

## INVESTIGATIVE REPORT

# Rearing Larvae of *Lucilia sericata* for Chronic Ulcer Treatment – an Improved Method

Hélène WOLFF and Carita HANSSON

Department of Dermatology, Sahlgrenska University Hospital, Göteborg, Sweden

**Larval therapy is a biological ‘dressing’ for chronic ulcers which is used for debridement, to initiate healing and to reduce bacteria and unpleasant odour. One condition for being able to employ larval therapy is access to disinfected larvae of good quality. We therefore started rearing *Lucilia sericata* larvae in 1998. Rearing necrophagous larvae in a hospital setting makes it essential to control odour. The improvement of the method includes the use of a controlled, humid, warm environment with artificial light and inexpensive disposable material, as well as the use of ready-made, constantly available and aseptically produced nutrition for feeding larvae and flies. With equipment such as a refrigerator and freezer, larval food is kept fresh and odour-free and chloramine solution is used to disinfect the eggs and thus the larvae. Adhering to a proposed weekly working schedule makes the rearing procedure effective and reliable. Key words: maggots; maggot therapy; larval therapy; debridement; bacteria; wounds.**

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Hélène Wolff, Department of Dermatology, Sahlgrenska University Hospital/Sahlgrenska/S, Gröna stråket 16, SE-413 45 Göteborg, Sweden. E-mail: helene.wolff@vregion.se

Larval therapy in wound care is an old method (1) that has recently been rediscovered. The debridement and wound-stimulating effects of the larvae were the first features to gain interest, but more recently interest has also focused on the antibacterial effect (2–10). In the early history of larval therapy, no sterilization was performed and some secondary infections with *Clostridium tetani* and *Clostridium perfringens* occurred (1). Today, the larvae are disinfected before use and toxic substances have to be avoided in the disinfection process. Continuous access to larvae of good quality facilitates the use of larval therapy and for this reason we started rearing our own larvae in 1998. Since then, we have improved the procedure. Our aim is a quick, easy, reliable, reproducible and non-odorous rearing process. We also want to produce large larvae because they are likely to become large, healthy flies capable of producing many eggs (11). Further, we want to rear micro-controlled *Lucilia sericata* larvae, which will effectively debride wounds, initiate a healing process

and at the same time reduce the bacterial load and odour. We present below a reliable, safe way of rearing larvae in a hospital-based insectary.

## MATERIALS AND METHODS

### *The fly species*

*Lucilia sericata*, the green blowfly, is the fly species that is most frequently used for larval therapy. One reason is that the larvae are necrophagous and only feed on dead tissue. Another reason is that *L. sericata* lay eggs and, unlike some other fly species, do not immediately lay larvae. Eggs are easier to disinfect than larvae. A further important factor is that *L. sericata* can survive in a germ-free environment, as well as in one with microbes (12). The *L. sericata* species is common in all European countries and in North America. The first *L. sericata* were purchased from the Biosurgical Research Unit at SMTL (Surgical Material Testing Laboratory, Bridgend, UK).

### *Food and water*

The flies are given caster sugar mixed with a low-calorie diet powder ‘Modifast’ – raspberry flavour (Novartis Consumer Health Care). Modifast contains protein, carbohydrates, fat, vitamins and minerals. The water bowls are covered with two layers of air-filled plastic and two layers of paper towel, enabling the flies to drink without drowning.

The larvae are fed meat, black pudding (Geås, Saltsjöbo, Sweden) and horse-blood agar plates (Oxoid).

### *The insectary*

We use a room which is large enough to harbour six wire-framed cages with 250–300 flies in each. Our space has an exhaust but no window and maintains a temperature of 25°C and a relative humidity of 40%. A day rhythm is accomplished with artificial light on a timer. Light is induced between 6 am and 3 pm. We use cages measuring 30 × 30 × 45 cm, as previously described by Sherman & Wyle (13), but detachable metal floors have been added. The cages are covered with a sleeve-shaped surgical dressing (T II, 20 M, Hospidana ApS, DK-4930, Maribo, Denmark) and marked with the date of birth of the fly population (Fig. 1) (1). A synthetic net covers the door of the insectary, preventing flies from escaping, and a lamp attracts and kills fugitive flies (Anticimex, Insect-o-cutor, Stockport, England, Mod. IND 40).

