EFFICIENCY OF SALT FLOTATION FOR EXTRACTION OF IMMATURE *CULICOIDES VARIIPENNIS* (CERATOPOGONIDAE) FROM MUD SUBSTRATES

BRADLEY A. MULLENS AND JOHN L. RODRIGUEZ
Department of Entomology, University of California, Riverside, CA 92521

ABSTRACT. Efficiency of direct salt flotation (NaCl or $MgSO_4$) for extraction of immature *Culicoides variipennis* from 30 ml aliquots of silt substrates was examined. When stirred at 5 min intervals for 15 min, $MgSO_4$ efficiency with immatures added to autoclaved mud was 50%, 72%, 88%, 100% and 100% for instars 1–4 and pupae, respectively. NaCl was quite toxic to instars 1 and 2, immobilizing them in 0.6 and 11.7 min respectively. Trials with freshly collected field mud confirmed that recovery in either salt increased with larval age, that $MgSO_4$ was superior for early instars, and that NaCl extracted more late instars in less time. Speed of larval extraction varied significantly on 2 different sampling days. Addition of the flocculant Separan® speeded settling of the sediments and made sorting and counting larvae much easier. Overall, $MgSO_4$ would be preferred for studies with all larval age classes, while NaCL would be preferred for quick extraction of late instars.

Extraction of immature ceratopogonids from soil substrates generally has proven to be a tedious and time-consuming process. Kline et al. (1975) reviewed methods which had been previously used, including sieve flotation, sand flotation, direct flotation and Tulgren funnels. They also compared the efficiency of these methods and rearing chambers for sampling salt marsh *Culicoides* in North Carolina. Since that time, 2 new methods of extracting salt marsh *Culicoides* have been described—an inverted funnel separator (Boreham 1981) and agar extraction (Kline et al. 1981). With the exception of one prior study (Davies and Linley 1966), work has dealt only with larvae in general, and not with specific life stages. Information on extraction efficiency for the various life stages of the target insect permits more detailed analysis of changes in larval age structure and dispersion patterns; it is also necessary for complete life table development. For these reasons we began studies to develop an accurate and time-efficient extraction method for larvae of *Culicoides variipennis* (Coquillett), a known vector of bluetongue virus in North American ruminants (Luedke et al. 1967).

MATERIALS AND METHODS

Because of availability and cost, we used NaCl and $MgSO_4$ (Epsom salts) in these trials. In the laboratory trials, surface mud (top 2 cm) was removed from the edge of a dairy lagoon near Norco, California, known to support large numbers of *C. variipennis*. This mud was returned to the laboratory and autoclaved. It was then homogenized, stored at 3–5°C, and was used for all laboratory extraction trials. Instars 2, 3 and 4, as well as pupae, were sieved from field-collected mud, backwashed into a white enameled pan, sorted to instar by head capsule size under a dissecting microscope, and used the same day. First instars were reared from eggs obtained from gravid females collected near the breeding site at dusk with a sweep net. The first instars were used < 1 day after eclosion from the egg. In both laboratory and field trials, 30 cc of mud was placed in a cup 7 cm tall × 6 cm diam; 30 immatures of the appropriate *C. variipennis* life stage were added to autoclaved mud in the laboratory trials. To this was added 70 cc of a saturated salt solution ($MgSO_4$ or NaCl) which contained 0.05 ml (2 drops) of a 0.5% solution of Separan® NP10[1], a commercial flocculating agent which greatly speeded settling of the sediment (Byrd et al. 1966). The *Culicoides*-sediment salt solution mixture was thoroughly stirred and watched continuously under a dissecting microscope at 12X. Live immatures floating/swimming to the top were continuously removed with fine forceps or a small pipette. Numbers were recorded by 5 min intervals for 15 min. Some trials were stirred again every 5 min, while others were not. From 10–21 "stir" and 5–20 "no stir" trials were conducted for each instar with the salts used.

