ARTICLES

A NEW METHOD FOR SAMPLING MANGROVE MUD FOR *CULICOIDES* LARVAE AND PUPAE, WITH NOTES ON FACTORS AFFECTING ITS USE

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ABSTRACT. A new, more effective technique for separating *Culicoides* immature stages from mangrove mud is described. This procedure utilizes an inverted funnel unit and hypersalinization of the mud sample. The effects of light, variations in salt concentrations, and frequency of decanting are reported and the method is compared to the previously used sand-layer technique. Best yields were obtained when the inverted funnel units were shielded from the light. A 150 g/l NaCl solution was optimal in achieving rapid and thorough separations of both larvae and pupae. It was found that decanting each unit 3 times at half-hour intervals after an undisturbed 1 hr period gave the quickest larval separation, but yields of over 90% could be obtained by decanting only once after a 3 to 4 hr quiescent period. The method gave significantly higher yields in less time than were obtained with the sand-layer method. The procedure may also find application in the separation of other motile invertebrates from littoral and benthic samples from a variety of habitats, ranging from freshwater to marine.

Many attempts have been made to develop a rapid, yet accurate procedure for separating *Culicoides* immatures from their natural breeding substrate. Although mangrove mud is one of the most difficult sources to sample, Bidlingmayer (1957) developed an effective method for sampling *Culicoides* breeding in these soils using a sand-layer process. This procedure requires the placement of about 700 ml of mud into a clean, quart-size (.95 liter) oil can followed by the addition of a 5 cm layer of builder's sand above the sample. Enough tap water is added to saturate the sand, and the samples are left to stand for 24 hr to allow for larval migration from the mud. The sand is then removed and washed using a 140 mesh sieve and the residue is placed into a black photo tray to provide a dark background so that the whitish larvae can be more easily seen. Using this method, agitation and decantation must be carried out at least 3 times to assure that all larvae have been found. Williams (1960) reported a modification of the technique using $MgSO_4$ flotation to facilitate the separation of fresh-water invertebrates from the sand layer after their migration from the soil sample.

Kline et al. (1975) discussed and compared various methods for *Culicoides* larval sampling. According to these authors, the 4 principal separation techniques are sieve-flotation, sand-flotation (modified sand-layer method as described above), direct $MgSO_4$ flotation and Berlese funnel migration. Using sieve-flotation and direct-flotation, the samples are processed immediately without waiting for larval migration. However, this is quite labor-intensive and requires considerable time to complete larval separation. Kline et al. concluded that of the 4 methods, the Bidlingmayer sand-layer technique was the best for the separation of *Culicoides furens* (Poey), while better yields of *C. hollensis* (Melander & Brues) were obtained using sieve flotation. The remaining methods were judged as being generally unsatisfactory for routine larval sampling.

Altman et al (1970) reported sampling for *Culicoides furens* and *C. guyanensis*

Floch and Abonnenc in Panama using the Bidlingmayer sand-layer technique. This procedure was tried by the author but it was considered too time consuming for processing large numbers of samples, and there were doubts about the thoroughness of larval separation.

METHODS & MATERIALS

In August 1976, experiments were initiated in the Panama Canal Zone to determine if a more effective technique could be developed for the separation of immature *C. furens* from the thick black mangrove mud where this species breeds. Several sites were surveyed and high larval densities were found at the Galeta Point tidal swamp near the town of Coco Solo. On each day of the trials, 20 or more 250 ml samples were collected from the upper 7.5 cm of soil about midway between high and low tide elevations. At the lab, twigs, roots and leaves were removed from the mud, and all of the samples were then combined and thoroughly mixed to provide for more uniform distribution of the larvae.

Larval separation was attempted using salt flotation in combination with an inverted funnel unit in an effort to improve the efficiency of this operation and to obtain increased yields. The separator unit (Fig. 1), was fashioned by snugly fitting a 105 mm diameter polyethylene funnel (hanger tab removed) inside a 1 qt tapered polyethylene "food saver" container. About 20 mm of the funnel stem was removed to widen the opening to a diameter of 12 mm. When in place, 3 cm from the bottom, the inverted funnel formed a chamber of approximately 450 ml capacity, and served to replace the sand-layer/mud interface of the Bidlingmayer separator unit.

