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Miscellanea

Non-Specific Esterases during Development of Culicine Mosquitoes

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Introduction

Former work had shown that non-specific esterase zymograms of female Culicidae were species- and subspecies-specific (FREYVOGEL et al., 1968). Since then, genetics of esterases in mosquitoes were studied by various authors (TREBATOSKI & CRAIG, 1969; SIMON, 1969; TOWNSON, 1971). Developmental variations of esterases isozymes were found in *Culex pipiens fatigans* (SIMON, 1969). Our present aim is to make a comparative study of zymograms during development of several Culicine species. Main emphasis will be put on the changes occurring and the specificity of zymograms in all stages.

Material and Methods

The following laboratory stock cultures were used throughout this work: *Aedes aegypti* (originally from former Belgian Congo), *Culex pipiens fatigans* (Bakersfield) and *Culex pipiens pipiens* (Paris, autogenous race). They are being maintained in our institute according to usual methods (26°C, 85 ± 5 % RH). All material used originated from mass breedings. Eggs were collected within 40 hours after oviposition. For *Aedes aegypti,* in addition, eggs in diapause, approximative 40–50 days after oviposition were used. The age of larvae and pupae was not determined further beyond the exact instar. Adults were taken within one day after emergence to make a comparison. For the number of experiments see figures 2–4.

From 10 to 65 mg fresh weight of live animals were brought into an all-glass grinder and were homogenized in 100 µl of ice-cooled distilled water with traces of phenylthiourea added, preventing melanization. There followed centrifugation (200 × g, 10 min).

For desalting the extract, the supernatant was pipetted into a tube containing 20 mg of dry Sephadex G-25, Medium (Pharmacia). This was allowed to swell for 10 min.; swelling was interrupted by centrifugation (3000 × g, 10 min) and the supernatant was ready for electrophoresis. The residue was applied directly onto another gel tube to indicate possible loss of enzyme activity during the Sephadex-treatment. This method allowed to remove most of the low-molecular substances which often caused unsatisfactory separation (tailing) of the enzyme bands.

In all experiments the esterase enzymes were separated by conventional disc-electrophoresis at 4–10 °C in a 10 % acrylamide gel (Eastman Kodak or Merck). The tube size was 70 × 3.5 mm. The electrode buffer was 0.005 M tris-glycine (pH 8.7) and bromphenol blue was the tracking dye. Electrophoresis time was about 1 hour (200 V dc, 0.7–1 mA per tube).

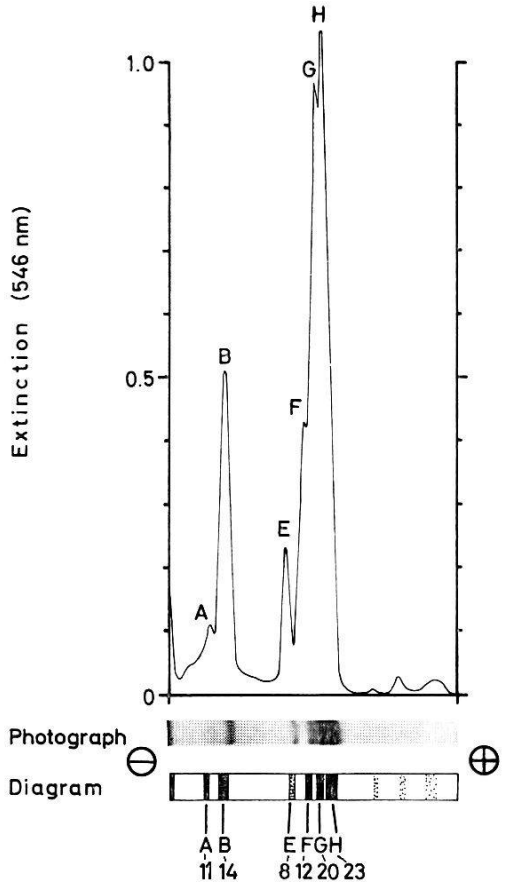

Fig. 1.