The field mud trials employed substrate freshly collected from the same area at the same dairy lagoon on 2 dates in August 1983, when all 4 larval instars were present. On each date the mud was thoroughly stirred in a 1 liter container, and 30 cc aliquots were quickly poured into 10 separate cups. These cups were randomly assigned to either NaCl or $MgSO_4$ treatments (plus Separan). The salt solution was added to the mud in a cup immediately before the 15 min extraction period. In this case the $MgSO_4$ and NaCl flotation trials were simultaneously conducted by 2 workers using the same timer and alternating the salts used each time. Larvae were extracted by pipette for 15 min under a dissecting microscope and stirred and

[1] Dow Chemical Company, Midland, MI 48640.

segregated by 5 min intervals as described above. After 15 min each sample was washed through a 200 mesh sieve, backwashed into a petri dish with 70% EtOH, and examined microscopically to determine recovery efficiency. Remaining larvae were identified and counted. *Culicoides variipennis* larvae were identified in all trials by their anteriorly narrowed head capsule, massive pharyngeal armature and size.

To determine the direct effects of the 2 salts on mobility of the larval instars, freshly sieved field-collected larvae (instars 2, 3 and 4), or reared (instar 1) larvae were individually placed in small watch glasses with a few ml of the salt solution. These larvae were observed continuously for the first hour and at 15 min intervals afterward. When a larva no longer responded to tactile stimulation with fine forceps, times were recorded and the larva was considered immobile.

RESULTS

LABORATORY TRIALS. The addition of the flocculant Separan greatly speeded sediment settling and made it easier to recover larvae from the salt surface without a great deal of debris. It had no discernible effect on the larvae.

Laboratory flotation trials with known numbers (30 larvae/trial) of the 4 larval instars indicated considerable differences. After the initial stirring of the mud-salt-larvae slurry, stirring the slurry again at 5 min intervals did not significantly increase recovery of instars 2, 3 and 4 or pupae in either salt (ANOVA F Test, $p > 0.05$), although restirring did tend to increase larval recovery in the $MgSO_4$. Restirring did make a significant difference ($p < 0.05$) for the 1st instars, however, increasing recovery from 28.7% to 50% in $MgSO_4$. Larval recovery in the $MgSO_4$ stirred at 5 min intervals improved with larval age (Table 1); it was 50%, 72%, 88% and nearly 100% for instars 1, 2, 3 and 4, respectively. NaCl was not tested on the early instars in these laboratory trials due to severe effects on these stages, as will be discussed later. For instars 3 and 4, however, NaCl increased recovery to 98% and 100%, respectively (Table 1). Pupal recovery was excellent in both salts, due partly to the natural buoyancy of the pupae.

Larval recovery with time also was affected by larval age and salt type (Table 1). The 1st instars were recovered in nearly equal numbers over the 3 consecutive 5 min intervals. Time interval effects were significant ($p < 0.05$) for the other instars and pupae in both $MgSO_4$ and NaCl, and later instars were recovered more quickly. Larval recovery in the first 5 min in $MgSO_4$ was 16%, 38%, 44%, 75% and 92% for instars 1, 2, 3, 4 and pupae, respectively. NaCl increased the speed of recovery for instars 3 and 4 to 78% and 92%, respectively, during the first 5 min. Larvae apparently were very irritated by NaCl and moved more rapidly than they did in $MgSO_4$.

The salts also were compared with regard to their direct effects on *C. variipennis* larvae (Table 2). In these trials, time required to cause immobility was fairly comparable to mortality time, as larvae generally failed to recover when removed from the salt into water. With the early instars, the severe osmotic differential in the saturated NaCl caused the larval hemocoel to collapse. Mortality times were significantly different for the 2 salts in each instar (*t*-test, $p < 0.01$).

FIELD TRIALS. Field-collected mud subjected

Table 1. Extraction efficiency of salt solutions for larvae of *Culicoides variipennis* (30 larvae/trial) stirred into sterile mud.