In operation, a 120 ml sample of the pre-mixed mud was placed into the container with 180 ml of either saturated

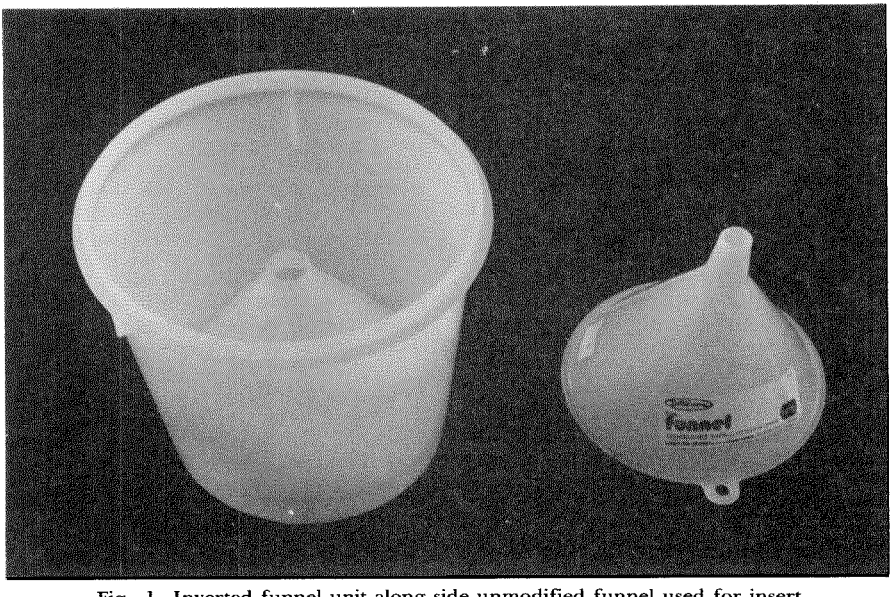

Fig.. 1. Inverted funnel unit along side unmodified funnel used for insert.

$MgSO_4$ or NaCl salt solution. This was gently stirred into the mud and the inverted funnel was press-fitted above the resulting slurry. More salt solution was then added to fill the lower chamber to the top of the funnel stem. While using a finger to close this opening and hold the funnel in place, the upper part of the unit was flushed out with tap water to remove any residual mud and debris. Next, seawater was added above the funnel to a level 3 cm above the tip of the stem.

Within a few minutes, many actively swimming *Culicoides* larvae could be seen exiting the highly saline conditions of the lower chamber into the marine (seawater) environment above. However, even after several hours, some larvae could still be seen swimming within the lower chamber and appeared reluctant to migrate farther.

RESULTS AND DISCUSSION

EXPERIMENT 1: EFFECTS OF LIGHTS ON LARVAL MIGRATION. Since initial trials of the unit were performed under strong fluorescent lighting, it was decided to determine if light might be affecting migration of larvae. Using 200 g/l NaCl solution, several units were prepared and placed in a dark cabinet while a parallel series was maintained under the strong lighting as before. Under dark conditions, over 80% more larvae were separated and the average rate of migration was nearly 6 times more rapid (Table 1).

The photonegative behavior observed was later used to good advantage in concentrating the separated larvae for counting. The inverted funnel separators were decanted into a white enameled pan and a circular fluorescent desk lamp was positioned a few inches above. Then a 4.5 cm black plastic disk attached to a large cork was floated in one corner of the pan. Within minutes, the entire collection of larvae would congregate under the disk. They could then be removed en masse to black porcelain depression plates for counting under a stereo microscope. The separator also gave unexpectedly good yields of *Culicoides* pupae due to the flotation action of the salt solution. These non-motile stages were found along the edges of the pan after decanting the units and were removed individually to rearing chambers. Due to the lessened mechanical handling of the sample, these pupae were usually in good condition and were successfully reared to adults for species determinations.

EXPERIMENT 2: EFFECTS OF SALT CONCENTRATION ON LARVAL MIGRATION. Due to the low relatively low cost and availability of table salt and its relative effectiveness in this technique, no further experimentation was conducted with $MgSO_4$. The optimal concentration of NaCl for use in the separator unit was determined using the range of concentrations shown in Table 2. Since seawater contains about 35 grams of salt per liter, 3 solutions were made at salinities lower than this value and 3 were made at higher concentrations. Beginning with 200 grams/liter NaCl, serial dilutions were made resulting in the range of salinities shown. Each was mixed with the mud samples as described and decanted after

Table 1. Effect of light on *Culicoides* migration in inverted funnel separation period 15 minute periods of larval migration.

