In Vitro and In Vivo Evaluation of Infestation Deterrents Against Lice

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ABSTRACT The human head louse is a cosmopolitan ectoparasite and frequently infests many people, particularly school-age children. Due to widespread pyrethroid resistance and the lack of efficient resistance management, there has been a considerable interest in the protection of uninfested people and prevention of reinfestation by disrupting lice transfer. In this study, two nonclinical model systems (in vitro and in vivo) were used to determine the efficacy of the infestation deterrents, *Elimax lotion* and *Elimax shampoo*, against human head lice or poultry chewing lice, respectively. With in vitro assessments, female head lice exhibited significantly higher avoidance responses to hair tufts treated with either of the test formulations, which led to significantly higher ovipositional avoidance when compared with female lice on control hair tufts. Additionally, both formulations were determined to be competent infestation deterrents in a competitive avoidance test in the presence of a known attractant (head louse feces extract). In in vivo assessments using a previously validated poultry model, *Elimax shampoo* was determined to be an efficacious deterrent against poultry chewing lice within *Menopon* spp. and *Menacanthus* spp.

KEY WORDS *Pediculus humanus capitis*, human head louse, *Menopon* spp., *Menacanthus* spp., poultry chewing lice

The human head louse, *Pediculus humanus capitis* (De Geer), is an obligatory hematophagous ectoparasite that frequently infests school-age children, their teachers and family members, and many others who have physically contacted infested people (Mumcuoglu 1996, Meinking 1999). Since the beginning of the modern era of synthetic insecticides, people have used a number of different pediculicides to control head lice (Durand et al. 2012). Most people find louse infestations intolerable and choose to use pediculicides, many of which pose a risk of adverse effects if not used properly. Misapplication and overuse may affect children in particular because of their small body size and higher sensitivity to chemicals (Goldman 1995).

Pediculicide sales in the United States were estimated to be >US$240 million per year in 1997 (Gratz 1997). This number increased to > US$350 million per year in 2003 (Jones and English 2003). The overall cost for dealing with head louse infestation (pediculosis),

including cleaning household items, visiting doctor's office or health clinic, purchasing prescription-based and over-the-counter medications, has been estimated at~US$1 billion annually (Gratz 1997, Clark et al. 2009). Furthermore, almost 10% of school children in the United States have experienced the long-term consequences of school absences due to the "No-Nit" policy and the implementation of ineffective control measures (Gratz 1997, Williams et al. 2001, Frankowski and Weiner 2002, Lebwohl et al. 2007). A more current economic burden of losing workdays in the United States alone has been estimated ~US$4–8 billion annually (Mumcuoglu et al. 2006).

The pyrethrins- and pyrethroids-containing formulations have been used extensively and intensely for >20 years (Durand et al. 2012), and clinical resistance has been widely reported as a consequence (Chosidow et al. 1994, Hipolito et al. 2001, Burgess et al. 2005, Hill et al. 2005). Louse resistance to pyrethroids under nonclinical settings has been documented in Czech Republic (Rupes et al. 1995), the United Kingdom (Downs et al. 1999), Denmark (Kristensen 2005), Israel (Mumcuoglu et al. 1995), the United States (Pollack et al. 1999, Lee et al. 2000, Lee et al. 2010), Argentina (Picollo et al. 1998), Japan (Tomita et al. 2003), and Australia (Hunter and Barker 2003).

Due to pediculicide resistance and the lack of efficient resistance management programs, there has been

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considerable interest in the protection of uninfested school children and prevention of reinfestation by interrupting lice transfer (Mumcuoglu et al. 1996, Semmler et al. 2012, Ketzis et al. 2014). Considering that head lice do not have wings or jumping legs, the mechanism used to transfer between hosts has been debated (Takano-Lee et al. 2005) and head-to-head transfer is generally accepted as a major mechanism of new infestation (Speare and Buettner 1999). Therefore, development of infestation deterrents that are safe to humans and can specifically discourage the transfer of lice from one host to another is a critical need in this niche market of affordable and effective louse control. Nevertheless, cost and time factors in clinical trials have been the major obstacles to researchers and sponsors who want to develop efficacious infestation deterrents. The current study provides data obtained from two nonclinical model systems (in vitro and in vivo) that can serve as efficient screening systems for developing deterrent formulations that are effective in the control of louse infestations.

Materials and Methods

In Vitro Avoidance Assays. *Human Head Lice.* - Permethrin- and DDT-resistant human head lice (*Pediculus humanus capitis*, SF-HL strain), which were originally collected from infested children in Plantation and Homestead, FL, and maintained on an in vitro rearing system at the University of Massachusetts-Amherst, were used for three bioassays: oviposition avoidance, hatchability determination, and competitive avoidance.

Test Products. Elimax lotion and *Elimax shampoo*, which contain oligodecene oil, sesame oil, and acrylate, were supplied by Oystershell Laboratories NV, Drongen, Belgium. For positive controls, OFF! Deep Woods Insect Repellent VIII (OFF!, SC Johnson, Racine, WI), which contains 25% *N,N*-diethyl-meta-toluamide (DEET), was purchased locally and used in the avoidance bioassays.

