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A methodology for the study of the life cycle of aquatic Chironomidae (Diptera)

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Summary

Methods for the analysis of life cycles of aquatic Chironomidae are described and discussed. Some needs and perspectives for the near future are indicated.
Additional Key-words : Chironomidae, life cycle, diapause.

Résumé

Des méthodes pour l'analyse des cycles de vie chez les Chironomidae aquatiques sont décrites et discutées. Des indications et des perspectives pour le proche avenir sont envisagées.
Mots-clefs : Chironomidae, cycle de vie, diapause.

Introduction

Most chironomid larvae have an aquatic mode of life. They are most common in benthic and epiphytic freshwater communities. Furthermore, about one fifth of all Belgian aquatic insect species are chironomids and densities of up to one hundred thousand larvae per square meter are by no means rare. Because of this quantitative importance in freshwater ecosystems, they form excellent subjects for ecological studies.
However, in spite of the fact that there is an extensive literature on their biology, accurate data from which life cycles of chironomids can be deduced appear rare. It is especially important to have a good understanding of the growth of chironomids when studying secondary production in lakes, ponds and rivers. However, in this type of research, there is a tendency to oversimplify the methodology : (1) larval growth is reduced to voltinism, (2) production estimates are mainly focused on the highly productive fourth instars, (3) laboratory data are applied to natural populations without additional justification.
At the Hydrobiology section of the K.B.I.N., life cycles of chironomids are analysed in their natural environment by following the larval populations in the course of time. Stagnant waters are preferred as study sites, because populations in such environments are better delimited and also because there is no drift. Thus far, two different sites have been studied : two trout-ponds at Mirwart (Belgian Ardennes) and the Blankaart reservoir (West-Flanders).

The present paper describes our methods and suggests a number of improvements over other existing methods. Some hypotheses and expectations for the near future are also given.

Life cycle characteristics

Voltinism and seasonality : Voltinism is one of the most striking features in the year cycle of chironomids. It appears from the literature that most species have more or less distinct emergence periods and that these periods are furthermore limited to certain seasons, which are typical of each species. In many taxa, the number of emergence periods is limited (uni-, bi- or trivoltine), but in some groups (e.g. the Orthocladiinae) multivoltine species are also common.
Growth and development : However, factors such as food, temperature, oxygen concentration and photoperiod significantly influence growth and development of chironomid larvae and consequently also the voltinism (PINDER, 1986). A rather constant voltinism pattern, typical of each species, is doubtlessly influenced by the recurrent year cycle of the (water) temperature. But fluctuations in the environmental factors, especially the extreme conditions, can still alter that pattern. A yearly diapause in a larval stage seems to be a key factor in determining the pattern of the life cycle of different species (GODDEERIS, 1983, 1986). The diapause is again species-specific, both for the development stage and for the season in which it occurs. Diapause in chironomids is mostly linked to overwintering, but can already commence in summer. Therefore, it determines the season(s) of possible growth and emergence. An example of specialisation in time-niche as a consequence of this diapause was observed at Mirwart, where the growth periods of different generations of four *Tanytarsus*-species groups appeared to alternate (GODDEERIS, 1987).
Synchronization : Moreover, the yearly diapause most probably directly causes the restricted emergence periods themselves (see above). From the beginning of the diapause, the whole population is settled in a distinct developmental stage (diapausing stage or stages), for-

ming a narrow(-er) larval cohort. Once growth and development start again, because of increase in water temperature and/or daylength in spring, the larvae in this narrow cohort will all be fullgrown and will pupate together in a distinct period. In multivoltine species, this first emergence period is reflected in the less synchronized second emergence period. Because of individual variation in growth and development of the larvae, the subsequent emergence periods will overlap rather quickly. However, the yearly diapause resettles and synchronizes the whole population again for the first emergence period of the next year.

Cohort versus generation : The terms "generation" and "cohort" are used as defined by DAJOZ (1974). A *generation* is formed by individuals of a population hatched during the same period (e.g. during, or just after an emergence period). A *cohort* is formed by individuals of a distinct and recognizable group in the population, because they went through the same event, which has caused the formation of that particular group (e.g. a diapause). A cohort can also be a generation, if the individuals of that generation are still recognizable as a single group. In this case, the causal event is only the fact that they hatched during the same

