, CC, BL	15B	20	22 (9)
		S, JN	79, ME, BL	13B	18	22 (18)
(2) Harper (1973)	Ple. – *Nemoura trispinosa* Claassen	S, JN	79, HW	16B	18	22 (18)
(3) Vaught & Stewart (1974)	Ple. – *Neoperla clymene* (Newman)	S	110?, WL	18–20M, 20–23F	[23]	[31 (26)]
		S	115?, HW	18–20M, 20–23F	[23]	[32 (28)]
(4) McClure & Stewart (1976)	Eph. – *Choroterpes mexicanus* Allen	S, JN, C	101M, 100F, R, H	16B	28M, 23F	28 (0M, 18F)
		S, JN, C	90M, 88F, SU, H	19B	19M, 20F	25M (24), 24F (17)
(5) Oberndorfer & Stewart (1977)	Ple. – *Hydroperla crosbyi* Needham & Claassen	S, JN, C	111, HW	12M, 14F	32	31 (3)
(6) Newell (1976), Newell & Minshall (1978)	Eph. – *Tricorythodes minutus* Traver	S, JN	67, EX, BL	19M, 23F	18	19 (5)
		S, JN	67, WN, BL	19?	19	19 (0)
		S, JN	ND, HW	6B	ND	—
(7) Snellen & Stewart (1979)	Ple. – *Perlesta placida* (Hagan)	S, JN	ND, HW	12–16B	ND	—
(8) Kondratieff & Voshell (1980)	Eph. – *Stenonema modestum* Banks	S, JN	34, HW	14–15B	10	9 (10)
(9) Cuffney & Minshall (1981)	Tri. – *Arctopsyche grandis* (Banks)	S, JN	47, HW	5B	10	13 (23)
(10) Sephton & Hynes (1982)	Ple. – 10 species	S, JN	—	—	—	—

*Abbreviations: B, sexes not separated; BL, body length; C, Cassie method; CC, Cape Crozier population; Col., Collembola; Eph., Ephemeroptera; EX, exuviae; F, females; H, head length; HW, head width; JN, Janetschek method using the JN gliding mean; M, males; ME, Mt England population; ND, data or plots not given; Ple., Plecoptera; R, overwintering generation; S, simple frequency method; SU, summer generation; Tri., Trichoptera; WL, wing pad length; WN, whole nymph.

† The number of found Janetschek periodic maxima peaks greater than zero was determined by counting these peaks: from published plots for papers 1, 2, 4, 5 and 9; from replotted data taken from the simple frequency plot for paper 6; and from replotted raw data for paper 8. The number of predicted peaks are determined by the regression equation, $y=0.28x-0.17$ (see Discussion). The number of found and predicted simple frequency peaks are listed for paper 3 in [ ]. The number in parentheses is the percentage difference between the found and predicted values.

logic and procedures used in evaluating these methods follows below.

While a cast body cuticle is proof of a moult and new instar, a peak or mode can only suggest the possibility of an instar. My analysis of frequency methods (simple frequency, Janetschek and Cassie) concerned the question of how reliably does a peak indicate an instar. The results–discussion begins with a comparison of the three methods to determine if the Janetschek and Cassie methods are significant improvements on the simple frequency method.

The next section concerns how sufficient overlap in size between successive instars can lead to false instar peaks or modes in frequency plots. The potential for overlap in mayflies and stoneflies is discussed in respect to their large number of instars and known developmental variability. The effect of different degrees of overlap on the number of peaks or modes in frequency plots was tested by creating appropriate simulated mayfly populations using generated normal distributions. These simulations (one normal distribution was created for each chosen instar) allowed me to test the resolving abilities of the frequency methods because the known number, location, shape and overlap of the distributions could be compared to their manifestations in the plots of the simple frequency (Fig. 6) and Janetschek methods.

The third section investigates the effect of the number of size classes on the number of peaks or modes in frequency plots. The number of size classes depends on the chosen size class interval and the size range of the organism; because the interval and range can vary within wide limits so must the number of size classes. A greater number of size classes partitions the data more, thereby potentially resulting in more peaks. Peaks would not indicate instars if the number of peaks can be altered simply by altering the number of size classes. The effect of the number of size classes was tested by varying the number of size classes for *Baetisca rogersi* and random number simulated mayfly populations (Table 2) and then counting the number of peaks in the plots using the same peak selection criteria. A linear regression was run on the number of size classes versus the number of peaks using the above data and that from the studies listed in Table 1.

The penultimate section concerns methods which have been or may be used to corroborate the results of frequency methods. Foremost of these methods is Dyar's 'law'. Modern use of Dyar's law is reflected in semi-logarithmic plots of instar frequency data as shown in Fig. 7. Instars are generally believed to be confirmed when the plot is relatively straight and uniform spacing exists between the designated instar data points. I test that hypothesis by analysing semi-logarithmic plots of the data from the normal distribution and random number simulations and from some of the studies listed in Table 1. Also the effect of the number of instars and size range of an insect on the Dyar's law mean progression factor (the reciprocal of Dyar's (1890) ratio) is studied (Table 3). Other corroborative methods discussed are supplemental rearing and best-fit analysis.

The final section provides conclusions and suggestions for future instar analysis of mayflies and stoneflies.

Methods

The plots of the simple frequency, Janetschek and Cassie methods were carefully analysed and compared for many of the studies listed in Table 1. Janetschek plots were traced and superimposed on the corresponding simple frequency plot to compare the relative position and prominence of peaks. Cassie plots were also traced and modes were independently determined. The simple frequency and Janetschek plots of Newell (1976) and Newell & Minshall (1978) were replotted (Fig. 1) from data taken from their simple frequency plots to demonstrate clearly the similarity between the two plots of their data. Kondratieff & Voshell's (1980) raw data (Kondratieff, pers. comm.) were used to plot the simple frequency and two sets of periodic maxima–minima values, calculated through use of two different gliding means, JN and JL, as described below.