Washing Solution. Sodium lauryl ether sulfate (SLES, ZETESOL NL-2U/ZETESOL 270, Zschimmer & Schwarz, Germany) was diluted to 12% (w/v) with distilled deionized water (ddH$_2$O) and used to wash all hair tufts.

Hair Tuft Treatments. A semicircular shaped (15 mm radius), flattened (∼2 mm thick) hair tuft (Sally USA, Denton, TX) or a rectangular hair tuft (10.75 by 25 mm, ∼2 mm thick) was placed on a glass Petri dish and treated with *Elimax lotion* (1 g /g hair tuft) or *Elimax shampoo* (1 g /g hair tuft) using a pipette gun to dispense the test formulation to saturate the hair tuft. Using sterile forceps, the hair tuft was gently rubbed into either *Elimax lotion* or *Elimax shampoo* using a circular motion for 30 s until complete hair tuft coverage was achieved and ensured by visual inspection under a stereomicroscope. The treated hair tuft was transferred to a clean Petri dish and incubated for 15 min at room temperature. A positive control hair tuft was treated with sufficient amount of OFF! (1 g OFF!/g hair tuft) as above for 15 min at room temperature. A negative

treatment control hair tuft was treated with sufficient amount of ddH$_2$O (1 g ddH$_2$O/g hair tuft) for 15 min at room temperature as described above for the *Elimax lotion*- or *Elimax shampoo*-treated tuft.

After the 15-min incubation, the treated hair tuft (either *Elimax lotion*, *Elimax shampoo*, OFF!, or ddH$_2$O) was completely submerged in a 250-ml beaker containing 100 ml of 12% SLES by using a weighted paper binder clamp. The beaker was placed on a magnetic stir plate (Nuova II, Thernolyne), and a magnetic stir bar (7.9 by 25.4 mm, diameter × length) was added to the beaker. Individual hair tufts were washed by stirring the 12% SLES for 30 s. The controlling knob of the plate was set at 4–5 to generate a consistent midspeed. The temperature of the 12% SLES solution was maintained between 25–30°C. This washing step was repeated two more times with fresh 12% SLES solution. Lastly, the treated and washed hair tufts were clamped, submerged, and rinsed in a beaker with 300 ml ddH$_2$O for 90 s using the stirring method described above for the SLES washing step. The hair tufts were air-dried at room temperature on a stack of filter paper (Whatman No. 1) for 1 h on a laboratory bench.

Oviposition Avoidance Bioassay and Hatchability. Freshly prepared hair tufts were used in each oviposition avoidance bioassay. A test arena was constructed on the blood-feeding membrane surface of an in vitro louse rearing unit (Yoon et al. 2006) by placing a test formulation (*Elimax lotion*- or *Elimax shampoo*-treated tuft) or a positive control (Off!-treated tuft) side by side next to a negative control (ddH$_2$O-treated tuft) as shown in Fig. 1. Adult female lice (at least 3 d old, 10 lice per test) were then placed on the boundary between treated (either *Elimax lotion*, *Elimax shampoo*, or OFF!) and negative control (ddH$_2$O-treated) hair tufts (Fig. 1). For control experiments, two semicircular hair tufts treated with only ddH$_2$O were used. The number of female lice and eggs on each semicircular hair tuft were counted under a stereomicroscope and recorded after 24, 48, and 72 h. All experiments (treatment and control) were performed in triplicate. Ovipositional avoidance values were calculated using equation 1.

$$\text{Ovipositional\ avoidance}(\%) = 100 - [\{(\text{nt}/\text{Nt})/(\text{nc}/\text{Nc})\} \times 100], \quad (1)$$

where nt = the total number of eggs on the treated tuft; Nt = the total number of eggs in the treated cup (treated plus control tufts); nc = the total number of eggs on the control tuft with the lower number of eggs; Nc = the total number of eggs in the control cup (both control tufts combined).

