

SOILING AVOIDANCE BY ANTS

By

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ABSTRACT

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Using both an 8-choice and a single-choice arena displaying organic muck soil and clay varying in moisture, we found that walking ants, *Pogonomyrmex occidentalis* and *Aphaenogaster rudis*, were strongly deterred by soiling treatments. By soiling we refer to any substance that clings to body surfaces and takes time and effort to remove. Organic muck soil with water content above 80 percent received one-third the number of ant visits recorded for dry soil. Clay at 50 percent water reduced ant visits by 80 percent relative to clay with 30 percent water or lower. No *P. occidentalis* walked over clay hydrated to 50 percent or higher. Moreover, the elapsed time for these ants to cross clay with soiling levels of moisture increased by about 10 fold compared to substrates hydrated less than 50 percent. These results clearly demonstrate, for the first time, that ants avoid getting dirty, something that was previously only assumed. Soiling substrates may be avoided by insects such as these ants, and thus might represent predator-free space. We recommend that additional research be directed to exploring practical uses of soiling substrates as protective barriers against pest insects.

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This thesis is dedicated to my parents and grandparents, many that you are, and to my husband, Peter Shaw. You are the sum of my parts and nothing that I have or will accomplish could be done without you by my side and in my heart.

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TABLE OF CONTENTS

LIST OF FIGURES	vii
INTRODUCTION	1
MATERIALS AND METHODS	6
Test Subjects	6
Substrates Tested	6
Organic muck soil.....	6
Modeling clay	6
Behavioral Arenas	7
Multi-Substrate Choice Arena.....	7
Single-Substrate Arena	8
Experiment 1 -- Multi-Substrate Choice Arena with <i>P. occidentalis</i>	9
Experiment 2 -- Multi-Substrate Choice Arena with <i>A. rudis</i>	10
Experiment 3 -- Single-Substrate Arena with <i>P. occidentalis</i>	10
Experiment 4 -- Single-Substrate Arena with <i>A. rudis</i>	11
Experiment 5 -- Quantifying Soiling as a Function of Clay Hydration	11
RESULTS AND DISCUSSION	12
Experiment 1 -- Multi-Substrate Choice Arena with <i>P. occidentalis</i>	12
Experiment 2 -- Multi-Substrate Choice Arena with <i>A. rudis</i>	12
Experiment 3 -- Single-Substrate Arena with <i>P. occidentalis</i>	14
Experiment 4 -- Single-Substrate Arena with <i>A. rudis</i>	15
Experiment 5 -- Quantifying Soiling as a Function of Clay Hydration	17
GENERAL DISCUSSION	18
APPENDIX.....	22
REFERENCES CITED	24

LIST OF FIGURES

Figure 1. Drawing of the 8-choice arena used to test visitation frequencies of ants to soil substrates at various hydrations. A = empty arena; B = arena with filled wells where the darkness level represents increasing percent water in organic muck soil. The light grey well represents only water. Batches of 25 ants were released at a time from the platform at the arena center	7
Figure 2. Drawing of the single-substrate arena used to test timing of ant touching and crossing of clay substrate at various hydrations. Individual ants were placed in the release chamber that was already open to a 9 cm runway leading to empty Petri dish #2.....	8
Figure 3. Panels A – C reveal frequency of observing harvester ants, <i>Pogonomyrmex occidentalis</i> , on wells of organic muck soil in an 8-choice arena at elapsed times of 5, 30, and 120 min, respectively. Panels D – F reveal data for the wood ant, <i>Aphaenogaster rudis</i> , under the same conditions as for A – E. In Panel D, means labeled with the same letter are not statistically different at $p = 0.05$. NS = not significant.....	13
Figure 4. Responses of the harvester ant, <i>Pogonomyrmex occidentalis</i> in the single-substrate arena of Figure 2 as influenced by water content of a clay substrate.....	15
Figure 5. Responses of the wood ant, <i>Aphaenogaster rudis</i> , in the single-substrate arena of Figure 2., as influenced by water content of a clay substrate.....	16
Figure 6. Amount of soiling of the end of a standard paper staple inserted into clay at various levels of hydration	17
Figure 7. Correlation between soiling index and proportion of harvester (A) and wood ants (B) crossing of hydrated clay strips.....	19
Figure 8. Correlation between soiling index and elapsed time for harvester (A) and wood ants (B) to cross hydrated clay strips	20

INTRODUCTION

Becoming dirty represents a fundamental problem for most organisms, as it does for man-made equipment like solar cells, atmospheric sensors, satellite dishes, ships, and airplanes (Amador and Hu 2015). By *dirt* I mean particles, liquids, and films, etc. that cling to surfaces more than briefly and that require time and energy for removal. Dirt can inhibit animal function by: 1) adding significantly to body weight, thereby reducing maneuverability and making locomotion more energetically costly (Amador and Hu 2015); 2) decreasing sensory input by immobilizing mechano-sensory hairs, blocking chemicals from reaching chemosensilla (Böröczky et al. 2013), or obstructing the field of vision; and perhaps most importantly, 3) substantially increasing the probability of infections by pathogens (Zhukovskaya et al. 2013) and colonization by endo- and ectoparasites (Gray et al. 2012).

It is not surprising, therefore, that animals allocate an appreciable and surprisingly consistent portion of their time budgets to dirt removal. For example, ants of the genus *Leptothorax* spend 5% of their lives grooming (Miramontes 2007), while birds on average spend 9% of their waking hours grooming (Cotgreave and Clayton 1994). That figure for cats is 4% (Eckstein and Hart 2000) for non-bored individuals. Some old world monkeys spend almost 8% of their waking hours grooming (Huang et al. 2007). Humans in the U.S.A., artificially assisted by mechanical devices (hair dryers, showers, etc.), spend 0.33% of their lives bathing and 3% cleaning their houses (US Department of Labor 2014, as cited by Amador and Hu 2015). Free-floating particulate matter has increased significantly since the start of the industrial revolution, leading to speculation that the time animals allocate to

grooming might be increasing (U.S. Environmental Protection Agency 2013; 2014; Amador and Hu 2015).