Different gliding means were used to observe their effect on the Janetschek plot. The gliding mean used by other investigators (Table 1) is described in the introduction and will be called the JN gliding mean. The second gliding mean will be referred to as the JL gliding mean and was calculated as

$$\bar{Y}_x=[(Y_x+Y_{x+1}+Y_{x+2}+Y_{x+3}+Y_{x+4})/5]$$

Most investigators (Table 1) using the Janets-

chek method have expressed serious concern over the 'loss of data' at both ends of the Janetschek plot because of the nature of the gliding means. Actually, there is no loss of data since the original simple frequency distribution can always be referred to. Also, all size class values can be preserved in some fashion, as was done in this study, simply by creating imaginary size classes of zero frequency value at both ends of the original simple frequency distribution. For example, the respective JN and JL gliding means for the last size class, $\bar{Y}_{xf}$, were calculated as

$$\bar{Y}_{xf}=[(Y_{xf-2}+Y_{xf-1}+Y_{xf}+0+0)/5]$$

$$\bar{Y}_{xf}=[(Y_{xf}+0+0+0+0)/5]$$

Six different body characters were measured for 178 female *Baetisca rogersi* nymphs collected by M. L. Pescador. To avoid complicating factors of sexual dimorphism, only nymphs mature enough to be sexed were used. These nymphs approximately represent instars 6–12 or 13 as determined by Pescador & Peters (1974) through rearings. The data for each character were analysed by the simple frequency, Janetschek and Cassie methods as indicated in Table 2A and plots were constructed similar to Figs. 2 and 4. Simple frequency and Janetschek values not plotted were examined by arranging each set of values in order of ascending size class and then choosing higher or positive values (peaks) which were bordered on both sides by lower or negative values. This made it possible to detect accurately the comparative location and number of simple frequency and periodic maxima peaks without actually plotting the data.

TABLE 2. Summary of analyses used to evaluate the simple frequency, Janetschek and Cassie instar determination methods*

Data source	Measurement character or no. of specimens	Size class interval (mm)	No. of size classes	No. of JN per. max. peaks > 0	Instar determination method			
					S	JN	JL	C
(A) *Baetisca rogersi* Berner	Head length	0.05	23	6	P	P	P	P
		0.03	37	10	P	P	P	P
		0.02	56	13	P	P	P	P
(178 female nymphs)	Head width	0.10	20	5	P	P	P	P
		0.05	40	12	P	P	P	P
		0.03	66	19	P	P	P	P
	Carapace length	0.10	56	15	P	P	P	—
		0.05	113	34	E	E	E	—
		0.03	188	53	E	E	E	—
	Carapace width	0.20	32	10	E	E	E	—
		0.10	64	18	E	E	E	—
		0.05	127	35	E	E	E	—
	Spine length	0.10	42	15	E	E	E	—
		0.05	84	25	E	E	E	—
	Abdominal width	0.20	24	6	E	E	E	—
		0.10	48	11	E	E	E	—
		0.05	96	19	E	E	E	—
(B) Random numbers†	500	0.06	22	6	E	E	E	P
		0.03	43	16	P	P	P	P
		0.02	64	20	P	P	P	P
		0.01	127	35	P	P	P	P
	1000	0.06	22	6	E	E	E	—
		0.03	43	11	P	E	P	—
		0.02	64	20	P	E	P	—
		0.01	127	39	E	E	E	—

* Abbreviations: C, Cassie method; E, examined (see Methods); JL, Janetschek method using the JL gliding mean; JN, Janetschek method using the JN gliding mean; P, plotted (see Methods); S, simple frequency method.

† Parameters and data not shown for the 2000, 3000, 4000 and 5000 specimens' cases; these are similar to those cases shown.

Various mayfly populations were simulated through use of computer-generated random numbers and analysed by two or more of the frequency methods as indicated in Table 2B. Each random number simulated the head length of an individual mayfly, and random numbers were generated between limits that corresponded to the approximate head lengths of the first and final nymphal instars of a real *Choroterpes mexicanus* population studied by McClure & Stewart (1976). All necessary calculations, plots and analyses were similar to those outlined for *Baetisca rogersi.*

The range of head lengths for the normal distribution simulations were also patterned after the *Choroterpes mexicanus* population. A normal distribution was created for each desired instar through calculations performed on a Texas Instruments 58C calculator using the Normal Distribution Program ML-14. Populations with 16, 19 and 25 instars were simulated, and the means of successive instars were separated by the following respective constant progression factors, 1.20, 1.16 and 1.12. The spread of each individual instar distribution was determined by a standard deviation which would yield a similar coefficient of variation (COV=s.d./$\bar{x}$×100%) for all the instars. The COV compares the relative variability of samples (Sokal & Rohlf, 1981) and so instars of a particular population in these simulations were relatively similar in head length variability. COV values of 5%, 7.5% and 10% were used. The 5% value was considered to be representative of insects whose instars are homogeneous, few and easily separable in a frequency plot (such as calculated from data in Poston, Hammond & Pedigo, 1977 and Roberts, Proctor & Phillips, 1978). The 7.5% and 10% COV values were chosen to show how a moderately greater degree of size variability could affect instar determination by frequency methods. Each instar of each simulated population was represented by about 100 specimens. Instar distributions of each population were plotted separately on the same simple frequency plot (adjacent or overlapping distributions were plotted in different colours) and then this plot was compared to a normal simple frequency plot of the same but combined data (Fig. 6).

Mean progression factors (the reciprocal of Dyar's (1890) ratio) were calculated for different numbers of instars for the same and different size ranges of an hypothetical insect, whose first and final nymphal instar head lengths were modelled after respective values of *Choroterpes mexicanus* (McClure & Stewart, 1976) (Table 3). Calculations were performed on the 58C calculator using the U.S. Method Compound Interest Program ML-18, since the progression factor is related to the idea of compound interest. The ML-18 program calculates the compound interest rate when the initial (first instar size) and final values (final nymphal instar size) and number of compounded periods (total number of nymphal instars minus one) are known. The compound interest rate multiplied by the size of the first instar yields the absolute increase in size between the first and second instars. The second instar size/first instar size=the progression factor. Final nymphal instar head lengths of 2000, 4000 and 8000 μm, which are not found in nature for *Choroterpes mexicanus,* were chosen to show the effects of a greatly increased size range on the progression factor.

Results and Discussion

Comparison of the simple frequency, Janetschek and Cassie methods

Many of the investigators listed in Table 1 apparently believed that the Janetschek and Cassie methods offered additional insights not provided by the simple frequency method alone so that 'true instar' peaks could be reliably delimited. Also, because all three methods were thought to be independent, similar results by two or three methods were considered as additional corroboration for their instar claims.

An analysis of all available plots of Table 1 studies (Fig. 1), of *Baetisca rogersi* and of random number data (Table 2, Figs. 2–5) revealed that peaks or modes occurred in the same size-class location in the final plots of all three methods. However, this is to be expected because of their similarity.