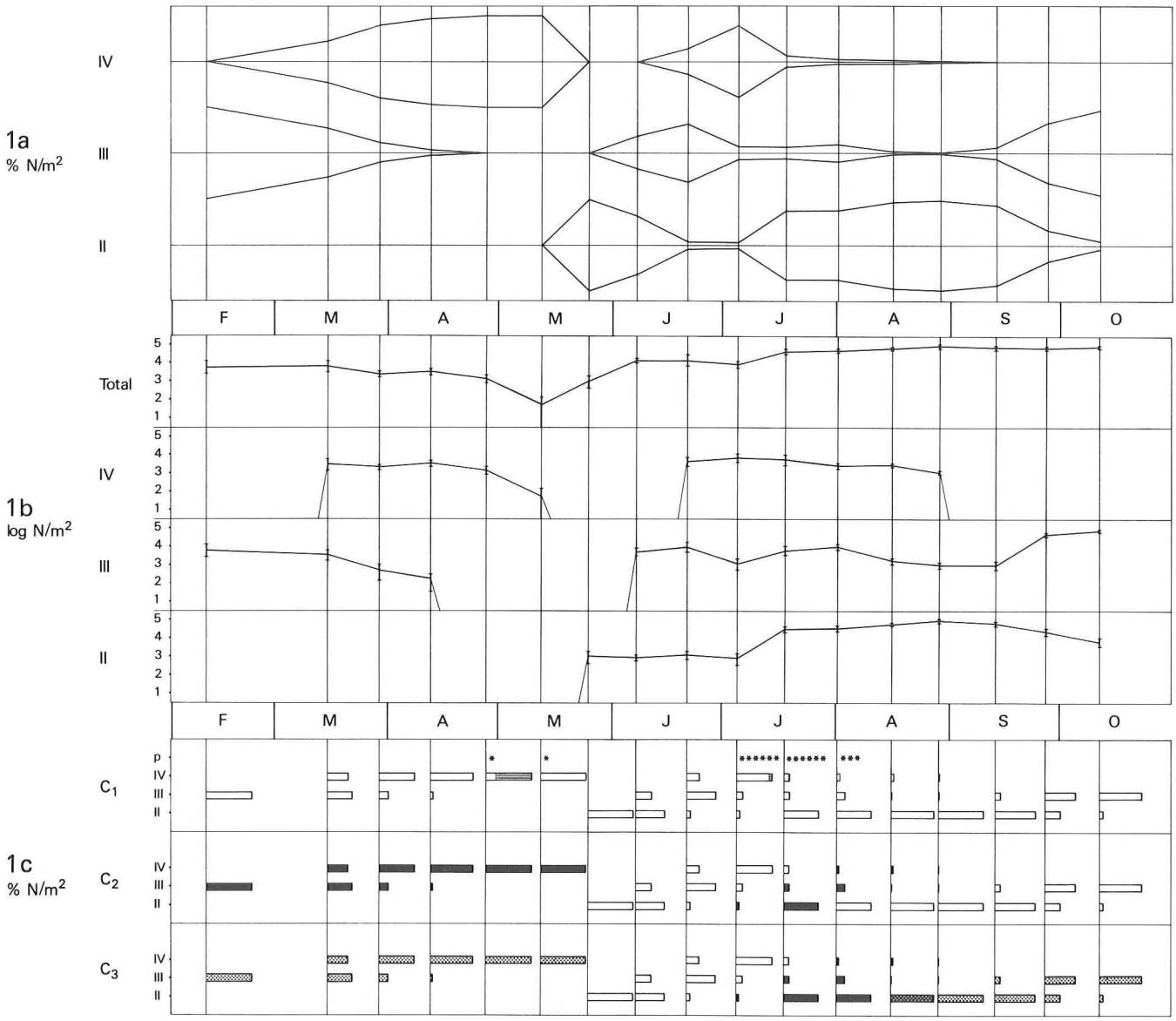

Figure 1
Tanytarsus debilis *(MEIGEN, 1830). Larval population dynamics in pond IV at Mirwart (Belgian Ardennes), 1977.* 1a: *kite diagram of the relative abundance of instars II, III and IV.* 1b: *density of instars II, III and IV and their total numbers in log N/m² ± 95% confidence intervals.* 1c: *block diagram of the relative abundance of instars II, III and IV:* $1c_1$ = *without any separation of generations, nor cohorts, the prepupating larvae shaded, total number of pupae in each sample indicated by asterisks;* $1c_2$ = *alternation of cohorts indicated by black and white;* $1c_3$ = *alternation of generations indicated by black, white and gray (= mixed generation). See also text for additional explanations.*

period. But if one part of a generation enters diapause and the other part does not, the generation is split into two cohorts. Then the non-diapausing larvae form a partial generation that emerges and their offspring links up with the diapausing larvae in a mixed generation (fig. 1c). Such phenomena seem to be common in chironomids and considerably complicate research on their life history.