The anatomical structures and behavioral programs involved in animal grooming have received considerable attention and the resultant body of data suggests that evolution has shaped organisms to stay clean. Considerable data on grooming behavior have been compiled for vertebrates. Rats and other rodents express an array of grooming behaviors often triggered by specific stimuli like: stress, awaking, preparing for sleep, and being introduced to a novel environment or new item within their environment (Delius 1970; Jolles et al. 1979). Grooming behaviors are usually stereotypical and are executed as a behavioral sequence carried out in a very similar manner every time they are observed. For example, rats wash their faces by using both forepaws to run over the fur, eyes, nose, and mouth. Rats also stereotypically scratch various body parts with their hind legs and use their mouth and tongue to clean their fur (Bolles 1960). Female rats that have recently given birth routinely self-lick their genital and mammary areas to deposit bactericidal saliva to these regions (Spruijt et al. 1992). This behavior preserves the health of pups not yet possessing the internal flora to help combat infection.

Many mammals, including rats, allogroom (Alcock 2013) where an individual grooms other members of a group. This type of grooming is perhaps the most rigorously studied of all the types of grooming in mammals. . Allogrooming occurs throughout social species and has significant implications in maintaining the social ties and rankings of individuals within a group. Social grooming is observed in animals ranging from insects to birds to primates and often includes a form of grooming that requires or makes use of particularly adapted grooming structures (Buettner-Janusch and Andrew 1962; Spruijt et

al. 1992). For example, the prosimians studied by Buettner-Janusch and Andrews (1962) use a lower incisor specifically for grooming; this comb-like incisor has not been observed to have any purpose in food consumption or manipulation. Likewise, some African antelopes have specialized teeth used mainly in allogrooming but also autogrooming, in addition to food manipulation (McKenzie 1990).

Arthropod grooming behaviors have received considerable study. Hymenopteran grooming sequences and processes have been extensively detailed by, e.g., Basibuyuk and Quicke (1994, 1995, 1999), Farish (1972), and Hlavac (1972). These studies offer a compendium of the movements associated with grooming, including stereotypical movements, common sequences of movement, and a repertoire of grooming behaviors reliably triggered by environmental factors (Farish 1972; Hlavac 1975; Basibuyuk and Quicke 1994; 1995; 1999). Understanding grooming behaviors specific to certain hymenopteran species was also helpful in constructing evolutionary phylogenies for similar insects (Hlavac 1975).

Grooming by mosquitoes has received some attention. For example, *Aedes triseriatus* mosquitos were induced to exhibit grooming repertoires after exposure to changes in airflow. Foreleg and hindleg tibial combs were employed as a replacement for more commonly used mandibular manipulation seen in insects with more conventional mouthparts (Walker and Archer 1988). Grooming behaviors were quite consistent in *A. triseriatus* allowing very accurate predictions to be made on future movements based on a present grooming behavior. Walker and Archer (1988) postulated that mosquito-grooming sequences are explicitly designed to remove dirt from the exoskeleton and to smooth scales and appendages so as to facilitate flight.

The conclusions about the importance of grooming on dirt removal arrived at for hymenopterans and mosquitoes are thought to hold for a wide range of arthropods, including Diptera and Blattodea. Drosophilids employ sweeping and rubbing movements using the legs (Szebenyi 1969) to remove yeast particles from the head, thorax, abdomen, wings, and legs. These movements have also been postulated to evenly distribute wax over the body (Szebenyi 1969). Other dipterans also utilize these behavior patterns in their grooming repertoire. Eight unique grooming behaviors were recorded from observations on *Calliphora erythrocephala*, including grooming performed using mouthparts and each leg (Dawkins and Dawkins 1976). When a stimulant is applied to the abdomen of *Periplaneta americana* it elicits an immediate grooming response from the cockroach in which the legs scrape and rub the affected area and the wings rub and jostle the stimulant until it is removed (Reingold and Camhi 1978). As in dipterans, the wax layer of the cockroach is also said to be distributed and smoothed by rubbing with the legs (Wigglesworth 1945). Decapod crustaceans are equipped with a series of combs and setal brushes to groom body parts of various detritus. Specialization of body parts for grooming behaviors has been noted in many species of crustaceans and observations have been made of these body parts scraping, combing, and removing unwanted substances from gills and other sensitive parts (Bauer 1981).

Given this background supporting the idea that grooming is critical to dirt removal, we were surprised to discover that data from behavioral studies quantifying the avoidance of dirt by animals is rare. In fact, the only such publication we located (Curtis 2007) entitled *Dirt, disgust, and disease: a natural history of hygiene*, was conducted on humans. Curtis (2007) collected data on what images elicited feelings of disgust in participants and

compiled cultural norms on disease transmission beliefs from around the globe. These findings highlighted human dirt avoidance intuition. In the current study, we directly tested the hypothesis that substrates that were highly soiling would influence avoidance by walking ants.

MATERIALS AND METHODS

Test Subjects

Multiple containers of harvester ants, sold as *Pogonomyrmex occidentalis*, each containing about 30 workers, were purchased from Life Studies LLC (Hurricane, Utah). Shipments were immediately refrigerated at 5° C to keep the ants dormant. Wood ant, *Aphaenogaster ruidis*, workers collected from the Waterloo Recreation Area, Jackson, Co, MI (latitude: 42.333, longitude: -84.373), were likewise separated into lots of about 30 individuals and refrigerated. Ants refrigerated for longer than one wk were briefly brought to room temperature ca. once per wk for a feeding of Orange Cube Cricket Diet (Fluker's, Baton Rouge, Louisiana) and peanut butter from a local food store.

Substrates Tested

Organic muck soil

Black soil very high in organic matter and comprised of only fine particles was collected from the Michigan State University Muck Experimental Farm, Laingsburg, MI (latitude: 42.834494, longitude: -84.398776) and dried at 60°C for 3 d in an industrial oven at the Michigan State University Agronomy Farm. Further heating resulted in no additional loss of mass. Dried soil was then dispensed into Ziplock (S. C. Johnson Co., Racine, Wisconsin) bags in lots of 900 g and stored at room temperature in the laboratory until hydrated to a defined level for the choice tests as explained under Experiment 1.

Modeling clay

The starting water content of 1.13 kg white, air-dry modeling clay purchased from Crayola (Bethlehem, Pennsylvania) was determined by drying three 2 g aliquots to constant weight.

