INTERACTIONS BETWEEN MOSQUITO LARVAE AND MUCILAGINOUS PLANT SEEDS. III. FACTORS INFLUENCING ATTACHMENT OF LARVAE TO SEEDS AND THEIR SUBSEQUENT MORTALITY [1]

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ABSTRACT. Various factors affecting attachment of mosquito larvae to mucilaginous plant seeds have been investigated together with the effects of these factors upon larval mortality. It has been shown that heat-killed seeds are able to entrap and cause the death of larvae after either brief or prolonged soaking and that sequential soaking and drying of the seeds did not remove their ability to subsequently entrap larvae. The pH of the medium in which attachment trials were performed influenced the percentage of larvae becoming entrapped. The attraction of larvae to seeds decreased with increasing age of larvae suggesting that the decreased attachment of larvae with age may have a basis in something other than mere physical size. Further attraction tests indicated that larval populations were homogenous with regard to individuals' responses to chemoattractant.

INTRODUCTION. The original observation of Reeves and Garcia (1969) that mosquito larvae, under laboratory conditions, become attached to certain species of mucilaginous plant seeds and subsequently died was investigated by Barber *et al.* (1974) to determine the chemical basis for this attachment. Page and Barber (1975) were then able to show that such larvae do not merely happen to attach to seeds as a result of random movement but that the seeds possess a chemoattractant that evokes a positive chemotaxis in the larvae. Implicit in each of the papers noted above was the idea that these mucilaginous seeds could possibly provide a biological control mechanism for mosquito larvae. The desirability of such a mechanism is obvious and while the efficacy of this particular mechanism is readily demonstrated in the laboratory, its practicality in the field is less clear at present.

It is, of course, just as important that pollution be minimal for biological control as for chemical control. The use of live seeds would constitute a form of biological pollution even though the seeds in question might come from plants which form a natural part of that particular ecosystem. Thus, it would be of some benefit if dead seeds were as effective as live ones, at least with regard to their entrapment of larvae.

Under field conditions it would be expected that the seeds would encounter a wide range of environmental conditions. These would include exposure to periods of both brief and prolonged soaking or drying and also random soaking and drying sequences. Similarly, the pH of the field water might be expected to vary from location to location and could directly influence the attachment of larvae to seeds. In terms of larval factors, the data of Reeves and Garcia (1969) obtained using 2nd and 4th instar larvae suggested that age was a determining influence in the entrapment of larvae by seeds. Whether this effect was due to the increasing size of the larvae with age, to decreasing positive chemotaxis or to other factors was not demonstrated. However, it is always possible that within any larval population, a certain percentage of the individuals show a positive response to the chemoattractant while the remainder are either indifferent to or are repulsed by it.

Inasmuch as each of the above factors could be expected to have some part in

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determining the efficacy of mucilaginous seeds in entrapping and killing larvae in the field, it was deemed appropriate to investigate them with a view to assessing the potential practicality of the use of mucilaginous seeds as a biological control mechanism for mosquito larvae, at least, insofar as certain field conditions could be tested in the laboratory. It is the results of such a study that are to be presented in this report.

MATERIALS AND METHODS. The seeds used in this study were *Capsella bursa-pastoris* (L) Medic (shepherd's purse) and were generously provided by Dr. W. H. Tallent of the Industrial Crop Laboratory, U.S.D.A., Peoria, Illinois. Live seeds were used as received; dead seeds were obtained by heat-killing at 130° C for 30 minutes.

Culex pipiens quinquefasciatus Say larvae were either collected in the greater New Orleans area or were raised from egg rafts kindly supplied by Drs. Harold C. Chapman and J. J. Petersen of the Gulf Coast Mosquito Research Laboratory, U.S.D.A., Lake Charles, Louisiana. Larvae were reared in aerated spring water and were fed ground Purina Dog Chow.