For demonstration of esterase activity the method given by MARKERT & HUNTER (cit. in MAURER 1968) was used. The quantitative estimation of the esterase bands was carried out by densitometry. The gels were analyzed on a photometer (Eppendorf) at 546 nm and the extinctions were recorded and integrated (W + W Recorder 4201). Finally, the approximate activity of each single band was calculated as percentage of total extinction per gel. In all cases, the corresponding values from the "supernatant" and the "Sephadex-gels" were added. For calculation of the relative mobility (rm) of esterase bands, the migration distance of the tracking dye was taken arbitrarily as 100 %. In all figures mean values are given.

The staining intensity of the bands in figures 1–4 is represented by different hatching and gives the optical impression of the actual gels. Very faint bands are weakly dotted. The rm values and the number of separated bands were obtained from densitometric graphs (fig. 1). Esterase bands are named by capital letters. As we did not homologize esterase bands among the species, the same letters do not necessarily mean the same enzymes: therefore, small letters, indicating the species, are added to the capital letters. Esterase bands were homologized within the species by mixing homogenates of different developmental stages in several possible combinations.

The protein content of the samples was estimated by the Biuret method. 20–40 mg of weakly anesthezised animals were homogenized in a buffered saline solution (0.65 % NaCl, 0.02 M tris-HCl, pH 8.6). Extinctions at 550 nm (photometer Eppendorf) were converted to mg protein taking a 1 % solution of bovine albumin (100 %, Pentex, Fluka) as a standard.

Results

A. Aedes aegypti (L.)

Fig. 2 is a diagram which schematically shows all non-specific esterase bands we were able to demonstrate in developmental stages of this species. Between 5 and 10 bands were found, the lowest number being obtained in eggs and pupae, the highest in larvae. Few bands remained very faint and inconspicuous and were seen in some cases only. They are marked by weak dots in the figure.

The relative extinction of each band, expressed as percentage of total extinction (cf. p. 2), is indicated by numbers at the right of each gel. From these it becomes evident that there are two main zones of activity, the one with a relative mobility (rm) of 0.2–0.3, the other with rm 0.6–0.7. In most stages bands Aa, Ba, Fa, Ga and Ha show rather high activities (10–50 %), whereas bands Ca, Da and Ea are often weak and less frequent. This applies to all stages with the exception of developing eggs. In all experiments some esterase activity is detected at the origin; it is comparatively low, again, with the exception of the developing eggs, where it amounts to 57 % of the total activity.

The zymogram patterns of the various stages examined do not differ much, with the exception of the developing eggs. Band Ba is shown to be composed of two substances in larvae I and II. Band Ga, too, is splitted in the diapausing eggs and larvae I. In adult mosquitoes bands Ga and Ha possibly overlap; with the methods applied they could not be separated in this stage. Band Ca is missing in developing eggs as well as in adult stages; also, it could not be demonstrated in larvae III. Band Da, too, failer to shop up in larvae III and pupae. As mentioned, the most striking difference is found between the two developmental stages of eggs. In eggs E, less than 36 hours old, embryogenesis is under way; eggs E_D are diapausing and contain larvae ready to hatch. In the former ones, band Ha is missing and band Ga shows a very low activity, whereas in the latter ones, the activities of these two bands increase up till 10 % and 50 % respectively. This is one of the most impressive events. The zymogram patterns of eggs E_D and larvae I, in fact,

