EXTRACTION OF LARVAE OF THE CERATOPOGONID BITING MIDGE, *CULICOIDES MISSISSIPPIENSIS*, FROM SALT MARSH SOILS WITH A NEW AGAR TECHNIQUE[1]

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ABSTRACT. Sand flotation was compared with layers of 1 and 2% (wt/v) agar for extracting *Culicoides mississippiensis* Hoffman larvae from samples of marsh soil. The 1% agar formulation extracted significantly fewer ($P<0.001$) larvae than the other 2 methods. In comparison with sand flotation, the 2% agar method produced overall a greater number of larvae, required less time to extract an equivalent number of larvae, was cleaner, and provided a quantitatively equivalent and less variable estimate of the total larval population.

Larvae of biting midges (*Culicoides* spp.) have been recovered from salt marsh substrate (soil) samples by the following methods: (1) sieve-flotation (Kettle and Lawson 1952, Wirth 1952, Kettle et al. 1956; Jamnback 1965); (2) sand flotation (Bidlingmayer 1957, Williams 1960); (3) direct flotation (Linley and Kettle 1964, Linley and Adams 1972); and (4) Berlese funnels (Jamnback and Wirth 1963, Jamnback 1965). Kline et al. (1975) found that all these methods were tedious, time-consuming, and had various other disadvantages.

Sand flotation, the most common method, is messy, and the extracted larvae are often damaged and unfit for use in rearing, insecticide, and pathogen studies. We needed a method of extracting healthy larvae that would be cleaner and quicker than sand flotation. Roberts (1966) and Kettle et al. (1975), respectively, successfully reared in agar media tabanid and culicoid larvae, so we decided to replace the sand with a layer of non-nutrient agar. The agar would allow the *Culicoides* larvae to move freely, and perhaps they would move from the soil samples up into the agar layer. If so, then the agar layer could be removed to an examining pan and broken apart in water, thereby freeing the larvae for easy removal with a pipette.

To test this possibility we collected several soil samples from known *Culicoides* breeding habitats at Yankeetown, Florida. A 0.8% (wt/v) agar solution was autoclaved for 15 min at 15 psi, allowed to cool to ca. 47°C, and then poured on to soil samples held in ca. 1-liter plastic containers. Three hundred ml of the solution were poured ca. 1 cm thick on each sample. After the agar gelled, the containers were covered and allowed to stand for 24 hr. When the lids were removed, several larvae, even though they are photonegative, were observed crawling on the surface. For easy removal of trapped larvae, the agar surface was rinsed with a small amount of filtered estuarine water, which was decanted into a pan; the larvae were then removed with a pipette. Most larvae remained in the agar layer and, as planned, were removed when this layer was broken apart. Unfortunately, this technique required considerably more time than sand flotation. However, the fact that some larvae were trapped on the surface of the 0.8% agar suggested that, with agar of optimum consistency, all the larvae in the substrate might crawl to the surface for easy recovery. We therefore, compared sand flotation with agar at 2 concentrations (1 and 2%). We counted the extracted larvae, observed their condition, and estimated larval densities.

MATERIALS AND METHODS

FIELD-COLLECTED SAMPLES. To test the feasibility of the agar techniques for

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routine sampling, we collected marsh soil samples from Yankeetown, Florida, during the winter and spring of 1978. With post hole diggers we took soil cores of ca. 0.8 liter (ca. 10 cm diam. × ca. 8 cm deep). Three adjacent samples were taken from areas that consistently produced many larvae of *Culicoides mississippiensis* Hoffman. Each sample was transported to the laboratory in a closed plastic container (16 cm diam x 11 cm deep). At the laboratory the soil was compacted to eliminate air pockets. A sample, chosen randomly, was used for each of the following treatments:

1. *Sand flotation method.* Samples in the field collection containers were covered with ca. 5 cm of prewashed sand, saturated with filtered estuary water. Lids were replaced and allowed to stand at room temperature (ca. 21°C) for 24 hr. Then the sand layer was removed to a 1-liter container. Saturated magnesium sulfate solution (500 ml) was added and the mixture thoroughly agitated. Then the sand was allowed to settle and the liquid was decanted into a black-painted porcelain enameled pan for examination. Because of the high specific gravity of the $MgSO_4$ solution, many of the white larvae floated on the surface and were easily seen against the black background; they were removed with a pipette. After removal of all visible larvae, the solution was returned to the container of sand and the mixture reagitated. This process was repeated until 3 consecutive negative collections occurred. With a stopwatch we measured actual processing time, from removal of sand from the container until the last larva was recovered. Each sample was then covered with a fresh layer of sand, saturated with filtered estuarine water and the whole process repeated. This procedure was repeated daily until no larvae were recovered for 2 consecutive days, or for a maximum of 9 days.