To determine the effect of the age of the larvae upon their attraction to *C. bursa-pastoris* seeds the bioassay methods of Page and Barber (1975) were used. These procedures were employed for very young larvae (1st instar), "middle-aged" larvae (2nd and 3rd instar) and "old" larvae (4th instar). All larvae had been raised from the same egg rafts and were therefore uniform with regard to origin. Two hundred larvae of a particular age were used in each attraction assay and all experiments were run in duplicate. Following each test using a particular age of larvae, those larvae which had been attracted (i.e. those in the +2 and +1 zones together with half of those from the starting zone) were removed using a pasteur pipette and were retested as a positive group exactly as before. Similarly, those larvae which had been repelled (i.e. those in the —2 and —1 zones together with the other half of those from the starting zone) were removed and retested as a negative group.

All measurements of the attachment of larvae to seeds and of their subsequent mortality were performed in 20 ml scintillation vials containing 15 ml of incubation medium (usually water but buffer, 0.1M $NaHPo_4$/0.5M citric acid, pH 2 to pH 8 in 1 pH unit increments, in the experiment involving the effect of pH upon larval attachment), 10 *C.p. quinquefasciatus* larvae of appropriate age and 10 *C. bursa-pastoris* seeds (live or heat-killed). The seeds and larvae were incubated together for various periods of time, and the numbers of larvae attached to seeds were recorded and also the numbers of dead larvae. Each experiment was performed in replicate and larvae which pupated during an experimental time period were discounted.

Using these methods, the effect of heat-killing seeds upon larval attachment and mortality was investigated over a 72 hr period during which time the seeds and larvae were incubated together.

The effect of presoaking the seeds, prior to their incubation with larvae, was similarily investigated. The preliminary results indicated that periods of up to 48 hr of presoaking of seeds did not significantly alter their ability to subsequently entrap and kill larvae. Therefore, using heat-killed seeds, to preclude germination, presoaking times of 1, 4, 7, 10 and 21 weeks were employed followed by incubation of larvae with the presoaked seeds; the percentages of larvae attached to seeds and the percentage of larvae dead were recorded after 24, 48, and 72 hours of incubation following the various presoaking periods.

The effect of sequential soaking and drying of the seeds upon their ability to entrap larvae was also tested. One such sequence consisted of 24 hr of soaking followed by 48 hr of drying at 27° C, followed by incubation of seeds with larvae for 24 hr, after which the number of larvae attached was recorded. Seeds were tested using this procedure after 1, 2, 3, 4 and 5 soaking and drying sequences. In addition to the above controlled sequences, one group of seeds was subjected to a random soaking and drying sequence covering 4 months; these seeds were then tested for entrapment of larvae.

All results obtained using treated seeds were compared with those obtained using untreated seeds.

RESULTS. The results given in Table 1 show an inverse relationship between larval age and positive chemoattraction. As the larvae became older their positive chemotactic response to *C. bursa-pastoris* seeds decreased. Thus, newly-hatched, 1st instar, larvae showed a 94% attraction (i.e. 94% of those larvae tested ended up in the +2 plus the +1 zones plus half of the number in the 0 zone). The "middle-aged," 2nd or 3rd instar, larvae showed a 79% attraction whereas the "old," 4th instar, showed only 64% attraction.

In any one age group, the larvae which had been attracted in the initial attraction test (the positive group) were retested under the same conditions. Similarly, those which had not been attracted in the initial tests (the negative group) were retested. Both positive and negative groups of larvae of all ages showed overall distributions, when retested separately, similar to those of the original groups in the initial tests (Table 1).

The effect of pH of incubating medium upon the attachment of larvae to seeds is shown in Fig. 1. It can be seen that, in the pH range studied, two optimal pH values exist for attachment of larvae to seeds, one at approximately pH 3 and the other at approximately pH 6. However, while a minimum in attachment can be seen at pH 4.5-5.0, the value obtained at that pH still represents a significant amount of attachment and it is suggested that only at very low or high pH's does the attachment of larvae to seeds become significantly decreased.