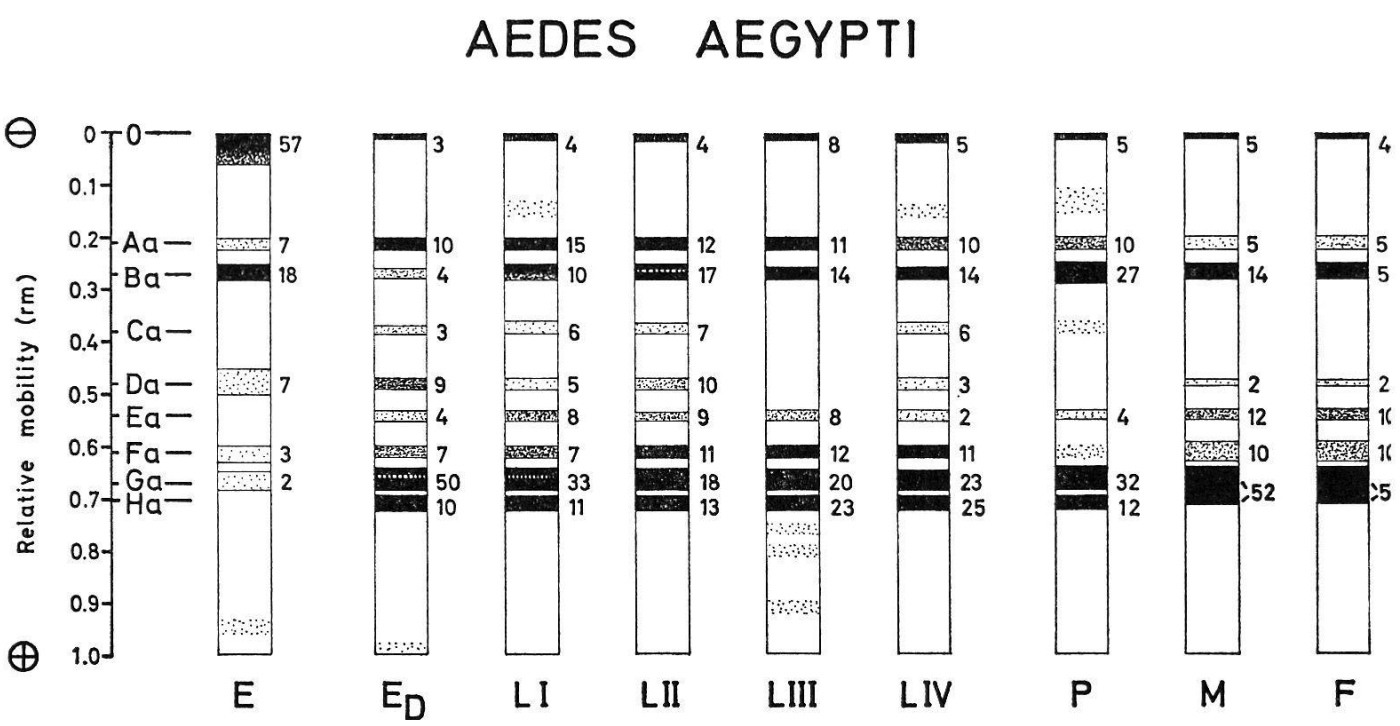

Fig. 2.

are almost identical, while the zymogram of eggs E is quite dfferent. The zymograms show their highest complexity during larval development, with only minor differences among its four stages. Changes do take place with pupation; the number of bands is substantially reduced. Pupae and teneral adults present close similarities. No sexual dimorphism can be demonstrated one day after emergence.

B. Culex pipiens fatigans Wiedem.

The results are shown in fig. 3 in the same way as for *Aedes aegypti*. From 6 to 12 esterase bands could be detected in this species. In eggs and pupae the bands are least numerous, while the maximum is found in larvae. The most conspicuous bands show a rm 0.3–0.5. The more prominent bands are Df, Ef, Ff, Gf and Hf, up to 34 %; all other ones are rather faint and of variable appearance. Esterase activity at the origin is comparatively low, including the egg stage. Bands Bf, If and Mf are found during larval development only. Band Af could not be detected in larvae IV, band Lf was not seen in pupae.

Band Gf is most conspicuous in eggs (31 % of total activity), well represented in pupae (20 %) and adults (14 %), but less prominent in the larval stages (6–7 %). The reverse seems to apply to band Hf. Band Nf could be shown from larva II to the pupal stage.