200 g/l NaCl	1	2	3	4	5	6	Endpoint
	Culicoides larvae						
Light	10	9	6	16	6	0	47 total
Migration rate per minute	.7	.6	.4	1.1	.4	0	0.5/min. average
Dark	89	55	65	41	5	1	256 total
Migration rate per minute	5.9	3.6	4.3	2.7	.3	.1	2.8/min. average

All experiments run at temperatures from 21 to 22° C.

Table 2. Effect of salt concentration on the rate of *Culicoides* larvae migrating per minute to upper chamber of separator unit.

G/l NaCl	1st 30 min.	2nd 30 min.	3rd 30 min.	4th 30 min.	Average rate per min.
			Hypomarine		
6.25	1.5	0.5	0.1	0.1	0.7
12.5	1.6	0.3	0.1	0.3	0.8
25.0	3.7	1.1	0.6	0.3	1.4
			Hypermarine		
50.0	7.5	4.1	2.9	1.8	4.1
100.0	9.5	5.0	3.1	1.0	4.7
200.0	9.1	7.0	1.4	0.6	4.5

30 min for 4 successive periods. As shown, rates for larval migration from the lower chamber were markedly lower at the hypomarine concentrations than for those units with the 3 higher salinity levels.

The 4-hr totals for a like series (Table 2a) showed great differences in larval yields from pre-mixed mangrove mud. At the two highest salinities, over 84% of the 4-hr totals were separated within the first 2 hr and over 95% were obtained within 3 hr. Similar yields were achieved in all of the hypermarine units after 4 hr. These results also appear to indicate that the pre-mixing of the mangrove mud was quite effective in providing a homogeneous source of *Culicoides* immatures. In a subsequent trial, (Table 3) after a 5 hr migration period, hypermarine units of 50 g/l and 200 g/l yielded 194 and 197 larvae respectively, while hypomarine units of 6.25, 12.5 and 25 g/l had produced only 12, 12, and 74 larvae. In this trial, the hypomarine units were then raised to salinities above that of seawater by adding 30 grams of salt through the funnel stem. After an additional 18 hr to allow for larval migration, the total separation was increased to 50, 38, and 128 larvae respectively. Only one additional larva was found in the other units.

After consideration of the above results, an intermediate concentration of

Table 2a. Numbers of *Culicoides* larvae and pupae separated from uniformly mixed mangrove mud samples at noted salt concentrations.[1]

Grams/liter NaCl	1st hour	2nd hour	3rd hour	4th hour	Total larvae	Total pupae
			Hypomarine			
6.25	60 + 8p	4 + 1p	3	25	92	9
12.5	52 + 6p	6	4	31	93	6
25.0	136 + 7p	12	17	16	181	7
			Hypermarine			
50.0	302 + 4p	111	67	61	541	4
100.0	340 + 10p	118 + 1p	62	25	545	11
200.0	376 + 14p	148	51	17	592	14

[1] Combined totals from four pints of pre-mixed mangrove mud using eight separator units for each concentration.

Table 3. Effect of salt concentration on the rate of *Culicoides* larval migration.

G/l NaCl	5 hrs.	After 5 hrs.	23 hrs.	Percent of total	Total
		Hypomarine			
6.25	12	30 g NaCl added	38	76%	50
12.5	12	30 g NaCl added	26	70%	38
25.0	74	30 g NaCl added	54	42%	128
		Hypermarine			
50.0	193	No salt added	1	2%	194
200.0	197	No salt added	0	0%	197

150 g/l NaCl was chosen as a standard solution for all further experiments.

EXPERIMENT 3: COMPARISON OF INVERTED FUNNEL UNIT WITH SAND-LAYER METHOD. Results of trials comparing the Bidlingmayer sand-layer method with the inverted funnel technique are summarized in Table 4. Mangrove mud negative for larval breeding used in these experiments was mixed to bring it to a similar consistency to that used in the earlier trials. A volume of 236 ml of mud was used in each quart-size oil can and 100 late instar larvae were gently mixed into the soil. Due to the volume restrictions of the inverted funnel unit, 2 units were used to process a like amount of mud and larval density. The sand-layer units were prepared as described earlier, and all units were placed in dark cabinets. Certain of the inverted funnel separators and sand-layer units were checked at the intervals shown while the rest were left undisturbed as a check on possible effects of multiple handling. The sand layer was processed without the aid of $MgSO_4$ flotation. Percent recoveries at 4, 5 and 24 hours are listed for each unit. Yields of 90% and above were obtained after 4 hr in all but one of the inverted funnel units, whereas the highest 4-hr recovery in the sand-layer units checked at that interval was 77%.

