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Environmental Factors Influencing the Growth of *Lucilia sericata* Larvae Used for Maggot Therapy under Laboratory Condition

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Abstract

Background: The larvae of *Lucilia sericata* are efficiently and widely used in maggot therapy. The aim of this study was to investigate some environmental factors that influence the mass rearing of *Lucilia sericata* as the most suitable candidates for maggot therapy in Iran.

Methods: This cross-sectional study was conducted in flies breeding insectarium of Tehran University of Medical Sciences. The best temperature for embryonic period and hatching was 27 °C with relative humidity of 80% and 16:8 light-dark periods. At the insectarium, food, water and a nest for laying eggs were provided for the flies and after oviposition, the eggs were transferred to a new rearing place and identification keys were used to identify the specimen. Four factors (temperature, humidity, photoperiod and diet) were studied for the maintenance and mass rearing of *Lucilia sericata* larvae under laboratory condition.

Results: The best temperature for embryonic period was 27 °C ($P<0.05$). The highest larval death (15.27%) was seen at the temperature of 23 °C and was statistically significant ($P<0.05$). The highest hatching rate (86.95%) was seen at relative humidity of 80% and was also statistically significant ($P<0.05$). The maximum batches of eggs laid (25 batches) occurred in 16:8 photoperiods. Significant difference was seen between the weight of larvae fed with burgers, fish, liver and blood agar ($P<0.05$).

Conclusion: In in-vitro condition, although temperature, humidity, light-dark period and diet had an effect on the growth of *L. sericata*, however, temperature and relative humidity were found to have more influence in the development of *L. sericata* larva.
developed in the left eye. Two patients had no family history suspicious for keratoconus.

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Introduction

Chronic wound is defined as wound that does not follow the routine stages and predictable time of the reparative process in order to produce an anatomical and functional integrity (1). Any wounds that do not heal within three months can be considered as a chronic wound, and may be due to an arrest in one or more phases of wound healing or when the body's ability to deal with the damage is overwhelmed by factors such as repeated trauma, continued pressure, ischemia, or illness (1, 2). Chronic wounds cause serious problems to the clients, healthcare system and the governments, because they require extra time for healing, are highly consumable and need huge financial budget. Severe burns, deep abscesses, cancers, bone infections, infected bedsores and poorly controlled diabetic mellitus can all progress to chronic wounds (3). A major risk factor for developing chronic wound is diabetic mellitus, due to neuropathy, which inhibits nociception and the perception of pain, thus patients may not notice small wounds to the legs and feet early to seek for medical intervention (4). Also, diabetes causes immunecompromised conditions and damage to small blood vessels, preventing adequate oxygenation of tissues, which ultimately leads to chronic wounds (5).

Fly larvae have long been used in treating chronic wounds and the secretions of fly larvae have been found to be very effective in rebuilding tissues and also reducing bacterial loads in patients with Methicillin Resistant *Staphylococcus Aureus* (MRSA). In addition, it can be used as a biological agent in cases where surgery and antibiotic use are impractical (6).

Maggot therapy is a micro debridement process, in which, larvae use their natural desire to devour dead and infected tissues killing bacteria that can prevent the reparative process without harming the healthy surrounding tissues (7). This treatment method was established in 1989 by Ronald Sherman (8). The larvae of *lucilia sericata* (green blow fly) flies are most efficient and widely used in maggot therapy and have been studied very well by many researchers in the field of maggot therapy and forensic entomology (7). This species was first used in 1931 by Bear and its medical use became noticeable in 1826 when Meigen removed some larvae from cavities of a patient's eye and face (8).

Lucilia sericata is a species that can be found in most parts of the world but it is mainly seen in southern hemisphere, including Africa and Australia. This fly has coastal distribution and prefers hot and humid weather (9). *Lucillia sericata* is an oviparous fly and lays eggs on corpses, open wounds of humans and animals as well as dirty or wet wounds of sheep. They have a complete metamorphosis (holometabolous), involving eggs, larvae, pupae and adult stage which can immediately identify dead tissues for feeding and oviposition (9). The adult females start laying eggs within 5-9 days after emerging from pupa in a suitable environment rich in protein (10). The oviposition behavior of *L. sericata* is dependent on light and temperature, so that egg lying generally occurs between 11-14 maximum sunlight hours. During this time, female flies need a temperature above 32 °C which stimulates oviposition (10). It usually takes 2-3 weeks to complete egg to egg cycle in this fly species.

