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METHODS FOR FIELD DETECTION OF RESISTANCE TO TEMEPHOS IN SIMULIIDS. LARVAL ESTERASE LEVEL AND TOPICAL APPLICATION OF THE INSECTICIDE TO ADULTS

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Two practical field methods for indirect detection of simuliid populations resistant to temephos are proposed. The first is based on high esterase activity in resistant larvae and involves adaptations of a filter paper test in which faintly stained spots indicate susceptible populations and strongly stained ones reveal populations resistant to temephos. The second is based on the resistance to the larvicide when adults are topically exposed, and involves the use of diagnostic doses obtained by the comparison between the LD_{50} *for susceptible and resistant populations. The relevance of such methods is discussed in order to help resistance detection in* Simulium pertinax *Kollar control programmes.*

Key words: Simuliidae – resistance detection methods – temephos – *Simulium pertinax*

The organophosphate larvicide temephos has long been considered the most acceptable insecticide for blackfly control in many countries, including Brazil. Its use as the main control agent in the largest vector control scheme in the world, the Onchocerciasis Control Programme of West Africa, has shown it was a highly efficient method for reducing human onchocerciasis. Although concerted control campaigns against this disease have not yet been conducted in South America (Shelley, 1988) temephos would be one of the larvicides of choice, should such campaigns begin.

Resistance selection to chemical insecticides in simuliid control programmes is quite probable being dependent upon many factors such as short intervals between applications during the whole year and over large areas. Therefore, some well documented cases of resistance to temephos have been reported in several countries. Different resistance levels, based on bioassay results against larvae, have been detected in onchocerciasis vectors of the *Simulium damnosum* complex from the Ivory Coast (Guillet et al., 1980) and Cameroon (Traoré-Lamizana et al., 1985) in West Africa, as well as in *S. oyapockense* s.l. (as *amazonicum*) from Guyana in South America (Rambajan, 1981).

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In Brazil, populations of the nuisance and non-vector blackfly *S. pertinax* occur frequently in sympatry and even in syntopy with some non-target species (Araújo-Coutinho et al., 1988) and various attempts have been made to control this species in the southeastern and southern states of this country. Resistance to temephos was demonstrated by means of field tests against larvae in the States of São Paulo and Rio de Janeiro (Andrade, 1989). In the southern States of Paraná, Santa Catarina and Rio Grande do Sul, the same situation has been assumed based on the failure of operational doses (Ruas Neto, 1984; Guimarães, 1986; C. M. Paz, pers. comm.).

Programmes for monitoring resistance before failures in control occur (Roush & Miller, 1986) or to detect cases where resistance has been already established (World Health Organization, 1980) require frequent attempts to develop new approaches (Brown & Brogdon, 1987). The organophosphate resistance mechanism in simuliids, as in mosquitoes, has been correlated to high esterase activity (Magnin et al., 1987). Field methods involving filter paper tests or spectrophotometry to detect different esterase phenotypes have until now only been proposed for culicids (Raymond & Mouchés, 1986; Hemingway et al., 1986). Based on the fact that larval resistance to temephos is reflected also in adults, Kurtak & Ouedraogo (1984) proposed a field bioassay using adults simuliids in order