Instar	Salt[1]	No. trials	Time interval[2]	$\bar{x}$ % extracted
1	$MgSO_4$	20	0–5 min	16.3
			5–10 min	16.7
			10–15 min	17.0
			Total	50.0
2	$MgSO_4$	20	0–5 min	38.0
			5–10 min	22.3
			10–15 min	11.3
			Total	71.6
3	$MgSO_4$	21	0–5 min	44.3
			5–10 min	30.3
			10–15 min	13.1
			Total	87.7
	NaCl	10	0–5 min	78.0
			5–10 min	18.7
			10–15 min	1.3
			Total	98.0
4	$MgSO_4$	15	0–5 min	74.9
			5–10 min	22.0
			10–15 min	2.7
			Total	99.6
	NaCl	20	0–5 min	92.2
			5–10 min	7.6
			10–15 min	0.2
			Total	100.0
Pupa	$MgSO_4$	10	0–5 min	92.3
			5–10 min	7.4
			10–15 min	0.3
			Total	100.0
	NaCl	10	0–5 min	96.6
			5–10 min	2.9
			10–15 min	0.0
			Total	99.5

[1] 70 cc saturated salt solution and 30 cc of mud. $MgSO_4$ only for instars 1 and 2 (see text).
[2] Slurry restirred at end of each 5 min interval.

Table 2. Mortality times of *Culicoides variipennis* larvae placed into saturated salt solutions.

Instar	Salt	No. tested	Time to death (minutes) $\bar{x} \pm$ s.d.	Range (minutes)
1	$MgSO_4$	28	28.1 ± 16.9	8–60
	NaCl	12	0.6 ± 0.2	0.5–1.2
2	$MgSO_4$	18	95.4 ± 38.5	38–170
	NaCl	15	11.7 ± 5.3	5–23
3	$MgSO_4$	23	89.3–42.3	30–165
	NaCl	20	31.1 ± 6.5	21–44
4	$MgSO_4$	14	193.0 ± 113.0	45–315 +
	NaCl	21	41.0 ± 11.4	11–58

to flotation in the 2 salts and restirred at 5 min intervals gave results very similar to the laboratory trials. The field trials differed, however, in that we were pipetting generally larger numbers of larvae from the surface of the slurry. We also were not certain how many larvae the slurry contained, and depended on sieving subsequent to the salt flotation to provide this information. On the first collection day (12 August), 54.7 ± 50.0 ($\bar{x} \pm$ s.d.) larvae/30 cc of mud were recovered in 15 min by the 2 salts; the second day (16 August), 192.1 ± 186.2 larvae/30 cc were recovered.

Results of these trials by time and instar over the 2 days are presented in Table 3. As in the laboratory trials, recovery efficiency increased with larval age. Recovery appeared to be considerably improved over the laboratory trials for instars 1 and 2. $MgSO_4$ generally resulted in recovery of somewhat higher numbers of the first 2 instars, while NaCl resulted in recovery of slightly higher numbers of instars 3 and 4; the differences, however, were not significant. Analysis of the distribution patterns of late instar recovery with time indicated that NaCl extracted these stages significantly faster than did $MgSO_4$ ($\chi^2, p < 0.05$), as was the case in the laboratory trials.

Table 3. Extraction efficiency of salt solutions for larvae of *Culicoides variipennis* in field-collected mud.

Instar	Salt[1]	# Trials	Time interval[2]	$\bar{x}$ no. extracted	% of total extracted/interval	
1	$MgSO_4$	10	0–5 min	12.5	63.1	
			5–10 min	4.2	21.3	
			10–15 min	3.1	15.7	
			Total	19.8	100.0	(80.8% recovery[3])
	NaCl	10	0–5 min	10.1	66.1	
			5–10 min	4.2	27.4	
			10–15 min	1.0	6.5	
			Total	15.3	100.0	(71.5% recovery)
2	$MgSO_4$	10	0–5 min	31.9	52.9	
			5–10 min	12.7	21.1	
			10–15 min	15.7	26.0	
			Total	60.3	100.0	(84.3% recovery)
	NaCl	10	0–5 min	44.8	68.8	
			5–10 min	16.0	24.6	
			10–15 min	4.3	6.6	
			Total	65.1	100.0	(81.2% recovery)
3	$MgSO_4$	10	0–5 min	66.9	54.3	
			5–10 min	37.4	30.3	
			10–15 min	19.0	15.4	
			Total	123.3	100.0	(92.9% recovery)
	NaCl	10	0–5 min	117.6	76.6	
			5–10 min	27.0	17.6	
			10–15 min	8.9	5.8	
			Total	153.5	100.0	(94.5% recovery)
4	$MgSO_4$	10	0–5 min	95.1	66.7	
			5–10 min	32.4	22.8	
			10–15 min	14.9	10.4	
			Total	142.4	100.0	(98.4% recovery)
	NaCl	10	0–5 min	135.5	84.3	
			5–10 min	21.2	13.2	
			10–15 min	4.0	2.5	
			Total	160.7	100.0	(98.6% recovery)

[1] 70 cc of saturated salt solution added to 30 cc of field-collected mud.
[2] Slurry restirred at end of each 5 min interval.
[3] Based on recovery of unfloated larvae with 200 mesh sieve.