Since the utilization of flies in the field of maggot therapy and forensic entomology requires maintenance and mass rearing of the flies in laboratory conditions, knowing the environmental factors that influence the growth of larvae are necessary and important. Some research centers in some countries produce and export larvae of flies to medical centers

within and outside their countries for utilization in maggot therapy. Unfortunately, despite the long history of this surgical treatment, this method in Iran is still new and very limited.

The purpose of this study was to investigate the various environmental factors that influence the proliferation and mass rearing of these flies as the most suitable candidates for maggot therapy in Iran.

Methods

This study was conducted in flies breeding insectarium of the Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Iran. The fly samples were collected using handheld net method and protein baits from five areas including: Tehran, Varamin, Roode-hen, Hashtgerd and Karaj cities in Tehran and Alborz provinces. The samples were then transferred into cage labeled with the location, temperature, humidity, height, collector and date of collection. At the insectariums, food (dry milk, protein and sugar) and water were provided for the flies and after oviposition, the eggs were transferred to new rearing place and identification keys were used to identify the specimens (11-13).

The temperature, humidity and light in the insectariums were stabilized for the purpose of this study in order to have accurate results. Four related temperatures 23°C, 25°C, 27°C and 30°C were examined in this study and in each temperature, egg masses were taken randomly and then after counting them, using binocular microscope, they were transferred into a container and incubated. The number of samples and the time of emergence from one stage to the next were recorded carefully in specific forms. The best humidity of breeding as well as the life cycle of this species was studied using three humidity levels of 30, 50 and 80% with random counting of eggs. In this study, three light-dark periods of 12:12, 10:14 and 16:8 were examined, and finally, in order to determine the best diet, four major regiments of food including liver, hamburgers, fish and blood agar were used. Since the life cycle; period of larval, pupal and adult stages are very important in various mean temperature and relative humidity, each stage was recorded separately. The samples from Hashtgerd were adapted very well to the insectariums condition, thus, the F3 and F4 generations of this strain were used for the rest of the study.

Data were analyzed with statistical methods (SPSS Version 16 and R2.12.). X^2 was used to establish significant association using 0.005 p-value at confidence interval of 5% and the optimal conditions for growth and rearing of the flies were obtained.

Results

Temperature

Fly numbers in a given locality vary with the availability of breeding places, sunshine hours, temperature and humidity. As shown in table 1, the number of eggs, rate of hatching and death of larval instars all varied with changes in temperature. At temperatures 23 °C and 27 °C, 54.96% and 97.27% of the eggs were hatched respectively with statistically significant difference ($P<0.05$), making 27 °C temperature to be the best temperature for embryonic and hatching period. Larval growth was examined and the rate of larval mortality at age I, II and III instar at various temperatures was calculated. More mortality was observed in the first and second larval instar which was associated with the high risk of survival at these stages of life. The highest larval death (15.27%) was seen at

the temperature of 23 °C (Table 1) and the lowest mortality (3.49%) was seen at the temperature of 27 °C with statistical difference between mortality rate in the control and treatment group (P<0.05).

Table 1. Different stages of *Lucilia sericata* (Hashtgerd strain) at various insectaria temperatures

Different stages of *Lucilia sericata*		Temperature (°C)			
		23	25	27	30
Number of eggs at F4 generation		131	159	147	151
Number of eggs hatched		72	141	143	138
Percentage of hatching		54.96	88.67	97.27	91.39
Number of unhatched eggs		59	18	4	14
The rate of deaths in larvae I, II [N (%)]		11 (15.27)	9 (6.38)	5 (3.49)	15 (10.86)
Number of larvae that reached III instar		61	132	138	134
Number of days III instar turned into pupa		8	6	5	3
Number of formed pupa		61	132	138	128
Number of days from pupa to adult		11	9	6	3
The rate of pupa not opened [N (%)]		15 (24.59)	12 (9.09)	7 (5.07)	32 (25)
The rate of emerged adults out of pupae [N (%)]	male	19 (41.30)	61 (47.28)	60 (45.80)	42 (43.75)
	female	27 (58.69)	68 (52.71)	71 (54.19)	54 (56.25)
Number of days from adult exit to pregnancy		14	10	8	5
Number of gravid females		5	14	23	16
Percentage of pregnancy		18.51	20.58	32.39	29.62
First time flies laid eggs after pregnancy		15	10	5	3

Our study found the susceptibility of young larvae to ambient temperature and lower temperature which play an important role in fostering. The period of transformation from 3rd instar to pupae at 23°C and 30°C temperatures occurred in 8 and 3 days respectively. There was no significant difference observed due to larval emergence at different temperatures (23 °C to 27 °C) or gender of flies (P>0.05). In mass rearing of flies, the number of days taken for mating to occur between flies that have emerged from pupae – (the first batch of eggs that started the next generation of breeding) is very important and was established in this study (14 days at 23 °C and 5 days at 30 °C). This change in temperature had an effect on the number of females that got pregnant. The lowest pregnancy rate occurred at 23 °C (18.5%) and the highest pregnancy rate was observed at 27 °C (33%).