From fig. 3 it is evident that, basically, there exist three zymogram patterns, one for eggs, a second for the four larval stages and the third for pupae and adults. The larval pattern is the most complex one. Again, no sexuel dimorphism was found in teneral adults.

C. Culex pipiens pipiens L.

In fig. 4 the results as worked out for this species are shown. The number of bands and their occurrence during development is similar to that in *Culex pipiens fatigans*. Their rm, in both species, is comparable in the pupal and adult stages, while the differences during larval development are more pronounced.

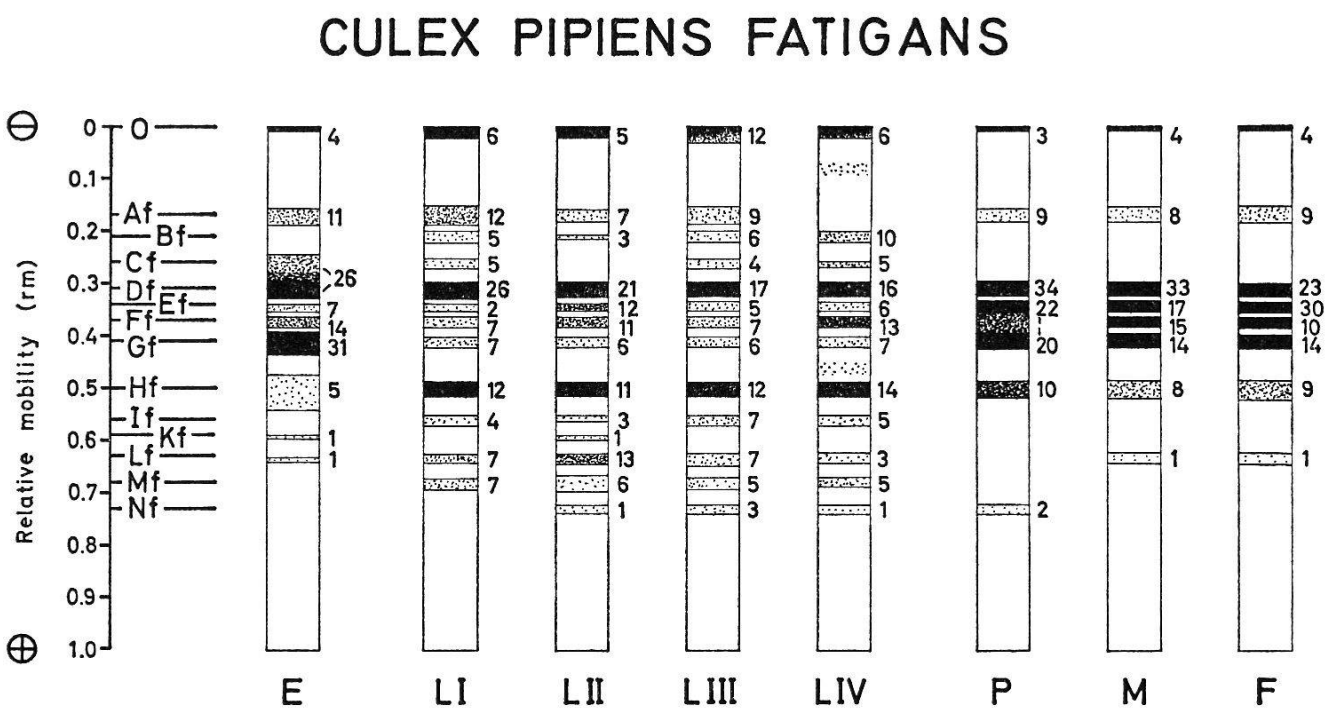

Fig. 3.