It can be seen from Table 2 that the heat-killing of seeds reduced their ability to entrap larvae very little. Thus, after 24 hr incubation, the heat-killed seeds had 14% fewer larvae attached than had the live seeds, after 48 hours they had 4% fewer and by 72 hr they equalled the live seeds in numbers of larvae attached. It should perhaps be noted that attachment of larvae, to untreated seeds at least, was not completely irreversible since the numbers of larvae attached after 48 and 72 hr of incubation respectively were smaller than 24 hr. The effect of heat-killed seeds on the mortality of larvae was slightly greater than it was upon the attachment of larvae; it was most evident over the shorter incubation periods (14% and 20% less than untreated seeds at 24 hr and 48 hr, respectively) but by 72 hr was more similar to the untreated seed kill, (cf. Table 2). It appears, therefore, that in the process of heat-killing, the seeds lost some of their ability to entrap larvae and also some of their larval-killing properties, but that in neither case did the loss amount to more than 10% of the untreated seed value obtained following a 72 hr incubation period.

Using heat-killed seeds, it was shown that relatively long periods of immersion in water did not eliminate the seeds' ability to subsequently either entrap or kill larvae (Table 3). While the results were not as internally consistent as one would have wished, it is perhaps not surprising when one considers the periods of time involved and the attendant problems of using "identical" larval populations throughout. Nevertheless, certain trends are evident; with increasing length of presoaking of seeds the attachment of larvae was reduced from approximately 80% to approximately 60% and the mortality of larvae was reduced by approximately 50%. The effect of presoaking of seeds was therefore greater upon the mortality of larvae than it was upon their attachment but in both cases, even following 21 weeks of pre-soaking, the seeds were still capable of entrapping and killing significant numbers of larvae (cf. Table 3).

The effect of sequential soaking and drying of seeds upon their ability to subsequently entrap larvae appeared to be relatively small. Thus, even after 5 sequences of soaking and drying covering 15

200 1st instar larvae

+2	+1	0	-1	-2
122	60	12	6	0

94% 6%

Retested

+2	+1	0	-1	-2
150	30	8	0	0

98% 2%

+2	+1	0	-1	-2
7	4	1	0	0

96% 4%

200 2nd and 3rd instar larvae

+2	+1	0	-1	-2
88	50	40	18	4

79% 21%

Retested

+2	+1	0	-1	-2
87	32	25	9	5

82% 18%

+2	+1	0	-1	-2
25	6	6	3	2

83% 17%

200 4th instar larvae

+2	+1	0	-1	-2
81	28	36	31	24

64% 36%

Retested

+2	+1	0	-1	-2
38	27	19	22	21

59% 41%

+2	+1	0	-1	-2
30	9	14	9	11

63% 37%

Table 1. The effect of age of larvae upon their chemotaxis and effect of previous exposure of larvae to chemoattractant, (200 larvae each, of different instars).

The numbers shown in the lower half of each box represent the percent of larvae occurring in the various zones (+2, +1, 0, 1—2) of the assay chamber. In all instances the seeds were contained in the area immediately to the left of the +2 zone.