Humidity

Another important factor in the maintenance and breeding of insects including flies is the relative humidity. For this purpose, the eggs laid by F3 Hashtgerd strains were selected and reared at 27°C and relative humidity of 30, 50 and 80% respectively. The results are shown in table 2. The highest hatching rate was 95.86 % with relative humidity of 80% while the lowest hatching rate was 43.82% with relative humidity of 30% and statistically significant (P<0.05). Relative humidity also affects larval growth and mortality. The mortality rate of larval instar I and II with a relative humidity of 30% was calculated to be 11.3%, while with higher humidity this rate declined, at relative humidity of 50% it dropped to 2.72% and was statistically significant (P<0.05). But the percentage of larval instar I and II death at relative humidity of 50 and 80% did not show a statistically significant

difference (P>0.05). The number of days required for III instar larvae to transform into pupae and also the length of pupal period at different relative humidity did not demonstrate much variation. After the pupal period, adult flies appeared, in this study at the relative humidity of 30%, 9.5 percent were still pupae, but at a higher relative humidity of 50% only 1.86 percent of pupae did not pupate into adults and this was statistically significant (P<0.05). There was no significant difference in the aspect of gender percentage between adults under different relative humidity (P>0.05).

Table 2. Different stages of *Lucilia sericata* (Hashtgerd strain) at various humidity

Different stages of *Lucilia sericata*		Humidity%		
		30	50	80
Number of eggs at F4 generation		162	138	145
Number of hatched eggs		71	110	139
Percentage of hatching		43.82	79.71	95.86
Number of unhatched eggs		91	28	6
The rate of death in larvae I, II [N (%)]		8 (11.26)	3 (2.72)	5 (3.59)
Number of larvae that reached III instar		63	108	134
Number of days III instar turned into pupa		5	6	4
Number of formed pupa		63	107	134
Number of days from pupa to adult		7	6	6
The rate of pupae not opened [N (%)]		6 (9.52)	2 (1.86)	3 (2.23)
Number of adults out of pupae	male	25 (43.85)	46 (48.42)	58 (44.24)
	female	32 (56.14)	49 (51.57)	73 (55.72)
Number of days from adult exit to pregnancy		7	7	5
Number of gravid females		18	25	32
Percentage of pregnancy		56.25	51.02	43.83
First time flies laid eggs after pregnancy		6	7	5

Light-dark Period

As shown in table 3, number of days required for III larval instar to become pupae did not show variation with different periods of light-dark compared to the difference that was seen between the day's pupae transformed to adult flies in 16:8 (light-dark) period and the other two periods. In each light-dark period, there was a percentage of pupae that did not transform into adult flies and this was more in the periods of 12:12, representing 12%. Adults that emerged from their pupal stages began to mate and lay eggs. The days required from the departure of adult fly to its pregnancy in 12:12 light-dark period was more and it reached almost 12 days. The number of females that were pregnant was only 29.54% in this period. While in 16:8 periods, the days reduced to half (6 days) and pregnant flies were 50% which represents the direct effect of light-dark on this period length and the number of pregnant flies. After pregnancy, flies laid eggs in suitable places. In selective light-dark period, different numbers of egg batches were laid and the maximum 25 batches were in 16:8 periods, most of the eggs were hatched in the same photoperiod. The embryonic period length was also dependent on light-dark period. Light-dark cycle of 16:8 had the lowest embryonic hatching which is very important in *L. sericata* breeding and maintenance in the laboratory (table 3). The developmental stages of flies from the transformation of III instar larvae to pupa and the emergence of adult from pupal

shell correlates with the fragile stages of development in other creatures where the risk of dying is very high from various factors. As shown in Table 3, at different light-dark periods, 50 III instar larvae were obtained and the highest pupae produced (96%) was in 16:8 light-dark periods.

