Egg Extraction Methods for *Bagrada hilaris* (Heteroptera: Pentatomidae) with Notes on Ovipositional Preferences[1]

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Abstract The ovipositional preference of *Bagrada hilaris* (Burmeister) was evaluated, and egg extraction methods from soil were compared. In a choice test between soil, plant material, and exposed plastic surfaces, females laid eggs only in the soil. Significantly more eggs were deposited in dry soil than in most soil. A significant preference for ovipositional depth within the soil was found, with the greatest proportion of eggs laid in the upper one-third (i.e., 0–0.4 cm) of the soil. Two egg extraction methods (i.e., wash and flotation) were compared for effectiveness, sampling time, and cost of setup. The wash method consisted of two types (wash methods 1 and 2, with or without a paint strainer, respectively), whereas the flotation method consisted of several solutions. The wash method (both types combined) was more effective in egg extraction than the flotation method from both small and large volume samples, but the two types differed from each other; method 1 had a higher recovery rate than method 2 for small soil volumes but a lower recovery rate than method 2 for large volumes. Total sampling time was shorter for the wash method than the flotation method and less expensive.

Key Words *Bagrada hilaris*, egg, extraction, oviposition, preference

Bagrada hilaris (Burmeister) is an Old World insect that recently has invaded the New World. Since arriving in California in 2008 (Arakelian 2010), it has become established as far east as Texas and as far south as northern Mexico (Bundy et al. 2018, Torres-Acosta and Sanchez-Peña 2016). It also has been reported from Hawaii (Bundy et al. 2018, Matsunaga 2014) and Chile (Faúndez et al. 2016, 2017).

Throughout its range, *B. hilaris* is an important pest of numerous vegetable crops, particularly among members of the Brassicaceae. In the Old World, it is one of the most important pests of oilseed brassica crops (Bundy et al. 2018, Sachan and Purwar 2007). In the New World, it quickly has emerged as one of the most important pests of cole crops, particularly in California and Arizona (Bundy et al. 2018, Palumbo et al. 2015).

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Bagrada hilaris deposits its eggs in a variety of places, including on host plants (stems, leaves, inflorescences, and seed pods), in detritus, on soil beneath plants, and in cracks in soil (Arakelian 2010, Batra 1958, Ghosal et al. 2006, Halbert and Eger 2010, Perring et al. 2013, Pruthi 1946, Rakshpal 1949, Reed et al. 2013).

During a study of the life history of *B. hilaris* on mesa pepperwort, *Lepidium alyssoides* A. Gray, in southern New Mexico by Taylor et al. (2015), eggs never were recovered from plants in the field. However, Taylor et al. (2014) reported that females display an unusual ovipositional behavior by depositing their eggs singly below the soil surface. Those eggs, when extracted from the soil, are covered with a layer of soil particles that adhere to the chorion (Taylor et al. 2015). As part of the process of describing the ovipositional behavior (Taylor et al. 2014) and the life history (Taylor et al. 2015) of this insect, several experiments were conducted to determine (1) ovipositional preference (i.e., ovipositional substrate, depth of egg deposition in soil, and soil moisture) and (2) effective methods of egg extraction from the soil. Presented here are the results of this study.