The Janetschek and simple frequency methods are distinguished by the use of gliding means. Apparently, gliding means were believed by the users of the Janetschek method to be the theoretical basis of how this method determined which peaks of the simple frequency distribution represented instars (e.g. Harper,

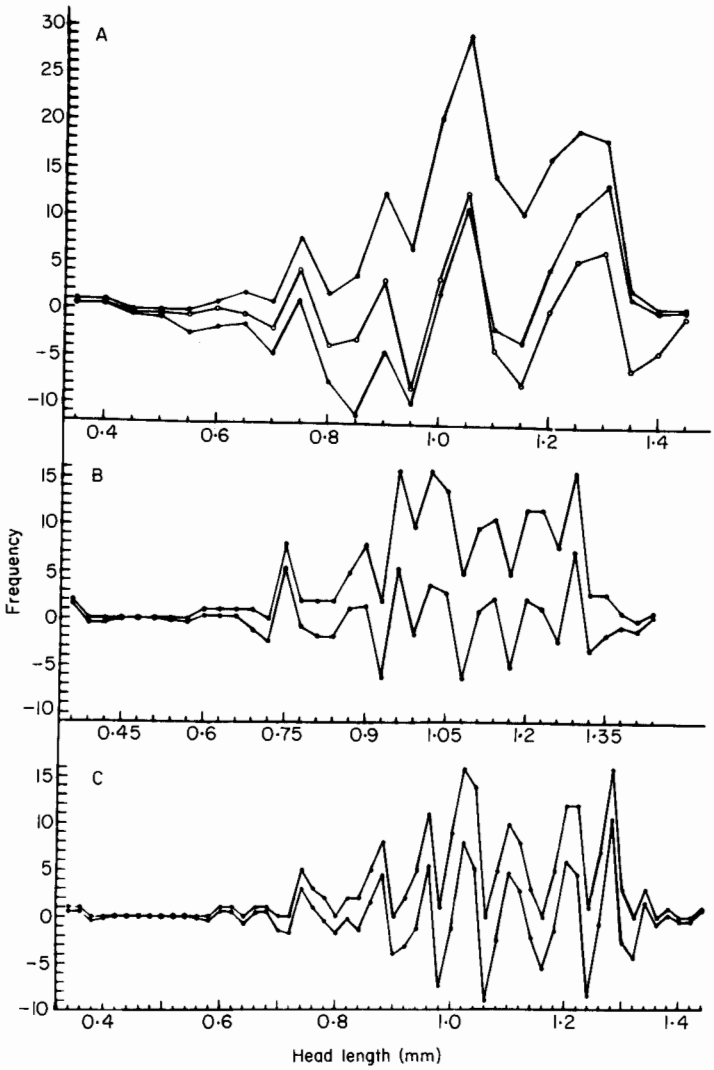

FIG. 2. Simple frequency and Janetschek periodic maxima–minima plots of size–frequency data from 178 female *Baetisca rogersi* nymphs (half-grown to mature) using three size class intervals: A, 0.05 mm (23 size classes); B, 0.03 mm (37 size classes); C, 0.02 mm (56 size classes). The top plot in each group is the simple frequency plot; the remaining two plots in A are periodic maxima–minima plots whose values were calculated with the JN (open dots) and JL (closed dots) gliding means; and the bottom plot in groups B and C is a JN periodic maxima–minima plot.

1973) and were considered so important that the investigators shown in Table 1 who published the Janetschek plot also published the gliding mean plot which is not used at all in determining instars.

The gliding mean plot does not show the general pattern of the population's size structure as Janetschek (1967) claims, because the population exists in reference to time. Rather, this plot shows the general pattern of combined size–frequency data which is organized without reference to time. More importantly, the subtraction of the gliding means from the simple frequency values cannot unmask the 'true instar' data from other 'non-instar' data, because the variability of the data is maintained in the gliding mean values. Comparing any two adjacent size classes indicates that the gliding means of the two size classes would differ slightly in most cases, since four of the five simple frequency values used in calculating either JN or JL gliding means would be the same for both size classes.

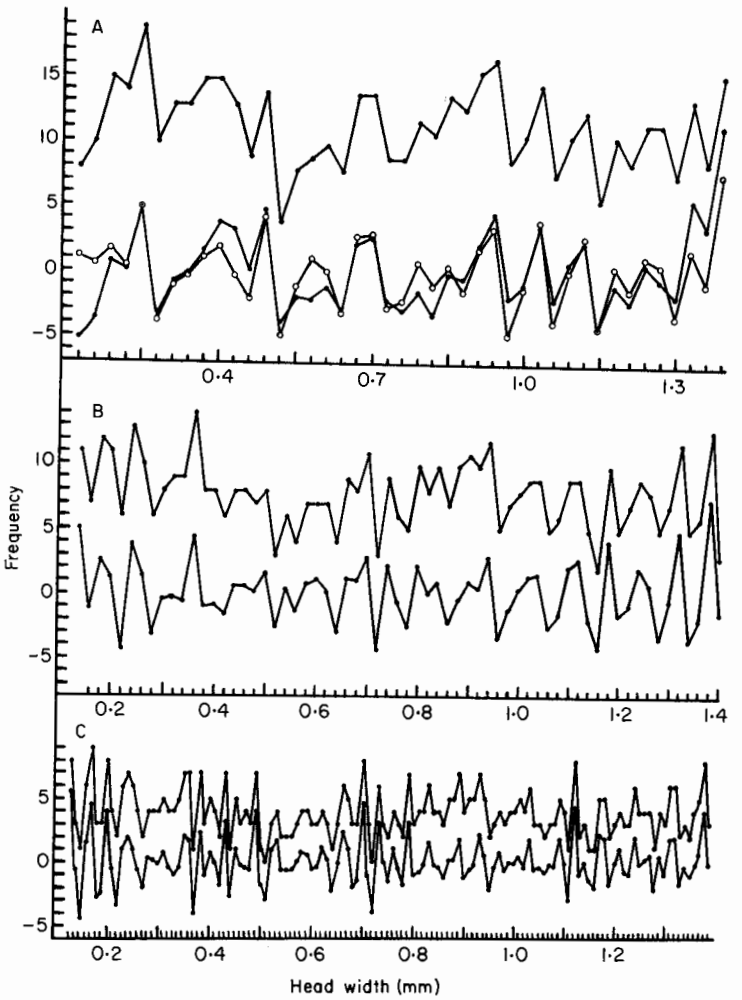

FIG. 3. Simple frequency and Janetschek periodic maxima–minima plots of size–frequency data from 500 random number simulated mayfly nymphs using three size class intervals: A, 0.03 mm (43 size classes); B, 0.02 mm (64 size classes); C, 0.01 mm (127 size classes). Rest of legend as in Fig. 2.

Subtracting the similar gliding means from the original frequency values results in maintaining the relative difference in magnitude between the two size classes, but the simple frequency plot must be displaced downward because each simple frequency value is now decreased by the amount of the corresponding gliding mean. Thus all periodic maxima–minima plots, including both JN and JL derived plots, observed in the present study for all data sources (published papers of Table 1, *Baetisca rogersi* and random number data) were identical in the location of almost all peaks and were even very similar in shape as well (Figs. 1–3; and see Fink, 1982).