Batches of clay were then adjusted by stirring in a desired amount of tap water into portions of the purchased starting material taken from a sealed, airtight container.

Behavioral Arenas

Multi-Substrate Choice Arena

We constructed a 96 cm diam behavioral arena (Figure 1) from 5 cm-thick Owens Corning (Toledo, Ohio) FOAMULAR pink Styrofoam insulation board cut with a router into 8 equal 3.5 cm-deep pie-piece-shaped wells with 1 cm dividers left intact. A 3 cm circle at the

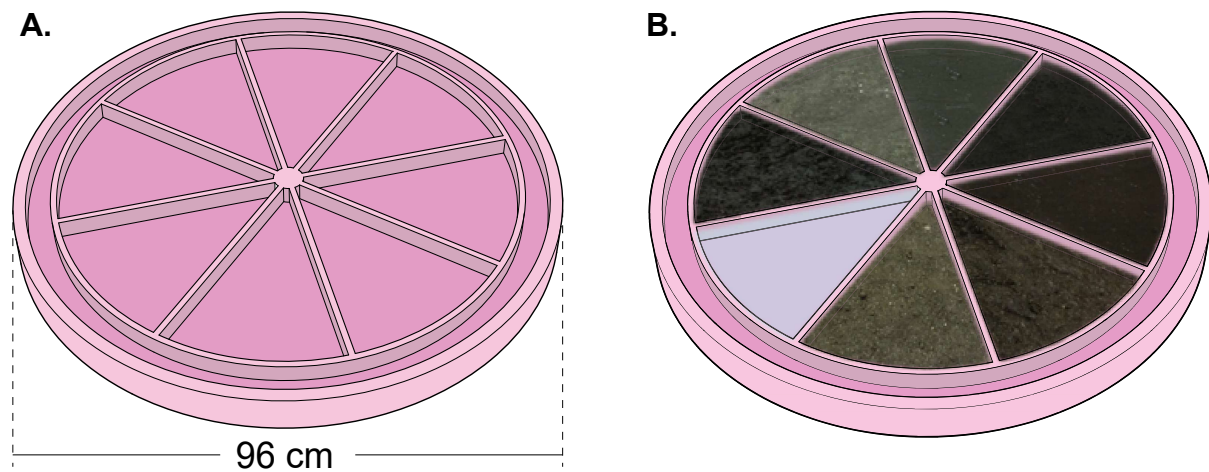


Figure 1. Drawing of the 8-choice arena used to test visitation frequencies of ants to soil substrates at various hydrations. A = empty arena; B = arena with filled wells where the darkness level represents increasing percent water in organic muck soil. The light grey well represents only water. Batches of 25 ants were released at a time from the platform at the arena center.

center of the arena, also left intact, served as a platform for release of 25 ants at a time. A 4 cm-wide water-filled moat confined ants to the choice section of the arena where they could visit any of the 8 wells filled with soil at varying states of hydration so as to *soil* the visitors to varying degrees

Single-Substrate Arena

This apparatus (Figure 2) was designed to force single ants into more extended interactions with given substrates than was achieved in the multi-choice arena. This

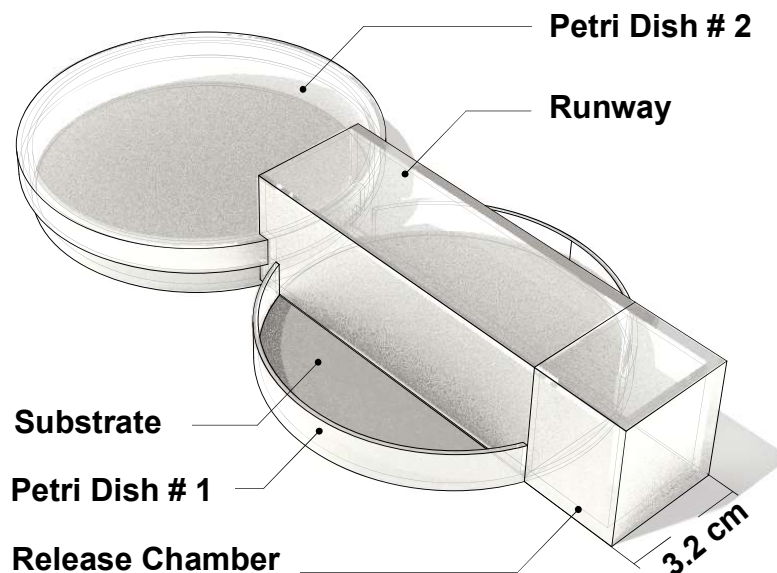


Figure 2. Drawing of the single-substrate arena used to test timing of ant touching and crossing of clay substrate at various hydrations. Individual ants were placed in the release chamber that was already open to a 9 cm runway leading to empty Petri dish #2.

arena allowed quantification of ants' willingness to walk across soiling substrates when no other passage led away from the confining 3.2 x 2.5 cm space of initial release (Figure 2). Leaving the release chamber required ants to traverse a 3.2 x 9 cm strip of clay substrate enclosed by a smooth plastic rectangular prism (3.2 cm OD x 2.5 cm ID) clear extruded square acrylic tubing (United States Plastic Corp., Lima, Ohio). The bottom edge of the rectangular tube was removed to allow its opened bottom to be positioned atop a 9 cm diam Petri dish filled with enough starting substrate that evaporation during an experimental run was inconsequential. Ants reaching the distal end of the runway found an empty 9 cm diam plastic Petri dish from which they rarely departed. The parts of this all-plastic apparatus were joined by hot glue. The port, allowing introduction of ants into the release chamber, and any gaps were sealed with Parafilm M (Sigma Aldrich).