Materials and Methods

Ovipositional preference. Petri dishes (8.6-cm diameter × 1.2-cm depth) were used as ovipositional arenas (Figs. 1A–C). These dishes, called BD Falcon compartment dishes or I-plates (Corning, NY), are split by a vertical plastic divider separating the arena into two equal halves. The arenas were modified by adding a thin layer of hot glue to the edge of the inner margin of the lid. The glue raised the lid approximately 5 mm above the top of the vertical divider, allowing the insects to roam freely on either side of the divider but preventing escape. Field-collected, nonhomogenized soil (35.0 g) was added to each half of the dish, and 2.5 ml distilled water was added to one half on day 1, thus producing moist and dry halves. Soil moisture content was calculated by weighing 35.0 g of soil to which 2.5 ml of distilled water was added (moist) or not (dry) and then placing the samples in a drying oven at 54.4°C for 5 days before weighing again. The following formula was used for percent moisture content (MC): $MC = (M–D)/D$ where M = weight of moist soil and D = weight of dry soil. The percent moisture content was approximately 9.0% and 1.0% for moist and dry soil, respectively. A leaf (approximately 2.5 cm) of organically grown kale, *Brassica oleracea* D.C. (Acephala group), was placed horizontally on the divider of the dish (one half was over the moist soil and one half over the dry) and replaced every other day. A field-collected mating pair of *B. hilaris* was added to the center of each leaf, and a strip of parafilm (Bemis NA Oshkosh, WI) was applied as an outside seal between the lid and bottom of the dish to prevent drying of the soil (Fig. 1B). Dishes were placed in an incubator at 25 ± 0.01°C under a photoperiod of 14:10 (light:dark) h. The arenas were checked daily for the presence of eggs on the surface of the soil, plant material, and petri dish with the aid of a dissecting microscope. Eggs were removed and recorded. After 5 days, the insects and plant material were removed, the soil divided into six even sections, top (depth, 0–0.4 cm), middle (depth, 0.5–0.8 cm), and bottom (depth, 0.9–1.2 cm) thirds for wet and dry soil, respectively, by carefully scraping each layer into a separate dish. Each layer was then washed through a handheld strainer fitted with mesh material (mesh size, 0.60 mm) to extract the eggs. The soil remaining in the

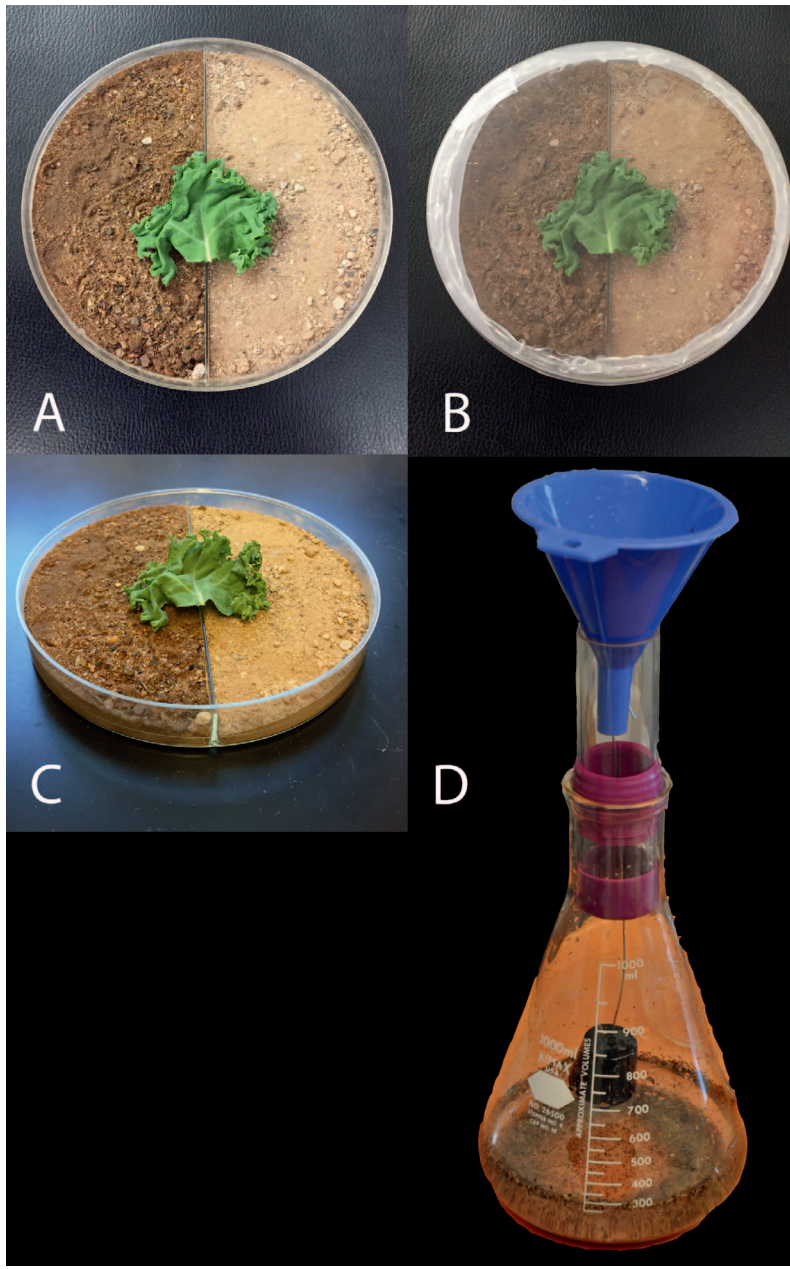

Fig. 1. Ovipositional arena and Erlenmeyer flask set up for egg preference and egg flotation studies, respectively, of *B. hilaris*. (A, C) ovipositional arena, lid removed; (B) ovipositional arena, lid sealed; and (D) Erlenmeyer flask.