Table 3. Different stages of Lucilia sericata (Hashtgerd strain) at various photoperiods (light-dark)

Different stages of *Lucilia sericata*		light-dark 12 :12	14 : 10	16 : 8
Number of III instar larvae		50	50	50
Number of days III instar became pupa		5	4	4
Number of pupae		44	47	48
Number of days from pupa to adult stages		7	5	4
Number of days from adult exit to pregnancy		12	8	6
The rate of gravid females [N (%)]		13 (29.54)	19 (40.42)	24 (50)
The day the first batch of eggs were seen		12	8	6
Number of hatching days		5	4	3
The rate of eggs hatched [N (%)]		5 (35.71)	9 (45)	16 (64)
The rate of pupae [N (%)]		44 (88)	47 (94)	48 (96)
Adults [N(%)]	Male	19 (43.18)	21(44.68)	17(35.41)
	Female	25 (56.81)	26(55.31)	31(64.58)
The rate of deaths during pupal period till egg laying time [N(%)]	Male	9(42.85)	7(46.66)	8(47.05)
	Female	12(57.14)	8(53.33)	9(52.94)
The rate of flies remaining [N (%)]	Male	10(43.47)	14(43.75)	9(29.03)
	Female	13(56.52)	18(56.25)	22(70.96)

Diet

Table 4 shows the results of morphometric studies conducted on F4 larvae of Hashtgerd strains which were fed with different diets and were eventually bred under laboratory conditions. A significant difference was observed between the weight of larvae fed by burgers, fish, liver and blood agar ($P<0.05$). Third instar larvae fed by burger recorded the lowest weight (2.48%) and the highest weight was seen with larvae fed by blood agar (5.08%). A significant difference was observed between body length and length of adult head capsule ($P<0.05$). Significant differences were also seen between head capsule length, thorax width and abdominal length in third instar larvae fed by burgers and blood agar ($P<0.05$). Using Kruskal-Wallis and proportion tests, a significant difference was observed between body weight and body length in larvae fed on blood agar and liver ($P<0.05$). A

significant difference was established between adult weights, adult body length and head capsule width of the samples that fed on liver and blood agar. The adult specimens which were fed on liver and blood agar had significant difference in thorax length and width, abdomen length and width, wing area and its length and width ($P<0.05$). In addition, the length of the hind tibia of adult flies that were fed on blood agar was more than others that were fed on liver. In addition, a significant difference was observed between the body lengths of adult flies emerging and fed on burger and blood agar during larval stage.

Table 4. Results of morphometric **studies** based on type of diet

Characteristics		Hamburger	Fish	Sheep liver	Blood Agar
Larvae	Weight (Milligrams)	2.48±0.02	2.79±0.18	3.76±0.04	5.08±0.19
	Length (Millimeter)	11.8±0.75	12.43±0.44	12.49±1.19	12.85±0.24
Adult	Weight (Milligrams)	2.51±0.04	2.69±0.03	3.84±0.56	5.33±0.05
	Length (Millimeter)	10.33±0.39	11.92±0.32	12.8±0.08	13.97±0.34
	Length Head Capsule (Millimeter)	1.37±0.25	2.43±0.37	2.98±0.55	3.46±0.63
	Width Head Capsule (Millimeter)	3.45±0.48	3.8±0.55	5.1±0.68	5.76±0.9
Thorax (Millimeter)	Length	3.86±0.58	5.71±0.55	6.86±1.03	7.02±1.08
	Width	2.89±0.49	4.51±0.63	5.99±1.05	5.88±1.02
Abdomen (Millimeter	length	3.34±0.6	4.48±0.78	5.58±1.06	5.99±1.04
	Width	3.63±0.68	4.92±0.86	6.62±1.01	6.96±0.93
Wing (Millimeter	Length	8.61±0.59	11.03±0.59	12.45±0.6	13.73±0.85
	Width	3.85±0.48	5.41±0.38	6.3±0.59	6.92±0.6
	Area (square millimeters)	3.09±0.45	5.04±0.45	5.78±0.57	6.32±0.82
	Length of second leg tibia (Millimeter)	1.81±0.49	4.09±0.63	5.1±0.89	5.29±0.92

Discussion

Fly samples were collected from 5 counties in two provinces (Tehran and Alborz), only the samples collected from Hashtgerd city in Alborz province were used for this study. This was due to the suitability and consistency of this strain to laboratory conditions including breeding and mass rearing. They were reared and maintained to fifth generation (F5) where various factors such as temperature, humidity, light-dark period and diet were studied on them.