Experiment 1 -- Multi-Substrate Choice Arena with *P. occidentalis*

This experiment tested the hypothesis that harvester ants, *P. occidentalis*, would avoid wells of muck soil hydrated so as to be sticky and soiling. The eight levels of hydration tested were achieved by adding tap water to dried muck soil so that filled wells contained: 0, 11, 35, 51, 72, 83, 90, and 100% water by weight. Initially, the positions of hydration treatments were randomly assigned to wells using a random numbers generator. A batch of 25 cold-sedated (Dronnet et al. 2005) ants was gently poured onto the central starting platform. We took great care never to agitate awakened *P. occidentalis*, because doing so caused them to become less sensitive to aversive substrates. For example, individuals dislodged from gripping the bristles of a small brush were willing to charge onto any substrate and even into standing water. At 5 min intervals during the first h and then at 30 min intervals up to 3 h, the frequency of ant visits onto each well was recorded during

direct observations. This experiment was conducted in a laboratory having normal fluorescent lighting overhead and a bank of windows along one side. To preclude any possible positional biases due to differential light stimuli, the choice arena was set upon a wheeled cart whose position with respect to the windows was randomized and then rotated into a new random position every 30 min. Nine replicates of this experiment using freshly prepared substrate treatments and fresh batches of ants for each replicate were accumulated through time. The randomization scheme for treatment positions was restricted so that a given treatment could never appear adjacent to that of a previous neighbor until all non-neighbors were exhausted. Because graphing of the continuous variable of substrate moisture against mean frequency of *P. occidentalis* visits proved linear, data for this randomized complete block experiment were examined statistically simply by regression analysis and testing of the significance of r values by the free software at: <http://www.danielsoper.com/statcalc/calculator.aspx?id=44>

Experiment 2 -- Multi-Substrate Choice Arena with *A. rudis*

This experiment used *A. rudis* and was conducted identically to Experiment 1 with the exception that a total of 5 replicates was accumulated through time. Because the outcomes for this randomized complete block experiment proved non-linear, results were analyzed by 2-way ANOVA (blocks and treatments) using SAS (SAS Institute, Cary, NC).

Experiment 3 -- Single-Substrate Arena with *P. occidentalis*

This experiment used harvester ants, *P. occidentalis*, responding in the single-substrate arena using modeling clay as substrate. Clay hydration levels were: 22 (directly from the manufacturer's container), 29, 35, 40, 44, 48, and 54% water by weight. One ant was tested at a time to one given treatment and the behavioral variables quantified during

the 30 min runs included: proportion of individuals crossing the substrate strip, elapsed time until successful crossing, time of first substrate touch, and elapsed time from first substrate touch until successful crossing of substrate. Individual ants were then tested to different treatments until all hydration treatments in the set were tested so as to complete one block wherein the order of treatments was randomized. Since 7 single substrate arenas were available, we structured the starting times to be offset so that some runs overlapped others with the provision that no observations at prescribed times were compromised. Twelve replicates of Experiment 3 were accumulated through time and the data were analyzed by regression analysis, as in Experiment 1, as outcomes proved linear.

Experiment 4 -- Single-Substrate Arena with *A. rudis*

This experiment was conducted identically to Experiment 3 with the only difference being that *A. rudis* was the test subject.

Experiment 5 -- Quantifying Soiling as a Function of Clay Hydration

To quantify soiling amount, I used a piece of thin metal as a surrogate insect body part. One of the short legs of an un-crimped chisel point standard paper staple (Model SB19 1/4CP; Stanley Bostitch, East Greenwich, RI) was inserted vertically into a small well of each clay treatment of Experiment 3 and 4 so that its full 0.64 cm length was submerged. After careful withdrawal, making sure no residue was lost, a soiled staple was weighed on a Satorius CP 1245 balance sensitive to 0.1 mg. Six replicate measures of soiling amount were taken of each of six hydration levels.

RESULTS AND DISCUSSION

Experiment 1 -- Multi-Substrate Choice Arena with *P. occidentalis*

Consistent with the stated hypothesis, *P. occidentalis* was observed less frequently on wetter organic muck soil that became more muddy and soiling rather than drier muck soil (Figure 3 A-C). The relationship between soil water content and observed visitation frequency proved linear and statistically significant at all times. Beginning at ca. 60 min, and increasingly thereafter, some *P. occidentalis* began excavating tunnels in the driest soil and, by 120 min, an average of 25% of all ants congregated in and around such tunnels. Such ants were no longer active participants in a choice test, thereby demonstrating a limitation of this type of behavioral assay. We also noted that at least 25% of these ants were at all times found on the Styrofoam dividers of the apparatus, demonstrating that these surfaces were not deterrent but perhaps preferred pathways for environmental explorations. At 2 h, 40% of the ants were observed on the dividers.

Experiment 2 -- Multi-Substrate Choice Arena with *A. rudis*

This wood ant did not respond linearly to increasing water content in organic muck soil (Figure 3 D-F), as did the harvester ant (Figure 3 A-C). Consistent with the stated hypothesis, the wood ant rarely visited water only or very wet organic soil. However, the response of *A. rudis* to muck soil moisture appeared to be bimodal; the frequency of visits was initially numerically highest to wells having 72% water (Figure 2 D). This value was statistically higher than that for 90% water, but not for any of the other treatments at an elapsed time of 5 min. Thereafter, the apparent preference for 72% water persisted. We speculate that this level of moisture was acceptable to *A. rudis* as it did