strainer after each layer was processed was washed into a dish and examined for eggs. The number of eggs in each layer then was recorded. Eight trials were conducted with five replications ($n = 40$). Previous work indicated that eggs are deposited in dry soil (Taylor et al. 2014, Taylor, unpubl. data), and we are aware of no evidence from the literature that they lay eggs in moist soil. Therefore, we felt it artificial and a poor use of limited resources to set up a true no-choice study (e.g., separate runs of the experiment using moist only and dry only soil). Moist soil was added as a curiosity. Our primary interest was depth of egg deposition.

Egg extraction. A colony of field-collected adult *B. hilaris* was maintained in an ovipositional cage in an incubator under the same conditions as those used for the ovipositional preference study (see above). The cage consisted of a terrarium (61-cm length × 32-cm width × 41-cm height) lined with paper towels and provided with leaves of organic kale (15–30-cm length). The leaves were inserted into a block of floral foam (7-cm length × 7-cm width × 5.5-cm height), which had been placed in a small plastic cup (350 ml) filled with distilled water and covered with paper towels to avoid spillage of excess water. The females were provided with nonhomogenized soil and strips of cheesecloth for oviposition. Soil was removed daily and rinsed through a fine mesh material (mesh size, 0.60 mm), and the remaining debris was placed into a sorting pan with water and examined for eggs. Cheesecloth strips, paper towels, and leaves also were examined for eggs. All eggs were gently removed with a fine-tipped paintbrush. The eggs then were placed in a petri dish on moistened filter paper and kept in refrigeration (approximately 1.6°C) until needed for the extraction experiment. A distinction was made between eggs laid in soil, which had a layer of soil particles adhering to the chorion (hereafter called "covered eggs"), and those laid on paper towels, leaves, or cheesecloth, which lacked soil particles ("clean eggs"). These soil particles potentially could affect the ability of the egg to float. Because the insects prefer to deposit eggs in soil (see Taylor et al. 2015 and this paper), the use of clean eggs is somewhat artificial, and an emphasis was placed on eggs that had been extracted from the soil (i.e., covered eggs).

Matteson (1966) described a technique using a wash method combined with a salt solution flotation to extract eggs from soil, and Heilman et al. (1983) described a simple wash method to extract insect larvae from soil. These methods were modified to develop flotation (multiple solutions) and wash methods (see below) for *B. hilaris*, which were tested and assessed according to percent egg recovery rate.

Both flotation and wash methods included two soil volumes: (1) small (approximately 268.0 g [one cup]; 20 reps) and (2) large (approximately 1.07 kg [four cups]; 10 reps) with 10 or 40 eggs per small or large volume, respectively, to help determine how much soil was needed for a reliable field estimate of egg density. These eggs were obtained from the laboratory colony. Eggs (covered or clean) of each density were placed on the surface of the soil and stirred in by hand with a metal rod in a large glass beaker (1,000 ml). Both methods involved the use of sieve washes.

The flotation method for egg extraction consisted of two steps: (1) a sieve wash to remove large debris and (2) a flotation solution to isolate the eggs. For this method, both covered and clean eggs were used to determine if the soil particles would impact flotation. The soil containing eggs was washed through a series of sieves (number 10 [2.0 mm], 20 [0.83 mm], and 35 [0.50 mm]) and then washed into an Erlenmeyer flask.

