

## Research Article

# Chronic Wounds, Biofilms and Use of Medicinal Larvae

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Chronic wounds are a significant health problem in the United States, with annual associated costs exceeding \$20 billion annually. Traditional wound care consists of surgical debridement, manual irrigation, moisture retentive dressings, and topical and/or systemic antimicrobial therapy. However, despite progress in the science of wound healing, the prevalence and incidence of chronic wounds and their complications are escalating. The presence & complexity of bacterial biofilms in chronic wounds has recently been recognized as a key aspect of non-healing wounds. Bacterial biofilms are sessile colonies of polymicrobial organisms (bacteria, fungus, etc.) enclosed within a self-produced exopolymeric matrix that provides high levels of tolerance to host defenses, antibiotics and antiseptics. Thus, there is a need for alternative therapies to reduce biofilms in chronic wounds. In this report, we present initial findings from in vitro experiments which show that larval debridement therapy with disinfected blow fly larvae (*Phaenicia sericata*) reduced total CFUs (6-logs) of planktonic and mature biofilms of *Pseudomonas aeruginosa* or *Staphylococcus aureus* grown on dermal pig skin explants by 5-logs after 24 hours of exposure, and eliminated biofilms (no measurable CFUs) after 48 hours of exposure.

## 1. Introduction

Chronic wounds are a significant health problem in the USA. Chronic wounds are those wounds which fail to progress as expected through the typical healing processes in a timely manner. Health care costs related to the management and treatment of chronic wounds in the USA exceeds \$20 billion annually [1–7]. For many health care providers, the treatment and management of nonhealing wounds are challenging. Traditionally, basic wound care has consisted of surgical debridement, manual irrigation, moisture retentive dressings, and topical and/or systemic antimicrobial therapy. Although there has been tremendous progress in the science of wound healing, the prevalence and incidence of chronic wounds and their associated complications continue to escalate [1]. The presence and complexity of bacterial biofilms in chronic wounds have recently been recognized as key

aspects of nonhealing wounds [8–20]. Bacterial biofilms are sessile colonies of polymicrobial organisms (bacterial, fungal, and possibly, viral) which are often symbiotic. These biofilm colonies produce a protective coating to protect the colonies from host defenses. The character of this protective substance unique to biofilms is dynamic, and the production of its components seems to be triggered by hostile environments in the wound bed (such as the presence of topical antibiotics). Biofilms have been shown to have survival and defense mechanisms that inhibit the healing aspects of inflammatory cells, resist antibiotics (topical and systemic) and other therapies, and initiate cell-to-cell communication pathways (quorum sensing) which facilitate new biofilm growth, resulting in recalcitrant nonhealing wounds [2].

With the increase of drug-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) [21], there is a need for innovative therapies in the treatment of

wound biofilms. Wound larval debridement therapy (LDT) has been shown to have promise in healing chronic wounds by eradicating biofilms. In this paper, we discuss the pathogenesis of chronic wounds with a focus on biofilms. We also discuss biofilm characteristics and the clinical relevance of LDT as an important treatment option for eradicating wound biofilms. We will also present preliminary findings from significant *in vitro* experiments demonstrating the effects of disinfected blow fly larvae (*Phaenicia sericata*) exposed to mature biofilm models (*Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilms attached to the dermis of pig skin explants).

## 2. Characteristics of Biofilms

A bacterial biofilm is characterized as an aggregated bacteria attached to a surface or formed at a surface interface and organized as a complex community embedded in a self-secreted extracellular polymeric substance (EPS) [2–20, 22–24]. These dynamic bacterial communities may consist predominately of single bacterial or fungal species or, more commonly, may be polymicrobial, containing multiple diverse species that are continuously changing [23]. Biofilms have been identified on various surfaces of the body including the teeth (plaque), endocardium, GI and GU mucosa, and nasal epithelium as well as foreign objects such as orthopedic prosthetics and invasive catheters [25–27]. Evidence suggests that biofilms are strongly associated with impaired wound healing in chronic skin wounds [6, 10, 12, 15, 16, 23, 24]. Wound biofilms trigger a chronic inflammatory response resulting in accumulation of neutrophils and macrophages surrounding biofilms. The neutrophils and macrophages secrete high levels of reactive oxygen species (ROS) that affect the biofilm and the surrounding tissues [15]. Inflammatory cells also secrete high levels of proteases (matrix metalloproteinases and elastase) that can help break down the attachments between biofilms and the affected tissue, dislodging the biofilms from the tissue [25, 28]. However, the ROS and proteases also have the capacity to damage the normal surrounding tissue, proteins, immune cells, and tissue cells, delaying healing. In vulnerable tissue, biofilms are created by planktonic bacteria attaching and forming a protective community before they are killed by the patient's immune system, antibiotics, or by debridement. Several conditions which impair the immune system or reduce the effectiveness of antibiotic drugs encourage the development and spread of biofilms in wounds. These include ischemia or necrosis of tissues, nutritional deficits or compromise, and comorbidities that impair the body's immune function, such as HIV, diabetes, major physical trauma, radiation treatment, or treatment with immune-suppressing drugs [1].