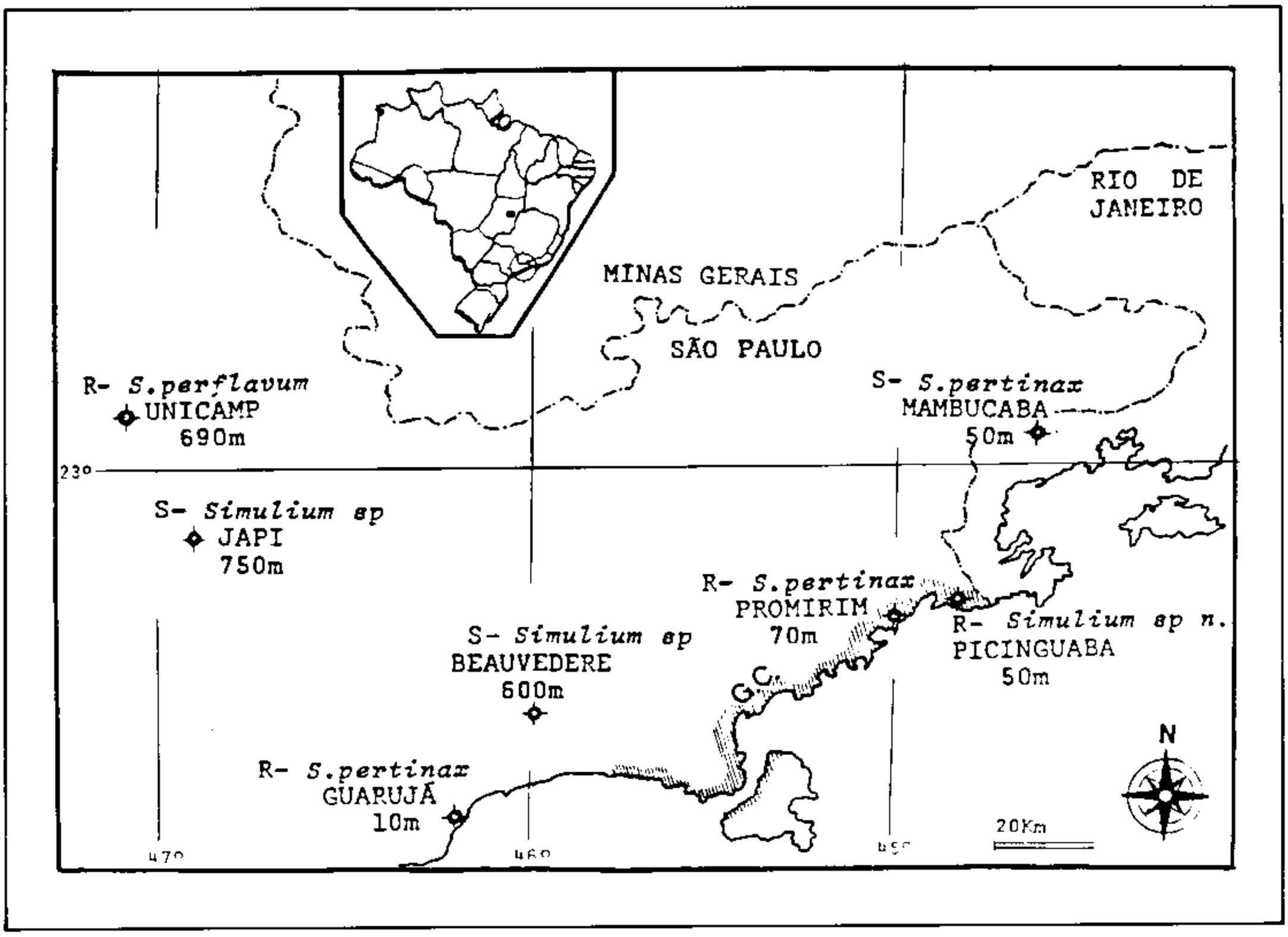

Fig. 1: collecting sites of simuliid species used in the present study. (R = population resistant of temephos; S = population susceptible to temephos; GC = area under government blackfly control).

to monitor resistance in the Onchocerciasis Control Programme in Africa. Resitance to temephos can only be suspected if no tests for resistance have been made.

Our aim in the present paper is to develop new approaches to detect temephos resistance among simuliids. The first is an adaptation from the filter paper method used for culicids and the other involves an improvement on the adult topical application method proposed by Kurtak & Ouedraogo (1984). The operational interest in *S. pertinax* control programmes is discussed.

MATERIALS AND METHODS

The study was divided into three parts: a preliminary survey to test whether differences in esterase levels occur between susceptible and resistant populations of *Simulium* larvae; field evaluation of a modified filter paper test for detecting esterase activity; and field evaluation of a topical application test for resistance to temephos. All blackfly populations had already been assessed as either susceptible or resistant to the larvicide under field conditions (Andrade, 1989).