Essentially, the subtraction of gliding means from the original simple frequency data is just a mathematical exercise which centres an altered facsimile of the simple frequency plot, the Janetschek plot, around a common horizontal axis. This may make the visual comparison of peaks slightly easier (Figs. 1–3) but a simple frequency plot, in which peaks are not easily compared, probably indicates developmental variability considerable enough so that all frequency methods would be unreliable.

A variation of the simple frequency method is the Cassie method although it produces a plot very different in appearance (compare Figs. 2

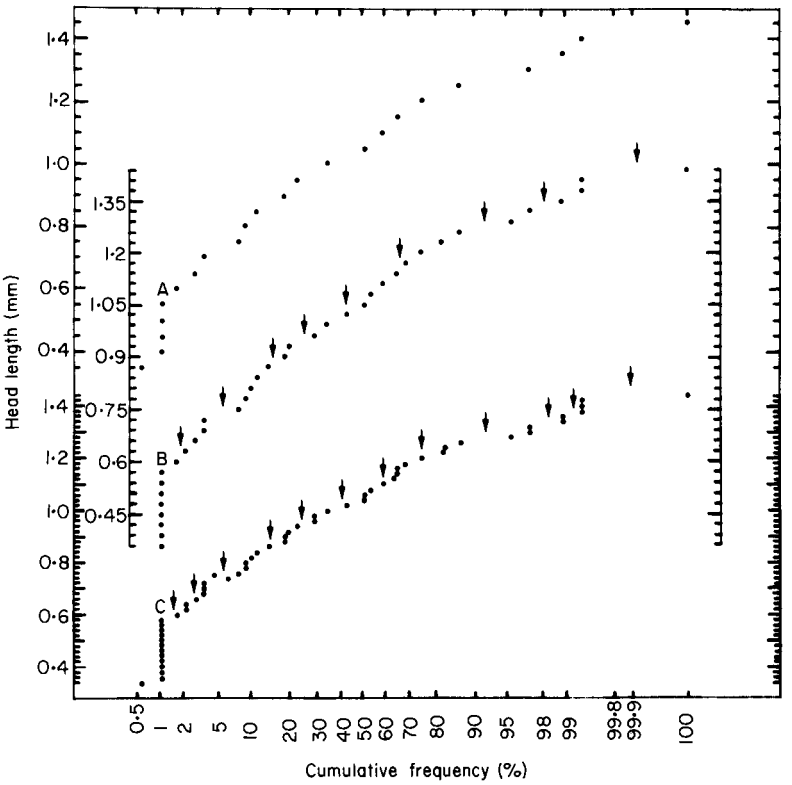

FIG. 4. Cassie probability paper plots of size–frequency data from 178 female *Baetisca rogersi* nymphs (half-grown to mature) using three size class intervals: A, 0.05 mm (outer top scale, 23 size classes); B, 0.03 mm (inner scale, 37 size classes); C, 0.02 mm (outer bottom scale, 56 size classes). Arrows point to modes for plots B and C (modes may be difficult to detect). Compare modes to peaks of simple frequency and Janetschek periodic maxima–minima plots of Fig. 2. The 99.99% axis is rounded off to 100%.

and 4, 3 and 5). Because cumulative frequency percentage values are calculated from simple frequency values, a mode, a 'sudden' increase in cumulative frequency percentage, must occur at exactly the same position as its corresponding simple frequency peak, which is a 'sudden' increase in frequency.

Due to the non-linear probability axis and curved pattern of the plot, the detection of modes and comparison of the relative magnitude of modes was much more difficult (or even impossible) than the detection and comparison of the corresponding peaks of the simple frequency and Janetschek plots. Modes could not be detected reliably for the larger size class interval random number data (Fig. 5), even though corresponding peaks were easily visible in the simple frequency and Janetschek plots (Fig. 3). To ensure that all modes were detected in any Cassie plot, it was necessary to compare them against peaks in the corresponding simple frequency and Janetschek plots. In papers 4 and 5 of Table 1, chosen Cassie 'instar' modes were the same as the chosen 'instar' peaks of the other two methods, but were often as distinct or even sometimes less distinct in appearance than modes which were ignored.

The Cassie method has been used primarily in fisheries biology and was not generally intended to be used for populations with very heterogeneous development nor for distributions containing more than a few component distributions (Harding, 1949; Cassie, 1954, 1963; Taylor, 1965; Cohen, 1966; Bhattacharya, 1967; Yong & Skillman, 1975). As will be shown below, mayfly and stonefly populations do not meet these restrictions.

The cause and importance of overlap

The analysis of polymodal distributions has been called '. . . a notoriously difficult mathema-