Immediately after the ovipositional avoidance bioassays, individual hair tufts with eggs were placed into new sterile Petri dishes, covered and moved to an incubator at 31°C and 70–80% relative humidity (RH). The number of first instars that hatched from the eggs were counted under a stereomicroscope and recorded over time until hatching ceased (∼7–10 d). Undeveloped
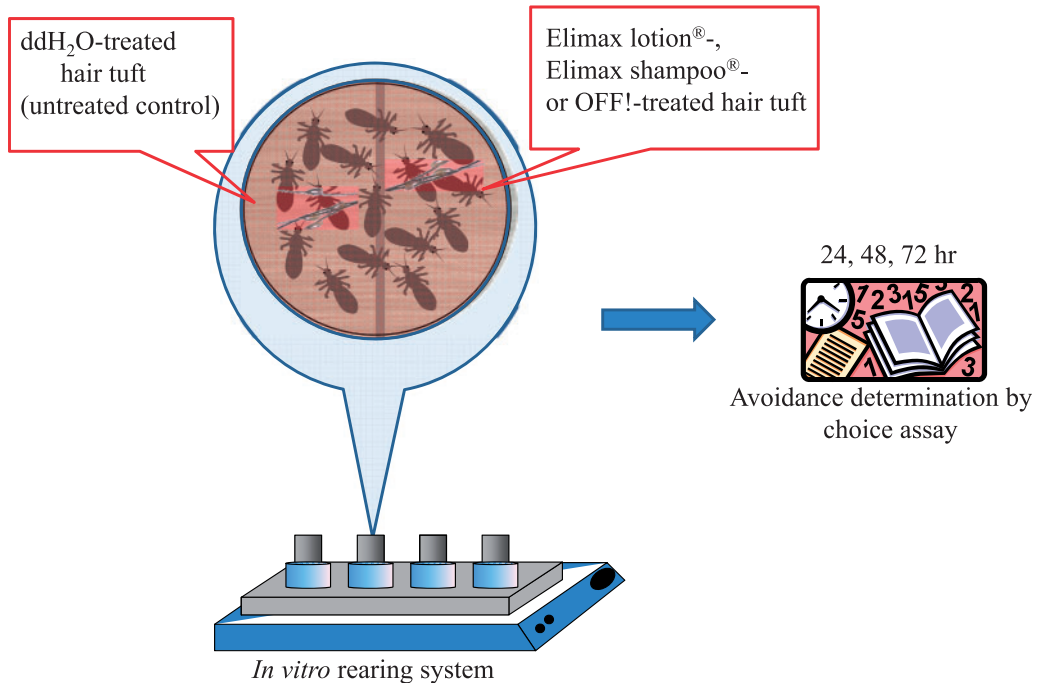
Fig. 1. An assembled test arena for the determination of ovipositional avoidance of female SF-HL in the presence of *Elimax lotion*, *Elimax shampoo*, or OFF! Insect Repellent VIII.

eggs and stillborn lice were recorded as dead. The hatchability of the eggs on each hair tuft was calculated using equation 2.

$$\text{Hatchability} = H/N \times 100, \qquad (2)$$

where H = number of eggs hatched, N = total number of eggs oviposited.

Competitive Avoidance Bioassay. The competitive avoidance was determined based on the experimental design described previously (Mumcuoglu et al. 1996) with modifications. The rectangular test arena (75 by 25 by 25 mm) was constructed from standard glass microscope slides and held together with epoxy glue (Fig. 2). Individual cotton pads (10.2 by 10.2 cm, VWR International, Radnor, PA), soaked with ddH$_2$O, ethanol, or hexane, respectively, were used to sequentially clean the test arena prior to each experiment. Four flattened hair tufts (18.75 by 25 mm) were prepared to fit the size of each of the four floor sections of the test arena (sections 1–4, Fig. 2). The rectangular hair tufts, which were fitted into the floor of the arena, allowed lice to move around freely without falling off or leaving the test arena.

The test formulation (either *Elimax lotion*, *Elimax shampoo*, or OFF!)-treated tuft was placed on section 2, and the two ddH$_2$O-treated control tufts were placed on sections 3 and 4 as shown in Fig. 2. An attractant-treated hair tuft was prepared as follows. Head louse feces collected from the in vitro rearing system was added to ddH$_2$O and mixed well to prepare 1% (w/v) suspension. This suspension was centrifuged
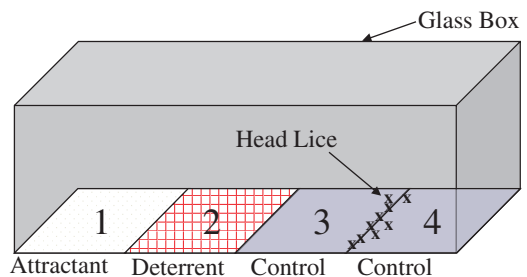
Fig. 2. An assembled test arena for the determination of competitive avoidance using an open-top glass box (75 by 25 by 25 mm).

(13,000 × g) for 1 min and the resulting supernatant was transferred to an amber glass vial and used as a 1% head louse feces extract. A hair tuft was treated with 1% head louse feces extract (1 g head louse feces extract/g hair tuft) for 15 min. The attractant-treated hair tuft was then transferred to a clean Petri dish, completely dried for 2–3 h at room temperature and placed on section 1 (Fig. 2).

Adult female lice (10 lice per trial, SF-HL) were placed on the boundary of the two control hair tufts (interface of sections 3 and 4, Fig. 2) and the arena was transferred to an incubator (31 ± 1°C, 70–80% RH) and kept in the dark. The number of lice found on each hair tuft was recorded after 30 min. Lice counted on the attractant hair tuft were added to the lice counted on the deterrent hair tuft and this total

number of females was used to determine competitive avoidance as determined using equation 3.

$$\begin{aligned} \text{Comparative} \quad \text{avoidance}(\%) \\ = 100 - [\{(n1/N)/(n2/N)\} \times 100], \end{aligned} \tag{3}$$

where N = total number of lice; n1 = total number of lice on the attractant- and deterrent-treated hair tufts; n2 = number of lice on the ddH$_2$O-treated control hair tufts.