Two-way ANOVA was used to examine the effects of collection day and extraction intervals (0–5 min, 5–10 min, 10–15 min) on larval recovery for each instar. The main effect of collection day was significant ($p < 0.01$) for instars 3 and 4 in both salts. This indicated that numbers of late instars, but not early instars, differed on the 2 days. The main effect of interval was significant ($p < 0.01$) for instars 3 and 4 in $MgSO_4$ and for all 4 instars in NaCl. This indicated that the 3 extraction intervals did not yield significantly different numbers of instars 1 and 2 in $MgSO_4$, but did for the other instar-salt combinations. The interaction between day and interval was significant for instars 3 and 4, but not for instars 1 and 2, for both salts. Examination of the data revealed that these late instars were extracted considerably faster on the second day (16 August) than on the first day (12 August) (Table 4).

DISCUSSION

Prior work had demonstrated that direct flotation with $MgSO_4$ would extract all 4 larval instars of *C. variipennis* successfully for voltinism and abundance studies (Mullens and Rutz 1983), but its exact efficiency was not determined. We chose to concentrate on developing a direct salt flotation method for several reasons. First, larvae of *C. variipennis* most often are found in shallow, vegetation-free sediment beds. This type of substrate is more amenable to direct salt flotation than are vegetation-rich substrates, since the plant debris often will not settle out and can screen the larvae from view. Second, we have found that sieving is an impractical way to extract the tiny first instars, which subsequently may have to be concentrated by salt flotation to be counted. Third, we felt direct flotation would be faster than other extraction methods for our conditions.

The laboratory and field trials agree in most respects. The apparent increase in recovery of the early instars (especially 1st instars) in the field mud may have been influenced by our failure to detect the tiny, dead larvae sieved out of mud samples which had already been subjected to salt flotation. In this case, the laboratory recovery rates of 50% for instar 1 and 72% for instar 2 are probably more accurate.

As regards direct application of these larval recovery data to individual field collections, the situation is less clear. Over time, they probably represent a good approximation of recovery of the different instars of *Culicoides variipennis* from silt substrates. Our 2 flotation trials on separate days, however, indicate that larvae may not be extracted with equal speed or efficiency on each day. Although the sampling was done in the same area at the same site and time of day only 4 days apart, substantial differences in larval density were evident. It is possible that larvae at higher densities may be extracted more quickly than at lower densities, but further work on this point is needed. Total recovery over 15 min did not vary as much on the 2 days; average total recoveries were within 20% of each other for each salt and instar combination.

Direct salt flotation is an efficient method of recovery for *Culicoides variipennis* immatures, made even more attractive by the relatively short period of time required to process samples. The flocculant Separan also made counting and sorting the larvae much easier, as far less sediment was removed when pipetting larvae from the surface of the slurry.

The results from both the laboratory and field trials demonstrate that larval recovery increases with age, as was the case with *Leptoconops bequaerti* Kieffer (Davies and Linley 1966). They also indicate that flotation with NaCl is a faster and more efficient method for extracting late instars. If, however, one desires to sample the entire larval population, $MgSO_4$ would be the salt of choice, due to the harsh effects of NaCl on early instars.

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Table 4. Efficiency of salt extraction for field-collected larvae of *Culicoides variipennis* on 2 different days.

Salt	Instar	Day[1]	$\bar{x}$ no. extracted/ 30 ml in 15 min	Total % recovery in 15 min	% Extracted in first 5 min
$MgSO_4$	3	1	46.4	75.9	40.1
		2	200.2	95.2	57.5
	4	1	25.8	96.3	34.9
		2	259.0	98.6	70.0
NaCl	3	1	84.0	84.7	68.6
		2	223.0	98.7	79.6
	4	1	28.0	90.3	62.1
		2	293.4	99.4	86.4

[1] Day 1 = August 12; Day 2 = August 16.

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