IN-VIVO STAINING OF *AEDES VIGILAX, AEDES AEGYPTI* AND *CULEX ANNULIROSTRIS* LARVAE WITH GIEMSA AND OTHER VITAL DYES

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ABSTRACT. Of 11 stains tested in the laboratory with larval *Aedes aegypti*, Giemsa at dosages of 6 mg/liter for 24 hr and 21 mg/liter for 3 hr proved most satisfactory for in-vivo staining. At these rates, Giemsa caused some mortality especially in first instars of *Ae. vigilax, Ae. aegypti* and *Culex annulirostris* but effectively was retained in all adults examined up to 3 days posteclosion. The method was deemed satisfactory for investigations of larval movement of *Ae. vigilax, Cx. sitiens* and *Ae. alternans* in salt marsh in southeast Queensland.

INTRODUCTION

Giemsa stain consists of a mixture of methylene blue and its oxidation products, the azures, in combination with eosin Y. Commercially-available brands are known to differ widely in their composition and dye content.

Data on the efficacy of Giemsa for marking living mosquito larvae have varied. Weathersbee and Hasell (1938) reported the usefulness of Giemsa for staining *Anopheles* larvae but Haddow (1942) could not repeat the results. Recently, Joslyn et al. (1985) reported successful marking of the salt marsh mosquito *Aedes sollicitans* (Walker) in which the Giemsa stain present in the 4th larval stage only was carried through to adulthood. The latter authors reported that this adult marking technique had obvious benefits over traditional mark-release-recapture methods, e.g., radioactive tracers, fluorescent dusts, because excess handling of adults was eliminated.

Our interest in vital staining of larvae developed through the need to trace the movement of larval *Aedes vigilax* (Skuse) through salt marsh at Coomera Island, 75 km south of Brisbane, Queensland. Eggs of *Ae. vigilax* hatch when they are inundated occasionally by high spring tides (new and full moon periods); several such floodings may occur within the one lunar period. Because newly hatched larvae are fragile and difficult to collect en masse, a direct method of staining the pools seemed most appropriate. Because of the daily flooding pattern, any such method had to be rapid. We also investigated vital staining in 2 other important vectors, *Aedes aegypti* (Linn.) and *Culex annulirostris* Skuse in the laboratory. In total, 11 dyes were evaluated for possible field use.

MATERIALS AND METHODS

Aedes vigilax larvae, from Redcliffe or Coomera Island salt marsh, and colonized *Ae. aegypti* and *Cx. annulirostris* larvae were tested at 27±1°C in 18 × 12 × 6 cm plastic containers containing 250 ml 50:50 saltwater-distilled water (*Ae. vigilax*), or in distilled water only. Powdered brewer's yeast and 'Dog Chow' was added daily at 0.1 g/tray as food. Adults were maintained on 15% sucrose solution at 27 ± 1°C.

Giemsa (Koch-Light Laboratories Ltd., BDH Chemicals Ltd.), methylene blue (BDH); Nile blue, Rhodamine B, Neutral red (Merck), Fluorescein isothiocyanate (BDH), Azure A,B,C. (Gurr Products 34100, 3374, 34167 respectively), vegetable clothing dyes (Dylon International Ltd.) and Cochineal (Queen Flavouring) were first evaluated with *Ae. aegypti* and the most effective dye was tested further on the other 2 mentioned species.

For preparation of stock solutions, 0.1 g methylene blue, Nile blue, Rhodamine B., Neutral red and the vegetable dyes were dissolved in 1 liter distilled water. As Giemsa did not easily dissolve in distilled water, it was prepared by mixing 1 g powder with 60 ml glycerol and after incubation at 60°C for 2-3 hr, 60 ml ethanol was added (Conn et al. 1960). Thus, each ml of stock solution, pH 7.06, contained 8.3 mg Giemsa powder.