A test formulation was considered a competent infestation deterrent of females if the competitive avoidance was >50% and a semicompetent infestation deterrent if the competitive avoidance value was >25.0% but <50.0%.

In Vivo Avoidance Assays. This assay was based on the method described previously for *Elimax lotion* (Ketzis et al. 2014). An outline of the in vivo design is presented in Table 5.

Animals and Housing. Thirty-three adult layer hens (31 white leg horn crosses and 2 Rhode Island red crosses) naturally infested with poultry chewing lice (*Menopon* spp. and *Menacanthus* spp., both type of hens had both species of lice) were acquired from a local supplier in St. Kitts, West Indies. None of the chickens had been treated with an insecticide previously. Individual chickens, identified by leg bands, were determined to be healthy by veterinary examinations. Chickens were group-housed in a 1.22- by 2.44-m pen with 4 perches. All husbandry procedures followed those described in the Ag Guide (Federation of Animal Science Societies [FAAS] Writing Committee 2010).

Test Products. Elimax shampoo and E004490 (the insecticidal oligodecene oil used in *Elimax shampoo*) were supplied by Oystershell NV. Dawn dish washing liquid (Proctor and Gamble, Cincinnati, OH) was purchased from a local supplier.

Treatments. The treatment procedure used was as per the proposed instructions for use on humans (Anonymous 2014). Six chickens, randomly selected from the 33 chickens, were treated with E004490 to kill all lice. E004490 was applied to individual chickens until fully covered (100–110 ml). The feathers were moved backwards and the skin and feathers were sprayed with E004490, using a hair dresser water spray bottle. After 15 min, the treated chickens were washed with Dawn dish washing liquid. Approximately 10 ml of dish washing liquid was mixed with water in a bucket, the chicken was placed into the bucket, and the feathers gently washed by hand. The washing liquid was rinsed off using a hose, the chickens were towel dried and gently air dried with a blow drier. Once chickens were completely dry, they were inspected to determine if all lice were killed. Lice-free chickens were assigned to the Group 2 (control group) and separately housed for 2 d. These chickens were examined for lice prior to the study start to ensure that no eggs had hatched and that they were still louse free.

After 2 d, the remaining 27 chickens were inspected to determine the number of lice on each chicken. Ten of the 27 chickens, randomly selected, were treated with *Elimax shampoo*. Treatment with *Elimax shampoo* consisted of spraying the formulation onto each chicken until the chicken was considered fully covered (100 to 144 ml of product per chicken was used depending on the size of the chicken). The feathers were moved backwards and the skin and feathers sprayed with the formulation until all feathers and skin were wet with the treatment. After 15 min, a small amount of water (<20 ml) was applied to the chicken to allow the soaping agent in the product to react. The chicken was gently washed by hand with the soap from the product and completely rinsed with water. The chickens were then towel dried and blow dried using cool air and assigned to the Group 1 (test group). The six chickens from Group 2 were rinsed with water and dried as above. The remaining 17 chickens without any treatments were assigned to the Group 3 (lice reservoir group). The timing of posttreatment intervals was begun at the time that rinsing commenced on Study Day 0.

Lice Assessment. All assessments were made by people blinded to the treatment for quantification of the deterrent properties over 7 d. The general design was based on the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Holdsworth et al. 2006) for assessing persistent activity of products for lice on cattle. To assess the number of lice on a chicken, five approximately 2- by 2-cm areas were inspected and the number of lice counted. The areas included the ventral and dorsal surface of each wing, the breast, the tail feathers, and the back. Initial lice assessments were performed on all chickens (Group 1, 2, and 3) 8 h ± 18 min posttreatment.

Study Days 1 to 7. Lice assessments were performed on all chickens (all groups) at +24 h, +48 h, +72 h, +96 h, +120 h, +148 h, and +172 h ± 1 h posttreatment.

Data Analysis. The arithmetic and geometric mean number of lice for each group are presented in Table 6. To calculate geometric means with zero counts, 1 was added to all of the numbers and then subtracted from the calculated geometric mean. Groups 1 and 3 were compared pretreatment to determine that they were not statistically different using a Kruskal–Wallis test. A Fisher's Exact test was used to compare the number of chickens with lice at each time point in Groups 1 and 2 using the total number of chickens infested at each time point as well as the accumulated number.

Results and Discussions

In Vitro Assessments. *Ovipositional Avoidance.* Female lice on the hair tufts treated with either *Elimax lotion*, *Elimax shampoo*, or OFF! exhibited significantly higher ovipositional avoidance responses compared with females on hair tufts treated with ddH$_2$O only (Table 1, Cochran-Mantel-Haenszel test, $P < 0.05$). This finding indicates that *Elimax lotion* and *Elimax shampoo* are efficacious deterrents to ovipositioning over a 72-h period.