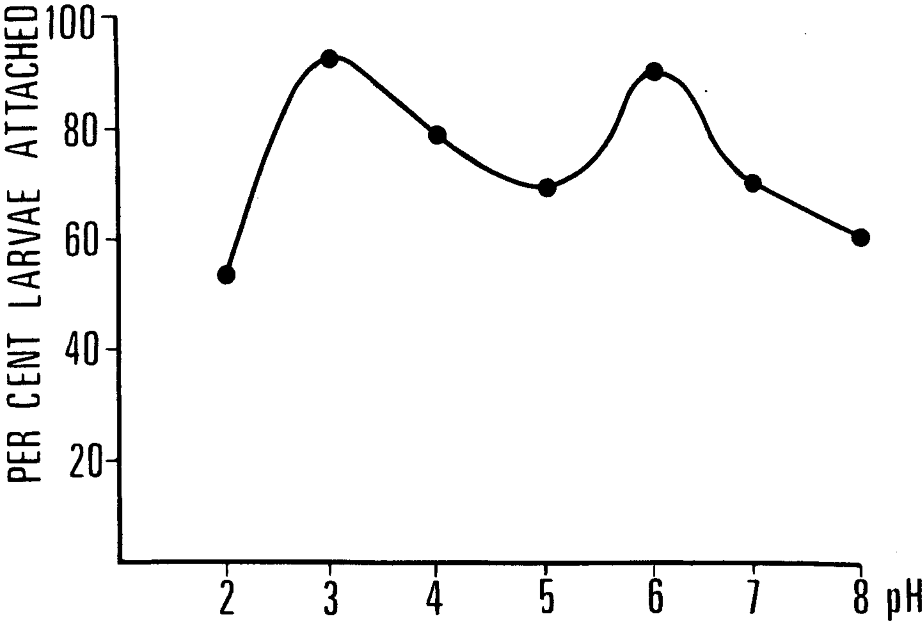

Fig. 1. Effect of pH medium upon the attachment of *C. p. quinquefasciatus* larvae to seeds of *C. bursa-pastoris*. Each point represents the mean of five replicates: incubation time of 6 hours.

days, the seeds were still able to entrap 72% of the larvae within 24 hours of incubation; this value compares favorably with the 72% and 86% values obtained using heat-killed, but otherwise untreated seeds (cf. Tables 2 and 3, respectively). However, when seeds were subjected to a random soaking and drying sequence covering 4 months, their ability to entrap larvae was reduced to 30% during a 24 hr incubation period (cf. the 72% and 86% values of Tables 2 and 3, respectively).

DISCUSSION. The various experiments described on the preceding pages were designed to test the effects of factors that could be expected to influence the entrapment (and mortality) of mosquito larvae by *C. bursa-pastoris* seeds in the field. To summarize, in advance, no results were obtained that would suggest that the seeds would be unable to entrap and kill larvae under a variety of field conditions. Certain factors had a quantitative effect but none was able to eliminate the ability of the seeds to entrap and kill larvae.

With regard to larval factors, the results of Table 1 show that as larvae proceed through successive instars they become less responsive to the seed chemoattractant. These results presumably reflect a greater nutritional requirement in the earlier instars; they also possibly shed some light on the observation of Reeves and Garcia

Table 2. Comparison between heat-killed and untreated *C. bursa-pastoris* seeds with regard to their ability to entrap and kill larvae of *C. p. quinquefasciatus* (5 replications).

	Incubation for:		
Seed treatment	24 hr.	48 hr.	72 hr.
	Percent attachment		
Heat-killed	72	74	80
Untreated	86	78	80
	Percent mortality		
Heat-killed	8	34	74
Untreated	22	54	84
No seeds	0	8	16

Table 3. The effect of pre-soaking, for various periods of time, heat-killed seeds of *C. bursa-pastoris*, upon their ability to subsequently entrap and kill *C. p. quinquefasciatus* larvae, (5 replications).

Seed Treatment	Percent attachment (a) and mortality (m) of larvae, incubated for the indicated periods of time, with seeds which had been pre-soaked for a varying number of weeks:					
	24 hr.		48 hr.		72 hr.	
	a	m	a	m	a	m
Seeds absent	–	0	–	8	–	16
No pre-soak	86	22	78	54	80	64
1 wk. pre-soak	60	8	60	30	62	54
4 wk. pre-soak	62	6	66	16	66	32
7 wk. pre-soak	60	2	68	14	64	30
10 wk. pre-soak	70	2	82	18	86	30
21 wk. pre-soak	40	10	66	26	72	32

(1969) that, in general, mucilaginous Cruciferae seeds were more effective in entrapping and retaining 2nd instar larvae than 4th instar larvae. While this observation could be interpreted in terms of the physical size of the larvae (i.e. fewer large larvae could "fit" on the relatively small seeds and those which did were more liable to be big enough and strong enough to escape) the results presented here indicate that the answer may lie in the indifference to chemoattractant that the larvae appear to acquire as they age. It is also true that these results draw attention to a source of experimental variation in studies such as this, since it would be extremely difficult to guarantee using the identical developmental stage of larvae in all experiments.