The Erlenmeyer flask (500 ml or 1000 ml, depending upon soil volume) (Fig. 1D) served as a container for testing multiple flotation solutions (see details below). The opening of the flask was fitted tightly with glass tubing open on both ends that was capped at the bottom with a hollow rubber attachment, through which a rubber stopper was suspended in the flask beneath the tubing by a thin metal wire. A mixing rod was placed in the flask with enough solution to fill the flask to approximately half way up the neck (approximately 250 ml or 500 ml, respectively). The apparatus then was placed on a stirring plate, and the solution was agitated for 5 min and allowed to settle for 10 min. The stopper then was pulled into place at the bottom of the glass tubing by the wire, forming a tight seal and trapping any material (including eggs) that had floated to the surface. This floating debris then was emptied into a petri dish lid, and the stopper and glass tubing was rinsed with fresh water to ensure that all debris was collected. The surface of the water in the flask also was checked for any missed floating debris, which, if found, was removed with a fine paintbrush. The remaining sunken material then was washed through a fine mesh material. All debris was then checked for eggs.

Multiple flotation solutions were tested. Concentrations of each solution were determined by adding increasingly concentrated solutions to a beaker containing 500 ml of fresh water and a known number of eggs (10–15). For each solution, solute was added to 100% water in 10 g increments until a maximum flotation of eggs was observed. Although never achieving 100% flotation, the following two solution concentrations resulted in the highest flotation rates and, thus, were used in the experiment: pickling salt (NaCl), 200 g NaCl to every 500 ml H_2O (6.85 mol/L) (93% flotation); and magnesium sulfate ($MgSO_4$), 340 g $MgSO_4$ to every 500 ml H_2O (5.65 mol/L) (50% flotation). Other additives to the NaCl solution, including several types of dish soap and Cascade, were tested in an attempt to remove soil particles from the egg surface with no apparent improvement in egg recovery, so were not used in this experiment. A sucrose solution of up to 700 g sucrose to 300 ml H_2O (6.82 mol/L) resulted in 40% flotation. However, because of the low flotation rate achieved, high concentration needed, and extended periods of time required to achieve measurable results, sucrose solutions were not tested further.

The wash method was tested to determine if this method alone was sufficient for egg recovery (i.e., flotation was not necessary). It was comprised of two types, with or without a paint strainer. Both types used sieves but differed in the number of sieves used (see below). Only covered eggs were used for this method because clean eggs are not found in soil.

The first type of wash method (i.e., method 1) used the three sieves (numbers 10, 20, and 35) detailed in the flotation technique above. Eggs of each density (10 and 40) were washed through the three sieves exactly as before, transferred to a pan of water, and examined for eggs.

The second type of wash method (method 2) used a commercially available paint strainer in conjunction with a single sieve to determine if a cheaper alternative with fewer steps was available. Soil containing eggs of each density (10 and 40) was washed through a number 10 sieve to remove larger particles, and the sieved eggs and remaining soil then were transferred to a large handheld strainer (13.0-cm diameter) fitted with mesh material (0.60 mm) cut from a 1-gallon (3.8 L) paint strainer (Paint USA, Highland Heights, OH). Soil caught in this material then was washed into a white pan and examined for eggs. In early trials using only the paint

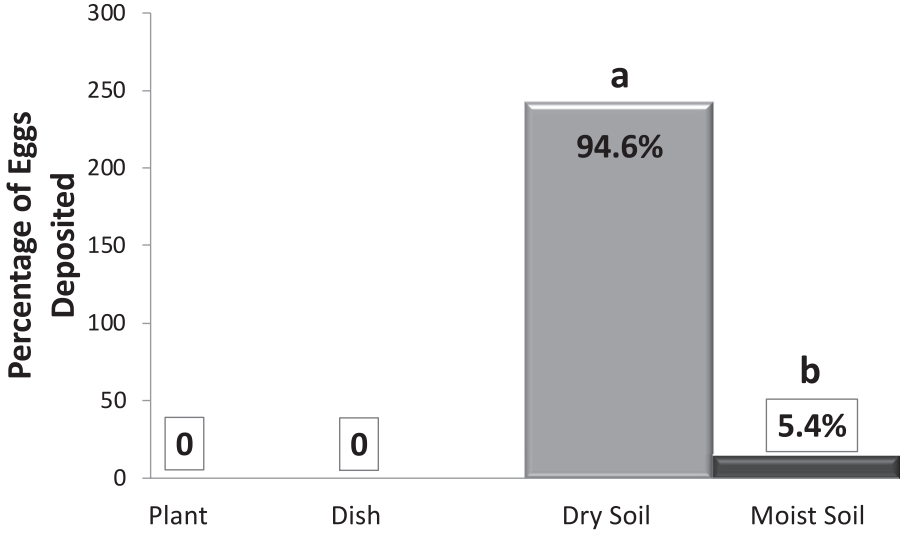

Fig. 2. Percentage of eggs of *B. hilaris* deposited in different substrates.

strainer, debris from the field (e.g., plant material and rocks) clearly hampered finding the eggs; therefore, the sieve was added to improve egg extraction.