Preliminary survey on esterase levels – In order to test whether the levels of esterase are different in populations of simuliids susceptible (S) and resistant (R) to temephos, collections of larvae were made from two localities; R-*S. perflavum* were collected from a lake outlet near Unicamp and S-*Simulium* sp from the Reserva Estadual do Japi (Fig. 1).

Estimation of esterase activity was based on the method of Magnin et al. (1987), except for protein quantification. Late instar larvae were collected in the field and then frozen in dry ice. In the laboratory, 25 larvae of similar size were homogenised in 2 ml of phosphate buffer (5 mM, pH 6.4) and precipitated by centrifuging at 10,000 rpm for 10 min. Five 0.5 μl replicate aliquots of the supernatant were incubated for 5 min with 750 μl of phosphate buffer and 100 μl of α- or β-naphthylacetate (10^{-3} M acetonic solution). After the enzymatic reac-

tion, 100 µl of Fast Garnett GBC salt solution were added, and 1 min later absorbance was read at 527 nm or 505 nm for α- or β-naphthylacetate respectively.

Filter paper test for esterase activity – Based on the preliminary survey on esterase levels, a filter paper test for esterase activity was evaluated in the field. Simuliid larvae were collected from the following four localities (Fig. 1): Guarujá-R-*S. pertinax;* Picinguaba-R-*Simulium* sp. n.; Mambucaba-S-*S. pertinax* and Beauvedere-S-*Simulium* sp.

This test was based on that used for individual mosquitoes by Pasteur & Georghiou (1981). In our study 0.2 ml of larvae was used (ca. 100 larvae) instead of individual insects due to the large variation in size of late instar larvae. Each suspension was produced by crushing the group of larvae against a fine metallic mesh in 0.5 ml of distilled water. Drops from the resultant water suspension were applied to Whatman no. 2 filter paper, and then immersed for 90 seconds in 5 ml of 1% acetonic α-naphthylacetate solution in 50 ml of phosphate buffer (pH 6.5). After this enzymatic reaction, the filter paper was blotted between tissue papers and transferred to staining Fast Garnett GBC salt solution (0.1%) for 1 min. Finally, it was fixed in 10 % acetic acid and allowed to dry. Larval extracts showing as dark spots indicated resistance to the insecticide, whereas light spots showed susceptible individuals.

Topical application of temephos – Collections of adult flies were made at three localities for this test (Fig. 1). Female R-*S. perflavum* were caught by sweep-netting a swarm in the breeding site at Unicamp. Female *S. pertinax* were collected from human bait by mouth aspirator at Promirim for a resistant and Mambucaba for a susceptible population. Twenty to 25 flies from each collecting site were kept in a cylindrical glass vial (8.0 cm x 1.5 cm diameter), which contained dry strips of filter paper previously soaked in 20% sucrose for feeding and resting. Flies were exposed to temephos shortly after collection. Each group was transferred to a cloth mesh fitted to the tip of a 12 V portable dust aspirator. Once the flies had been immobilized, 0.5 µl of a freshly prepared temephos-acetone solution was applied either dorsally or ventrally on each adult, depending upon its relative position. The application was made with a microcapillary tube, spending no more than 1 min to treat each group. After application, adults were re-collected from the cloth mesh by mouth aspirator and transferred to the vials. Mortality was recorded 3 and 5 h after application, and results expressed in terms of LD_{50} and D_{99} were analyzed by log-probit regression, using a PC-microcomputer and a BASIC programme developed by the first author.

Also, two diagnostic doses (DD) were established for routine evaluation of temephos resistance in *S. pertinax* populations, using 20 to 25 flies from each of the collecting sites at Promirim and Mambucaba.

RESULTS

Preliminary survey on esterase levels Comparison of esterase activity between R-*S. perflavum* and S-*Simulium* sp populations, showed higher absorbance levels for α- as well as β-naphthylacetate in the resistant group. For these two substrates, the absorvance relation between R- and S-larvae was calculated as 1.22 and 1.12 for α- and β-naphthylacetate respectively, indicating a higher increase of the A-type esterases.