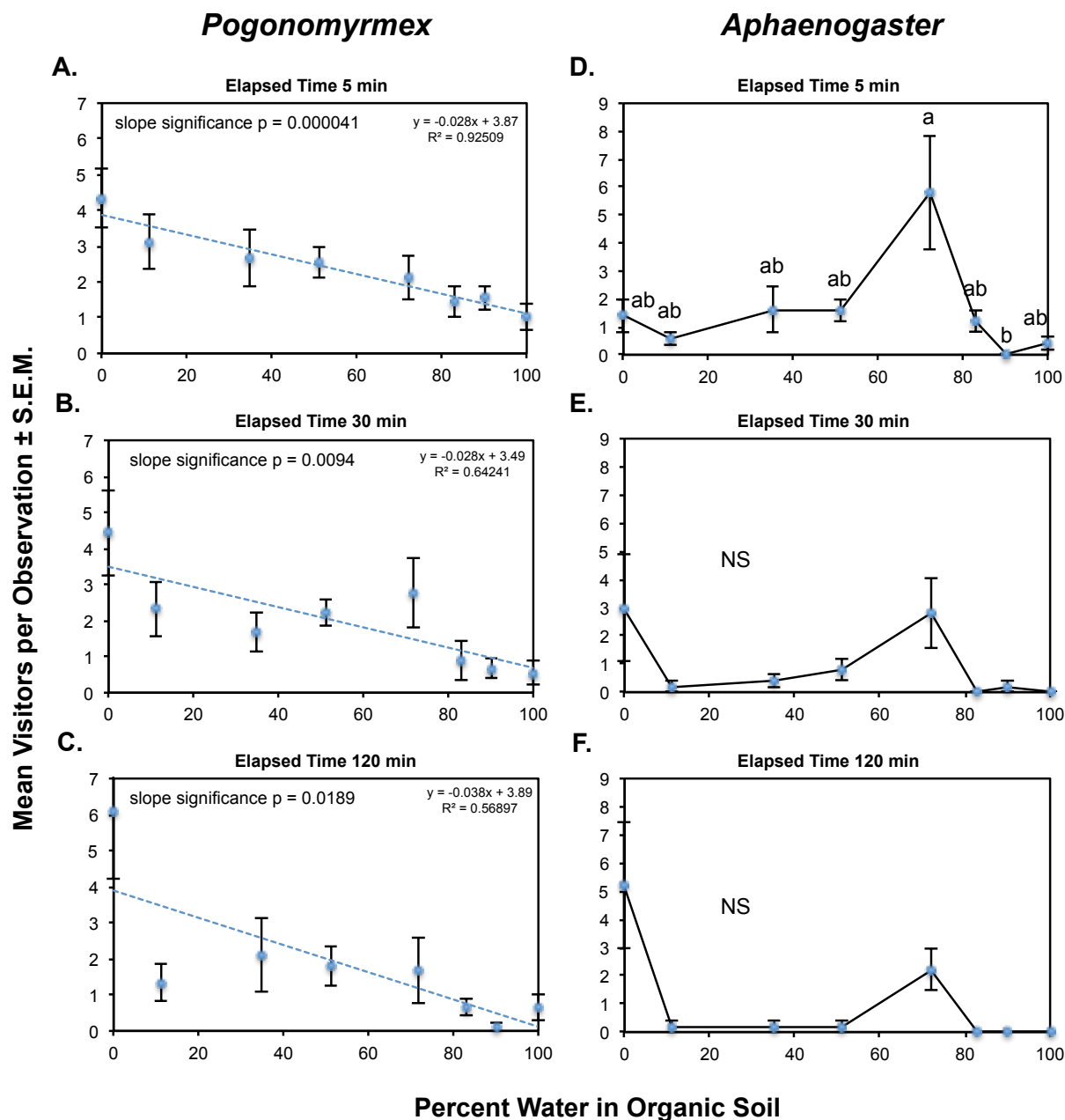


Figure 3. Panels A – C reveal frequency of observing harvester ants, *Pogonomyrmex occidentalis*, on wells of organic muck soil in an 8-choice arena at elapsed times of 5, 30, and 120 min, respectively. Panels D – F reveal data for the wood ant, *Aphaenogaster rudis*, under the same conditions as for A – E. In Panel D, means labeled with the same letter are not statistically different at $p = 0.05$. NS = not significant.

not soil the ants to an unacceptable level and provided a high humidity level like that of their normal nests in damp decaying matter. Alternatively, this treatment might have been an acceptable site for drinking, as during the experiment ants were observed to place mouthparts on this substrate. Visitation to the driest soil rose with time, as *A. rudis* also began excavating tunnels in the driest soil. No statistically significant differences were documented at elapsed times of 30 and 120 min. Statistical power in differentiating among muck soil moisture treatments tested in this multi-choice arena proved weak for this ant because of: scatter in the data, lack of a consistent pattern across data that would permit more than point by point statistical comparisons, and the consistent finding that more than half of these ants were always found on the Styrofoam dividers rather than any soil treatment. Interactions among ants via pheromone trails could not be ruled out in this choice arena. This finding provided more impetus for employing a more behaviorally directed bioassay and testing ants singly, i.e., the single-substrate arena of Figure 2.

Experiment 3 -- Single-Substrate Arena with *P. occidentalis*

Wetted clay proved highly deterrent to this harvester ant. The proportion of individuals crossing the wetted substrate dropped precipitously, linearly, and highly significantly as water content increased from 30 to 55% (Figure 4 A). No ants crossed a strip of clay wetted to 55%. Moreover, the elapsed time until ants were willing to cross the clay strip increased linearly and highly significantly with percent water (Figure 4 B). Clearly, wetted and sticky clay was highly aversive to *P. occidentalis*. There was no significant difference in the elapsed time until *P. occidentalis* first touched any of the clay treatments of Experiment 3 (Figure 4 C), suggesting that differences in these treatments were not detectable at a distance but required touching which would then be accompanied

by soiling. Because the interval until first touch was short, the relationship between percent water in clay and elapsed time for crossing was little different from that for percent water in clay and elapsed time from the first touch until crossing (Figures 4 B vs. D), also highly significant statistically.