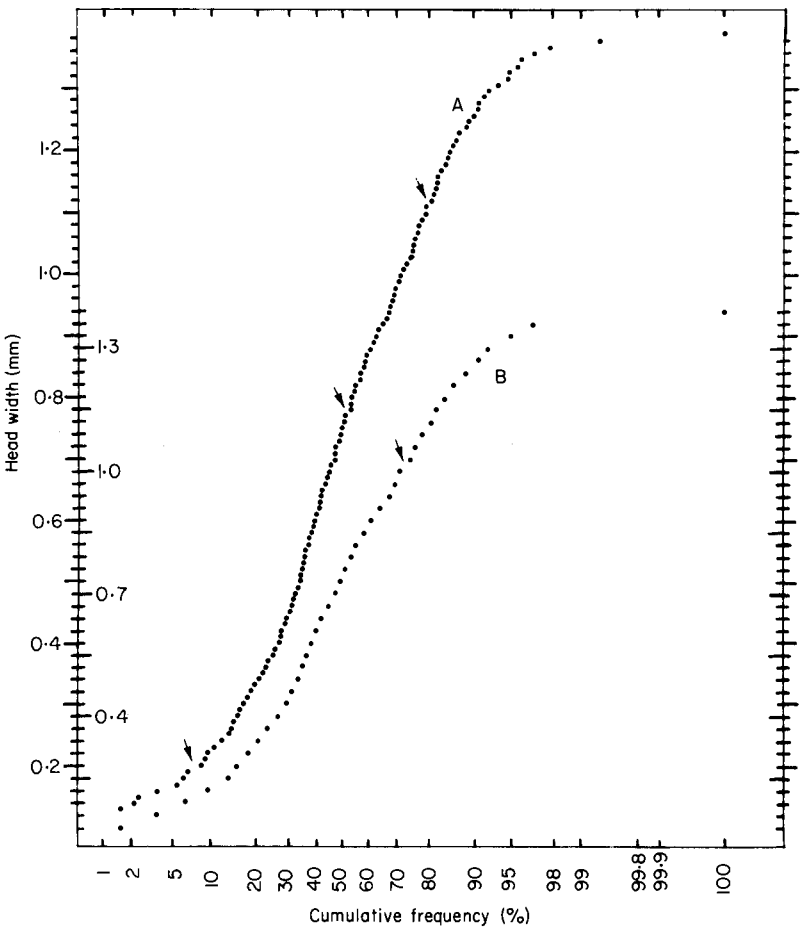

FIG. 5. Cassie probability paper plots of size–frequency data from 500 random number simulated mayfly nymphs using two size class intervals: A, 0.01 mm (outer scale, 127 size classes); B, 0.03 mm (inner scale, 43 size classes). Arrows point to some of the modes present in the plots. Compare modes to peaks of simple frequency and Janetschek periodic maxima–minima plots of Fig. 3. The 99.99% axis is rounded off to 100%.

tical task.' (Harding, 1982), the work has been proceeding on this topic since at least 1894 (Pearson, 1894). Overlap of the size distributions of adjacent components (e.g. age classes, instars) is the reason for this difficulty.

Linear measurements, such as head width and body length, are usually or approximately normally distributed (Snedecor & Cochran, 1967) and in a frequency plot of known and characterized instars, a mean determines the location of each instar distribution along the abscissa, while the standard deviation, a measure of variability, determines the dispersion of the distribution on either side of the mean. Overlap between the distributions of adjacent instars can occur through a suitable combination of closely spaced means and wide dispersions. Because instars of field populations are not known or characterized, overlap becomes very important since it can result in an accumulation of frequency values that produce peaks which do not represent instars.

The average progression factor refers to the geometric separation between the mean measurement values of adjacent instars. Overlap thus becomes more likely with low progression factors. The average progression factor is reduced when the number of instars is increased for the same size range (Table 3), and the progression factor is affected much more by the

TABLE 3. Effect of the number of instars on the mean progression factor for hypothetical insects

No. of instars	4	8	12	16	19	25	16	16	16
Head length (μm)									
First instar	90	90	90	90	90	90	90	90	90
Final instar	1390	1390	1390	1390	1390	1390	2000	4000	8000
Mean progression factor*	2.49	1.48	1.28	1.20	1.16	1.12	1.23	1.29	1.35

* Values shown are rounded off.

number of instars than the size range (Table 3). Progression factors for mayflies and stoneflies are lower than those of most other insects because these aquatic insects moult about 3–5 times more. The more closely spaced instars of mayflies and stoneflies might be resolved by frequency methods if the instars varied little in size.

The overwhelming evidence for mayflies and stoneflies, however, indicates considerable developmental variability (Rawlinson, 1939; Hunt, 1953; Trost & Berner, 1963; Pescador & Peters, 1974; Snellen & Stewart, 1979; Grant & Stewart, 1980; Humpesch, 1981), and has been most clearly documented in studies which have been able to rear separately, under constant conditions, individual specimens throughout their entire immature life (Degrange, 1959; Brittain, 1973; Cianciara, 1979; Clifford, Hamilton & Killins, 1979).

Greater variability probably occurs under natural conditions because individuals would be exposed to a greater range of environmental conditions due to different microhabitats and prolonged oviposition and egg hatching periods. In the studies listed in Table 1 heterogeneous development is indicated by the presence of a large range of size classes per sampling date (papers 2–9). This is further reflected by simple frequency plots (papers 1, 2, 4–6, 8, 10; Fig. 1) which are far more complex than the plots of those lepidopteran species whose instars could not be resolved by the simple frequency method in the studies of Gaines & Campbell (1935) and Schmidt *et al.* (1977).

Other examples of the variability of mayfly and stonefly development include observations, for many species, of large size differences of imagoes collected at different times during the emergence period (Khoo, 1968; Sweeney & Vannote, 1978; Illies, 1979; papers 5 and 7 of Table 1), and sexual size dimorphism (Hunt, 1953; Brittain, 1973; Pescador & Peters, 1974; McCafferty & Huff, 1978; papers 3 and 5–8 of Table 1). The sexes, unfortunately, cannot usually be routinely identified until relatively late in the life cycle when external genitalia or secondary sexual characters are sufficiently distinct. Sexual dimorphism may have confused frequency data for studies listed in Table 1, and also led several investigators (papers 3, 5 and 6) to conclude that females, because they were larger, moulted more often than males. However, an alternative hypothesis is that females moulted about as frequently as males but increased more in size after some moults. Frequency instar analyses may be further compounded if different body parts grow at different rates, thereby possibly leading to different instar determinations depending on the body part selected. Allometric growth has been demonstrated in the mayflies *Leptophlebia cupida* (Say) (Clifford, 1970a, b; Clifford *et al.*, 1979) and *Baetisca rogersi* (Savage & Fink,

TABLE 4. Results of normal distribution simulations

COV (%)	Size class interval (mm)	No. of size classes	No. of instars	No. of peaks
5	0.01	147	16	16
5	0.01	145	19	18
5	0.01	149	25	34+
5	0.02	74	16	12–13
5	0.03	49	16	11–12
7.5	0.01	154	16	35
10	0.01	159	16	32
5	0.01	147	16 & 19	14