The female ovipositional avoidance of either *Elimax lotion*- or *Elimax shampoo*-treated hair tufts was also significantly higher than that of OFF!-treated hair tufts

Table 1. Comparative ovipositional avoidance of female SF-HL on either *Elimax lotion*-, *Elimax shampoo*-, or OFF!-treated hair tufts determined by choice assays performed on the in vitro rearing system

Treatment[a]	Wash[b]	No. of ddH$_2$O rinses[c]	Time	Nc[d] or Nt[e]	nc[f] or nt[g]	% OA[h]
				Nc	nc	
ddH$_2$O (Control group)	12% SLES	1 (90 s)	24 h	124	51	–
			48 h	318	135	–
			72 h	488	218	–
				Nt	nt	
OFF!*	12% SLES	1 (90 s)	24 h	172	7	90.1
			48 h	366	32	79.4
			72 h	576	58	77.5
				Nt	nt	
*Elimax lotion**,+	12% SLES	1 (90 s)	24 h	174	0	100
			48 h	358	0	100
			72 h	557	1	99.1
				Nt	nt	
*Elimax shampoo**,+	12% SLES	1 (90 s)	24 h	133	0	100
			48 h	353	0	100
			72 h	503	0	100

[a] Individual hair tufts were treated with ddH$_2$O, OFF!, *Elimax lotion*, or *Elimax shampoo* (1 g treatment/g hair tuft) for 15 min.
[b] Individual hair tufts were washed using three separate 100 ml 12% SLES baths (stir bar method).
[c] Individual hair tufts were rinsed using a 300 ml ddH$_2$O bath (stir bar method).
[d] Nc = the total number of eggs in the control cup (both control tufts combined).
[e] Nt = the total number of eggs in the treated cup (treated plus control tufts).
[f] nc = the total number of eggs on the control tuft with the lower number of eggs.
[g] nt = the total number of eggs on the treated tuft.
[h] OA (ovipositional avoidance, %) = $100 - [\{(nt/Nt)/(nc/Nc)\} \times 100]$
* % Avoidance is significantly different than control (Cochran-Mantel-Haenszel test, $P < 0.05$).
+ % Avoidance is significantly different than OFF! (Cochran-Mantel-Haenszel test, $P < 0.05$).

(Table 1, Cochran-Mantel-Haenszel test, $P < 0.05$), suggesting that *Elimax lotion*- and *Elimax shampoo*-treated hair tufts are more efficacious deterrent to oviposition than OFF! under the aforementioned experimental conditions.

Avoidance Response of Female Lice. Female lice exhibited significantly higher avoidance responses to the hair tufts treated with either *Elimax lotion*, *Elimax shampoo*, or OFF! compared with the female avoidance responses to the control ddH$_2$O-treated hair tufts (Table 2, Cochran-Mantel-Haenszel test, $P < 0.05$). Although data collection was not based on the continuous monitoring of female movements on the hair tufts, the data from the three time points certainly provides a reasonable basis for the ovipositional avoidance reported and discussed above.

Female avoidance responses to either *Elimax lotion*- or *Elimax shampoo*-treated hair tufts were also significantly higher than those determined using OFF!-treated hair tufts (Table 2, Cochran-Mantel-Haenszel test, $P < 0.05$), indicating that both *Elimax lotion* and *Elimax shampoo* are more effective female deterrents compared with OFF! under the experimental conditions used in current investigation.

Hatchability. Because the ovipositional avoidance values of female SF-HL in tests with either *Elimax lotion*- or *Elimax shampoo*-treated hair tufts were higher than 99.5% (Table 1), there were very few eggs, if any, laid on the test formulation-treated hair tufts. With such a small sample size, statistical analysis of hatchability was not feasible (Table 3). The hatchability values obtained from hair tufts treated with OFF! (trial 3 data excluded, as only one egg laid on the OFF!-treated hair tuft) were significantly lower than those

obtained from ddH$_2$O-treated hair tufts (Table 3, *t*-test, $P < 0.05$), suggesting that the OFF! product may contain an ovicidal agent. In fact, DEET often exhibits insecticidal activity and one of the nonvolatile ingredients, isopropyl myristate, has been reported as an effective agent removing hydrocarbons from head lice (Burgess et al. 2008, Barnett et al. 2012). It is possible that these two compounds in the OFF! product may be responsible for the ovicidal response, but further investigation is necessary to confirm this speculation.