### *Production of eggs*

At the age of 1–2 weeks, the flies can lay eggs (12, 13). Meat is used to make the flies fertile and a folded slice of meat is put on a piece of paper in a Petri dish. A disposable plastic coffee cup with an opening cut into the brim is inverted over the meat to create a protected area for the flies to lay (blow) their eggs

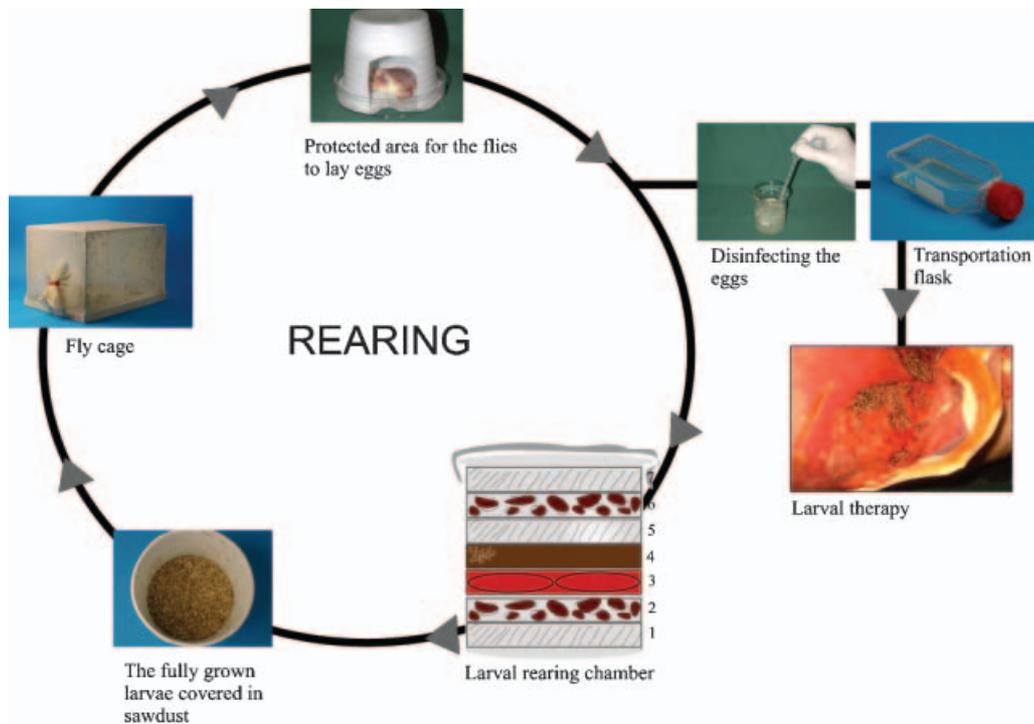


Fig. 1. The procedure for rearing *L. sericata* larvae for larval therapy. Description of the rearing chamber: [1, 5 and 7] polyurethane foam strips with a thickness of 2 mm, [2, 6] pieces of black pudding, [3] two horse-blood agar plates, [4] meat with non-disinfected eggs on it. A loosely woven fabric and a plastic lid with a large opening are used to seal the chamber.

(Fig. 1) (13). After about 30 min, when the flies have been presented with the meat, they start to lay eggs. The cup is then removed within the hour to prevent the eggs drying out. A disposable plastic inoculation loop (10  $\mu$ l) is used to collect the eggs and put them into a beaker of cold water.

The eggs can either be used for rearing non-disinfected larvae/flies for further egg-production (I), or be disinfected for rearing larvae for larval therapy (II) (Fig. 1).