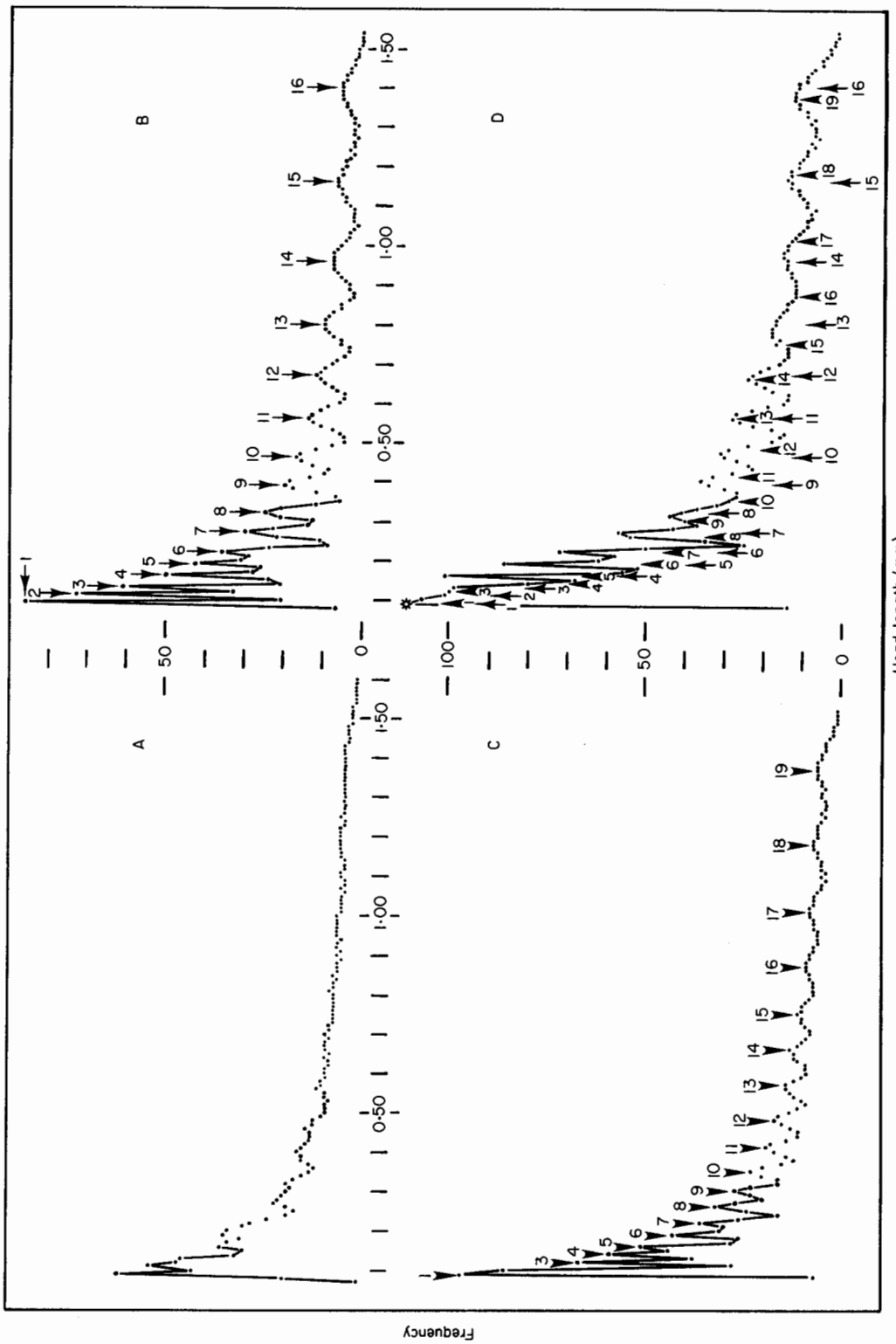

FIG. 6. Simple frequency plots of size–frequency data from normally distributed, simulated mayfly populations (approximately 100 nymphs per normally distributed instar). A, 16 instars, 7.5% COV; B, 16 instars, 5% COV; C, 19 instars, 5% COV; D, 16+19 instar subpopulations, 5% COV for each subpopulation. Arrows indicate the mean head length of respective instars for the 16 instar subpopulation; arrow heads indicate the same for the 19 instar subpopulation.

unpublished data). In Chironomidae, certain body measurements resulted in easily analysable frequency plots while others yielded more ambiguous plots (Soponis & Russell, 1982 and pers. comm.).

The normal distribution simulations indicated that accurate frequency method instar determination for arthropods with large numbers of instars is possible only within narrow limits of instar size overlap. Instars were resolved only when the total number of instars and COV (the coefficient of variation) were respectively less than or equal to 19 and 5%, and the number of size classes was not reduced from 145–147 (Table 4, Fig. 6). Exceeding those values yielded erroneous results due to sufficient overlap between adjacent instars. Increasing the number of instars alone from only 16 to 19 resulted in a greater overlap between the normally distributed instars, and the overlap for 19 instars and 5% COV approached the overlap for 16 instars and 7.5% COV. Increasing the number of instars to 25 with a 5% COV resulted in an instar determination almost 10 instars higher than the actual number (Table 4). When COV was increased from 5 to 7.5 or 10% for the 16 instar population, an instar determination value about double the actual number resulted (Table 4, Fig. 6A). COV values for instars of other insects and arthropods between 6% and 7.5% or higher are not uncommon (such as calculated from data in : Schroeder, 1968; Ward & Cummins, 1978; Nair, 1978; Mizell & Nebeker, 1979; and reported by Soponis & Russell, 1982). COV values calculated for the instars of the seven *Nemoura avicularis* Morton females reared by Brittain (1973) ranged from 4.8% to 14.2%, with an average of 8.9%. Clifford *et al.* (1979) reported COV values for *Leptophlebia cupida* nymphal instars as high as 13.

The normally distributed instar data were further manipulated, by mixing equal numbers of 16 and 19 nymphal instar populations (each with 5% COV, and with first and final nymphal instars of the same size), to simulate instar number variability within a population. Essentially this would serve to increase the COV values for many of the instars thereby resulting in greater overlap, probably inaccurate results (i.e. number and location of peaks) and a more complex plot. Generally this occurred; while instars were accurately represented in the separate plots of each subpopulation (Figs. 6B, C), the combined plot showed only about 14 peaks (Fig. 6D). Many of the peaks in the combined plot actually represented two groups of nymphs which differed in the actual number of instars by up to three (Fig. 6D). Unexpectedly, the combined plot was simple in appearance and therefore gave no indication that its peaks did not accurately reflect instars.

The relative complexity of the original simple frequency plot is likely to indicate the reliability of frequency methods. Very simple plots with large, clearly separated distinct peaks might indicate the presence of fairly homogeneous instars, whereas complex plots indicate heterogeneous development. Gaines & Campbell (1935) and Schmidt *et al.* (1977), however, found that even simple looking frequency plots could be misleading. Vaught & Stewart (1974) believed they had correctly determined instars for 737 *Neoperla clymene* stonefly nymphs through use of the simple frequency method for both wing-pad length and head width data, since the number of peaks was the same in both plots and peaks were quite distinctive. However, the plots of the two measurement characters were very different in shape despite being based on the same specimens; for example, there were about twice as many ninth instar head width specimens as there were ninth instar wing-pad length specimens. Thus, all peaks in the two plots do not indicate instars. The conclusion from the normal distribution simulations and the above studies is clear: the accuracy of frequency methods cannot be evaluated based on the frequency plot alone.