Table 4. Comparison of inverted funnel separator with sand-layer separator. Percent recovery of known numbers of *Culicoides* larvae.

Inverted funnel separator (50 late instar larvae per 118 ml mud sample)			
Run no.	4 hours	5 hours	24 hours
1	92%	100%	100%
2	90	92	96
3	96	100	100
4	94	100	100
5	80	88	88
6	94	96	98
7		88	88
8		90	96
9		88	98
10		84	92
11		96	100
12		96	98
13			96
14			94
15			100
16			100
17			96
18			96
Sand layer separator (100 late instar larvae per 236 ml mud sample)			
Run no.	4 hours	5 hours	24 hours
1	67%	79%	84%
2	77	80	80
3	72	78	78
4		64	69
5		74	79
6		74	77
7			68
8			72
9			76

A paired t-test was calculated for the 24 hr totals for both methods, with the combined sums from the 2 inverted funnel units used to compare like volumes and larval populations in the sand-layer units. The difference between the 2 methods proved to be significant at the .001 level (t_{11} (.001) = 4.437).

EXPERIMENT 4: THE EFFECT OF FREQUENT DECANTING ON RATE OF LARVAL SEPARATION. Nine pairs of inverted funnel units were prepared using pre-mixed mangrove mud having a relatively high larval density, and the units were decanted and checked at the intervals shown in Table 5. Percentages of the 24 hr totals are listed for each pair (236 ml of mud), with the boldface figures indicating the initial check of previously undisturbed pairs. All of the disturbed units yielded more larvae than those undisturbed units decanted for the first time. The agitation of the slurry during decanting appears to significantly increase the rate of larval separation. Therefore it is advisable to decant each unit 2 to 3 times at half-hour intervals after allowing a 1 hr undisturbed period. If this cannot be done, then it would be best to leave the units undisturbed for from 3 to 4 hr to obtain maximum separation.

CONCLUSIONS

The above experiments indicate that the inverted funnel separator is a valuable

Table 5. Effect of decanting frequency on separation of *Culicoides* larvae from pre-mixed mangrove mud using inverted funnel separator.

	Percent of 24 hour totals from 236 ml mud samples (2 units/run)								
Run no.	½ hour	1 hour	1½ hours	2 hours	2½ hours	3 hours	4 hours	5 hours	24 hour total
1	**37%**	75%	90%	97%	99%	99%	100%	100%	(178)
2		**66%**	91%	99%	99%	99%	100%	100%	(191)
3			**83%**	92%	99%	99%	100%	100%	(224)
4				**86%**	96%	100%	100%	100%	(156)
5					**92%**	98%	100%	100%	(172)
6						**97%**	99%	99%	(187)
7							**94%**	99%	(190)
8								**91%**	(211)
9									**(136)**

Boldface indicates units decanted for first time after undisturbed period.

tool for use in sampling for *Culicoides* immatures from mangrove mud habitats. Seawater was used in the above trials due to its availability, but NaCl solutions of about 35 g/l should work very well. The 150 g/l salt solution added to the mud sample worked well in these experiments but somewhat lesser concentrations may work just as effectively. Although the separators were placed in a dark cabinet during this study, covering each unit with a black plastic bag or a tent of aluminum foil should provide adequate light shielding.

The stimulation of *Culicoides* larval migration through the establishment of hypermarine conditions in the lower chamber of the separator unit greatly facilitates the handling of large numbers of field samples and the technique also yields good numbers of pupae through flotation. Such inverted funnel processing, as compared with other larval separation techniques, should give more rapid results with much less manipulation of mud samples and provide an average of 90% or better sampling accuracy. The technique may also be applied to the separation of other classes of motile invertebrates from littoral and benthic samples taken from marine, estuarine or even freshwater habitats.

ACKNOWLEDGMENTS

The author wishes to thank Mr. Pastor Chaves and Mr. Inocencio Leguia for their valuable assistance in this study. I am endebted to Mrs. Claire Joplin for her consultation on statistical testing. I also express my gratitude to Dr. D. C. Baerg for his many valuable editorial suggestions, and to Dr. B. N. Chaniotis, Dr. H. A. Christensen and Maj. H. J. Harlan for their kind review of the manuscript.

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