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Larval insect identification by cellulose acetate gel electrophoresis and its application to life history evaluation and cohort analysis

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Abstract. Early instar larvae that lack distinguishing structural characters can be identified quickly and easily with cellulose acetate gel electrophoresis. Examples are given from the genera *Ameletus* (Ephemeroptera) and *Coenagrion, Enallagma,* and *Lestes* (Odonata). We tested 28 or 29 enzymes which yielded up to 29 scorable loci; of these only six or seven were necessary to achieve species separation at three or more loci. The technique facilitates the search for structural characters, confirms species separation based on morphology, and allows the construction of complete life histories and life tables.

Key words: electrophoresis, larval identification, life history, Ephemeroptera, Odonata.

The elucidation of aquatic insect life histories is beset with sampling difficulties. Zelt and Clifford (1972), Suter and Bishop (1980), and Mutch and Pritchard (1982) showed that erroneous conclusions were frequently drawn from data collected with samplers of too coarse a mesh size. In addition, the last authors pointed out that early instars were often misidentified because of the lack of easily observed distinguishing morphological features. To separate small larvae of the stonefly *Zapada columbiana* (Claassen) from two other sympatric congeners, Mutch and Pritchard (1982) reared all three species from egg to adult in the laboratory to find distinguishing characters. Even so, they still found it difficult to distinguish *Z. columbiana* larvae with head widths of 0.22 mm or less from newly hatched *Z. cinctipes* (Banks) larvae. This is a problem, not only for benthic ecologists but for entomologists in general, especially if life table work is being undertaken. Most insects encountered in aquatic ecological work are larvae because the larval stage lasts longer and larvae occur earlier than adults on the survival curve.

In our studies of populations of *Ameletus* mayflies in the Rocky Mountains of Alberta, we have found that as many as 12 species of the genus can occur at a single site. In the Elbow River drainage in the Bow-Crow Forest of the eastern Rocky Mountain foothills in southwestern Alberta, where we have compared life histories, seven species (*A. celer* McDunnough, *A. cooki* McD., *A. similior* McD., *A. validus* McD., and three undescribed species, to be described by Zloty as *A. "majusculus"*, *A. "bellulus"* and *A. "pritchardi"*, are found at Ford Creek, a eurythermal, subalpine, 2nd-order stream (50°48'N, 114°41'W), and three of these (*A. celer, A. similior* and *A. "majusculus"*) also occur at a stenothermal site near the source of the Elbow River (50°38'N, 115°00'W). After rearing adults from late instar larvae, we isolated morphological characters of the larvae that would separate these species. We were able to find characters that were useful for most instars but were unable to distinguish between species in the first few critical instars.

Similar problems arose in our work with lestid and coenagrionid damselflies at a pond on the University of Calgary campus (51°5'N, 114°7'W), in spite of the Odonata being better known than any other group of aquatic insects in Canada (Walker 1953, 1958, Walker and Corbet 1975). Here we encountered five species of Coenagrionidae (*Coenagrion resolutum* Hagen, *Enallagma boreale* Selys, *E. cyathigerum* Charpentier, *E. ebrium* (Hagen), and *E. hageni* Walsh) and four species of Lestidae (*Lestes congener* Hagen, *L. disjunctus* Selys, *L. dryas* Kirby and *L. unguiculatus* Hagen). Three of the species of *Lestes* are currently morphologically separable only by the very dubious characteristic of labial length: width ratio (Walker 1953, Cannings and Stuart 1977), and Walker (1953) could only suggest that a 6-segmented versus a 7-segmented antenna might separate larvae of the genera *Coenagrion* and *Enallagma*. However, this character is

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not stable enough to work even for last instar larvae and, because segments are added during development, certainly does not work for earlier instars. Baker and Clifford (1980) encountered similar problems and found characters that would separate larvae of the two genera in Alberta. We found that these characters worked quite well for medium- and large-sized larvae at our study site but were inadequate for the separation of small larvae. Within *Enallagma*, characters on the caudal lamellae are used to separate species, but these are notoriously variable and lamellae are often missing or distorted through regeneration. Furthermore, no characters are known that will separate female larvae of *Enallagma ebrium* and *E. hageni* (Cannings and Stuart 1977).