Larvae were exposed to various concentrations of stains or dyes from 0.1 to 500 mg/liter for 1–24 hr by (1) mixing the dyes with water in larval trays to the desired concentrations and adding larvae immediately thereafter, and (2) by adding larvae to the trays 3 hr after a yeast-dye mixture had been introduced. Usually 25 *Ae. vigilax* and 50 *Ae. aegypti* or *Cx. annulirostris* larvae were tested per tray and each combination (dye, immersion time) was replicated 4 times. Following immersion, larvae were rinsed and transferred into distilled water to which larval food had been added as above. This was done in order to simulate (1) the daily flushing of salt marsh pools, and (2) the stain settling into the mud substrate of pools. Larvae were then reared and examined daily for dye visibility and mortality. Trays of unstained larvae were maintained as controls.

When appropriate concentrations of acceptable dyes had been established, stained larvae

were then reared to adulthood and the presence and duration of adult staining established by crushing on microscope slides (Joslyn et al. 1985) or later, by crudely macerating the mosquitoes in 24-well Lucite™ plates used for serology. One drop of alcohol was added as it sometimes obviated microscopic examination (20X) and prevented tissue from sticking to the crushing rod.

Giemsa was then tested with *Ae. vigilax* in small-medium sized pools at Coomera Island. The volume of salt marsh pools was estimated by multiplying surface area by average pond depth. To dose salt marsh pools with Giemsa, a bucket of water was drawn from the pool and the appropriate volume of stock solution added to the bucket. The bucket was then emptied into the pool at various points. Further stirring was necessary to ensure that the stain had mixed throughout. The efficacy of the direct staining method was checked by dipping for larvae, just prior to tidal flushing the next day.

RESULTS

From screen testing with *Ae. aegypti*, Giemsa, Azure B and methylene blue were the only stains tested which were retained from larval to adult stages. However for methylene blue, larvae were stained satisfactorily when yeast was added. Giemsa was most effective at 6 mg/liter for 24 hr 10 mg/liter for 6 hr and 21 mg/liter for 3 hr. At the latter 2 dosages, mortality of *Ae. aegypti* was reduced while dye and retention in larval tissue varied little (Table 1). Mortality of II-IV instar *Ae. vigilax* and *Cx. annulirostris* was greater than that of *Ae. aegypti*.

Only IV instar *Cx. annulirostris* larvae and pupae were examined for retardation as a result of staining with Giemsa. The mean duration ±1 standard deviation in days for larvae and pupae respectively were as follows: (control) 4.97 ± 0.79, 2.81 ± 0.6; (21 mg/liter for 3 hr) 5.36 ± 0.87, 3.48 ± 0.82; (10 mg/liter for 6 hr) 5.88 ± 1.01, 3.59 ± 0.8; (6 mg/liter for 24 hr) 6.29 ± 1.01, 3.74 ± 0.74.

At the above dosages, blue coloration of adults was visible in the intersegmental neck membrane and between abdominal sclerites I and II. Giemsa was also evident in the posterior sternites of all of 411 *Ae. aegypti, Ae. vigilax* and *Cx. annulirostris* examined microscopically at 1 day posteclosion (Table 2). With squashing, stain was evident in 100% *Ae. aegypti, Ae. vigilax* and *Cx. annulirostris* (6 mg/liter for 24 hr) for a further 2 days. At 10 mg/liter (6 hr) and 21 mg/liter (3 hr) respectively, 15 and 25% *Cx. annulirostris* were unstained by day 3. Microscopic examinations and squashing became more necessary as time progressed and staining weakened. All *Ae. aegypti* and *Ae. vigilax* were negative by 8–11 days depending on the dosage, and after 7 days with *Cx. annulirostris.*

With methylene blue, some adult *Ae. aegypti, Ae. vigilax* and *Cx. annulirostris* retained stain for up to 7 days but squashing was necessary even in 1 day-old adults, e.g., at 0.5 mg/liter (24 hr) and 1.0 mg/liter (6 hr), stain was visible without squashing only in 33–68% of those marked; at 3 mg/liter (2 hr), in 22–38%. At these dosages, stain was detected in all of 452 *Ae. aegypti* and 421 *Cx. annulirostris* for 3 days but for only 1 day in 80 *Ae. vigilax.* None of a further 2,553 mosquitoes retained methylene blue beyond 7 days. Mortality of larval *Ae. aegypti,* other than instar 1 in all concentrations except 3 mg/liter was minimal with retention varying between 1 day (first instar) to 4–6 days for IV instars.