*I. Rearing non-disinfected larvae/flies for further production of eggs.* The majority of the eggs are harvested and put into a beaker of water, but some are left on the meat. The meat with the remaining eggs is put into a rearing chamber. The chamber is made of a small plastic bucket with a tight-fitting lid into which a large hole is cut. Inside the bucket, strips of polyurethane foam form the bottom layer. Crushed black pudding forms the next layer and two horse-blood agar plates form the third layer. The following layers are composed of meat with eggs and polyurethane foam strips, black pudding and more polyurethane foam strips. The last layer of the rearing chamber consists of a loosely woven fabric which is kept in place by the lid. Taken together, there are seven different layers in the chamber, each sprinkled with water (Fig. 1). The chamber is then left in the insectary for 4 days to allow the larvae to reach adulthood and stop feeding. After that time, the larvae need a dry place in order to pupate. For this reason the contents of the chamber are sieved through a strainer into a plastic bucket. The negatively phototactic larvae move rapidly into the bucket when the light is switched on and the slippery inside of the bucket prevents them from escaping. The larvae are then covered with sawdust and left in the bucket in the insectary for 6 days for the pupa-forming process (Fig. 1). The pupae can be used at once for breeding flies, or they can be stored for subsequent use at a temperature of 4°C for at least a month.

Each fly population is discarded every 6 weeks. As we have six cages, we are able to put an entire pupa population into an empty, clean cage once a week (Table I).

*II. Disinfecting the eggs for breeding larvae for larval therapy.* The eggs in the water are separated from one another with a disposable plastic pipette (Fig. 1). The healthy eggs sink to the bottom of the beaker and are thereby easily recognized. The others are drawn into a glass pipette on a tube connected to the water tap and discarded. The procedure is repeated, until the water looks clean. Afterwards the eggs are rinsed three times with 0.25% chloramine replacing the water. They are then put in a sterile polystyrene tissue culture flask (PE Vented Cap, Canted Neck, 25 cm<sup>2</sup>, Sarstedt). Chloramine solution 0.25% is added until the tissue culture flask is full. The flask is shaken hard, at first by hand and then on a cradle for 20 min before the solution is removed with a glass pipette. The procedure is repeated three times. The entire disinfection process takes about 1.5 hours.

The disinfected eggs are then put into a transportation and storage flask. In this flask, the larvae hatch during the first day and feed. About 200 eggs are put in each prepared flask, which consists of a tissue culture flask filled with a mixture of 1 ml of BHI (Brain Heart Infusion) broth (Difco) and 1 ml of EX-Agar Standard. The solutions were dissolved in a water bath before they were mixed. The EX-Agar Standard is made of 10 g/l meat-extract L29 (Oxoid), 10 g/l bacteriological peptone L34 (Oxoid), 5 g/l NaCl and the rest is agar (Acumedia). The cap on the transportation and storage flask is closed gently after the eggs have been put inside, allowing air to penetrate it. The flasks are left at room temperature for 24 h, while waiting for the test results of the disinfection control, before they can be used for larval therapy (Table I).

Table I. Working schedule ( $\approx 20$  working h/week) for rearing fly eggs and preparing larvae ready for larval therapy

Monday	Tuesday	Wednesday	Thursday	Friday
Egg harvesting			Egg harvesting	
Disinfecting the eggs			Disinfecting the eggs	
Starting disinfection control	Finishing the disinfection control		Starting disinfection control	Finishing the disinfection control
Discarding the oldest fly population	Sending larvae for larval therapy			Sending larvae for larval therapy
Putting pupae in the refrigerator at 4°C as a back-up			Putting pupae in a new clean cage	
Emptying the oldest of two rearing chambers into a plastic bucket and covering the larvae with sawdust			Emptying the oldest of two rearing chambers into a plastic bucket and covering the larvae with sawdust	
Making a new larval rearing chamber			Making a new larval rearing chamber	
Changing food and water bowls	If needed, extra black pudding is added to the rearing chambers	If needed, extra black pudding is added to the rearing chambers	Changing food and water bowls	If needed, extra black pudding is added to the rearing chambers

To delay maturation, the larvae can be stored in a refrigerator at a temperature of 4°C for at least 5 days.

#### Experiments on adult larvae and flies

To investigate whether adult larvae can pupate in a fluid, we performed tests on three occasions, putting about 20 adult larvae in meat stock, in 0.25% chloramine solution and in phosphate-buffered saline for 6 days.