Statistical analyses. Ovipositional preferences of *B. hilaris* females were compared between moist and dry soils and for depth preference within the soil overall (moist and dry combined) for various levels on or within the soil (surface; or top, middle, or bottom third) by using a one population *t*-test (SAS Institute Inc. 2013). To determine if the insects showed a preference for moist or dry soil, a proportion (number of eggs in dry soil/total number of eggs in dry + moist soil) was determined for each petri dish containing eggs; this was compared against a proportion of 50%, which would be expected if the insects showed no preference. To test for statistical differences in the proportion of eggs at each of the four levels on or within the soil, a proportion (number of eggs per level/total eggs per dish) was determined for each petri dish containing eggs; this was compared against a proportion of 25%, which would be expected if the insects showed no preference. The egg extraction techniques were compared with a generalized linear model with a binomial response. Contrasts were performed for overall covered versus clean eggs, wash method 1 versus wash method 2, NaCl covered versus clean, $MgSO_4$ covered versus clean, and wash (methods 1 + 2) covered versus flotation (NaCl + $MgSO_4$) covered for 10 eggs and 40 eggs, respectively. The level of significance was set at 0.05.

Results

Ovipositional preference. In the choice test in petri dishes between soil, plant material, and exposed plastic surfaces (lids and sides), 40 females laid 257 eggs in 19 of the 40 dishes, all of which were in the soil (Fig. 2). In additional tests, females exhibited a significant preference for dry soil over moist soil (243 and 14 eggs,

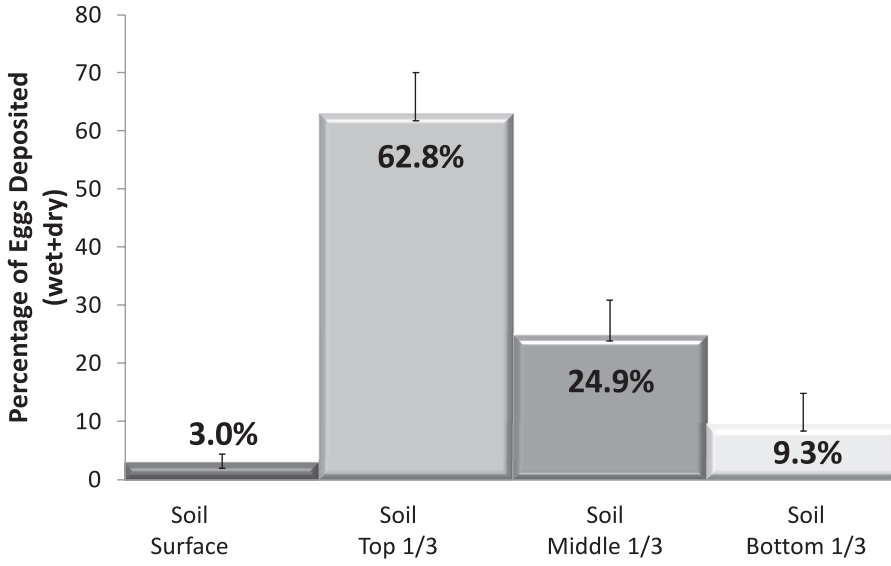

Fig. 3. Percentage of eggs of *B. hilaris* deposited at different depths of moist and dry soil.

respectively) for oviposition in the dishes ($t = 7.1$; df = 18; $P < 0.0001$), with an average proportion of 94.6% dry/total eggs per dish. Of the total eggs laid in the soil (dry and moist combined), those deposited on the surface (3.0%), top one-third (62.8%), and bottom one-third (9.3%) were significantly different ($t = 2.11$, df = 18, $P = 0.0001$; $t = 8.60$, df = 18, $P < 0.0001$; and $t = 1.71$, df = 18, $P = 0.0105$, respectively) from what would have been expected with no ovipositional preference (i.e., 25% per level) (Fig. 3); those laid in the middle one-third (24.9%) of the soil were not significantly different ($t = 4.12$; df = 18; $P = 0.983$).