## 3. Biofilm Mechanisms

It has been suggested that the processes employed by biofilms include molecular mechanisms which enable bacteria to attach to host cells and inject proteins to reorganize host cellular pathways [25, 26]. For some bacterial species, the injected bacterial proteins reorganize the host cellular

cytoskeleton and prevent migration and mitosis, and inhibit apoptosis [27, 29–33]. As bacteria begin to form a biofilm, their molecular mechanisms may attract other bacteria to form a sustainable polymicrobial system [25, 26]. A biofilm colony is thought to possess an expanded diverse gene pool representing numerous species of bacteria [16, 34]. Long-term biofilm survival is often directly related to the genetic diversity of the biofilms, resulting in chronic infections that become recalcitrant to treatment. Survival of a bacterial biofilm requires gene expression to ensure attachment to the host, cellular senescence of the host to prevent shedding and to cause local inflammation, and stimulation of the production of plasma in the wound bed to nourish the biofilm colony [35].

Microorganisms that have the ability to form biofilms also possess quorum-sensing molecules to direct the focus and organization of the biofilm [26, 36]. Directed secretion of molecules and organization of the colonies in biofilms maximize the availability of nutrients and other essential molecules while minimizing the opposing effects of waste products, toxins of competitors, and other environmental hazards on the biofilms. Polymicrobial biofilms likely incorporate quorum-sensing molecules that can regulate pathways and also perform bidirectional signaling [26]. Biofilm organisms have the ability to sense and communicate with many quorum-sensing pathways. Biofilms have numerous defenses and can be resistant to treatment, limiting the effectiveness of antibiotics [26]. Antibiotics and antiseptics kill single bacteria very easily, but the biofilm barrier blocks most antibiotics and antiseptics from reaching the bacteria, particularly towards the center of the wound matrix [26]. Wound biofilms are resistant to antibodies, antibiotics, disinfectants, and phagocytic inflammatory cells. There is strong clinical evidence suggesting that larvae therapy, a less costly continuous debridement therapy, may be useful in eradicating wound biofilms [37–39].

## 4. Larvae Background

Maggot or larval debridement therapy (MDT or LDT) has been utilized for medical purposes for hundreds, if not thousands of years [40–42]. Surgeons since the 1700s have documented that the larvae of certain common blow flies or greenbottle flies (*Phaenicia sericata* and *Lucilia sericata*) remove only dead tissue while promoting healthy tissue in the wound bed, helping wounds heal faster [42]. The lifecycle of the typical fly larvae is about 10–14 days from the point of hatching until becoming an adult fly. However, larvae need to pupate before maturing into an adult fly; medicinal maggots are both physically and reproductively sterile, and because they are maintained in a moist environment, they are never allowed to pupate. In addition, these fly larvae will not burrow into or remove healthy tissue; they will only degrade, liquefy, and ingest dead tissue [40, 41]. Interestingly, while the larvae secretes an enzymatic substance which may also have natural antimicrobial properties [41], they do not excrete any waste product back into the wound. Prete (1997) suggested that their secretions also stimulate the growth of granulation tissue in the wound bed [42].

The medicinal use of fly larvae to remove necrotic tissue has been referred to as biosurgery, maggot debridement therapy (MDT), larval debridement therapy (LDT), or just larval therapy. Typically, the larvae are only 2 mm long when first applied to the wound bed. They are applied using 5–10 larvae per square centimeter and are usually left in the wound for up to 4 days. These therapeutic larvae essentially continue to ingest necrotic tissue (and wound waste, such as bacteria) until they have grown to more than 4–5 times their original size, about 3–4 days, at which point they are removed and/or replaced with new larvae. Larvae applied in such a fashion have been known to ingest up to 15 grams of necrotic tissue per day [38, 40–43].