Filter paper test for esterase activity – The filter paper tests showed that larvae of *S. pertinax* and *S.* sp n. resistant to temephos, from Guarujá and Picinguaba respectively, produced strongly stained spots. Larvae from populations of *S. pertinax* and *Simulium* sp, susceptible to temephos collected at Mambucaba and Beauvedere respectively, produced faintly stained spots in the test (Fig. 2).

Topical application of temephos – Previous work established some improvements on the methodology proposed by Kurtak & Ouedraogo (1984). The use of temephos-acetone solution was more acceptable than alcohol due to a fast evaporation rate in the aspirator system. Acetone only induced a low mortality in the control group, ranging from 2 to 6% after 14 h, while starvation of adult flies, provoked a higher mortality in the same period, varying between 30 and 40%. The mortality progression for the higher doses of temephos showed that a 3 h period was sufficient to detect the whole insecticidal effect, and that after this period the mortality in the control group was insignificant, avoiding needs for any corrective formula.

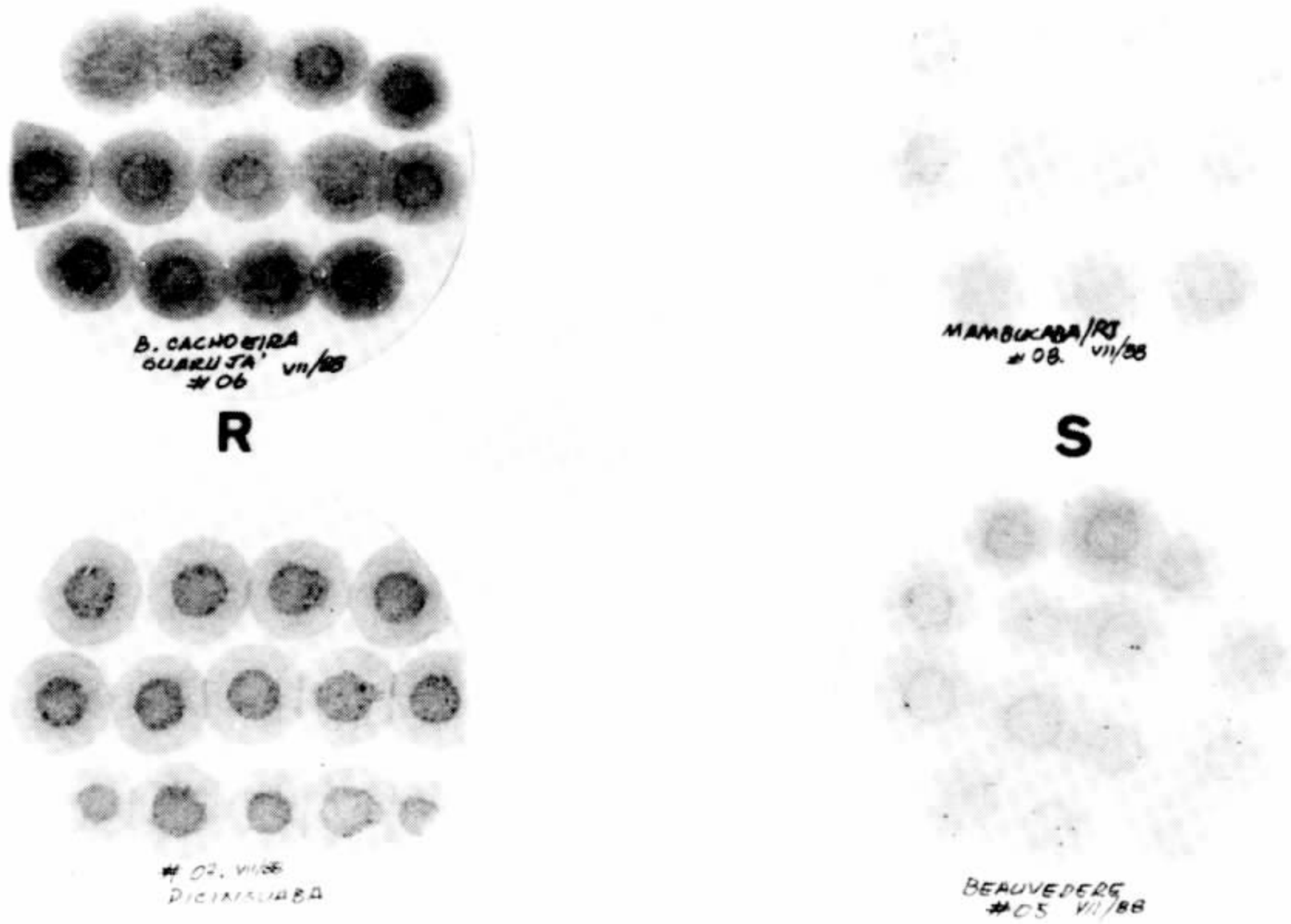

Fig. 2: esterase filter paper test for resistant (dark spots) and susceptible (faint spots) simuliid larvae from four localities (B. Cachoeira/Guarujá – *S. pertinax;* Picinguaba – *Simulium* sp n.; Mambucaba/RJ – *S. pertinax;* Beauvedere – *Simulium* sp).

TABLE I

LD_{50} and LD_{99} (ng/adult) with confidence limits (C.L.) and slope (b) in 3 populations of adult simuliids after 3 h exposure to a topical application of temephos

	R-*S. perflavum* (Unicamp)	R-*S. pertinax* (Promirim)	S-*S. pertinax* (Mambucaba)
LD_{50}	314.8	401.5	48.4
C.L.	205.7-481.9	333.9-482.8	30.6-76.6
LD_{99}	5,808	14,666	2,139
C.L.	3,562-9,467	12,196-17,636	1,150-3,979
b	1.8	3.8	2.0

TABLE II

Mortality of resistant and susceptible populations of *Simulium pertinax* adults after topical treatment with two diagnostic doses (DD) of temephos