To investigate whether the temperature changed in the food substance during larval feeding, the temperature was measured in the rearing chambers on two occasions: before feeding (the black pudding had just been taken out of the refrigerator) and after 4 h of feeding. The test was repeated three times.

To investigate whether the flies could become fertile and lay eggs on substrates other than meat, the adult flies were presented with both black pudding and meat.

*Disinfection control and bacterial cultures:* For aerobic bacterial cultures, horse-blood agar plates (Oxoid), Drigalski agar and Staphylococcal agar 110 (Difco) were used. For fly water cultures, a TGEA (tryptone glucose yeast extract agar) (Oxoid) plate was added. For the anaerobic cultures, horse-blood agar plates were used.

Some of the eggs from each flask that are going to be used for larval therapy are always taken aside for disinfection control. In the disinfection control about 20 disinfected eggs from each flask are streaked over a horse-blood agar plate which is incubated for 24 h at a temperature of 37°C under aerobic conditions.

The effectiveness of the disinfection was assessed by comparing disinfected eggs and larvae with non-disinfected eggs, larvae (whole and squashed), fly drinking water and meat.

The agar plates were streaked with approximately 20 newly hatched larvae (from disinfected eggs) on 15 different occasions. The agar plates were incubated at 37°C aerobically and anaerobically for 2 days.

Agar plates were each streaked sporadically with one drop of the fly drinking water and rolled over with small pieces of meat. They were also incubated at 37°C aerobically and anaerobically for 2 days.

Occasionally, the agar plates were also streaked with either squashed disinfected newly hatched larvae (brought up from disinfected eggs) or with squashed non-disinfected larvae (of different developmental stages) and then incubated for 2 days in a thermostat at a temperature of 37°C aerobically and anaerobically.

## RESULTS

### General performance of the improved rearing of larvae

Using mainly aseptically produced food and only a small amount of fresh meat, the rearing technique was made odour-free.

Keeping the environmental temperature and humidity at a constant level, and following a weekly working schedule, the larvae could be produced efficiently (Fig. 1; Table I).

### Specific findings about rearing conditions

When larvae feed, there is a temperature elevation from 4° to 34°C in the food during a period of 4 h at an environmental temperature of 25°C.

We found that adult larvae do not pupate in a fluid and that black pudding makes the flies produce eggs just as meat does.

The mixture of BHI and EX-standard Agar used in the transportation flasks was found to keep the larvae alive, moist and easy removable (Fig. 1; Table I).

### Bacterial contamination

Only rarely did the disinfected eggs show any growth of bacteria on the horse-blood agar plates after incubation at 37°C for 24 h under aerobic conditions, but cultures of newly hatched larvae (from the same eggs) regularly showed growth of at least one spore-bearing bacterium after 2 days at 37°C in the thermostat. In 14/15 cases, growth of *Bacillus subtilis* was detected, *Bacillus pumilus* in 2/15 and *Paenibacillus pabuli* was present in 1/15. The spore-bearing bacteria were typed at the Culture Collection of the University of Göteborg (CCUG) and they were each assigned a CCUG number: *B. subtilis* (CCUG 48577), *P. pabuli* (CCUG 48662) and *B. pumilus* (CCUG 48773).

In the fly drinking water and on the non-disinfected eggs, spore-bearing bacteria, coagulase-negative staphylococci, streptococci, enterococci, *Bacillus cereus*, *Aeromonas* species and *Proteus mirabilis* were found. Squashed non-disinfected larvae revealed the same bacteria, including *Proteus mirabilis*, but squashed disinfected larvae displayed no growth other than the spore-bearing bacteria. Bacterial cultures from the meat presented for the flies to lay eggs on showed bacteria from the normal human skin flora.

### DISCUSSION

We have gradually improved the quality of the larvae and the rearing procedure. A clean, odour-free environment is not only important for the personnel, it also appears to increase the performance of the larvae and the flies (visible estimation of size and mobility of the flies).