We resorted to electrophoresis to solve these problems. Over the last 20 years, electrophoresis has been applied to many areas of biology (Richardson et al. 1986), perhaps most successfully for distinguishing between closely related species (Ferguson 1980). The problem that we have investigated here is similar to the latter, in that morphological differences are not available to identify species in the usual way. But additionally, the identification of earlier instar larvae of insects has the problem of the very small size of the specimens. However, advances in electrophoresis technology, especially through the use of cellulose acetate gels, has resulted in a dramatic increase in sensitivity; even individual eggs and small larvae of two species of *Heliothis*, not distinguishable by their morphology, have been identified by this technique (Daly and Gregg 1985).

Although there are few reports of electrophoresis on dragonflies (Anderson et al. 1970, Knopf 1977, Schott and Brusven 1980, Maibach 1985), a considerable amount of electrophoretic work has been done on mayflies (e.g., Matha and Sula 1984, Söderström and Nilsson 1986, Sweeney et al. 1986, Zurwerra et al. 1987, Funk et al. 1988, Hefti et al. 1988, Sweeney and Funk 1991), and electrophoresis was used by Kownacki and Starmach (1984) to identify young larvae. However, most of this work has been done to collect genetic data and assess phylogenetic relationships between species, and the usefulness of the technique to ecologists has not been sufficiently emphasized. It is our purpose here to point out the usefulness of the technique in that most important step in much ecological work—the identification of species. Given correct identification throughout the life cycle, life history patterns can be properly determined and, if samples are taken quantitatively, life tables can be constructed. Furthermore, the technique can be used to detect parasitism as we shall show in the case of nematode parasitoids in *Ameletus*. In this paper, we illustrate the application to life history evaluation with four of the species of *Ameletus* from Ford Creek.

Voucher specimens are currently held in the Aquatic Invertebrate Collection at the University of Calgary.

Methods

Species identification

Adult insects were obtained by 1) aerial netting of imagoes; 2) allowing mayfly subimagoes, picked individually from rocks, to transform in the laboratory; and 3) rearing from larvae. Larvae were collected with a D-frame net which had an inner bag with a mesh size of 0.8 mm and an outer bag with a mesh size of 0.2 mm; mayfly larvae were collected by disturbing the substrate upstream of the net, and damselfly larvae were obtained by sweeping through aquatic vegetation. The contents of the fine net were returned to the laboratory in water-filled plastic bags, and small larvae were removed under a magnifying lamp in the laboratory. Larvae collected by the coarse net were sorted in the field and transferred alive to the laboratory. Adult and subimago mayflies were kept alive by transporting on ice, and damselfly adults were brought over the short distance from the pond to the laboratory in envelopes. All insects required for electrophoresis were dry-frozen in 1.5 ml polypropylene centrifuge microtubes (Fisher Scientific Ltd.) and stored individually at −86°C in a Revco Ultima 1090 Chest Freezer. We kept known adults of both sexes, samples of morphologically unidentifiable larvae, and a range of morphologically identifiable larvae to monitor changes in enzyme composition during development.

Of the various electrophoretic techniques available, we favor cellulose acetate gel electrophoresis, because it has a number of useful characteristics, not the least of which is its sensitivity, which allows analysis of very small quantities of extract (0.5–2 μL), allowing the

characterization of individual larvae, even of 1st-instar larvae. We followed the methods described by Hebert and Beaton (1989), but took some staining protocols from Packer and Owen (1989) and Richardson et al. (1986). Just before electrophoresis, samples were partially thawed and larvae with head widths >0.8 mm were individually macerated in distilled water with a glass rod. To ensure an appropriate concentration of the homogenate, the amount of water added was equal to larval body volume. The samples were then centrifuged at 10,000 rpm for 90 s and the supernatant was transferred into sample wells by an Eppendorf digital pipette (0.5–10 μL). Small larvae, with head widths <0.8 mm, were placed individually directly into sample wells and were homogenized with a metal spatula. Electrophoresis was carried out at room temperature (21°C) at 250 volts and 8 mA/plate for 60 minutes. The single buffer system used was CAM buffer (8.4 g citric acid, 10 mL N-(3-aminopropyl)-morpholine in 1 L of double-distilled H_2O, pH 6.1).