2. *Agar method.* We compared two agar concentrations (1 and 2%) to determine whether concentration significantly affected the number of larvae that moved up into the agar layer. Soil samples were retained in their field containers. Agar was prepared and allowed to cool to about 47°C, and then 300 ml of either 1% (wt/v) or 2% agar were poured onto the samples. After the agar gelled the container lids were replaced. After 24 hr, 200 ml of filtered estuarine water were poured onto the surfaces, swirled back and forth several times, and then decanted into a black-painted porcelain enameled pan. Larvae were removed with a pipette. After removal of all visible larvae, the 200 ml of estuarine water was again poured into the container and the process repeated until 3 consecutive trials were negative. Actual processing time, from the moment water was first poured onto the agar until removal of the last larva, was recorded. This procedure was repeated daily until no larvae were recovered for 2 consecutive days or for a maximum of 9 days.

Laboratory samples. Field-collected samples, which contained unknown numbers of larvae, were unsuitable for tests of the relative efficiency of each method. Therefore, plastic laboratory containers were set up with known numbers of larvae. The samples contained 25 (5 replicates) or 50 (5 replicates) 3rd or 4th stage larvae which were added to specially prepared soil in the plastic containers. For each container, 254 grams of field-collected marsh soil was air-dried and then held at 50°C for 24 hr to assure that no viable *Culicoides* eggs or larvae remained. The soil was then saturated with filtered estuarine water and allowed to stand for 24 hr. Then the *Culicoides* larvae were added and *Panagrellus* sp. nematodes were liberally provided as food. Three treatments (sand flotation, 1 and 2% agar) were set up and processed exactly as the field-collected samples were.

RESULTS AND DISCUSSION

Larvae recovery. Recovery (number and percentage) of larvae was greatest from the 2% agar (Table 1). Analysis of variance (ANOVA) of these data by sample source was not significant for the field-collected material but was for the

Table 1. Recovery of *Culicoides mississippiensis* Hoffman larvae from field-collected marsh soil samples and laboratory samples.[a]

Method	Mean recovery of larvae from: Field samples[b] (No. collected)	Laboratory samples[c] (% recovered)
1% Agar	52.4a	43.4b
2% Agar	73.3a	88.1a
Sand flotation	67.0a	79.3a

[a] Means in the same column followed by different letters are different at the 0.05 level (Ducan's multiple range test).
[b] Based on 40 samples.
[c] Based on 5 samples with 50 larvae and 5 samples with 25 larvae.

laboratory samples ($p \leq 0.001$). Possibly differences were nonsignificant for field samples because of the high variability introduced by the heterogeneity of soil and numbers of larvae. The 2 techniques, sand flotation and 2% agar, that recovered the large numbers of larvae, did not differ significantly ($P = 0.05$, Duncan's multiple range test) for either field or laboratory samples.

Possibly the difference in recovery of larvae from laboratory samples between 1% agar and the other techniques was related to the properties of the agar. We speculate that the agar layer interferes with oxygen exchange between the soil and air, and that the interference was greater by 2% than by 1% agar. Possibly low oxygen or even anaerobic conditions in the soil force the larvae to move upward, penetrate the agar, and eventually burrow through to the surface. Larval penetration of the soil-agar interface is probably achieved by the larvae using the denser substrate to push against. The photonegative larvae then could become trapped on the agar surface, even after light enters when the container lid is removed, because they could not reenter the agar layer due to the excessive surface tension and because the air-agar interface would not provide them with any dense substrate to push against. Only those larvae close to a burrow were able to escape; the others were easily rinsed off the surface.

Daily recovery rates (Table 2) were greater for 2% than for 1% agar, and recovery was more extended (9 vs. 5 days). Perhaps 1% agar was a less effective barrier than 2% to oxygen exchange, and fewer larvae left the soil. Deterioration of the 1% agar gel also might be a factor. Both agar gels deteriorated (i.e., became mushy) progressively; 1% agar broke down in only 3–5 days, and 2% agar lost its firmness after 10–14 days. We speculate that the softer 1% agar allowed easier access to the photonegative larvae when the container lid was removed, especially with time, than the 2% agar. In order to make this determination, several 1% and 2% agar layers from the field-collected samples were removed and examined after their allotted sampling time had elapsed. Many larvae were consistently recovered from the 1% agar layers, but only an occasional larva from the 2% agar layers.

TIME REQUIREMENTS. There are 4 basic steps required to obtain biting midge larvae: (1) collection of soil samples; (2) preparation of samples for larval extraction; (3) actual processing; and (4) clean-up. The time required is constant

Table 2. Recovery of *Culicoides mississippiensis* Hoffman larvae by sand and agar methods (cumulative % recovery in parentheses) from laboratory samples.