In one of our previous studies (14) on Laboratory colonization of *L. sericata* Meigen (Diptera, Calliphoridae) strain from Hashtgerd, Iran the best temperature for rearing Hashtgerd F3 larvae generation under laboratory conditions was reported to be 27 °C in which this present study also observed the same temperature (14). Therefore, the suitable temperature for mass rearing in Tehran was found to be 25 to 27 °C which agrees with previous studies (15- 19). These results emphasized that changes in temperature was found to be an effective agent in changing the duration of larval period.

The relative humidity also has an important role in rearing larvae in the laboratory and average egg hatching rate was calculated using 3 humidity levels, 47, 49, and 71%. At RH of 47% only 4% of the eggs were hatched whereas lower than 50% and over 50% eggs were hatched at RH of 49% and 71% respectively. This result is supported by a previous study, where none of the eggs of *L. sericata* was hatched below 50% humidity (14). In a study conducted on larvae and adults rearing of F3 generation of Hashtgerd strain, the most suitable relative humidity was found to be 80% and 50% respectively (14). It is remarkable to note that the required relative

humidity for adult flies is much less than those of the larvae and our results completely involved to the natural biological process of flies. In addition, temperature and light-dark period had influence on diapauses of *L. sericata* III Instar larval stage (20, 21).

Another important environmental factor in breeding and maintenance of the samples was the light-dark period. The growth and reproduction of *L. sericata* flies was found to be directly related to light-dark period temperature, shade, sunlight and habitat. The result that corresponds to light-dark period in nature was 16:8 through flies' season of activity (22).

Appropriate diet was considered as another important factor in the growth and reproduction of animals. For each species of animals, including insects, there is the need for nourishment in a proper way for survival. Since flies have complete metamorphosis (Holometabolous), the required food for their larval period is quite different from their adults, and because of that in the laboratory where flies are bred, this point should be observed in future investigation. Since the adult flies in nature are used to various proteins, water and sugar; in this survey, in order to maintain the flies, the afore-mentioned materials were used and they served as a source of proper nutrients for the adult flies (23- 25).

One of the other important factors for mass rearing in the laboratory is a suitable source of food for larvae. In this study, four different types of food were used for feeding the larvae including hamburger, fish, liver and blood agar. Feeding with blood agar had the best result in larvae growth. That is, the effect could be seen in length and body weight of larva and also, weight of adults that emerged from pupa fed by this regimen. Studies conducted by researchers from other countries used different types of food. In one study by Zhang et al (2009), 4 different regimens of diet were used for larvae; Regimen 1: containing water, propionic acid, agar powder, barley powder, yeast and dry milk; Regimen 2: containing water, agar powder, barley powder, yeast and dry milk; Regimen 3: containing water, agar powder, barley powder in small amounts, yeast and dry milk; and Regimen 4: containing water, agar powder, barley powder in large amounts, yeast and dry milk. These 4 diets were compared with beef liver to see which one could influence larval growth and to see whether change in the amount of fat, protein and water had a direct effect on the growth process of larvae (26). Another study by Arrese and colleagues (2010) showed that sterols are vital ingredients in the diet of *L. sericata*, because they turn into cholesterol in the fly's body (23). Dry milk is a convenient source as well (23, 24). In another similar study, the diet of beef was tested in comparison with calf's liver and chicken. It was observed that the growth of larvae fed on beef and chicken was more than larvae that was fed on calf's liver (25).

Also, other studies (27, 28) showed that protein in flies′ diet has an important role in speeding up growth; the size of larvae at the same age fed on liver was two times bigger than the larvae that were not fed on liver (27). Comparing the weight of larvae fed by blood agar and larvae fed by hamburger, fish and liver shows a remarkable difference. The difference was due to the balanced diet. Also, comparing the length and weight of adults obtained from these larvae shows a distinct difference among the 4 types of diet, and this shows that blood agar diet primarily and liver diet in the next step had the best efficacy in producing a larger larva. In our study, we observed a significant difference between weight of III instar larvae fed on hamburger and blood agar using Kruskal-Wallis test and Proportion test.

Conclusion

In in-vitro condition, although temperature, humidity, light-dark period and diet had an effect on the growth of *L. sericata*, however, temperature and relative humidity were found to have more influence in the development of *L. sericata* larva. The best temperature for embryonic period and hatching (97.27%) was 27 °C with statistically significant difference (P<0.05). The highest larval death (15.27%) was seen at the temperature of 23 °C and was statistically significant (P<0.05). The highest hatching rate (95.86%) was seen at relative humidity of 80% and was also statistically significant (P<0.05). The maximum batches of eggs laid occurred in 16:8 photoperiods. A significant difference was observed between the weight of larvae fed by burgers, fish, liver and blood agar (P<0.05).

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