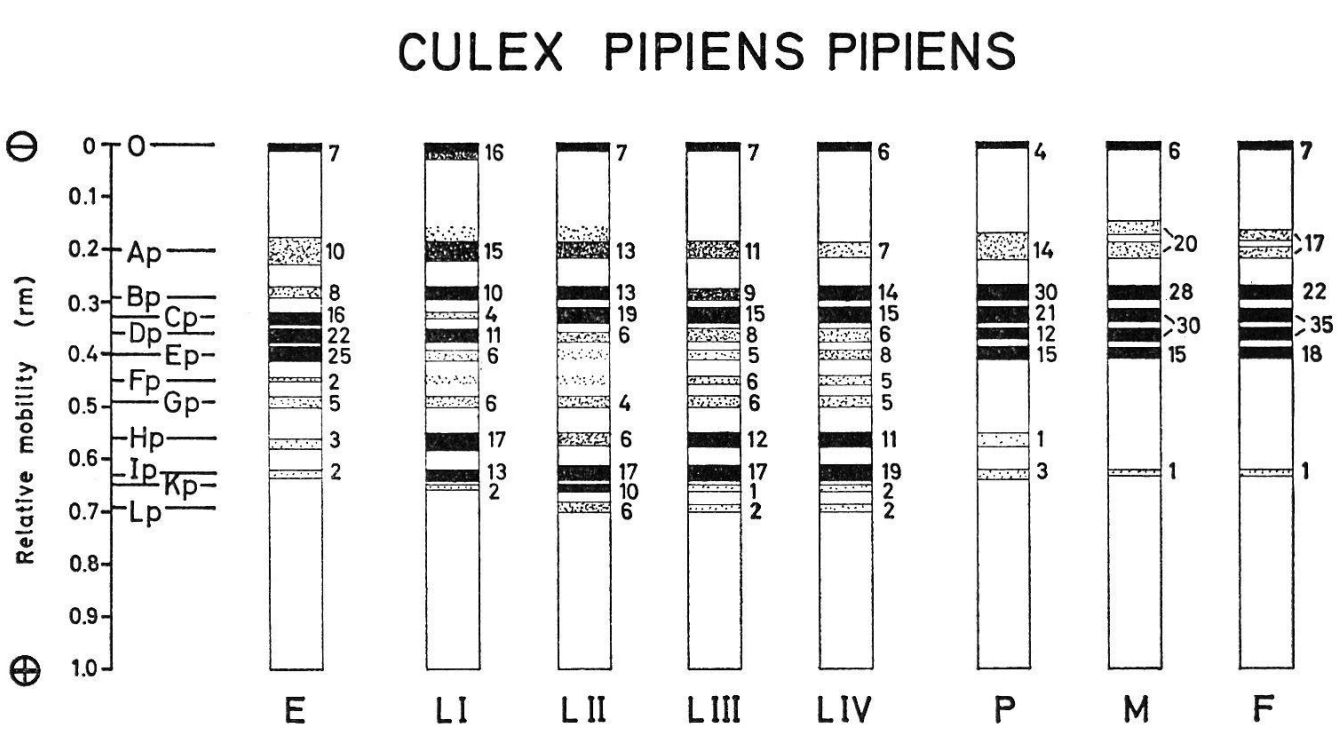

Fig. 4.

In eggs, pupae and adults the bands of highest intensity (10–30 % of total activity) show a rm 0.3–0.4; in the larvae, in addition to this group, a second group of prominent bands (11–19 %) has a rm 0.55–0.65.

Esterase activity at the origin is low. Band Ap clearly appears as double-band in the adult stages; in the eggs, larvae I and II and in the pupae double-bands may be assumed; in larvae III and IV band Ap, however, appears singly. Bands Fp and Gp are missing in the pupae and adults, band Hp in adults only. Band Kp is found in larvae only, band Lp from larva II to IV. Band Ep (15–25 % of total activity) is quite conspicuous in eggs, pupae and adults, while it is much weaker in all larvae (5–8 %). Band Ip, on the other hand, shows the reverse pattern, 1–3 %, versus 13–19 %. Also in this species, three zymogram patterns show up during development. With respect of the larvae, the closest resemblance is observed between stages III and IV. Teneral adults exhibit no sexual dimorphism.