Dr. John F. Zacharias (1837–1901), a Confederate American Civil War surgeon, is recognized as the first healthcare provider in the USA who intentionally applied maggots for wound care/debridement purposes. He noted that “maggots could clean a wound better in one day” than any other agent they had at their disposal [36]. He credited maggots with saving many soldiers’ lives. Likewise, Dr. William S. Baer was an orthopaedic surgeon in WW1 who recognized the efficacy of maggots on the battlefield to “clean up” compound fractures and large flesh wounds, recognizing that maggots prevented sepsis in two battlefield cases which otherwise would have certainly been fatal [36, 42]. Maggots as a medical treatment impressed Dr. Baer immensely, and in 1929, he started conducting research at Johns Hopkins University using maggots he found in the neighborhood or raised on a windowsill. Two of his patients contracted tetanus from contaminated maggots (one died), so he developed sterile maggot-raising procedures. He used maggot therapy in 21 patients with chronic osteomyelitis who had not responded to other treatment. He demonstrated rapid wound debridement of necrotic tissue, a return of the wound bed to an alkaline pH environment, the reduction of bacteria, reduced odor levels, wound closure, and complete healing of the osteomyelitis infections within six weeks [36, 43].

With the development of antibiotics in the 1940s and various skin and wound antiseptics, the use of LDT declined. Arguably, one of the biggest reasons LDT may have lost favor in clinicians’ eyes was not ineffectiveness, for they remain a most effective form of debridement, but rather, the “yuck factor.” Patients, their caregivers, and clinicians found it distasteful to apply small squirming worms that could crawl out of a wound. Even Dr. Baer said that “the sight was very disgusting and measures were taken hurriedly to wash out these abominable looking creatures [36].”

With the advent of antibiotic-resistant organisms and increasing drug sensitivities, there was a renewed interest in maggot therapy in the 1980s [40]. The U.S. Food and Drug Administration (FDA) cleared medicinal maggots (*P. or L. sericata*) for debriding nonhealing necrotic skin and soft tissue wounds including diabetic foot ulcers, pressure ulcers, nonhealing surgical or traumatic wounds, and venous stasis ulcers. In the USA, larval therapy with maggots is classified as a medical *device* [40]. However, in Europe, Canada, and Japan, maggots are classified as medicinal *drugs*.

Maggots used in the USA for larval debridement therapy are all processed under controlled laboratory conditions

and are sterile (free of disease as well as unable to reproduce). Larval debridement of nonviable tissue within chronic wounds results partly from necrotic tissue and wound waste being liquefied by the proteolytic digestive enzymes (along with bacteria and biofilm) which the larvae ingest. As such, larval therapy is a most efficient and noninvasive method to debride a wound without the pain, bleeding, or inflammatory response associated with debridement [40].

Unfortunately, current larval debridement methods available in the USA have not addressed the “yuck factor” of free-roaming maggots in open wounds (or the patient’s aversion to the sight of maggots). This may explain why, despite the clinically proven effectiveness of larval therapy to aid in the healing process, many US clinicians do not use this method of wound treatment. Many nurses, doctors, caregivers, and patients have voiced an aversion to handling maggots or having to “count the number that go into a wound or come out of a wound.”

## 5. Materials and Methods

*Mechanisms of action.* Studies demonstrate that LDT works by mechanical as well as enzymatic debridement, has antibacterial properties, and stimulates wound healing [40–43]. However, the exact mechanisms of action require further exploration. Recently, the University of Florida Wound Research Laboratory conducted several in vitro experiments to demonstrate the efficacy of larval exposure to biofilm and document the results.

The In-Vitro Experiment Protocol included.

*Step 1.* Three 35 mm diameter pigskin explants were inoculated with *Pseudomonas aeruginosa* (PA01) bacteria, and three additional 35 mm pigskin explants were inoculated with *Staphylococcus aureus* (SA35556) bacteria. The pigskin explants were maintained on soft agar in a 90 mm Petri dish for 3 days. At the end of the 3 days, these explants represent a chronic wound biofilm model (demonstrating fully mature biofilm colonies on the explant). This model has provided consistent results and has been used successfully in several other in-vitro biofilm studies by the Wound Research Laboratory at University of Florida.