Just as the larval population in the field could be expected to vary if only with regard to the age of individuals, so too will field conditions such as the pH of the water in which the larvae are found. The results of Fig. 2 show that the seeds were effective, in terms of attachment, throughout the pH range tested, though there were maxima in attachment at pH 3 and pH 6. It is tempting to suggest that the increase in attachment in going from pH 5 to pH 3 represents protonation of carboxyl groups in the mucilage of the seeds (cf. Barber *et al.*, 1974), which results in an increased negative charge and an increased larval-seed attachment. Indeed the pH range used was selected with the thought that it might yield some insight into the mechanism of larval-seed attachment, though it was also recognized that it did not accurately represent the range in pH's of the habitats in which mosquitoes normally breed (e.g. Petersen and Chapman, 1970). It is evident, however, that the attachment process is a complex multifactorial one which presumably involves both the seeds and the larvae.

With the knowledge that heat-killing the seeds did not significantly diminish their efficacy with regard to the entrapment and killing of mosquito larvae (Table 2), a series of experiments involving pre-soaking and soaking and drying of dead seeds was conducted to determine the effects of these treatments upon larval entrapment and mortality. These treatments correspond to conditions which might be imposed upon the seeds in the field. Sequential soaking and drying had no effect upon the seeds' ability to entrap larvae, at least following treatment with 5 such cycles covering approximately 2 weeks. However, when seeds were treated with a random soaking and drying sequence covering 4 months, only a 30% entrapment value was obtained. This may relate to a loss or chemical alteration of seed chemoattractant with repeated soaking and drying. Alternatively, it could involve a gradual loss of structural integrity of the mucilage to the point that it was no longer able to retain entrapped larvae. It is, however, remarkable that, even following 4 months of this relatively harsh treatment, the seeds still possess a recognizable pellicle and retain some ability to entrap larvae.

The results of pre-soaking seeds for varying periods of time, with no intermittant drying, indicate that while the seeds retain the ability to entrap larvae, they gradually lose the ability to kill the entrapped larvae (Table 3). One explanation could be that with prolonged soaking the seeds lose the toxic factor that they appear to possess (Page and Barber, 1974). Supavarn *et al.* (1974) have shown that adult plants of certain members of the Cruciferae contain materials with larvicidal properties. Thus, while a live seed might contain toxin-like components which are continuously produced as the seed germinates and the plant grows and develops, dead seeds would contain only a finite amount of toxin-like component(s) which would be progressively leached out in water. Alternatively prolonged soaking could lead to gradual deterioration of the mucilage until it is unable to irreversibly entrap larvae.

Extrapolating from the results of this investigation, it appears to be theoretically possible to disperse heat-killed *C. bursa pastoris* seeds in potential mosquito breeding sites having a wide range of pH values. These seeds would be resistant to prolonged soaking and/or repeated desiccation and rehydration and would subsequently still be able to entrap larvae when they first appeared in the environment and when they were most susceptible to the seeds' chemoattractant and to being entrapped and killed by the seeds. Many other environmental factors, affecting seed-larvae interactions, remain, even though the efficacy of *C. bursa-pastoris* seeds with regard to the mortality of *C. p. quinquefasciatus* larvae in the laboratory has been indisputably proven. Only following a consideration of all possible factors and rigorous field tests will it be possible to assess the value of these seeds as an actual means of biological control of mosquito larvae.

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