Misidentification can occur if enzyme mobilities change during development, but the statistical aspects of electrophoretic determination make complete assurance that changes do not occur between unidentifiable and identifiable larvae and adults a very difficult goal to attain. However, if a large number of specimens are tested and no changes in enzyme mobility between adults and morphologically identifiable larvae are found, and if small larvae with electrophoretic profiles matching each of the known adult profiles are found, we may assume that there are no size-associated changes in the mobilities of diagnostic loci. Clearly, the more fixed differences between species that are used, the lower is the probability of a mismatch occurring. Therefore, we have strived to obtain differences between species at three or more loci.

Life-history elucidation

Having characterized the species, the technique can then be applied to field populations. To distinguish cohorts for life-history elucidation or life-table analysis, it is necessary to count the number of larvae in each age class in samples taken at different times of year. Our procedure is to take each field sample in which small unidentifiable larvae occur, and to determine electrophoretically the proportional representation of each size class of each species in subsamples of larvae. The number of larvae in each subsample will depend on the number of species present and their relative abundance. If some species are rare, then a larger subsample size will be necessary to properly assess them. Clearly, a compromise will usually be struck between the amount of effort and the amount of data obtained. In our work we use a minimum sample size of about 200 larvae if only two species are present, but up to 400 larvae if three or more species are involved. As a particular system becomes more familiar, so the efficiency with which these numbers can be processed increases. For example, when one knows the actual mobilities of enzymes, it is possible to double or even triple the number of individuals processed on a single gel by starting runs at two or three different places on the gel. Also, the number of loci can be reduced, even to one in some cases, when species are far apart phylogenetically and many runs have revealed complete consistency in mobility with no alternative electromorphs. Working at this efficiency, a sample of 400 larvae can be processed in a single day.

Results

Species identification

No changes in enzyme mobility between adults and morphologically identifiable larvae were found in any species. Small larvae with electrophoretic profiles matching each of the known adult profiles were found for all species.

Of the 28 enzymes tested for *Ameletus*, 21, representing 27 presumptive loci, were useful to us (Table 1). The seven species of *Ameletus* are sufficiently far apart phylogenetically that they were separable at three or more loci by using only six of these enzymes (seven loci)—Aconitase-1, Aconitase-2, Esterase-5, Glucose phosphate isomerase, Phosphoglucomutase, Adenylate kinase, and Lactate dehydrogenase. The relative mobilities of these seven electromorphs and the characterization of each species are given in Table 2. Thus, *Ameletus "bellulus"* and *A. cooki* are separated by Pgm, Acon1 and Acon2, while *A. celer* differs from *A. similior* at Est5, Acon2, Ldh and Gpi.

In addition to the D electromorph at the Gpi locus, many specimens of *A. celer* showed a slower electromorph (designated C in Table 2)

TABLE 1. The enzymes tested for species separation of *Ameletus* larvae.