Life cycle analysis

Adults : Voltinism in chironomids is often directly measured by the use of emergence traps, i.e. by monitoring the population dynamics of the adults. Distinct emergence peaks are thought to correspond with different generations; the lapses between the peaks would then occur during the larval development. Day-degree requirements for larval development and temperature at which larval development stops (development zero temperature) have been calculated for several species in this way (MUNDIE, 1957). However, fluctuations in sampling efficiency, emergence success, predation on pupae, etc. hamper the interpretation of such results. Furthermore, hypotheses on larval growth deduced in this way remain unreliable, because all measurements are indirect and because factors such as diapause are not taken into account.

Weight : Reliable data on the growth rate of larvae in their natural habitat have been obtained from populations of univoltine species by following the mean individual weight in time (a.o. CHARLES *et al.,* 1974). However, in such investigations and without additional corrections, growth is underestimated in periods of natality and emergence because small and young larvae are continuously added and because of the loss of the fullgrown larvae respectively. For the same reasons, growth is underestimated in species where voltinism is not absolutely limited to one generation but where a partial generation is added. When the mean weight method is used, diapause is completely overlooked and at the onset of the diapause, the growth rate of the non-diapausing larvae is biased, because the weight of diapausing and non-diapausing larvae are simply lumped together.

Instar analysis : For bi- or multivoltine species, it is necessary to separate the larvae of the different generations to obtain information on their growth. This can be done by instar analysis (EDMONDSON, 1971). The growth of the head capsule in chironomids is discontinuous and increases immediately after each moult. The four larval instars can be distinguished from each other by the size and the shape of the cephalic structures. Monitoring the number of prepupating larvae (recognisable by the swollen thorax) and pupae can also yield interesting data. However, it is often difficult to clearly separate generations, because they can overlap, especially when their number per year increases.

In a simple instar analysis diapause is either not detected or at most superficially indicated.

Figure 1 illustrates some of these difficulties in an example : the life cycle of *Tanytarsus debilis* in a pond at Mirwart in 1977. Relying on superficial analysis, one could conclude that *Tanytarsus debilis* is bivoltine, as the proportional analysis of the instars shows two main peaks in one year (fig. 1a). However, analysis of the absolute numbers of the third instar larvae reveals three statistically significant peaks (fig. 1b) (a third partial generation is also observed in a length-frequency analysis). The second decline in the number of third instars in July in this analysis would probably not have been detected if the samples had been collected one week later or earlier. It thus appears that reliable data on densities of younger (I-III) instars are also quite essential.

Laboratory cultures : Especially for multivoltine species, a number of authors deem information about larval growth obtained through laboratory cultures necessary (MACKEY, 1977 ; WATERS, 1979). In our opinion, the applicability of such data to field situations is, to say the least, doubtful, especially when any kind of control is missing. In laboratory cultures, differences in specific growth rates of up to 500 % have been measured (ANDERSON & CUMMINS, 1979); these differences were furthermore due to alterations in the quality of food only.

Length frequency distributions : Life cycle data obtained with the methods described above remain speculative. We started with the assumption that the distinct emergence periods, when present, must be clearly reflected in the structure of the larval populations, even in multivoltine species. From the measurements of the changes in this population structure (accurate density and instar analysis, length frequency distributions, development of the imaginal discs - see below) we should be able to deduce the pattern of growth and development in relation to environmental factors.

In the length frequency distributions, larvae are arranged in length classes of 1/4 mm (fig. 2). Length growth in chironomids is approximately linear (KONSTANTINOV, 1958 ; MACKEY, 1977), especially when the growth of the first instars and prepupating larvae is not considered. The first instar is the dispersion stage and has a planctonic mode of life ; growth probably only starts when the larvulae are well settled. Each length class therefore has about the same duration, and this is important in the interpretation of the frequency distributions.

It is quite useful to incorporate an instar analysis in the length frequency distributions, as demonstrated in figure 2.

Length frequency analysis : In the length frequency distributions a displacement of a cohort over the abscis (length) in time is an indication of larval growth ; confirmation is furthermore obtained when those animals moult to the following instar. However, a

displacement of the mean length could for example also be effected by a selective mortality. As long as the peak of the frequency distribution remains in the youngest instar classes, one may expect an increasing natality (as long as there is no diapause in that instar). Once the peak of the histogram moves to higher length classes, the natality peak of that cohort has passed. When no more small larvae are observed, we can assume that there is no more (or little) natality.