*Step 2.* In the control, colony forming units (CFU) of bacteria were determined before and after high antibiotic treatment (a wash of gentamycin, stimulating the free floating bacteria to develop into a fully mature biofilm colony  $>10^5$  CFU).

*Step 3.* Punch biopsies (5 mm) were obtained from each explant for plating, CFU counts, and SEM (electron microscopy photos) at 24 hours prior to the addition of larvae to the Petri dishes.

*Step 4.* Eight-layer cotton gauze moistened with 3 mL 0.9% NaCl was applied on top of the biofilm models.

*Step 5.* At 3 days, the explants (with 3-day biofilm) were changed from atop soft agar to atop saline moistened gauze in the Petri dish.

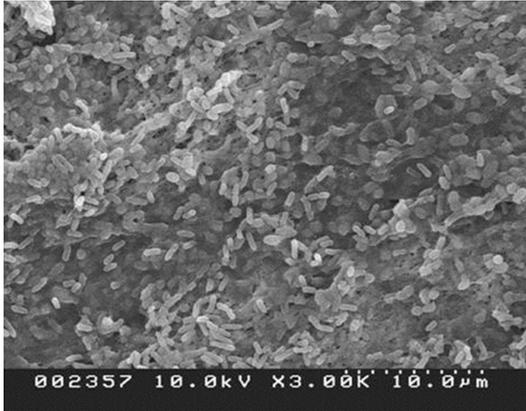


FIGURE 1: Three-day PA01 biofilm on pig explant (before larvae are applied).

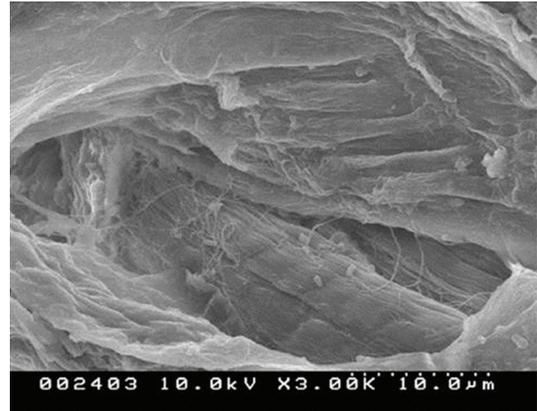


FIGURE 2: PA01 biofilm on explant (24 hours after larvae are applied).

*Step 6.* Thirty live *L. sericata* larvae were applied to the top of each pigskin explant/mature biofilm model, then the Petri dish lid was set in place and sealed with parafilm.

*Step 7.* Punch biopsies (5 mm) were obtained from each explant for plating, CFU counts, and SEM (electron microscopy photos) at 24 and 48 hours after the addition of larvae to the Petri dishes.

Figures 1–4 are scanning electron microscope photo documentation of the punch biopsies obtained from the 35 mm round pigskin explants (chronic wound model) inoculated with either *P. aeruginosa* (PA01) bacteria or *S. aureus* (SA35556) bacteria. Figures 1 and 3 demonstrate the mature biofilm colonies present on the pigskin explants. Figures 2 and 4 demonstrate complete eradication of the biofilm (the pigskin explant was left intact) within 24–48 hours of exposure to the maggots (Table 1). The bacteria had not reappeared on the explants after 48 hours. This is the first paper, to our knowledge, that showed LDT specifically and preferentially removing biofilm attached to non-viable dermal tissue. Additionally, there was no evidence that the maggots had ingested any of the pigskin explant or each other.

## 6. Results

Results are illustrated in Table 1 and Figure 5.