Enzyme	Abbreviation	Enzyme commission number	Detected loci	Scored loci	Stain recipe[a]
Aconitase hydratase	ACON	4.2.1.3	2	2	1
Adenylate kinase	AK	2.7.4.3	2	1	1
Alcohol dehydrogenase	ADH	1.1.1.1	1	0	1
Arginine kinase	ARK	2.7.3.3	2	1	1
Diaphorase (NADH)	DIA	1.8.1	1	1	2
Diaphorase (NADPH)	DIAPH	1.6.99	1	0	2
Esterase	EST	- - - -	5	2	1
Fumarate hydratase	FUM	4.2.1.2	1	0	1
Glucose-6-phosphate isomerase	GPI	5.3.1.9	1	1	1
Glutamate oxaloacetate transferase	GOT	2.6.1.1	2	1	3
Glyceraldehyde-3-phosphate dehydrogenase	G3PDH	1.2.1.12	1	1	1
Glycerol-3-phosphate dehydrogenase	αGPDH	1.1.1.8	1	1	1
Hexokinase	HK	2.7.1.1	2	1	1
β-Hydroxybutyrate dehydrogenase	HBDH	1.1.1.30	1	0	3
Isocitrate dehydrogenase	IDH	1.1.1.42	2	1	1
Lactate dehydrogenase	LDH	1.1.1.27	1	1	1
Leucine aminopeptidase	LAP	3.4.11.1	2	2	1
Malate dehydrogenase	MDH	1.1.1.37	2	2	1
Malic enzyme	ME	1.1.1.40	1	0	1
Peptidase leucyl-alanine	PEP-la	3.4.11.13	2	2	1, 3
Peptidase phenylalanyl-proline	PEP-pp	3.4.13.8	1	1	1, 3
Phosphoglucomutase	PGM	5.4.2.2	1	1	1
6-Phosphogluconate dehydrogenase (NADP)	6PGDH	1.1.1.44	1	1	1
Phosphoglycerate kinase	PGK	2.7.2.3	1	0	2, 3
Pyruvate kinase	PK	2.7.1.40	2	1	3
Superoxide dismutase	SOD	1.15.1.1	2	1	1
Trehalase	TRE	3.2.1.28	2	2	1
Xanthine dehydrogenase	XDH	1.2.1.37	1	0	1

[a] 1—Hebert and Beaton 1989; 2—Packer and Owen 1989; 3—Richardson et al. 1986.

due to the presence of a nematode parasitoid (Fig. 1).

For the damselflies, we scored 28 loci in *Enallagma*, 29 in *Coenagrion*, and 23 in *Lestes* (Table 3). *Coenagrion resolutum* is genetically well separated from *Enallagma* (Zloty 1992) and so can be differentiated at most of the 28 loci. But it was necessary to run six loci to get three fixed differences between *E. cyathigerum* and the other two species of *Enallagma*, and the 28 scored loci in total revealed only one fixed difference between *E. ebrium* and *E. hageni* (Table 4). The four species of *Lestes* were separable on the three-or-more criterion by using the five loci shown in Table 5.

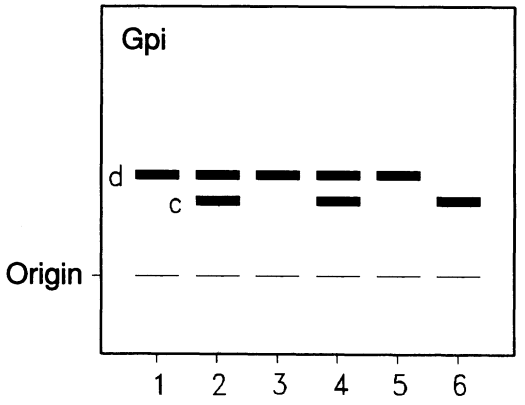

FIG. 1. Glucose-6-phosphate isomerase (Gpi) occurs as two electromorphs in some larvae of *Ameletus celer*. Electromorph d is produced by the mayfly and electromorph c is produced by a nematode parasitoid. *Ameletus celer* larvae in positions 2 and 4 are parasitized. The nematode alone is at position 6.

Life-history elucidation

During 1989 we took samples at monthly intervals from Ford Creek, and four species of *Ameletus* were common enough to determine

TABLE 2. Electrophoretic characterization of seven species of *Ameletus* at seven loci. R_f values (relative mobilities in mm with *Ameletus "majusculus"* as the standard (100)) are shown in parentheses.