Azure B, a component of Giemsa, behaved similarly to methylene blue as the blue color was visible (without squashing) only in 20–42% of 273 males and females 1 day after emergence and had completely disappeared after 7 days. At the dosages necessary to produce good

Table 1. Mean percent mortality and retention time of different instars (I–IV) of *Aedes aegypti, Aedes vigilax* and *Culex annulirostris* stained with Giemsa (without yeast).

Dosage mg/liter (h)	Species*	Mortality I	II	III	IV	Retention (days) I	II	III	IV
6 (24)	*Ae. aegypti*	18	13	5	2	1	3	6	8
	Ae. vigilax	—	15	10	8	—	3	10	12
	Cx. annulirostris	—	15	12	7	—	3	6	8
10 (6)	*Ae. aegypti*	5	3	2	1	1	2	3	7
	Ae. vigilax	—	13	10	8	—	3	5	8
	Cx. annulirostris	—	5	3	3	—	2	3	4
21 (3)	*Ae. aegypti*	7	4	0	0	1	2	4	6
	Ae. vigilax	—	10	6	4	—	3	10	12
	Cx. annulirostris	—	10	8	5	—	2	5	6

* Assessments based on an average of 4 replicates each of 50 *Ae. aegypti* and *Cx. annulirostris* but of 25 with *Ae. vigilax.*

Table 2. Percentage dye visibility in adults (♀) emerged from early fourth instar larvae stained with Giemsa.

			Ae. aegypti				*Ae. vigilax*				*Cx. annulirostris*			
				Percentage				Percentage				Percentage		
Conc (mg/liter)	Staining time (hrs)	Days after emerged	Total no.	+(ve) visual	+(ve) squashed	−(ve)	Total no.	+(ve) visual	+(ve) squashed	−(ve)	Total visual	+(ve) visual	+(ve) squashed	−(ve)
6	24	1	56	100	0	0	32	100	0	0	52	100	0	0
		2	55	50	50	0	28	80	20	0	50	40	60	0
		3	54	28	72	0	29	65	35	0	48	10	90	0
		4	53	5	92	3	28	22	68	10	45	1	78	21
		5	54	0	70	30	26	8	74	18	46	0	50	50
		6	45	0	41	59	27	0	70	30	38	0	32	68
		7	48	0	29	71	26	0	62	38	40	0	16	84
		8	42	0	10	90	26	0	45	55	36	0	0	100
		9	36	0	1	99	25	0	22	78	—	—	—	—
		10	36	0	0	100	25	0	11	89	—	—	—	—
		11	—	—	—	—	25	0	2	98	—	—	—	—
		12	—	—	—	—	23	0	0	100	—	—	—	—
10	6	1	52	100	0	0	35	100	0	0	50	100	0	0
		2	50	40	60	0	30	81	19	0	49	37	63	0
		3	50	11	89	0	29	50	50	0	50	4	81	15
		4	49	0	78	12	30	15	77	8	45	0	63	37
		5	48	0	50	50	28	5	80	15	43	0	42	58
		6	48	0	36	64	29	0	68	32	42	0	28	72
		7	42	0	25	75	28	0	45	55	40	0	17	83
		8	39	0	5	95	28	0	28	72	38	0	0	100
		9	40	0	0	100	25	0	10	90	—	—	—	—
u		10	—	—	—	—	26	0	5	95	—	—	—	—
		11	—	—	—	—	24	0	0	100	—	—	—	—
21	3	1	54	100	0	0	32	100	0	0	48	100	0	0
		2	50	35	65	0	30	60	40	0	40	12	88	0
		3	50	5	95	0	31	50	50	0	48	0	75	25
		4	49	0	80	20	30	10	85	5	45	0	50	50
		5	45	0	49	51	29	2	78	20	40	0	32	68
		6	42	0	32	68	29	0	50	50	42	0	26	74
		7	42	0	28	72	30	0	39	61	40	0	18	82
		8	39	0	2	98	28	0	20	80	38	0	0	100
		9	32	0	0	100	26	0	9	91	—	—	—	—
		10	—	—	—	—	25	0	3	97	—	—	—	—
		11	—	—	—	—	26	0	0	100	—	—	—	—