Competitive Avoidance. All three formulations (*Elimax lotion*, *Elimax shampoo*, and OFF!) were determined to be competent deterrents at the end of the initial 30-min assay period (Table 4). Female avoidance (%) determined for OFF! treatment was significantly decreased from 75.0 to 22.2% at the end of the next 30-min period (*t*-test, $P < 0.05$). In a similar comparison, the avoidance values for either the *Elimax lotion* or *Elimax shampoo* formulations did not change at the end of the second 30-min period. Interestingly, the mean avoidance value ($69.1 \pm 10.3\%$) obtained from the assays with *Elimax lotion*-tufts was significantly higher than the avoidance value ($22.2 \pm 19.2\%$) obtained from assays with OFF!-tufts (ANOVA with Tukey's test, $P < 0.05$). This result shows that *Elimax lotion* and *Elimax shampoo* elicited more consistent competitive avoidance over the 1-h duration of experiment, suggesting that both *Elimax lotion* and *Elimax shampoo* may be better deterrents against female head lice. Further research, including clinical trials, is necessary to confirm the above suggestion.

In vivo Assessments. All of the chickens initially had heavy louse infestations associated with the wings, feathers, and body. For those in Group 2, no live lice

Table 2. Comparative avoidance of female SF-HL on either *Elimax lotion*-, *Elimax shampoo*- or OFF!-treated hair tufts determined by choice assays performed on the in vitro rearing system

Treatment[a]	Wash[b]	No. of ddH$_2$O rinses[c]	Time	Nc[d] or Nt[e]	Nc[f] or nt[g]	% FA[h]
				Nc	nc	
ddH$_2$O (Control group)	12% SLES	1 (90 s)	24 h	30	8	–
			48 h	27	10	–
			72 h	29	12	–
				Nt	nt	
OFF! *	12% SLES	1 (90 s)	24 h	30	4	50.0
			48 h	30	3	73.0
			72 h	30	5	59.7
				Nt	nt	
Elimax lotion*,+	12% SLES	1 (90 s)	24 h	30	0	100
			48 h	30	0	100
			72 h	30	0	100
				Nt	nt	
Elimax shampoo*,+	12% SLES	1 (90 s)	24 h	30	0	100
			48 h	30	0	100
			72 h	30	0	100

[a] Individual hair tufts were treated with ddH$_2$O, OFF!, *Elimax lotion*, or *Elimax shampoo* (1 g treatment/g hair tuft) for 15 min.
[b] Individual hair tufts were washed using three separate 100 ml 12% SLES baths (stir bar method).
[c] Individual hair tufts were rinsed using a 300 ml ddH$_2$O bath (stir bar method).
[d] Nc = the total number of females in the control cup (both control tufts combined).
[e] Nt = the total number of females in the treated cup (treated plus control tufts).
[f] nc = the total number of females on the control tuft with the lower number of eggs.
[g] nt = the total number of females on the treated tuft.
[h] FA (female avoidance, %) = 100 − [{(nt/Nt)/(nc/Nc)} × 100]
* % Avoidance is significantly different than control (Cochran-Mantel-Haenszel test, $P < 0.05$).
+ % Avoidance is significantly different than OFF! (Cochran-Mantel-Haenszel test, $P < 0.05$).

Table 3. Comparative percent hatchability of SF-HL eggs oviposited on differentially treated hair tufts

Treatment[a]	Wash[b]	No. of ddH$_2$O rinses[c]	Trial	% Hatchability	
				ddH$_2$O	ddH$_2$O
ddH$_2$O (Control group)	12% SLES	1 (90 s)	1	96.1 (74/77)	83.6 (56/67)
			2	78.9 (56/71)	87.3 (62/71)
			3	88.5 (108/122)	91.3 (73/80)
				OFF!	ddH$_2$O
OFF! (25% DEET)	12% SLES	1 (90 s)	1	86.5 (32/37)[d]	92.6 (138/149)
			2	85.0 (17/20)[d]	90.6 (145/160)
			3	100 (1/1)	89.2 (140/157)
				Elimax lotion	ddH$_2$O
Elimax lotion	12% SLES	1 (90 s)	1	ND[e]	89.8 (177/197)
			2	ND[e]	96.4 (135/140)
			3	100 (1/1)	95.5 (210/220)
				Elimax shampoo	ddH$_2$O
Elimax shampoo	12% SLES	1 (90 s)	1	ND[e]	95.0 (151/159)
			2	ND[e]	96.3 (130/135)
			3	ND[e]	97.6 (204/209)

[a] Individual hair tufts were treated with ddH$_2$O, OFF!, *Elimax lotion*, or *Elimax shampoo* (1 g treatment/g hair tuft) for 15 min.
[b] Individual hair tufts were washed using three separate 100 ml 12% SLES baths (stir bar method).
[c] Individual hair tufts were rinsed using a 300 ml ddH$_2$O bath (stir bar method).
[d] Hatchability (trial 3 data excluded) on the OFF!-treated hair tuft was significantly lower that on treated hair tuft (*t*-test, $P < 0.05$).
[e] ND, not determined due to the lack of oviposition of eggs on the hair tuft.

were found after the E004490 treatment, but their infestation levels prior to lice removal indicated that they were capable of having heavy louse infestations. Groups 1 (treated with deterrent) and 3 (reservoir for lice) were not statistically different in regards to their louse counts pretreatment (Kruskal–Wallis test; $P = 0.688$; 0.687 adjusted for ties), indicating that all of the chickens were able to maintain equivalent levels of louse infestations (Table 6).