Between the natality peak and the emergence peak of a cohort, the *length growth* of the larvae can be related to temperature by measuring the displacement of the mode of the frequency distributions over different sampling dates. The displacement of the largest and smallest larvae (the extremities in the frequency distributions) also gives information on larval growth.

Emergence periods are indicated by the presence of mature larvae and pupae, and by the appearance of a new cohort approximately at the same time as the cohort of large larvae disappears.

Phenomena of *diapause* are indicated by a sudden stop on the otherwise linear growth in a distinct development stage ; usually the population evolves from a continuous structure with all instars to a discontinuous structure, where only the diapausing and younger development stages are present. Confirmation of diapause is given when the growth arrest occurs every year at approximately the same time in the same stage. In this context, it is important to have information on more than one waterbody during one year, or on more than one year cycle in one locality.

Imaginal discs : Diapause phenomena are hardly detectable in length frequency distributions when they occur in the fourth instar. Growth and development are not absolutely correlated and most often, discontinuities in development are not easily observed in the length frequency distributions. An analysis of the development of imaginal discs is advisable then.

The development of the imaginal discs has been described in *Chironomus*-species (WUELKER & GOETZ, 1968 ; INEICHEN *et al.,* 1983). Nine substages in the fourth instar are established and the applicability of this subdivision is demonstrated both in laboratory cultures (FISHER, 1974 ; INEICHEN *et al.,* 1979) and in field situations (KRUEGER & NEUMANN, 1983 ; BUTLER, 1987). These imaginal discs are easy to observe in euparal microscopical slides as described below, when the larvae are mounted with a lateral side up.

However, it is necessary to re-establish the different substages for each species, because the morphology of some discs and consequently their development features can be quite different. It is furthermore important to define substages of approximately the same duration, otherwise an observed accumulation of larvae in a substage could be an artefact, rather than a developmental characteristic. To establish nine substages that are homologous between most species, the development of the leg discs seems to give the best results.

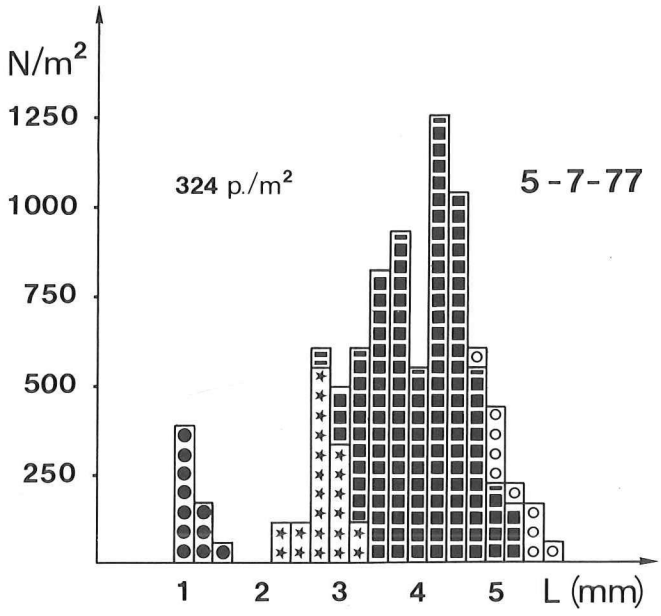

Figure 2
Tanytarsus debilis *(MEIGEN, 1830). Length frequency distribution in pond IV at Mirwart (Belgian Ardennes) on July 5th, 1977. Full dots = instar II; asterisks = instar III; full squares = instar IV; open dots = prepupating larvae; p = pupae.*

Difficulties in separating the substages are as follows: the continuous character of imaginal disc development (which renders every attempt to construct clearly delimited artificial substages difficult), the variable synchrony of the development of the different discs, and the deformations due to fixation. Observation of the structures can be difficult, but is considerably facilitated by colouring.

Sampling program

A well adapted sampling program allows one to obtain the desired information about populations in the most economic way. It requires answers to questions about size, number and location of the samples and frequency of sampling, while avoiding unnecessary work. Statistical principles that are to be considered while sampling benthic organisms are given by ELLIOT (1977) and PREPAS (1984). In life cycle studies, however, many factors that should determine the sampling strategy are in fact those fluctuating factors that are to be elucidated by the program itself. Furthermore, a sampling program for benthos in general greatly depends on practical and environmental circumstances, such as the available time and sampler devices for a given substratum.