staining (6–21 mg/liter), mortality was greater but retention times were similar. In contrast to methylene blue, Azure B solution could be used by direct addition to water.

Neutral red, Nile blue and Rhodamine B produced dark staining of *Ae. aegypti* only when used with yeast with low-moderate mortality with retention times of 1 day or less. Azures A and C produced only a light stain but were not evaluated with yeast. With these latter stains, however, 50–90% of II to IV instar *Ae. aegypti* died when treated at rates of 6 mg/liter for 6 hr or greater.

Fluorescein at dosages of 200 mg/liter for 6 hr to 8000 mg/liter for 24 hr was unsatisfactory as only 55–60% and 80% respectively of III and IV instar *Ae. aegypti* and *Cx. annulirostris* fluoresced when examined under ultraviolet light. Mortality of the above larvae ranged from 10 to 50%; survivors retained fluorescein for ≤ 2 days.

Cochineal and vegetable dyes were completely unsatisfactory as they only colored the alimentary tract at various intensities for 1 day or less.

Giemsa added to 7 small-moderate capacity salt marsh pools marked II-IV instar *Ae. vigilax*, *Ae. alternans* Westwood and *Cx. sitiens* Wiedemann with varying degrees of success. Whereas a dosage rate of 6 mg/liter marked 0–56% larvae sampled by dipping, higher concentrations of 10–20 mg/liter marked all larvae sampled 1 day later (Table 3). In 2 trials at 6 and 18 mg/liter, self-marked adult *Ae. vigilax* and *Ae. alternans* were reared from larvae stained at instars III and IV. Total adult staining was achieved at 18 mg/liter. On staining, salinities and temperature of the pools were 14–20% and 24–27°C.

DISCUSSION

Giemsa, particularly at dosages of 6 mg/liter for 24 hr and 21 mg/liter for 3 hr. was the most effective stain for larval instars of *Ae. aegypti*, *Ae. vigilax* and *Cx. annulirostris*. The stain caused minimal mortality and could be detected in all adults examined for 3 days posteclosion. Subsequent field trials demonstrated that 2 additional salt marsh species, the predacious *Ae. alternans* and another species *Cx. sitiens* could also be stained effectively. Other vital stains had limited application because of the mortality they caused and poor retention of stain (e.g., neutral red, Nile blue, Rhodamine B, Fluores-

Table 3. Field evaluation of Giemsa in salt marsh pools at Coomera Island.

Approximate pool capacity (liters)	Concentration (mg/liter)	Species	Instar	% staining after 1 day	
				Larvae	Adults
10	20	*Ae. vigilax*	II–IV	100	
10	12	*Ae. vigilax*	II, IV	100	
		Cx. sitiens	III	100	
70	6	*Cx. sitiens*	II	0	
230	10	*Cx. sitiens*	II, III	100	
50	6	*Ae. vigilax*	III	56	50
250	18	*Ae. vigilax*	IV	100	100*
		Ae. alternans	IV	100	100
270	6	*Cx. sitiens*	II, III	52	

* The stain was visible in 64% of *Ae vigilax* 3–4 days posteclosion.

cein isothiocyanate) or simply because they had to be applied using pre-stained yeast which was impractical for field usage (e.g., methylene blue).