	Status and population origin							
	R- Promirim				S- Mambucaba			
DD ng/adult	50		400		50		400	
Replicate	A	B	A	B	A	B	A	B
% mort. (3 h)	0.0	0.0	0.0	0.0	31.5	72.7	84.2	72.2
% mort. (5 h)	0.0	8.7	27.3	45.5	84.2	86.4	94.7	100.

Table I shows the lethal doses for R-populations (*S. perflavum* at Unicamp and *S. pertinax* at Promirim) as well as the S-population (*S. pertinax* at Mambucaba). The differences between LD_{50} and LD_{99} values were considered to be significant, or not, depending on overlapping existance on their confidence limits ($P < 0.05$).

It was observed that there were no significant differences between the two R-LD_{50}, while the S-LD_{50} was significantly lower. The comparison between slopes for R- and S- *S. pertinax* curves, in terms of Z value (Kurtak & Ouedraogo, 1984), showed a significant difference ($P < 0.05$). The same is not true when S- *S. pertinax* and R- *S. perflavum* slopes are compared. Considering only the intraspecific relationship, high LD-resistance factors (RF) for both LD_{50} and LD_{99} were observed. The RF-LD_{50} and LD_{99} were calculated as 8.3 and 6.9 respectively.

The diagnostic doses, closely related to R-LD_{50} and S-LD_{50} were established as 400 and 50 ng/adult. Table II presents the results obtained for R- and S- *S. pertinax* populations with the use of the diagnostic doses.

The susceptible *S. pertinax* females from Mambucaba (Table II) showed, mortalities ranging from 31.5 to 84.2 % after 3 hrs for the two diagnostic doses. For the same period, females from the resitant population failed to show any mortality. The observed mortalities 5 hrs after application also indicate the same difference in the sensitivity level among the 2 assayed populations. While mortalities ranging from 0.0 to 45.5% were obtained in the resistant group, higher mortalities, ranging from 84.2 to 100 % were observed in the susceptible group.