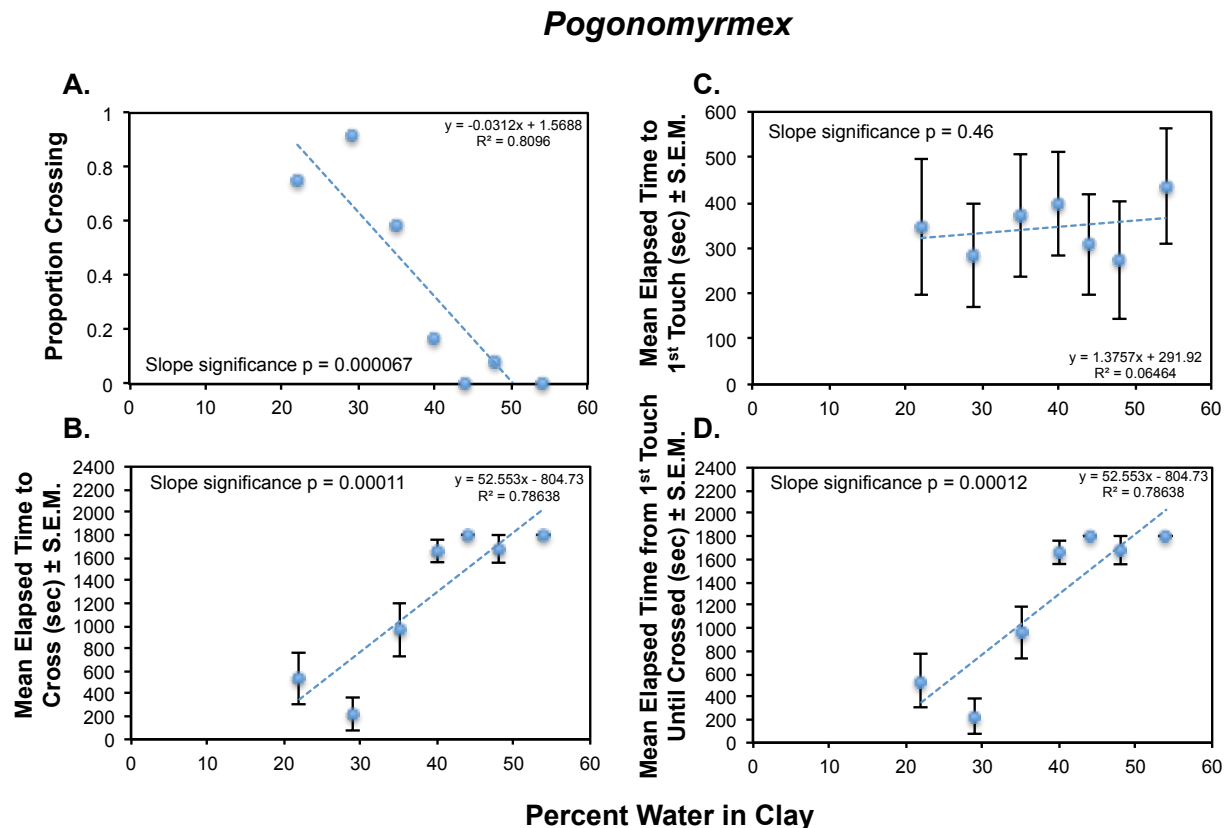


Figure 4. Responses of the harvester ant, *Pogonomyrmex occidentalis* in the single-substrate arena of Figure 2 as influenced by water content of a clay substrate.

Experiment 4 -- Single-Substrate Arena with *A. rudis*

Responses of the wood ant to clay hydrations were virtually identical to those for the harvester ant in the single-substrate arena. The only notable difference was higher

variability in the elapsed time until the first substrate touch for *A. rudis* vs. *P. occidentalis* (Figure 4 C vs. 5 C). The higher variability in the data for the wood ant was due to the occurrence of a few individuals who remained quiescent in the release chamber for a considerable time before commencing locomotion. Nevertheless, this occurrence had little impact on the overall outcome.

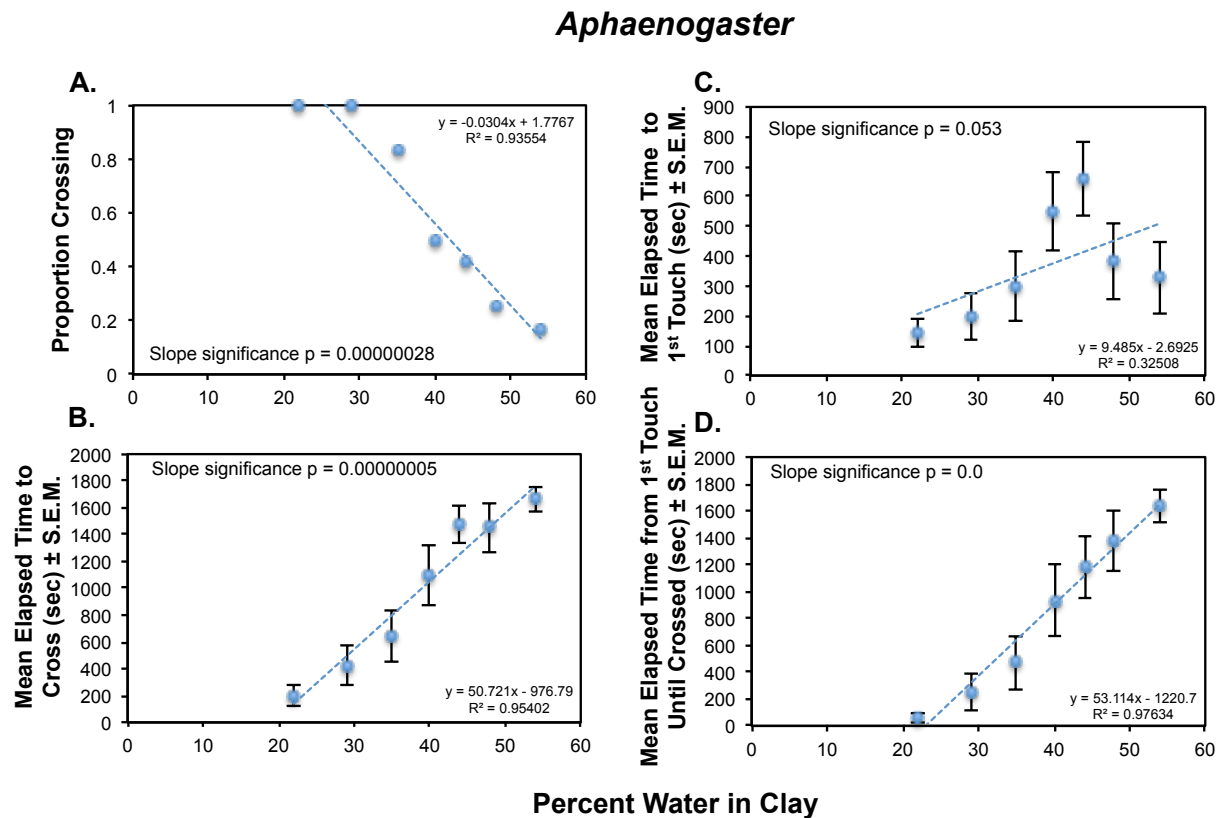


Figure 5. Responses of the wood ant, *Aphaenogaster rudis*, in the single-substrate arena of Figure 2., as influenced by water content of a clay substrate.