The study of a highly dynamic taxocoenosis like a chironomid community is even more complicated. The most appropriate sampling scheme at a given locality for a certain population or species is not necessarily suitable for another population, and can even turn out to be inadequate one week later.

It is not surprising, then, that sampling programs for the life cycles of benthic organisms are always compro-

mises between practical factors and theoretical considerations.

Number and size of samples : An average of thirty larvae from each population (or cohort) seems to be a minimum to construct valid length frequency distributions. A sample of thirty larvae of a small species (fullgrown larvae with a length of 6 mm are very common in chironomids) give a mean length with confidence limits of ± 0.25 mm when half the length range (or 2.75 mm) is occupied by 95 % of the cohort in a normal distribution ; 0.25 mm is the range of the length classes used. When the whole length range is occupied by the larvae of one cohort, four times more larvae have to be measured to obtain the same confidence limits of ± 0.25 mm. In general, length measurements of thirty larvae of a cohort yield confidence limits of maximum ± 10 % of the length of the fullgrown larvae.

At Mirwart, an average of four hundred chironomid larvae was obtained from each of the two ponds by sampling 180 cm^2 of sediment surface. In summer-time, when growth and development may be intensive, about one thousand or more larvae were collected from the same surface. These numbers were supposed to be sufficient to follow the year cycle of the dominant species. As the distribution of benthic organisms is furthermore rather clustered, the reliability of the density estimations is greatly increased by subdividing the total surface sampled in as much sample units as possible. For example, it is better to take 5 samples of 20 cm^2 than one sample of 100 cm^2. However, the size of a sample unit also has its lower limits ; for example with decreasing size the edge effect errors are increasing. At Mirwart, 12 sample units of 15 cm^2 were taken in each pond at each sampling date. The standard error of the total density varied around 20%.

Sample devices : In shallow waters, plexi tubes are very useful sampling gear for soft sediments (CHODOROWSKI, 1971). At Mirwart, a tube with a diameter of 4.4 cm was driven from a boat into the sediment, until the rocky subsoil was reached (sediment layer ± 10 cm). The tube length has to be somewhat shorter than the water depth. Then a core can easily be pulled out of the sediment by closing the upper aperture of the tube with the palm of the hand. It is advisable to have a series of tubes with length differences of 30 cm at hand, in order to be able to sample at different water depths.

In the Blankaart reservoir (depth up to 5 m), the observed densities were lower than at Mirwart. Therefore, and because the size of the reservoir (60 ha) was larger, the number of units at each sampling date was elevated to twenty. The samples were taken with a Petite Ponar grab (16.8 x 14.4 cm ; Wildco-Saginaw U.S.A.), a device mostly used for sandy sediments (DOWNING, 1984). The sampled surface being too large, only 1/8 of each unit (= 30 cm^2) was processed.

Distribution of samples : The samples were taken in a stratified random way, which maximizes the accuracy of the density estimates. At Mirwart, each pond was divided into six equally sized strata and two sampling units were taken from each stratum at random. A single random sample unit was taken from each of twenty equally sized strata in the Blankaart reservoir. With this method, each point of the bottom surface has an equal chance of selection, but the chance that the whole sample is taken from one part of the pond or reservoir only, is eliminated.

Sampling frequency : It is difficult to formulate general recommendations for sampling frequency, because this aspect greatly depends on the life cycle characteristics of the species under consideration. It is clear that for a univoltine species, one does not have to sample as frequently as for a trivoltine species. Furthermore, it should here be stressed that analysing growth and development of the larvae involves more than simply filling in the periods between emergence peaks.

MUNDIE (1957) stated that his monthly sampling procedure was insufficient to adequately monitor densities of the larvae of species (-groups). At Mirwart, the monthly (in summer even threeweekly) sampling of 1976 also proved to be inadequate for initial analysis of multivoltine species. When growth is continuous, four samples for every generation seem to be a minimum for analysis independently from information of other generations. With fewer samples, there is a risk that the generation is sampled only once in full development and not detected in the other samples. In general, a fortnightly program is a good approach, with lapses of three to four weeks in late autumn and winter. A more intensive sampling is only recommended in special cases, for example the first generations of multivoltine species (quadrivoltine or more); from the fourth generation onwards, overlap becomes too apparent anyway to allow distinction of the following generations.