The flies do not need natural daylight but develop in a satisfactory way with artificial light controlled by a timer. Increasing the humidity to 40% in the insectary helps prevent the eggs from drying out. Keeping the environmental temperature at a constant level also enables us to predict when the eggs can be harvested, as the developmental time from egg to adult fly is highly temperature-dependent (Fig. 1; Table II) (11, 14). We noted that the larvae in the rearing chamber developed better on polyurethane foam than on the sawdust, which has been used previously (13). The sawdust often becomes moist when the larvae secrete profusely in order to liquefy the food, which affects the pupation process negatively. In an *in vitro* experiment, we were able to show that adult larvae do not pupate in solutions. Even if the larvae managed to stay alive with their tails and breathing spiracles above water, for as long as it usually takes to form a pupa, they did not pupate. Probably their skin could not harden to a pupa shell in the fluid. Sawdust is still used to cover the larvae shortly before pupation, because at this stage they have stopped feeding and secreting and want to bury themselves in a dry environment.

Table II. Time from the egg stage to the other developmental stages of *Lucilia sericata* at a temperature of 25 °C

Developmental stage	Time (days)
Egg	0
Instar 2 larva (the skin has been changed once)	≈1
Instar 3 larva (the skin has been changed twice)	≈4
Pupa	≥7
Fly	≥12

The oldest fly population in the insectary is discarded every 6 weeks. The cage is cleaned in the laboratory dishwasher and the covering dressing is disposed of. New flies are not added to old populations in the way other fly-keepers have previously done (13). We want to secure gender equality in the cages and therefore we put an entire pupa population into an empty, clean cage. The flies do not all emerge from the pupae at the same time, first the males come out and then the females, but in a whole pupa population there is always a 1:1 gender ratio. Females need to mate with several males before they are able to lay eggs; they are also more likely to lay eggs if other females do the same (15). Probably the latter is due to a better survival expectancy of the larvae if there are many of them (15). The most fertile period for the flies is at the age of 2–4 weeks (13) and after 6 weeks of age, the male flies start to die. Subsequently only small, female flies are left, if the population is too old. In conclusion, a new pupa population every 6 weeks means that there are many fertile flies at the same time in a cage and most probably good egg production as well.

One of our previous findings (unpublished data) is that large larvae are the most efficient at debriding wounds. The size of both the flies and the larvae is dependent on food and water (11, 12). The two different developmental stages, larva and fly, have quite different needs; it is almost as if they were two different species. The main function of the larva is to feed and the main function of the fly is propagation (16). The larvae store most of the necessary nutrients for the fly to rely on when laying eggs. Protein and vitamins are necessary for egg production (11, 13). We give the flies sugar and a powder that is commonly used for under-nourished elderly people, containing both protein and vitamins (Modifast). The flies also need an abundance of water (11). Changing the food and water bowls twice a week instead of once increases the size of the flies (visible estimation). Feeding the *L. sericata* larvae fresh, aseptically produced food like black pudding and blood agar plates instead of meat not only increases the size of the larvae, it also reduces the smell (Table I). The blowfly larvae cannot ingest solid food particles and the food is therefore liquefied by proteolytic enzymes in the larval secretion (13, 17, 18) and then further digested by several enzymes in the larval intestines (19). In the past,

easily digestible putrefied meat, which is already partly degraded by bacteria, has been used to feed the larvae, as well as raw or cooked liver, cooked brain, rat or chicken fetuses (11–13, 20–24), but autoclaved fish or beef has not been employed, as young larvae have difficulty digesting coagulated proteins (12). One way of limiting the odour from putrid meat has been to confine flies, larvae and food in a plastic bag (13). Instead, we feed the larvae inexpensive, ready-made, aseptically produced black pudding, which has been cooked in a closed, heat-resistant plastic bag to a core temperature of 90°C and then vacuum-sealed. The pudding is preserved with the substance nitrate and is simply stored in the refrigerator. It is easy to obtain and undemanding for the larvae to digest. Feeding the larvae the black pudding repeatedly in small portions instead of all at once also keeps the odour under control (Table I). The larvae digest a large amount of food in a short time (23). The elevated temperature in the food when larvae feed probably facilitates the metabolism of the larvae (12). Temperature effects on the larval food similar to those observed by us have been seen when the larvae of the housefly (*Musca domestica*) feed (24). Black pudding and meat can both be used to make the flies fertile and to make them produce eggs, but eggs are easier to remove from meat. We cut the meat in slices and keep it odour-free and available in the freezer at –18°C.