Experiment 5 – Quantifying Soiling as a Function of Clay Hydration

Soiling as measured by increase in weight if a staple end inserted into small batches of clay increased linearly and highly significantly with percent water (Figure 6). This outcome bore a striking resemblance to that for increase in elapsed time until ant crossings of clay strips in Experiments 4 and 5 (compare Figures 4 B and 5 B with Figure 6).

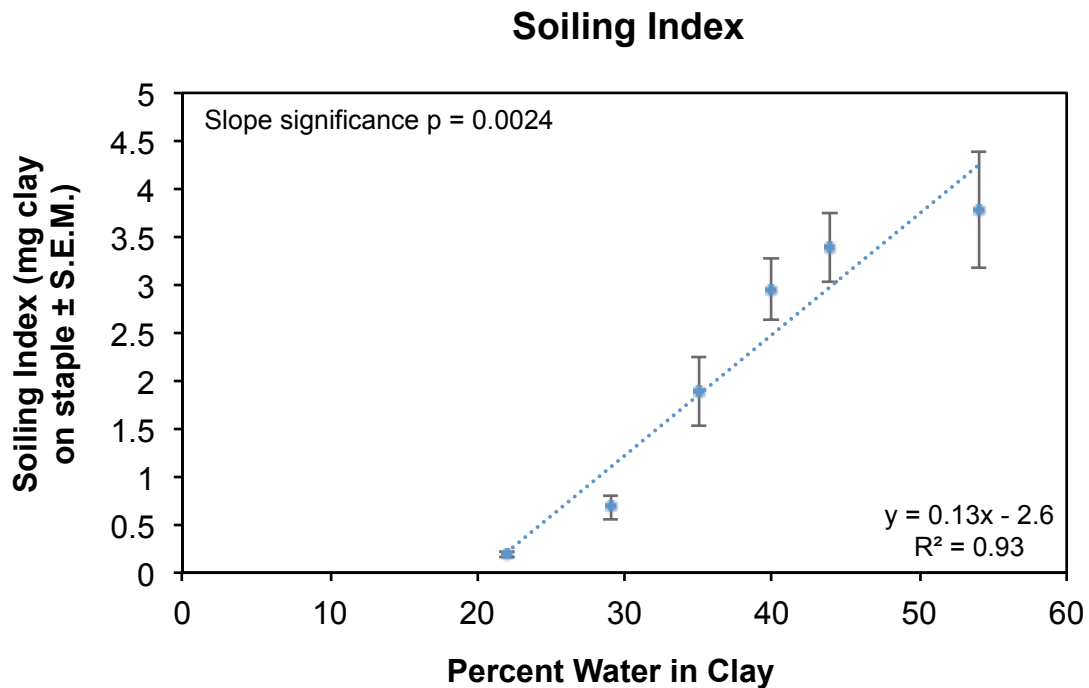


Figure 6. Amount of soiling of the end of a standard paper staple inserted into clay at various levels of hydration.

GENERAL DISCUSSION

This research clearly demonstrated that wet and sticky substrates were aversive to both *P. occidentalis* and *A. rudis*. Numerous times during the multi-substrate arena tests, we observed that ants of both species venturing onto sticky substrates became soiled and immediately groomed once they reached a less soiling substrate. Moreover, ants in the single-substrate tests using clay were occasionally observed to run from the release chamber onto very wet clay and become temporarily stuck. When successfully withdrawing from such a substrate, grooming time was required before ants resumed locomotion. On the other hand, ants on less sticky substrates were not observed to become soiled and spent considerable time walking and resting.

We had originally postulated that very dry soils might also be repellant to ants, both because of their very low humidity and the possibility that particles might adhere to body parts due to, e.g., static charges (Edwards 1962, Vaknin et al. 2000) and van der Waals forces (Kesel et al. 2004). However, no data were obtained for either ant species supporting any aversive effect for organic soil heated so as to become dehydrated beyond any naturally attainable level.

Both ant species responded similarly to hydrated clay in the single-substrate arena. However, *A. rudis* crossed wet clay at a somewhat higher frequency than did *P. occidentalis* (Figures 5 A vs. 4 A). As already noted above, we speculate that the wood ant is slightly more tolerant of moist substrates, like those in which it nests (Lubertazzi 2012), than is the harvester ant, a desert species (Cole 1994).

The data of Figure 6 are critical to making the case that soiling is responsible for the measured aversion of these ants to hydrated soils. Indeed, the correlation between soiling index and proportion of ants refusing to cross such a clay strip is remarkably high (Figure 7) as is that for soiling index and elapsed time for crossing hydrated clay strips (Figure 8).