During the treatment applications, the lice tended to move away from the area of application (Group 1) and stopped moving quickly (Groups 1 and 2). *Elimax shampoo* (Group 1) washed off of the chickens relatively easily resulting in very clean chickens; E004490 (Group 2) left a slight oily film on the chickens for ~24 h. This difference posed a challenge in regards to blinding. Therefore, all chickens were thoroughly examined for lice (e.g., additional locations examined)

Table 4. Competitive avoidance (%) of SF-HL females (10 lice per trial) on the glass arena with hair tufts treated with ddH₂O, an attractant (1% feces), and a test formulation (OFF! Deep Woods Insect Repellent VIII, *Elimax lotion*, or *Elimax shampoo*)

	Trial 1	Trial 2	Trial 3	Mean[a,b] ± SD
OFF!				
30-min avoidance	75.0	75.0	75.0	75.0 ± 0[a]
60-min avoidance	33.3	0	33.3	22.2 ± 19.2[A]
Elimax lotion				
30-min avoidance	33.3	100	57.1	63.5 ± 33.8[a]
60-min avoidance	75.0	75.0	57.1	69.1 ± 10.3[B]
Elimax shampoo				
30-min avoidance	57.1	75.0	75.0	69.1 ± 10.3[a]
60-min avoidance	75.0	57.1	33.3	55.2 ± 20.9[A,B]

[a] A formulation is considered to be a competent deterrent to infestation when % avoidance is >50.0 %.

[b] 30-min avoidance means followed by the same lower-case letter are not statistically different by ANOVA ($P > 0.05$); 60-min avoidance means followed by the same upper-case letter are not statistically different by ANOVA ($P > 0.05$).

Comparative avoidance (%) = $100 - [\{(n1/N)/(n2/N)\} \times 100]$, where N = total number of lice; n1 = total number of lice on the attractant- and deterrent-treated hair tufts; n2 = number of lice on the ddH₂O-treated control hair tufts (Fig. 2).

Table 5. In vivo study design

Time point	Procedures
Day -3	Chickens arrived; veterinary examination
Day -2	Group 2[a]: E004490 used to remove lice
	Group 2 housed separately until Day 0
Day 0	Lice on all chickens counted
	Group 1[b]: *Elimax shampoo*, applied
	Group 2: rinsed with water
	Groups 1, 2, and 3 housed together
Day 0 + 8h	Lice counted
Days 1–7 (+24 to +172)	Lice counted
Treatment	
Group 1[b]	100–144 ml *Elimax shampoo*, applied
	15 min wait
	Small amount of water used to create suds
	Rinsed with water
	Dried
Group 2[a]	100–110 ml E004490 applied
	15 min wait
	Soap used to wash off E004490
	Rinsed with water
	Dried

[a] Chickens treated with E004490 according to the instructions (Treatment).

[b] Chickens treated with *Elimax shampoo* according to the instructions (Treatment).

whenever a count of zero was determined. Following treatments, all of the chickens (Groups 1, 2, and 3) were housed together and shared the perches in a common pen. After treatments, none of the chickens were avoided by the other chickens, indicating that there was sufficient and equal opportunity for lice to transfer from chicken to chicken and no pecking order appeared to influence contact between the chickens.

Average louse counts per group over time post treatment and the number of infested chickens in each group are presented in Tables 6 and 7, respectively. All chickens in Group 3 (reservoir group) were infested with lice at all of the time points post treatment (3 to 93 lice per chicken). All six Group 2 chickens, used to demonstrate natural reinfestation without protection, had lice within 8 h of being exposed to the Group 3 chickens and continued to have lice during the 7-d experimental time period, except for one chicken at +24 h. While the infestation levels were low (1–7 lice per chicken), the Group 2 chickens consistently had lice.

Group 1 chickens, treated with *Elimax shampoo*, had low louse counts (0 to 3) during the 7-d experimental period (Fig. 3A). At 8 h after being exposed to the lice-infested Group 3 chickens (reservoir group), two of the 10 Group 1 chickens had one louse each. From 24 to 72 h after being exposed to the Group 3 chickens, only 1 of the 10 Group 1 chickens had lice (1 louse). At 4 d (96 h) after treatment and exposure to the Group 3 chickens, the Group 1 chickens began to become as infested as the Group 2 chickens (Fig. 3B and C).

The proportion of infested chickens in Groups 2 was significantly higher than that of infested chickens in Group 1 during a period from 8 to 72 h (Fig. 3C). In other words, the Group 2 chickens were infested faster than the Group 1 chickens during the 8–72 h period. By 96 h posttreatment and onward, the chickens in Group 1 appeared to be able to maintain an increased number of lice, indicating that the deterrent properties of *Elimax shampoo* may no longer be effective.