Effect of the number of size classes

All linear regressions of number of size classes versus number of periodic maxima peaks greater than zero resulted in very high positive correlation coefficients ($P<0.001$). The correlation coefficient (r), r^2, N (number of data points), and the predictive regression equation ($y=mx+b$) are: *Baetisca rogersi* data, 0.98, 0.96, 17, $y=0.28x-0.25$; random number data, 0.99, 0.99, 24, $y=0.31x+0.09$; data from Table 1 studies (excluding Vaught & Stewart, 1974, and Sephton & Hynes, 1982), 0.92, 0.85, 12, $y=0.26x-0.68$; above data sources combined, 0.97, 0.94, 53, $y=0.28x-0.17$. The number of size classes alone accounted for 85–99% of the total variation in the number of peaks or modes. Thus, the number of peaks or modes in the plots

of all frequency methods can be varied greatly simply by altering the number of size classes. For the *Baetisca rogersi* data (only female nymphs in the upper 50 percentile size range were used), the number of periodic maxima peaks greater than zero varied from 5 to 53 as the number of size classes was varied from 20 to 188 (Table 2A, Fig. 2). Rearing (Pescador & Peters, 1974) and Palmen body (Fink, unpublished data) data indicated about 12–13 instars for the entire nymphal stage. The number of periodic maxima peaks greater than zero for the random number data varied from 6 to 41 as the number of size classes was varied from 22 to 127 (Table 2B, Fig. 3). Decreasing the number of size classes also resulted in a decrease in the number of peaks in the frequency plots of the 16 instar, 5% COV normal distribution simulation (Table 4). In the published studies (Table 1) the predicted number of periodic maxima peaks greater than zero differed by an average of only 14% from the number of actual peaks when the combined data source regression equation was used (Table 1).

Some may argue that only 'significant' peaks

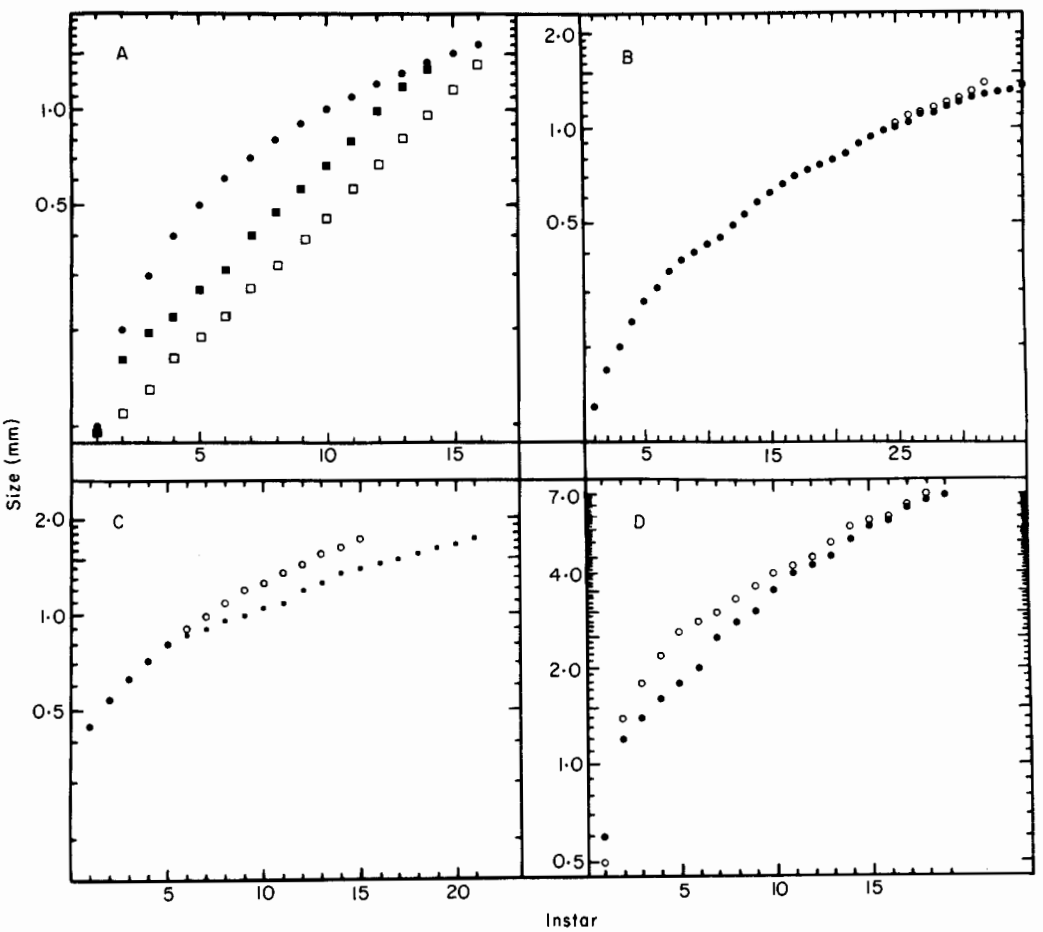

FIG. 7. Dyar's law plots from instars determined through frequency methods. A: dots, hypothetical mayfly population with the same arithmetic progression factor between successive instars; open squares, hypothetical mayfly population with the same geometric progression factor between successive instars; closed squares, plot of the 16+19 instar mayfly subpopulations normal distribution simulation (see Fig. 6D). B: plot of a random number simulated mayfly population with 127 size classes and $N=500$ (see Fig. 3C); closed dots, all periodic maxima peaks greater than zero plotted as instars; open dots, only large peaks plotted as instars (previous closed dots were large peaks). C: plot of *Gomphiocephalus hodgsoni* (Cape Crozier population) instars as determined by Janetschek (1967), open circles; all periodic maxima peaks greater than zero are indicated by the closed squares. D: plot of periodic maxima peaks greater than zero of Fig. 1; open dots, exuviae; closed dots, whole nymphs.

should be chosen since they may be more likely to indicate instars. However, the normal distribution simulations indicated that it is impossible to be certain that any peak indicates an instar. An examination of Figs. 3B and C shows that, ignoring very small peaks in both periodic maxima–minima plots, increasing the number of size classes from 64 to 127 results in almost doubling the number of 'significant' peaks. Even data from a caddisfly population (paper 9 of Table 1), presumably with only five real instars, showed a size class effect: periodic maxima peaks numbered at least 10. Had these data come from a mayfly or stonefly population, they would have been interpreted as indicating at least 10 instars. Newell & Minshall (1978) may have selected a very low number of size classes for their head width data with the result that only six 'instars' were determined (this value was viewed as unrealistic by the authors). The effect of the number of size classes can be overcome only by a very homogeneously developing population.