	Acon1	Acon2	Ak	Est5	Ldh	Gpi	Pgm
Ameletus "bellulus"	B (98)	B (102)	A (96)	D (104)	A (97)	A (99)	F (105)
Ameletus celer	A (96)	A (100)	C (100)	D	C (101)	C/D (101)	D (103)
Ameletus cooki	A	A	A	D	A	A	E (104)
Ameletus "majusculus"	C (100)	A	C	A (100)	B (100)	B (100)	A (100)
Ameletus "pritchardi"	A	B	B (98)	C (103)	A	D (103)	B (101)
Ameletus similior	A	B	C	C	D (102)	E (105)	D
Ameletus validus	A	A	C	B (102)	B	B	C (102)

TABLE 3. The enzymes tested for species separation of damselfly larvae.

			Enallagma		*Coenagrion*		*Lestes*		
Enzyme	Abbreviation	Enzyme number	Detected loci	Scored loci	Detected loci	Scored loci	Detected loci	Scored loci	Stain recipe[a]
Aconitase hydratase	ACON	4.2.1.3	1	0	1	0	—	—	1
Adenylate kinase	AK	2.7.4.3	1	1	1	1	—	—	1
Alcohol dehydrogenase	ADH	1.1.1.1	1	1	1	1	1	1	1
Aldolase	ALD	4.1.2.13	1	1	1	1	1	1	3
Arginine kinase	ARK	2.7.3.3	2	1	2	1	2	1	1
Diaphorase (NADH)	DIA	1.8.1	1	1	1	1	1	1	2
Diaphorase (NADPH)	DIAPH	1.6.99	1	0	1	0	3	2	2
Esterase	EST	- - - -	2	2	2	2	2	1	1
Fumarate hydratase	FUM	4.2.1.2	1	1	1	1	—	—	1
Glucose-6-phosphate isomerase	GPI	5.3.1.9	1	1	1	1	1	1	1
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	1	1	1	1	1	1	1
Glutamate oxaloacetate transferase	GOT	2.6.1.1	2	2	2	2	2	2	3
Glyceraldehyde-3-phosphate dehydrogenase	G3PDH	1.2.1.12	1	1	1	1	1	1	1
Glycerol-3-phosphate dehydrogenase	αGPDH	1.1.1.8	1	1	1	1	1	1	1
Hexokinase	HK	2.7.1.1	1	0	1	0	—	—	1
β-Hydroxybutyrate dehydrogenase	HBDH	1.1.1.30	1	0	1	0	—	—	3
Isocitrate dehydrogenase	IDH	1.1.1.42	2/3	2/3	3	3	2	1	1
Lactate dehydrogenase	LDH	1.1.1.27	1	1	1	1	1	1	1
Leucine aminopeptidase	LAP	3.4.11.1	1	0	1	0	—	—	1
Malate dehydrogenase	MDH	1.1.1.37	2	2	2	2	2	1	1
Malic enzyme	ME	1.1.1.40	1	1	1	1	—	—	1
Mannose phosphate isomerase	MPI	5.3.1.8	1	1	1	1	—	—	1
Peptidase leucyl-alanine	PEP-la	3.4.11.13	1	0	1	0	2	2	1, 3
Peptidase phenylalanyl-proline	PEP-pp	3.4.13.8	1	1	1	1	1	1	1, 3
Phosphoglucomutase	PGM	2.7.5.1	1	0	1	0	1	1	1
6-Phosphogluconate dehydrogenase	6PGDH	1.1.1.44	1	1	1	1	1	1	1
Phosphoglycerate kinase	PGK	2.7.2.3	1	0	1	0	—	—	2, 3
Pyruvate kinase	PK	2.7.1.40	2	1	2	1	2	1	3
Sorbitol dehydrogenase	SDH	1.1.1.14	1	1	1	1	—	—	3
Superoxide dismutase	SOD	1.15.1.1	1	1	1	1	—	—	1
Trehalase	TRE	3.2.1.28	1	0	1	0	1	1	1
Triose phosphate isomerase	TPI	5.3.1.1	1	1	1	1	—	—	1
Xanthine dehydrogenase	XDH	1.2.1.37	1	1	1	1	—	—	1

[a] 1—Hebert and Beaton 1989; 2—Packer and Owen 1989; 3—Richardson et al. 1986.