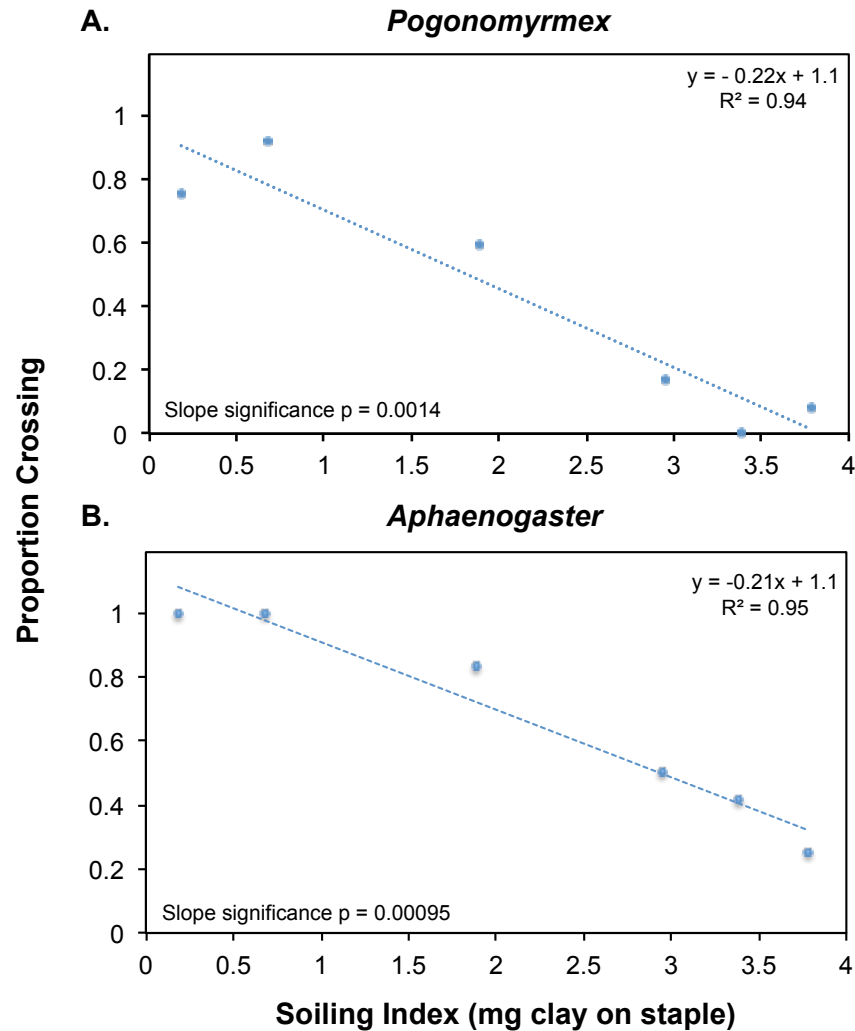


Figure 7. Correlation between soiling index and proportion of harvester (A) and wood ants (B) crossing of hydrated clay strips.

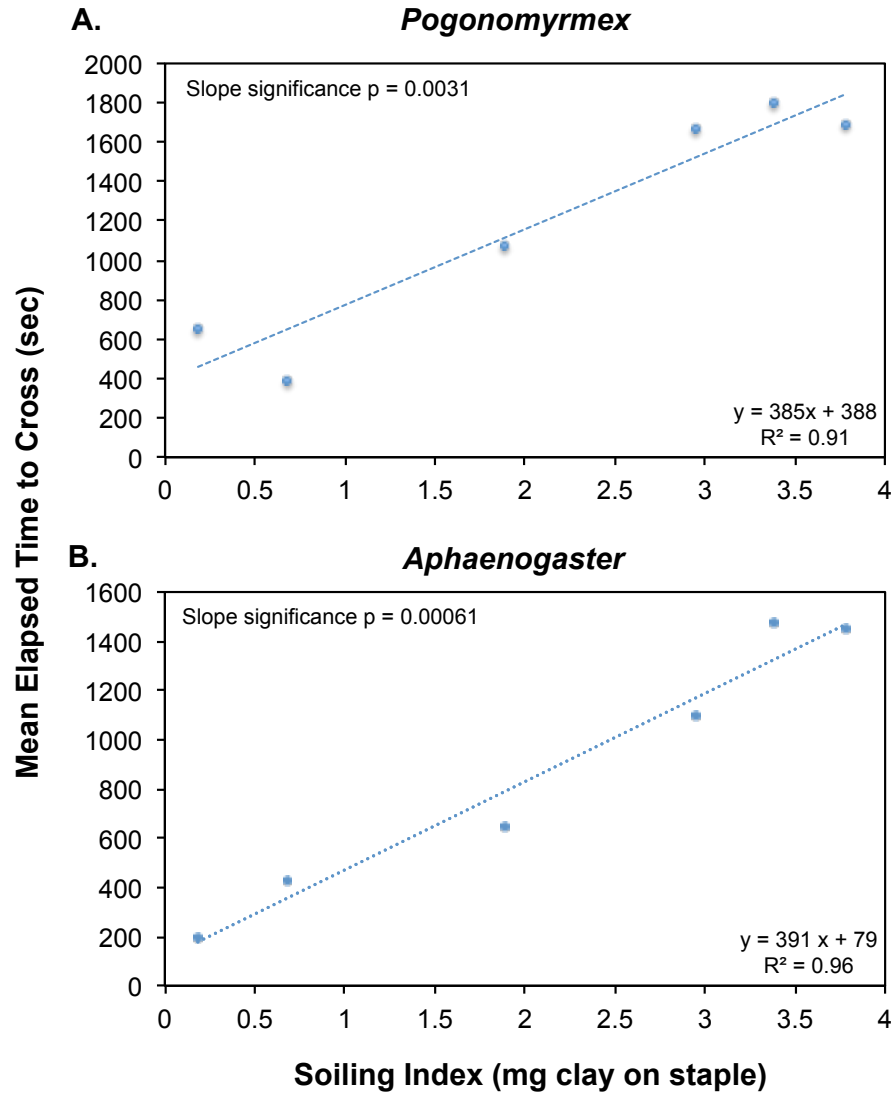


Figure 8. Correlation between soiling index and elapsed time for harvester (A) and wood ants (B) to cross hydrated clay strips.

In both cases there is a pronounced dosage-response effect. However, water alone must also be acknowledged as highly aversive because it supports very little weight and visitors

become submerged. We suspect the overall aversive effect to very wet soil result both from soiling and sinking.

Documentation that ants strongly avoid soiling substrates might have relevance to both basic and applied biology. For example, we suggest that wet and sticky substrates might represent predator-free (Barrios-O'Neill et al. 2015) or predator-reduced space. For example, Miller et al. (2007) report that the malaria mosquito, *Anopheles gambiae*, preferentially deposits its eggs on wet mud at the edge of the puddle habitats for its larvae rather than in the water itself where aquatic spiders are observed to forage. Their speculation that sticky mud might be more predator-free than water of drier soils is corroborated by the current data. Wet and sticky substrates might find applications as barriers protecting resources like garden plantings and even homes from visitation by walking insect pests. Evolution has already exploited this phenomenon as evidenced by the protection offered by sticky exudates from plant trichomes (Belcher and Thurston 1980; Krimmel and Pearse 2013) and sap exudates from wounded plants (Wilson and Wilson 1977; Sanderson and Farr 1960).

APPENDIX

APPENDIX

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2016-06

Author and Title of thesis:

Author: Casey Ruth Marie Shaw

Title: Soiling avoidance by ants

Museum(s) where deposited:

Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

<u>Family</u>	<u>Genus-Species</u>	<u>Life Stage</u>	<u>Quantity</u>	<u>Preservation</u>
Formicidae	<i>Pogonomyrmex occidentalis</i>	adult	10	pinned
Formicidae	<i>Aphaenogaster rudis</i>	adult	10	pinned

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