In Vitro and In Vivo Assessments to Evaluate Deterrent Formulations Against Head Lice. Based on results from in vitro and in vivo assessment, *Elimax lotion* and *Elimax shampoo* can be considered as effective infestation deterrents against head lice. In the in vivo study, *Elimax shampoo* effectively killed the

Table 6. Average and mean lice counts for Group 1 (*Elimax shampoo*, infestation deterrent), Group 2 (re-infestation control group), and Group 3 (lice reservoir group)

| Group	Average lice counts (geometric mean[a])Hours posttreatment								
	Pretreatment[b]	+8	+24	+48	+72	+96	+120	+148	+172
1	33 (28)	0.2 (0.2)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.8 (0.6)	0.5 (0.4)	0.7 (0.5)	0.7 (0.5)
2	0 (0)	2 (2)	2 (3)	3 (3)	2 (2)	2 (2)	2 (2)	2 (1)	2 (2)
3	30 (26)	35 (30)	32 (27)	33 (27)	28 (23)	32 (23)	28 (19)	26 (16)	29 (17)

[a] Geometric mean (GM) calculation: when a count contained zeros, 1 one was added to each number, the GM calculated and then 1 subtracted from the calculated GM.

[b] Groups 1 and 3 were not statistically significantly different ($P = 0.688$; 0.687 adjusted for ties; Kruskal–Wallis) pretreatment.

Table 7. Number of chickens infested in Group 1 (*Elimax shampoo*, infestation deterrent) and Group 2 (re-infestation control group) posttreatment

Group	Number in group	Number of positive chickens (accumulated number of positive chickens)							
		+8	+24	+48	+72	+96	+120	+148	+172
1	10	2	1 (3)	1 (3)	1 (3)	5 (6)	5 (8)	5 (9)	5 (9)
2	6	6*	5*	6*	6*	6	6	6	6

An asterisk indicates a significant difference in infested:noninfested chickens between Groups 1 and 2 ($P < 0.01$; Fisher's Exact test).
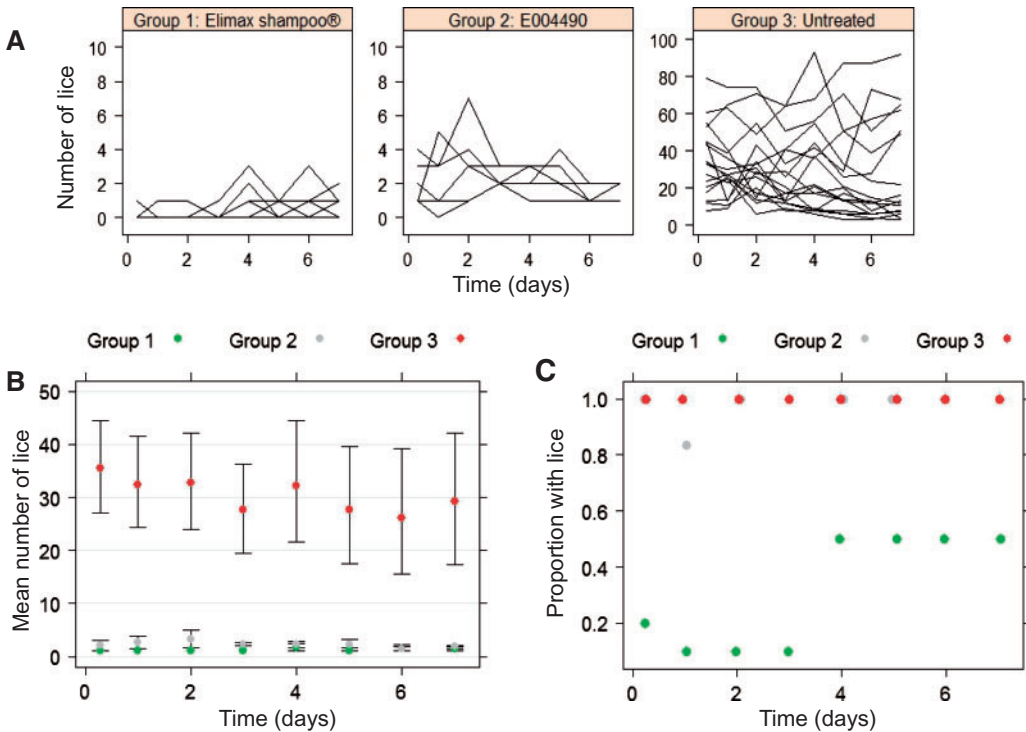
Fig. 3. Analysis of data obtained from in vivo avoidance assays. (A) Changes in number of lice determined on individual chickens superimposed by treatment group. (B) Average ($\pm$SD) number of lice for lousy chickens. (C) Proportion of lousy chickens. (●) group 1 treated with *Elimax shampoo*, (●) group 2 treated with E004490, and (●) group of lousy chickens.

existing chewing lice on chickens and provided significant protection from reinfestation for 72 h.

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