Egg extraction. The two wash methods (1 and 2) consistently were more effective in extracting eggs than flotation methods for both small and large soil volumes ($P < 0.0001$, each) (Figs. 4, 5) but differed in their effectiveness between each other. Specifically, wash method 1 had a higher recovery rate than the wash method 2 for small soil volumes (98.0% and 91.0%, respectively; $P = 0.0058$) (Fig. 4), but a lower recovery rate than method 2 for large soil volumes (87.3% and 93.3%, respectively; $P = 0.0069$) (Fig. 5). Overall, for the flotation methods, significantly more ($P < 0.0001$, both sizes of samples) clean eggs were recovered than covered eggs. The NaCl flotation method had a significantly higher recovery rate for clean eggs than covered eggs for both the small volume (76.0% and 16.5%, respectively; $P < 0.0001$) and large volume (68.3% and 28.3%, respectively; $P < 0.0001$) samples. Similarly, the $MgSO_4$ flotation method had a significantly higher recovery rate for clean eggs than covered eggs for small volume samples (65.0% and 17.0%, respectively; $P < 0.0001$) and large volume (71.8% and 50.3%, respectively; $P < 0.0006$) samples. However, there was a higher recovery rate for covered eggs at the large volume compared with that of the small volume samples.

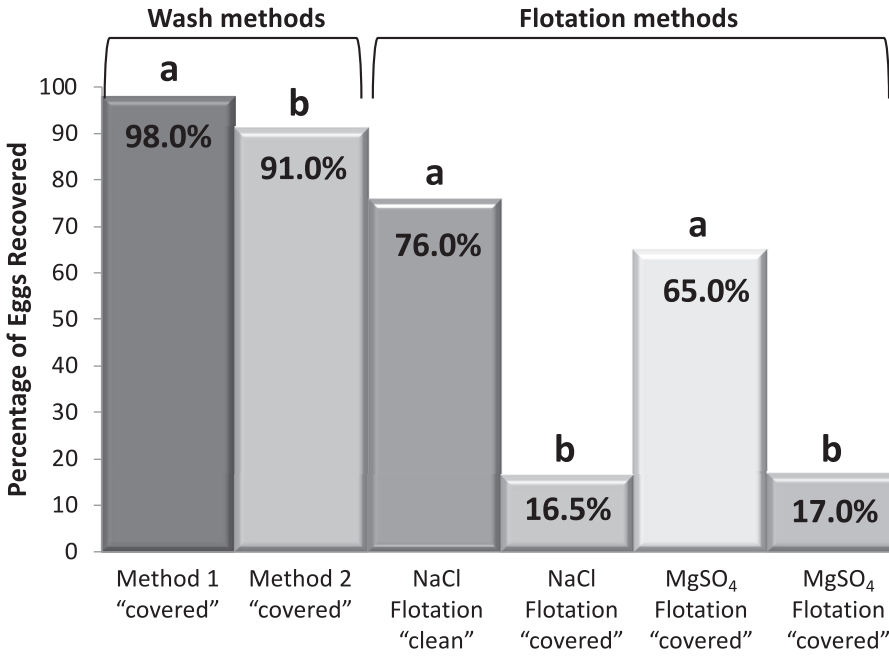

Fig. 4. Percentage of eggs of *B. hilaris* recovered in small volume samples (268 g) by each extraction technique.

Discussion

Given a choice, *B. hilaris* females prefer soil over plant material for ovipositional sites, which supports previous research (e.g., Taylor et al. 2014, 2015), and dry soil over moist soil. Also, the majority of eggs are laid beneath the surface within the top 0.4 cm. These results are consistent with the female's ovipositional behavior while inserting eggs in the soil (Taylor et al. 2014).

Our results show that the two wash methods (1 and 2) were more effective in egg extraction than the flotation methods. However, they varied in their effectiveness depending upon the soil volume used.