The effects of Giemsa are inconsistent, but this may be due to the variety of commercial stains available, the duration of staining and the buffers used. For example, Saal (1964) evaluated 27 commercial Giemsa samples by spectrophotometry, chromatography and for ability to stain *Babesia* and found considerable variation. The BDH (Gurr) formulation used in our study contained 26.5% eosin, 10.5% methylene blue and 63.0% Azure I (A,B with methylene violet and azure C removed). Weathersbee and Hasell (1938) often used higher Giemsa concentrations for up to 120 hr in Grubler's stock solution (pH 6.9–7.0) to permanently stain eclosed *Ae. aegypti* for up to 13 days. Our data indicate that adults progressively lose the stain. Haddow (1942) was unable to repeat the findings of Weathersbee and Hasell (1938). R. Washino (personal communication) noted that Giemsa stock solution required buffering with 25 mM phosphate solution but Joslyn et al. (1985) used deionized distilled water for preparation of Giemsa and successfully used this stock solution at 0.1 mg/liter (0.001%). Our stock solution, 42 times more concentrated than that of Washino, confers advantages for field application as less has to be added to pools.

By our methods, Giemsa was retained for a much shorter period in adult *Ae. aegypti*, *Ae. vigilax* and *Cx. annulirostris* than for either *Anopheles* spp. (Weathersbee and Hasell 1938) or *Ae. sollicitans* (Joslyn et al. 1985). Because our aim was to develop suitable means for marking larvae directly in salt marsh pools which were reflooded daily by spring tides, it was necessary to expose larvae to higher concentrations of Giemsa for only 3–24 hr; other workers allowed larvae to remain in substantially lower concentrations until adult emergence. For larval population studies, increased mortality of first and second instars and their short-term retention of stain create unresolvable problems. In addition, some growth retardation was evident in *Cx. annulirostris* larvae stained as IV instars with Giemsa at all dosage levels, as has been noted for several vital stains including Giemsa, methylene blue, neutral red and Nile blue (Weathersbee and Hasell 1938, Peters and Chevone 1968, Chevone and Peters 1969, Barbosa and Peters 1970). It is presumed that Giemsa would similarly effect *Ae. vigilax*.

However, for the evaluation of larval movement of species throughout a salt marsh, quick staining of III-IV instar larvae at dosages of 21 mg/liter for 3 hr is quite adequate. The bright blue larvae seem to move normally and therefore, should be easily recaptured as larvae or pupae. It is just as obvious, however, that because the emerging adults do not remain permanently stained, our direct staining method has limited usefulness for studies of adult dispersal.

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References Cited

Barbosa, P. and T. M. Peters. 1970. Retardation of growth rate in *Aedes aegypti* (L.) larvae exposed to vital dyes. J. Med. Entomol. 7:693–696.

Chevone, B. I. and T. M. Peters. 1969. Retardation of larval development of *Aedes aegypti* (L.) by the vital dye, Nile blue sulphate (A). Mosq. News 29:243–251.

Conn, H. J., M. A. Darrow and V. M. Emmel. 1960. Staining procedures, p. 187. Williams and Wilkins Company Baltimore, MD.

Haddow, A. J. 1942. The mosquito fauna and climate of native huts at Kisumu Kenya. Bull. Entomol. Res. 33:91–142.

Joslyn, D. J., L. B. Conrad, and P. T. Slavin. 1985. Development and preliminary field testing of the Giemsa self-marker for the salt marsh mosquito *Aedes sollicitans* (Walker) (Diptera: Culicidae). Ann. Entomol. Soc. Am. 78:20–23.

Peters, T. M. and B. I. Chevone. 1968. Marking *Culex pipiens* Linn. larvae with vital dyes for larval ecological studies. Mosq. News 28:24–28.

Saal, J. R. 1964. Giemsa stain for the diagnosis of bovine babesiosis. I. Staining properties of commercial samples and their component dyes. J. Protozool. 11:573–582.

Weathersbee, A. A. and P. G. Hasell. 1938. Mosquito studies on the recovery of stain in adults developing from anopheline larvae stained *in vitro*. Am. J. Trop. Med. 18:531–543.