In the disinfection process, it is essential to clean the fly eggs from the sticky albumin mass by which they are covered and in which bacteria are trapped (1, 11–13, 25). To separate the eggs, we use water and mechanical manipulation and we then use chloramine solution for disinfection (26). This disinfectant is effective against bacteria, viruses and fungi of all kinds and is also inexpensive and often used for cleansing wounds. Others have previously used disinfectants like hydrochloric acid, mercuric chloride, formalin, sodium hypochlorite (Bleach™, Clorox™), Dakin's solution™ (containing 1% sodium carbonate) and Lysol brand disinfectant™ (containing phenyl phenol) (11–13, 20, 22, 24, 25). Compared with these, chloramine solution is more environmentally friendly, even though allergic reactions to chloramines have been reported (27). However, in comparison with its frequent use, the risk of sensitization appears to be low (26).

Even if chloramine solution is effective in eradicating the potential wound pathogens found on the non-disinfected larvae, *B. subtilis* was regularly found on the disinfected larvae and sometimes other spore-bearing bacteria, such as *Paenibacillus pabuli* or *B. pumilus* as well. It is very difficult to eradicate all bacterial spores unless the eggs or larvae are autoclaved, but this process would, however, kill them. It should be remembered though, that these spore-bearing bacteria are not pathogens. Nevertheless, the disinfection control of the eggs is essential and there should be no bacterial growth

on the horse-blood agar plates after 24 h, if the larvae are to be used for larval therapy.

Microbes in the fly population are of particular importance (12). Wild flies always harbour spore-bearing bacteria (11) and the larvae of the housefly, for example, cannot survive in a microbe-free environment (12, 24). We have cultured 3-day-old fly drinking water in which the flies not only drink, but also regurgitate and make their droppings (12). The cultures revealed a high level of bacteria, many of which were also found on the non-disinfected eggs.

The commensals of the larvae are probably of some assistance for them when digesting food and inactivating other bacteria (20, 28–30). Squashed, disinfected larvae displayed no bacterial growth other than spore-bearing bacteria, suggesting that they have lost some of their commensals. Squashed non-disinfected larvae (newly hatched, as well as adult ones) revealed the same bacteria as those in the fly drinking water and on the non-disinfected eggs, including *Proteus mirabilis*. *P. mirabilis* is regarded as an important commensal in the screw-worm (*Cochliomyia hominivorax*), which is the larva of a closely related fly species (29). As *P. mirabilis* is present on non-disinfected eggs, larvae could easily pick up *P. mirabilis* as a commensal, but we are not able to determine from our cultures whether *P. mirabilis* was there as a commensal or only present on the body surface of the larvae.

On the one hand, it is vital to ensure that the fly population is as healthy as possible, which probably means using a non-sterile rearing technique. On the other hand, we need to guarantee that no pathogens are introduced into the wound when the larvae are used for larval therapy. To minimize the risk of contaminating the insectary with anaerobic bacteria such as *C. tetani* and *C. perfringens*, we have been feeding the flies and larvae mainly aseptically produced food, and also been inbreeding the flies since we started in 1998. Inbreeding the flies for a longer period of time does not appear to be disadvantageous in any way (11, 15).

In conclusion, the procedure has proven to be reliable, reproducible and odour-free. Our larvae are large and efficient and lay a lot of eggs, which are harvested on a regular basis. Our routine disinfection control of the eggs is sufficient to reveal the usual potential wound pathogens and we have had no incident of infection after larval therapy since we started rearing larvae in the late 1990s (6). The larvae have, however, probably lost their commensals in the disinfection process and further studies are needed to compare the efficacy of larvae with and without commensals.

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