Sampling day	Percent recovery by indicated method: Sand flotation	1% Agar	2% Agar
1	72.1 (72.1)	27.4 (27.4)	42.2 (42.2)
2	6.8 (78.9)	10.4 (37.8)	29.8 (72.0)
3	0.4 (79.3)	2.6 (40.4)	7.4 (79.4)
4	0.0 (79.3)	1.4 (41.8)	3.4 (82.8)
5	0.0 (79.3)	1.6 (43.4)	2.6 (85.4)
6		0.0 (43.4)	1.4 (86.8)
7		0.0 (43.4)	0.6 (87.4)
8			0.3 (87.7)
9			0.4 (88.1)

for collection of the samples but varies with method for the other 3 steps.

The actual preparation time required per sample was ca. 1–2 min for sand flotation and ca. 30–45 secs. for the agar methods. The difference in time was that compaction of the soil samples can be done while the agar is cooking and cooling, but with the sand flotation, compaction and addition of sand cannot be done simultaneously. The importance of this time differential increases with the number of samples.

Actual processing of the samples can be sub-divided into 3 steps: (1) removal of larvae from container; (2) picking larvae from the enameled pan; and (3) resetting those samples that are to be continued in the test. The time is constant among methods for step 2 so any time differential between methods is caused by steps 1 and 3.

In the flotation method the sand layer is transferred to another container where a flotation liquid is added. Transfer, shaking, and decanting requires ca. 2–3 min per sample, whereas the agar surface can be rinsed in ca. 30 secs. Regardless of method, each sample may require the process to be repeated 6 times before the necessary 3 consecutive negative observations occur. That could bring the difference between methods to 12 min per sample. In our ecological study of larval habitats, we routinely process 50 samples per wk. In the flotation method resetting of samples takes extra time; on each day a soil sample was used in this comparative study, it was covered again with a new layer of sand that was then saturated with filtered estuarine water. This step required 2–3 min per sample. In contrast, agar layers once established lasted throughout the study.

Clean-up requires at least twice as long for the sand flotation as for the agar method. The salt solution and sand inevitably end up all over the work area, on the processor's clothes, and on the floor. With the agar method, only water is spilled.

Analysis of variance of actual processing time (excluding resetting time) for the laboratory studies showed a highly significant difference ($p \leq 0.001$) between the mean times required per sample by the sand flotation and agar techniques. Mean time required significantly ($P = 0.05$, Duncan's multiple range test) differed among all 3 treatments (13.3, 18.1, and 23.7 min., respectively, for 1% agar, 2% agar, and sand flotation).

QUALITY OF LARVAE. Larvae recovered by the agar method appeared to be healthier and had a lower mortality rate (<1% at 24 hr collection) than larvae extracted by sand flotation (ca. 6% mortality). Therefore, we thought that the larvae recovered by agar were more desirable for use in our rearing, insecticide, and pathogen studies.

ESTIMATION OF NATURAL POPULATIONS. If a particular technique recovers a certain proportion of the total larvae within a sample, then the inverse of that proportion times the number recovered would provide an estimate of the total number within the sample. Table 3 presents the mean percentage recovered after 1 day of extraction by sand, 1 and 2 days of extraction by agar, the standard error of the mean ($S\bar{x}$), the inverse of the percentage recovered (multiplier) and the 95% confidence interval about the multiplier. For

Table 3. Variability of population estimates based on sand flotation or 2% agar techniques.

Technique	Mean recovery rate (%)	$S\bar{x}$	Multiplier	Multiplier fiducial limits (95%)[a]
Sand flotation (1 day)	72.1	6.6	1.39	1.15–1.75
2% Agar (1 day)	42.2	4.5	2.37	1.91–3.13
2% Agar (2 days)	72.0	2.2	1.39	1.30–1.47

[a] Based on t_9, .05 = 2.262 and laboratory samples.

example, multiplication of the number of larvae actually recovered by the sand flotation method for 1 day by 1.39 provided an estimate of the total number of larvae in the sample. Data obtained by the 2% agar method varied less than that obtained by sand flotation. Variability of data by the 2% agar method was reduced further by processing the samples for 2 consecutive days (Table 3). Our data also showed that a 2-day extraction with 2% agar recovered the same percentage of larvae (72%) as sand flotation in 1 day, but only required ca. 62% as much actual processing time.

Thus, the 2% agar method outperformed the 1% agar and sand flotation methods of extraction for *Culicoides* larvae. In comparison with the other methods, the 2% agar method recovered a greater number of larvae (Table 2), required less actual processing time to obtain the same number of larvae, produced healthier larvae, was cleaner, and provided a less variable estimate (Table 3) of larval population densities.

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