Sample treatment : It is important that the samples are fixated *as soon as possible* in order to avoid further development, especially of diapausing stages. Our samples were transported in frigo-boxes and sieved before fixation, the day after sampling. A *mesh size* of 70 μm was used to retain the first instars, the width of the head capsule being the critical dimension for living larvae (MUNDIE, 1971). Before sieving, some formaldehyde was added to activate the larvae, driving them out of their tubes (BRITT, 1955). A higher efficiency can be reached by sieving the samples a second time (but this time with dead animals) over 125 μm mesh (STOREY & PINDER, 1983). When the first instars cannot be identified and are of no importance, it is advisable to focus processing on second instars by using a larger mesh size. This saves considerable time and effort.

Our samples were *fixated* by adding ethanol 90°, the remnant of water diluting the sample to approximately 70°. Some drops of concentrated "Rose Bengale" were added to colour animals bright red, which is useful when sorting the larvae. In ethanol, the larval body also stretches very well, which is an additional advantage for

length measurements. However, in large or quite organic samples, it appears advisable to add some formaldehyde to the ethanol up to 4 % to assure all round preservation of the sample (LADLE *et al.,* 1984). Before *sorting* the larvae, the samples were fractioned by sieving over 2.5, 0.38 and 0.07 mm mesh size. Sorting was done under stereo-microscope, the 2.5 and 0.38 mm residue with a magnification of 6 x, the 0.07 mm residue with a magnification of 12 x. A benzene floatation technique was applied to the 0.07 mm fraction (MUNDIE, 1957 ; KARLSSON et al. 1976). In summer and autumn, the first and second instars were so numerous at Mirwart, that only the larvae of a subsample of half the 0.07 mm fraction were sorted. From the Blankaart, subsamples of 1/8 of the large PONAR-samples were treated and only 1/4 of the 0.07 mm residue of these subsamples were sorted in summer and autumn.

All larvae were mounted in *microscopical slides* with euparal as medium. An advantage of such permanent slides is the possibility to individually number every larval specimen, which allows separate identifications, measurements and control later on. For example, in our study it was possible to make the determination of the imaginal disc substage ten years after the Mirwart material was collected. The slides were made cover-glass on cover-glass ; observation from the both sides is thus made possible. Before mounting, the larvae were identified as far as possible and also separated in size classes (small-medium-large).

The *orientation* of the larvae depends on identification characteristics. Tanytarsini were mounted on their lateral side, because the important antennae are then quite visible. The head capsule of Chironomini-larvae, on the other hand, was cut off and mounted dorso-ventrally. In euparal, the larvae shrink ; the Tanypodinae larvae even have to be pierced prior to mounting. A shrinkage coefficient was taken into account in the length-weight diagrams established on living larvae. However, the larvae in euparal were still longer than the living ones, due to the stretching of ethanol fixation.

Parallel *rearing* was effected to obtain a checklist of the fauna. Unknown mature larvae, recognizable by their swollen thorax, were individually reared to obtain a larva-pupa-adult sequence for identification.

Length measurements (top of the head to anus) were made with a curvimeter from projections of the microscopical slides at a magnification of 100 x.

Perspectives

It was already mentioned in the introduction that there is a real paucity of data to elucidate the life cycle of chironomids. Diapause in chironomids is probably much more common than is thought to date. Because diapause is also species-specific, varying between the developmental stage(s) and between the season(s) in which it occurs, it could govern the life cycle pattern of each species. Therefore, the occurence and ecological significance of diapause phenomena in different taxa are to be determined. In this context, life cycle strategies in species from lentic and lotic habitats are to be compared, as the hibernating conditions are quite different. It would also be interesting to investigate the life cycles of the same species in different geographic regions and under different climatic conditions.

Specific threshold temperatures for pupation have been proposed as the causal factor of the distinct and synchronized emergence periods (MUNDIE, 1957 ; REISS, 1968 ; LAVILLE, 1971), the overwintering cohort only commencing emergence when the threshold temperature is reached. The specific emergences in spring indeed occur at approximately the same specific temperature at different depths, in different lakes and over different years. However, this hypothesis has never been tested on larval populations, and the specific threshold temperatures cited in the literature are by no means constant. The yearly resettlement of the larval population in a narrow cohort by a specific diapause is at least an equally valid hypothesis to explain the causes of distinct and specific emergence periods.

The induction, course and release of diapause in chironomids are still not understood. The photoperiod is known to be a key factor in diapause phenomena, but other factors such as temperature, food and even endogenous induction should also be considered (INEICHEN *et al.,* 1979).

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