Discussion

In the present paper we give not more than an inventory of non-specific esterases in all developmental stages of three laboratory-bred species or subspecies of Culicine mosquitoes, as far as these can be separated with the technique employed using only one gel system and substrate. In order to obtain a more accurate picture of the actual number of esterases or their isozymes, additional techniques, such as two-dimensional electrophoresis and inhibition experiments, should be applied. Comparisons of the various zymogram patterns obtained within the limits of the present work, however, are permissible. Also, by use of the method of mixing homogenates of different stages of one species, in contrast to our preliminary note (FREYVOGEL & BRIEGEL, 1971), we are able to show that quite a number of bands which seemed to undergo frequent changes, are homologous throughout considerable parts of development.

The distribution of the demonstrated esterase activity on the gels and, especially, their staining patterns are clearly different for each species and subspecies examined. In *Aedes aegypti* two zones of major activity are observed, with rm 0.2–0.3 and 0.6–0.7. In *Culex pipiens fatigans* there is only one such zone, with rm 0.3–0.5, while in *Culex pipiens pipiens* two zones are found, with rm 0.3–0.4 and 0.55–0.65 for larvae and the slower one only for pupae and adults. Thus we are able to confirm the usefulness of non-specific esterase zymograms carried out under identical experimental conditions as an accessory tool for taxonomy, down to the level of subspecies (FREYVOGEL et al., 1968; FREYVOGEL & MCCLELLAND, 1969), this applying equally to adult mosquitoes as to developmental stages. As far as imagines are concerned, their age must be taken into account; it has been demonstrated for *Culex pipiens fatigans* that slight changes do occur in the esterase zymograms with age (SIMON, 1969). If one compares the imaginal zymograms in the present work with those described in a previous paper (FREYVOGEL et al., 1968), two zones of man activity, with similar rm values were found in *Aedes aegypti* (more clearly with JD-strain); one zone of main activity, with an intermediate rm value, was observed for adult *Culex pipiens*. The differences encountered can be explained by the differences in age of the imagines used and by differences of strains (FREYVOGEL & MCCLELLAND, 1969).

Developmental changes in the protein constitution of the mosquito *Armigeres subalbatus* were shown to occur by DESOWITZ, 1969. In *Culex pipiens fatigans* developmental variation were also demonstrated for esterase isozymes by SIMON (1969). In principle, our results agree with those of the last author except for two observations: we find a close similarity of zymograms of pupae and adults and no sexual dimorphism. Otherwise there are characteristic zymogram patterns for each of the major stages of development, this being true for both genera examined so far.

With respect to the egg zymograms of the two *Culex pipiens* subspecies one has to be aware of autogeny in *C. pipiens pipiens,* as opposed to *C. pipiens fatigans*. In the eggs of *C. pipiens pipiens* being matured without any blood meal, yolk synthesis and also protein composition is different from that in *C. pipiens fatigans* (BRIEGEL, 1969). This could, in part, explain the difference in the esterase patterns. With reference to *Aedes aegypti,* the strong difference between developing and diapausing eggs is remarkable and even more striking than that between eggs an larvae I in *Culex pipiens*. We think these conspicuous changes in the zymogram patterns to be an indication for important alterations in metabolism during embryogenesis as opposed to larval life.

No major changes seem to occur in the zymograms of the four larval stages. There appear, however, a number of temporary bands, often characteristic for three or four larval instars only. In *Aedes aegypti* this applies to band Ca, although, unexpectedly it could not be shown in larvae III. It also applies to bands Bf, If and Mf in *Culex pipiens fatigans*. In addition, in this species, band Nf is temporary, too, but it persists to the pupa stage. In *Culex pipiens pipiens* bands Kp and Lp, similarly, are of temporary nature, besides bands Fp, Gp and Hp which are already found in eggs and of which the last persists into the pupal stage. In addition to these temporary bands, in each *Culex pipiens* subspecies a pair of bands may be said to exist, which is present in all stages examined, but which switches intensity of activity during larval life. This seems to be correct for the pair Gf/Hf in *Culex pipiens fatigans* and, possibly, for the pair Ep/Ip in *Culex pipiens pipiens*. No such relationship can be observed in *Aedes aegypti* zymograms. A second important change in the zymograms occur during transition from larvae IV to pupae. Almost half the bands disappear. It is surprising that the number of esterases is substantially lower in pupae, where in the context of metamorphosis metabolic activity must be particularly high. It is noteworthy