TABLE 4. Electrophoretic characterization of four species of Coenagrionidae at six loci. R_f values (relative mobilities in mm with *Coenagrion resolutum* as the standard (100)) are shown in parentheses.

	Got1	Got2	Idh1	Idh2	Ldh	Gpi
Coenagrion resolutum	A (100)	C (100)	A (100)	A (100)	A (100)	C (100)
Enallagma cyathigerum	B (105)	A (96)	B (104)	B (101)	C (105)	B (99)
E. ebrium	B	B (98)	C (105)	B	B (103)	A (98)
E. hageni	B	B	C	B	B	B

life histories. However, morphologically unidentifiable larvae with head capsule widths <0.8 mm were present in every sample; there were 278 such larvae out of a total of 406 in the May sample, 83 of 204 in June, 65 of 181 in July, 289 of 376 in August, 522 of 587 in September, and 347 of 421 in October. After electrophoretic determination of these small larvae, the growth patterns of each of the four species could be determined as shown in Fig. 2. Thus, it appears that all four species have univoltine life cycles. *Ameletus "bellulus"*, *A. celer*, and *A. "majusculus"* are winter species, sensu Clifford (1982), with eggs hatching without delay, whereas *A. similior* is a summer species, the eggs spending the winter in diapause.

Discussion

Proper species identification, especially of the larvae of insects, is a serious barrier to progress in ecology. This barrier has three components: 1) it is impossible to know how much work has *not* been done because of taxonomic uncertainty, 2) studies that stop short of species identification are of limited use unless voucher specimens are deposited and later can be correctly identified, and 3) studies in which species are misidentified fall into the same category or worse if the misidentifications are accepted.

A selection of ecological publications on North American *Ameletus* illustrates the problem. Some authors (e.g., Grant and Mackay 1969, Doherty and Hummon 1980, Hill and Knight 1988) were unable to put species names on most of their *Ameletus*, whereas of those that used species names (Hill and Knight 1987, Benton 1989, Delucchi and Peckarsky 1989, Matthews and Tarter 1989, Benton and Pritchard 1990, Ward and Stanford 1990, Giberson and Mackay 1991), only Hill and Knight (1987) state how larvae were identified, in this case by rearing. Furthermore, all of the latter group had only a single species at a site and, given the state of taxonomic knowledge of this genus, and the fact that few, if any, sites have only one species of *Ameletus*, the validity of many identifications might be questioned. Certainly, the identifications of Benton (1989) and Benton and Pritchard (1990) are confused, as our work at the same sites has shown. Thus it is essential that authors take more care over their identifications and state how identifications were made.

In this paper we have described the application of a relatively simple technique that can be used to give proper identifications of all developmental stages in taxonomically difficult groups. Without it we would have been unable to do any meaningful ecological work on the *Ameletus* mayflies or the coenagrionid and lestid damselflies in our study sites, nor on the Costa Rican *Hetaerina* (Zloty and Pritchard 1993). We urge all researchers working on small larvae of insects in sites where several congeneric species

TABLE 5. Electrophoretic characterization of four species of *Lestes* at five loci. R_f values (relative mobilities in mm with *Lestes congener* as the standard (100)) are shown in parentheses.

	Got1	Got2	Idh1	Idh2	Gpi
L. congener	A (100)	B (100)	D (100)	C (100)	A (100)
L. disjunctus	B (103)	B	B (97)	A (93)	C (104)
L. dryas	B	A (98)	A (92)	B (94)	B (101)
L. unguiculatus	B	B	C (98)	B	A