The flotation methods were much less effective for egg recovery, particularly for eggs covered in soil particles after deposition. Although still significantly lower than that for clean eggs, we noted a much greater level of recovery for covered eggs by using the $MgSO_4$ flotation method for the large volume than the small volume samples. It is unclear if this has real world meaning or is just an artifact of sampling. Ideally, a method that could strip the soil particles from the egg surface during extraction and, thus, allow better flotation could make these techniques a more viable option, as our study showed decent recovery rates for clean eggs.

Calculating the sampling time, cost of set up, and efficacy of egg recovery is useful for the comparison of wash method 1, wash method 2, and flotation methods (Table 1). The total sampling time (including washing and counting of eggs) was approximately 25 min for the flotation methods and approximately 15–20 min for

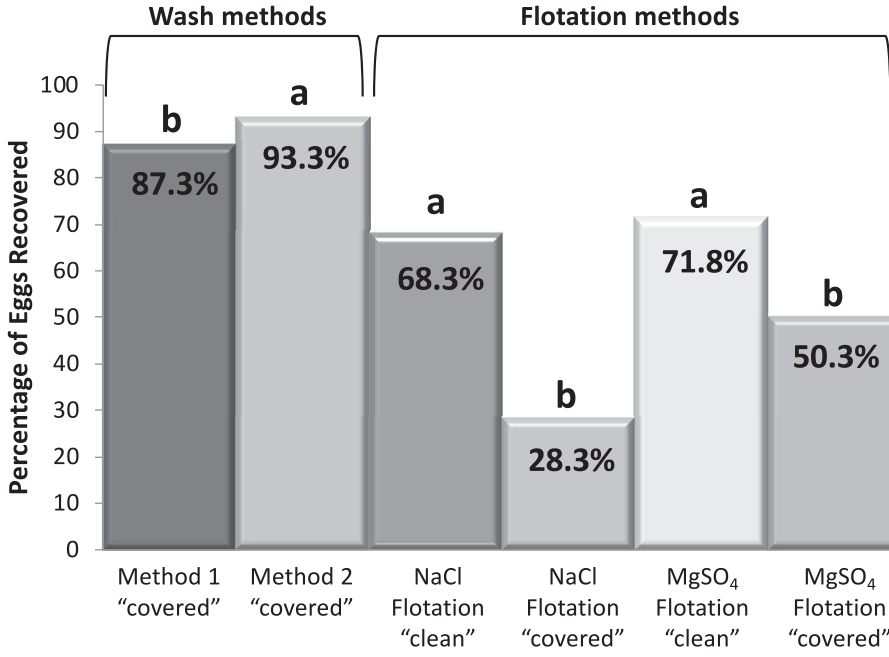

Fig. 5. Percentage of eggs of *B. hilaris* recovered in large volume samples (1.07 kg) by each extraction technique.

both wash methods. The initial setup cost for the flotation methods is markedly greater than that of the wash methods. We estimate that the material required for the flotation method (e.g., flask, beaker, stirring plate, mixing rod, rubber stopper, and solutions) cost approximately $388 USD, the wash method 1 materials (e.g., three sizes of sieves and a white pan) $171, and the wash method 2 materials (e.g., sieve, pack of paint strainers, mesh colander, and a white pan) $87 USD (without the sieve, this method could cost $30 USD). The efficacy of egg recovery was low to moderate for the flotation methods and high for the wash methods. Clearly, the

Table 1. Overall comparisons of egg extraction methods for *B. hilaris*.

Method	Startup Cost ($)*	Time/Sample** (min)	Efficacy†	Ranking
Wash method 2	87/30	15–20	High	1
Wash method 1	171	15–20	High	2
Flotation	388	25	Low-moderate	3

* Based on cost of equipment and supplies needed. Note: wash method 2 costs are given, respectively, with and without a number 10 sieve.
** Time includes a 10-min egg count time for each method.
† Based on the proportion of eggs recovered for each method.

wash methods outperformed the flotation methods in all categories. Although both wash methods appear equally effective, wash method 2 is less expensive.

The results of our study should prove useful to researchers who wish to search efficiently for eggs of *B. hilaris* in the soil. The soil wash methods are viable options for estimating populations of this highly mobile insect. Also, they could prove useful for recovery of the various egg parasitoids in the soil that remain poorly known for this unusual invasive pest species and may be important for its management.

Acknowledgments

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