Figures 1 through 4 show scanning electron microscope (SEM) photographs of punch biopsies obtained from 35mm diameter pigskin explants (chronic wound model) on which mature biofilms of either *P. aeruginosa* (PA01) bacteria or *S. aureus* (SA35556) bacteria were grown. Figure 1 (PA01) and Figure 3 (SA3556) show the mature biofilm colonies present on the pigskin explants before exposure to LDT. There are clear structural features that are characteristic of biofilm community structures. For example, in Figure 1, numerous rod-shaped bacteria are present within the sheet-like structure of the exopolymeric matrix of the biofilm that collapsed during the fixation and dehydration of the SEM sample. A few *P. aeruginosa* rods are visible on the surface

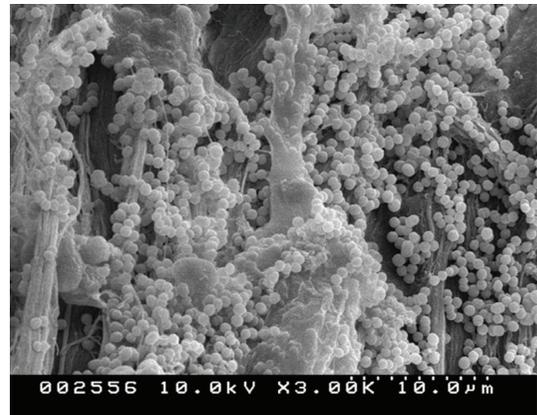


FIGURE 3: Three-day SA35556 biofilm on pig explant (before larvae are applied).

of the biofilm matrix. Similarly, Figure 3 shows numerous spherical shaped *S. aureus* bacteria embedded in the biofilm matrix, and some bacteria that are attached to the surface of the pig skin dermis that are not yet enclosed in a matrix.

Figures 2 and 4 demonstrate no detectable biofilm or planktonic bacteria on these areas of the surface of the pig skin explants after 24 hours of exposure to the medial maggots. The rope-like structures of collagen fibers are still visible, showing the effective debridement accomplished by the LDT.

Quantitation of levels of planktonic and biofilm bacteria in the treatment groups are presented in Table 1. Three days after inoculating the pig skin explants, there were high total bacterial counts on the explants, with approximately 3.4 million CFUs of PA01, of which 300,000 CFUs were in the biofilm and were tolerant to 24 hours of exposure to high levels of antibiotics. Similarly, there were 11.3 million CFUs of total SA35556, of which 430,000 CFUs were in the biofilm and were protected from 24 hours of exposure to antibiotics. After 24 hours of exposure to medical maggots, the levels of both bacteria were 1.7 to 3.3 CFUs per explant, which represents approximately 5-log reduction of total bacteria. After 48 hours of exposure to LDT, no bacterial growth (0 CFUs) was

TABLE 1: Bacterial CFU/mL at 24 hours and 48 hours after larvae exposure.

Bacterial strain	Total bacterial count (including free-floating planktonic)	Antibiotic-tolerant biofilm	1 day (24 hours after larvae treatment)	2 days (48 hours after larvae treatment)
PA01	3.4E06 ( $3.4 \times 10^6$ )	3.0E05 ( $3.0 \times 10^5$ )	3.3E00	0.0E00 (eradicated)
SA35556	1.13E07 ( $1.13 \times 10^7$ )	4.3E05 ( $4.3 \times 10^5$ )	1.7E00	0.0E00 (eradicated)

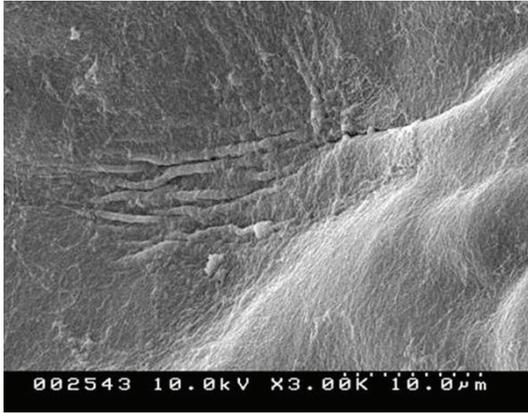


FIGURE 4: SA35556 biofilm on pig explants (24 hours after larvae are applied).