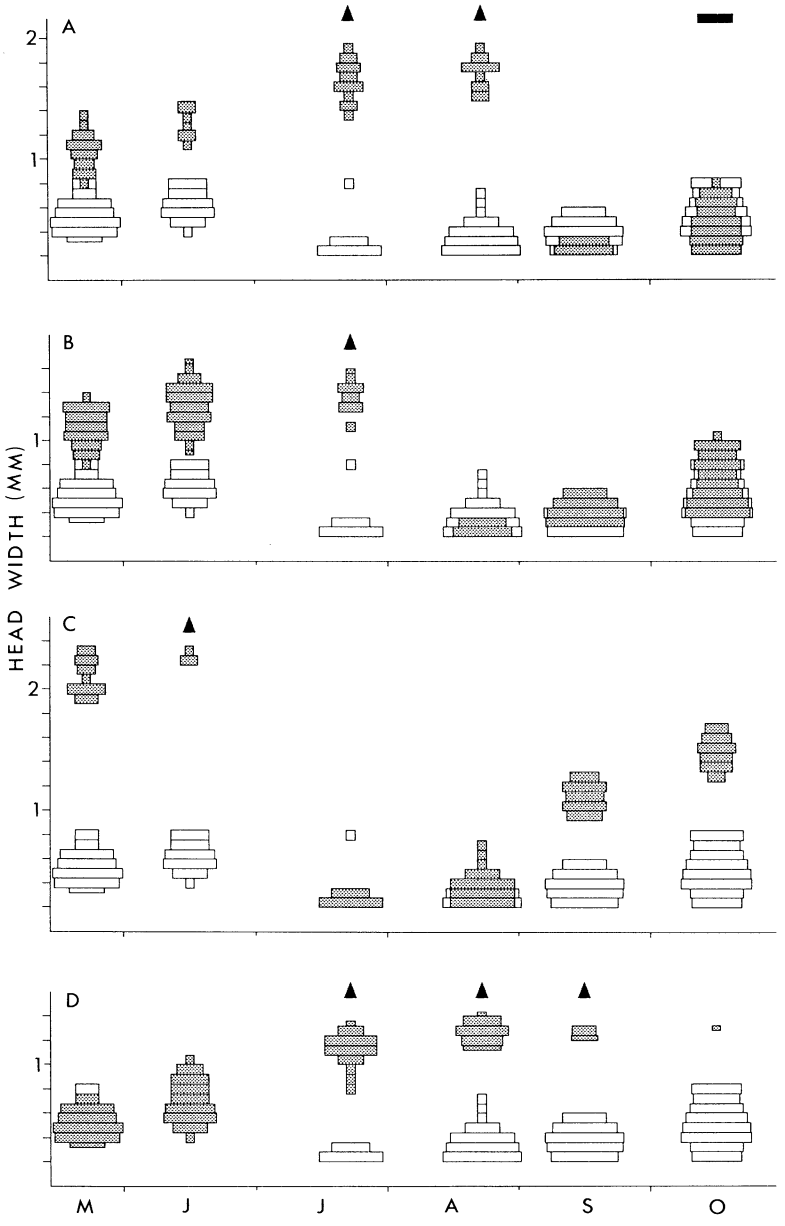

FIG. 2. The size distribution of *Ameletus* larvae in Ford Creek between May and October, 1989. Open boxes represent morphologically unidentifiable larvae; shaded boxes represent larvae of A) *A. "bellulus"*, B) *A. celer*, C) *A. "majusculus"*, and D) *A. similior*, those with head widths <0.8 mm having been identified by electrophoresis. Head widths were measured to the nearest 0.04 mm and grouped into classes of 0.08 mm. Arrows represent emergence of adults. The solid bar is equivalent to $\log_{10}10$ individuals.

occur to confirm their identifications with this or a similar technique.

A further application of the technique became apparent when analyzing the data from *Ameletus celer*. When large, the nematode is usually visible through the larval cuticle of late-instar mayflies, when it occupies much of the body cavity, but it is invisible when smaller. Electrophoresis thus allows us to determine the incidence of parasitism throughout the life cy-

cle. We shall elaborate on this application of the technique in a future publication which will compare the life histories of species of *Ameletus* in Ford and Elbow Creeks.

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