that in developing eggs, during embryogenesis, the number of esterase bands, too, is comparatively low, at least in *Aedes aegypti* and *Culex pipiens fatigans*. As to *Culex pipiens pipiens* the number of bands is the same as in larvae I, but their activities resemble rather the ones found in eggs of *Culex pipiens fatigans* than the ones in *Culex pipiens pipiens* larvae I. Thus, in the three species and subspecies investigated, differentiation processes during embryogenesis cause an increase of the number of esterase bands; also their intensities change considerably. Complex esterase pattern is seen in the larvae, where growth is the dominant process. Also distinct changes from larvae IV to pupae are seen, especially in *Culex pipiens* species. Recent experiments (unpublished) indicate that in ageing females of *Aedes aegypti* the number of bands with esterase activity increases again.

These observations may add new questions with reference to the function of nonspecific esterases, which are, as yet, poorly known.

Acknowledgements

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ERRATA

to

HANS BRIEGEL & THIERRY A. FREYVOGEL

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number 3, 1971

p. 291 Line 13 from below: read *2000* × g instead 200.
p. 293 Line 7: read *p. 292* instead p. 2.
p. 296 Line 8 from below: read *persist* instead of exist.

p. 292 Insert explanation of fig. 1 as follows:

Non-specific esterase zymogram; α-naphthyl-acetate; *Aedes aegypti* larvae III: Photograph of actual gel, corresponding densitometric scan and resulting diagram as shown in fig. 2. For further explanation see text p. 292.

p. 293 Insert to fig. 2 the following values and the explanation:

	E	E_D	L I	L II	L III	L IV	P	M	F
Approximative age (d)	1	40	1–3 *	5–8 *	9–11 *	12–17 *	18–19 *	1 **	1 **
Number of experiments	10	7	4	3	6	6	6	1	1
Number of bands	5	9	9	9	6	8	5	6	6
Protein (mg/gel)		4.50	2.05	2.02	1.23	1.47	1.54	0.80	0.89

* After hatching. ** After emergence.

Fig. 2. Aedes aegypti: diagram of non-specific esterase zymograms in all stages of development. (Numbers to the right of the columns indicate relative extinction; see text p. 292). E = eggs in embryogenesis; E_D = eggs in diapause; L I–IV = larvae, 1st to 4th instar; P = pupae; M = male imagines; F = female imagines.

p. 294 Insert to fig. 3 the following values and the explanation:

	E	L I	L II	L III	L IV	P	M	F
Approximative age (d)	1	1–3 *	5–7 *	8–11 *	12–15 *	16–18 *	1 **	1 **
Number of experiments	9	5	5	7	4	8	1	1
Number of bands	8	11	12	12	11	6	7	7
Protein (mg/gel)	3.07	0.91	1.21	0.91	1.93	1.33	1.67	1.93

* After hatching. ** After emergence.

Fig. 3. Culex pipiens fatigans: diagram of non-specific esterase zymograms in all stages of development. (Legend as for fig. 2.)

p. 295 Insert to fig. 4 the following values and the explanation:

	E	L I	L II	L III	L IV	P	M	F
Approximative age (d)	1	1–3 *	5–7 *	8–11 *	12–15 *	16–18 *	1 **	1 **
Number of experiments	3	5	3	3	4	6	2	2
Number of bands	9	9	9	11	11	7	6	6
Protein (mg/gel)	2.94	0.51	0.62	1.20	2.69	3.51	1.04	2.22

* After hatching. ** After emergence.

Fig. 4. Culex pipiens pipiens: diagram of non-specific esterase zymograms in all stages of development. (Legend as for fig. 2.)

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