Random number simulations were used in the present study to show that random data produces frequency and Dyar's law plots which are indistinguishable from those of real populations when size sampling bias is acknowledged for real populations (compare Figs. 1–3 and 7). Data from both real and random number populations produced approximately evenly spaced peaks (Figs. 1–3) which seemed to indicate instars when actually these peaks were the direct result of size class data partitioning. Essentially, frequency instar analysis of real and random number mayfly or stonefly populations is reduced to a size-class effect, because a reasonable number of instars can be produced merely by selecting a certain number of size classes.

Dyar's law, supplemental rearing and best-fit analysis

Dyar's (1890) 'law' or 'rule' is popularly invoked to corroborate instars, including those instars determined through frequency methods (papers 1–4, 6 and 10 of Table 1). Unfortunately, Dyar's law has been misunderstood by many. Departures from Dyar's law should be expected to be commonplace since growth of many animals through time is not constant (Simpson, Roe & Lewontin, 1960), especially for many mayflies and stoneflies whose relatively long immature life extends over different seasons. Thus for these insects a straight line (semi-logarithmic) plot should not be expected (Fig. 7A). Fortunately, the usefulness of a Dyar's law plot in instar analysis depends not on the straightness of the plot but rather on the uniformity of spacing between 'instar' data points; if a large gap between two data points is found then it may indicate the presence of additional instars. However, a Dyar's law plot does not confirm the existence of any of the instars. Gaines & Campbell (1935) did not recommend using Dyar's law to corroborate instars since it may indicate instars that do not exist. A Dyar's law plot (Fig. 7A) of the normal distribution simulation (Fig. 6D) gave no indication of the missing instars nor did it indicate that an 'instar' data point actually was the result of as many as two instars. A Dyar's law plot does not corroborate instars determined through frequency methods because the uniform spacing of Dyar's law data points is the direct result of the uniform spacing of peaks in frequency plots of almost all data sources, whether or not peaks indicate instars (Fig. 7). Dyar's law plots even 'corroborated' different numbers of instars of the same frequency data, when different peak selection criteria were used in the frequency plots (Figs. 7B, C). Dyar's law plots from most frequency analyses would be expected to show a uniform spacing of data points that appear in shape to be somewhere between the smooth curve of perfect arithmetical intervals and the straight line of perfect geometric intervals (Fig. 7A). A Dyar's law plot based on frequency data should not be used to determine the general growth pattern of a population (e.g. see Vaught & Stewart, 1974) since the shape of the plot is determined by the estimated instars which may not be instars at all. Also it is generally meaningless to compare mean progression factors for different species since these factors are the direct result of the number of instars and the total size range of the species (Table 3).

Supplemental rearing has been cited by some of the studies listed in Table 1 (papers 4, 5 and 8) as additional support for their instar claims; however, none of these studies presented any data which would indicate the adequacy of their rearing programme. Only a rigorous rearing programme could verify or determine instars and instar size variability on a population level,

which is exactly what investigators have sought to avoid by using frequency methods.

Harding (1949) showed a way to lend additional credence to the possible existence of component groups in frequency distributions. By trial and error, Harding chose appropriate normal distributions for each supposed component group and then formed a simple frequency distribution that was compared to the original simple frequency distribution through a chi-square best-fit analysis. Those normal distributions which provided the best-fit were then believed to offer the most likely solution. This process has been automated by several iterative computerized versions (e.g. Yong & Skillman, 1975; Brassard & Correia, 1977; Dahlberg, 1978) The Yong & Skillman (1975) method generates polynomial equations. A polynomial that provides the best-fit to the original simple frequency distribution is chosen as the most likely solution.

However, a good fit of the proposed distribution to the original distribution does not prove the existence of component groups because as Simpson *et al.* (1960, pp. 377–378) eloquently expressed, the danger of fitting mathematical functions to biological data '. . . lies in the extraordinary power of a mathematical function to fit observations. If enough parameters are introduced into the equation of a curve, that curve will, by suitable choice of parameter values, fit any set of observations perfectly . . . Thus, the establishment of a satisfactory mathematical model should not be confused with an elucidation of the underlying biological process.' The specific problem in instar determination is that a mathematical function or series of normal distribution will certainly be found that matches the frequency data very well, including the false peaks.

The future of mayfly–stonefly instar analysis

Many questions remain unanswered in mayfly and stonefly biology, and much of it concerns just how these insects develop. Following development through life-history diagrams and morphological stages has given us a broad but schematic picture, that only hints at the intricacies and causes of development. Because moulting is an integral process of development in arthropods, correlating the amount or degree of development per instar with environmental factors will help us understand better how the environment affects these organisms. We need to know why individuals may pass through different numbers of instars or increase in size or change in morphology at a rate significantly different from other individuals and how important environmental factors such as food, temperature and photoperiod affect instar number and development, and the eventual initiation of adult tissue development. Few studies have linked development with instars, and even fewer studies have then correlated instar development with environmental factors, as Cianciara (1979) did so carefully. While a number of rearing studies have provided important data on development, too few specimens were usually reared to determine accurately within population variability, which must be known before different populations under different conditions can be compared. Paradoxically, the variability of development and instar number that we wish to study in mayflies and stoneflies is the very reason that frequency methods cannot be used. To continue to use frequency methods for instar determination invites great confusion in the literature due to the proliferation of inaccurate and unsubstantiated results.

The primary alternative to frequency methods is rearing, which ideally should involve the rearing of a large number of specimens individually from egg to adult. If rearing throughout the life cycle is not possible then rearing of individuals from one distinct morphological stage or uniform size to another could yield important results on the variability of the number of moults and relative change per moult. Indiscriminate resupplying of nymphs from the field to replace dead specimens may not be very useful, however, since it may be difficult to compare the growth of nymphs that differ initially. Rearing experiments could yield much information on the interaction between development, instars and environmental factors manipulated experimentally. Obviously rearing is not without detractions, these being the difficulty of success and the time needed to monitor and maintain rearings. However, it is doubtful that most investigators use equipment expressly designed to rear such small aquatic insects where the conflicting needs of ease of observation and simulation of important environmental needs are sufficiently balanced to ensure routine success.

The Palmen body method (Fink, 1980;

Degrange, 1959) provides a potentially most valuable alternative for determining instars of mayflies. Whereas results from rearing may not be representative of those in the field, the Palmen body method can be utilized directly on field and laboratory populations. Accurate observations of the number of cuticular cylinders (where each cylinder indicates an instar) for some Heptageniidae and certain large mayflies can be made from wholemount Palmen bodies, while counts of the cylinders of most mayflies must be made from thin sections (Fink, unpublished data).

In summary, the future of understanding development in mayflies and stoneflies, and other arthropods which display some of the characteristics discussed in this text, lies in the use of the direct instar determination methods of rearing and the Palmen body (mayflies only).

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