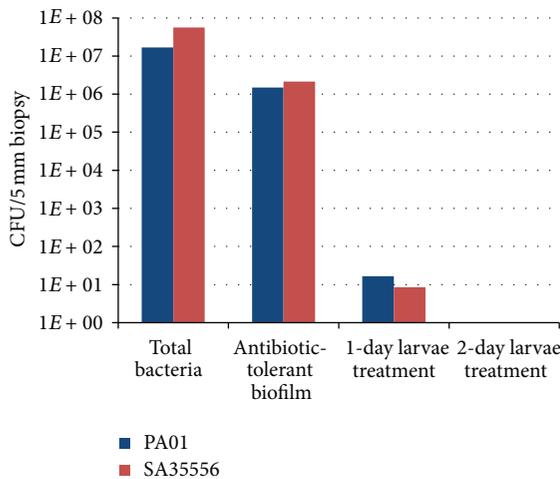


FIGURE 5: Graph of bacterial CFU before and after larval exposure.

recovered from the processed pig skin explants, indicating total removal of the planktonic and biofilm bacteria.

### 7. Discussion and Conclusions

As demonstrated, LDT shows promising effectiveness at eradicating bacterial biofilm from chronic wounds. In light of multidrug-resistant organisms [44] and drug allergies/sensitivities as well as the pain associated with traditional debridement procedures, larvae therapy may indeed be one of the most effective tools in the clinician’s arsenal for treating chronic, non-healing wounds. More research is

warranted to further investigate the clinical efficacy of this treatment and explore the exact mechanisms of action with regards to wound healing and the effects of larvae on the microenvironment of the wound bed. Future research is also needed to explore ways to utilize this treatment in a more aesthetically acceptable manner. Potential limitations of the experiments reported in this paper include the fact that they were conducted in a controlled in-vitro setting without a human host wound environment, limiting their generalizability. Currently, this study is being repeated in living human wound models to validate similar findings regarding the larvae’s ability to eradicate biofilm within a chronic wound environment. In addition, further research should address questions regarding the length of time larvae debridement therapy should be conducted to achieve maximum therapeutic results.

### 8. Pearls for Clinicians

*Clinical Indications for Larval Therapy.* For debriding non-healing full thickness skin or soft tissue wounds with necrotic or non-viable tissue wound types that may benefit from larval therapy include; diabetic or neuropathic foot ulcers, venous stasis ulcers (where compression may be delayed a few days/weeks), pressure ulcers, and non-healing traumatic or postsurgical wounds.

*Contraindications.* Blind tunnels or fistulas which lead to internal organs; wounds with necrosis around major blood vessels; patient allergy to fly larvae or to products used in larvae cultivation (soy proteins and/or brewer’s yeast); in or near eyes; upper GI tract or respiratory tract; wounds not exposed to the outside air; wounds that must be covered with completely occlusive dressings or compression/direct pressure such as sitting surfaces (situations where the larvae would be killed, compressed, suffocated, etc.). *Precautions.* Patients with coagulopathies (monitor closely for bleeding) or severe arterial insufficiency. See manufacturers’ insert for full listing of contraindications or precautions [43]. *How to order.* At present there are limited resources where disinfected larvae or medical maggots may be purchased in the USA (more sources are available in the European market). Larvae used for this study were kindly provided by Monarch Labs [45] 17875 Sky Park Circle, Suite K, Irvine, CA 92614, USA.

*Applying and Removing LDT.* Follow universal precautions and good clinical practice as you would for any dressing change. Suggestions unique to larval therapy: keep the shipping container with the larvae at about 8–10 degrees Celsius. Check manufacturer guidelines about storage—they typically should be placed in the wound within one day. You may wish

to use a “cage” to keep the larvae within the wound bed or “window” the wound edges with hydrocolloid dressing cut to the exact size and shape of the wound. Place larvae in wound bed, some recommend counting each one as it goes in and counting them as they are removed. They may be applied on a moist 4“x4” cotton gauze and place larvae side down in the wound, secured with a nylon stocking or netting. The dressing should remain slightly moist but avoid excessive moisture as it will drown the larvae. Avoid putting direct tight pressure over the larvae and avoid overpacking the wound. The larvae will expand in size, and if there are too many, this may cause some discomfort to the patient. When removing them, place the old dressing/larvae in alcohol or other cleaning agent (to kill them) or sealed biohazard bag and dispose in biohazard container. Document number larvae in and out, number of days in wound, appearance of wound bed before and after LDT, and standard wound monitoring documentation.

*Considerations When Using Live Biological Dressings.* Consider placing a note on the patient’s door (“Biological Therapy in Progress”) or otherwise give advanced notice to caregivers that larval therapy is underway so they are not surprised/caught unaware and demonstrate a negative reaction.

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