DISCUSSION

Higher esterase levels among resistant populations of simuliids as described by Magnin et al. (1987) were confirmed in the present study, where R- *S. perflavum* from Unicamp was compared under laboratory conditions with S- *Simulium* sp from Japi.

The adapted filter paper test proved to be a practical field test for simuliid resistance detection. The routine test can be performed by a technician spending no more than 5 min after collection of larvae. In this way, many breeding localities can be checked daily. Moreover, such a test can utilize as a standard, spots previously obtained from populations already recognized as R- or S-. While the original test (Pasteur & Georghiou, 1981) permits the individual detection of resitant phenotypes, useful for monitoring programmes (Roush & Miller, 1986), our adapted test is veveloped to verify the presence or absence of resistant phenotypes at high frequency among the collected samples.

Topical application assays against *S. pertinax* adults (Table I) showed essentially 8-fold increase in the LD_{50} (i.e. RF-LD_{50}). According to Brown (1986) an approximate 2-fold increase in adult LD_{50} of mosquitoes is accompanied by a 10-fold increase in larval LD_{50}, and a 4-fold increase in adult LD_{50} by a 100-fold increase in larval LD_{50}. An exaggerated high resistance level in *S. pertinax* to the larvicide temephos can therefore be suggested. The increase in the slope of the *S. pertinax* dose-mortality curve (Table I), as discussed by Burges (1971), also confirms that it is a well established resistance, which produced a reduction in the genetic variability.

The responses to the two proposed diagnostic doses can be interpreted in comparative terms. The observed percent mortality after 3 and 5 h (Table II), clearly indicate the actual status of *S. pertinax* populations from Promirim and Mambucaba, despite small variations occurring among replicates. Each test (application in ca. 50 flies) requires no more than 5 min after adult collection and can be performed under field conditions by two technicians. The use of an aspirator for imobilization permits total recuperation of the flies after treatment, avoiding individual manipulation by forceps and the CO_2 treatment proposed by Kurtak & Ouedraogo (1984), which made them eliminate non-recovered individuals. Furthermore, the use of temephos-acetone solution which evaporates faster than alcohol solution under natural conditions, as well as a comparatively shorter time to obtain results (3 and 5 h vs. 24 h) was more reliable. Moreover, field application just immediately following adult collection proposed in our technique avoids further stress, and can permit much more precision than ca. 20%, assumed by Kurtak & Ouedraogo (1984) due to their application method.

Chemical insecticides have been systematically used against *S. pertinax* larvae by the government in the northern littoral areas of São Paulo State (Fig. 1) for more than 30 years. From 1971 to 1984 DDT was replaced by the organophosphate temephos (Abate^{R}) with applications at 22 day intervals. Since then the interval between insecticide application was reduced to 15 days based on longevity data on *S. pertinax* larvae. This attempt was made in order to restore the efficiency of the government control campaign (Araújo Coutinho, 1988). The continuous occurrence of biting populations of *S. pertinax* at nuisance levels since 1984 (Andrade & Castello Branco Jr, in press), clearly suggested resistance.

On the other hand, the existence of simuliid populations resistant to temephos near Unicamp was not suspected. *S. perflavum*, which reveals high level of resistance, is not a target species and was never submitted to temephos treatments. Cross-resistance would be the cause of this phenomenon, since many agricultural chemical insecticides are being used in the region. Such a phenomenon has already been reported in blackflies (Kurtak et al., 1987; Lies, 1988).

In Africa, once simuliid resistance was detected to temephos and chlorphoxin, these chemicals were suspended and replaced by the large-scale use of products based on *Bacillus thuringiensis* var. *israelensis* (BTI). After a screening programme in a search for possible alternative larvicides, Kurtak et al. (1987) indicated that temephos should be used if resistance does not occurs. In Brazil, there is a tendency to control blackflies on a regional basis (Habib, 1988). In the southeastern states, the introduction of new insecticides has been delayed where temephos resistance is evident. The Methodologies proposed in the present work could be utilized directly to map the localities where simple or even cross-resistance is already established at high phenotype frequences, thereby improving *S. pertinax* control programmes.

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