Agitation of the Bacterial Biofilm and its Effects on the Hatch Rate of *Lucilia cuprina* Eggs (Diptera: Calliphoridae)

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Abstract: Blow flies are important for their use in Maggot Debridement Therapy (MDT) to treat patients' wounds. Many different sterilization techniques are used on their eggs in a process to reduce the transfer of bacteria from the maggots to the patients. Agitation can be used to disrupt the biofilm of bacteria found on an egg's surface. In this study, *Lucilia cuprina* eggs were collected and the effects of agitation on hatch rates was tested. After collection, eggs were put on filters, covered in an insect saline solution, and put through two treatments: (1) a control with no agitation soaking in saline for five minutes and (2) an agitation treatment at 5 rpm soaking in saline for five minutes. Afterwards they were rinsed with leftover saline and plated in nutrient agar cups to observe the hatching process. Agitated eggs had significantly lower hatch rates than the non-agitated control treatment. These data showed that agitation had an adverse effect on mortality of the fly eggs, but provided no information on the effect on bacterial life. The importance of this study cannot be fully understood without knowing how agitation affects the bacteria found on the tested fly eggs.

Keywords: *Lucilia cuprina,* Fly Eggs, Bacteria, Agitation, Hatch Rate

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Lucilia cuprina (W.) (Diptera: Calliphoridae) have been used in Maggot Debridement Therapy (MDT) in place of the more abundant temperate *Lucilia sericata* (M.) (Diptera: Calliphoridae) with similar results reported on their effectiveness in the treatment of wounds (Paul et al. 2009). Understanding more about *L. cuprina* will allow doctors to better utilize them for procedures in the parts of the world where they thrive. Sterilization of the eggs of *L. cuprina* is a vital pre-requisite to ensure the safe usage in MDT of sterile maggots on patients' wounds and their health (Yeong et al. 2011). Without proper sterilization of eggs, bacteria can transfer from the egg cases to the larval epidermis and patients being treated can develop infections.

Bacteria adhere to natural and synthetic surfaces as a survival mechanism because nutrients in aqueous environments have the tendency to accumulate near surfaces

(Busscher and van der Mei 2012). While on the surface of insect eggs, bacteria form a biofilm made from sessile cells in a gregarious nature that provide protective advantages which allow them to remain in favorable environments (De Rienzo et al. 2015). Biofilms are made of embedded cells in an extracellular polymeric substance (EPS) matrix composed of polysaccharides, extracellular DNA (eDNA), proteins, and lipids. eDNA mainly hold together young biofilms (PAO1) while polysaccharides serve primarily as a structural scaffold for the biofilm (PA14) (Petrova and Sauer 2012). Bacterial cells organized in biofilms are more than ten times more resistant to antibiotics than in their planktonic (solitary) state (Rodriguez-Martinez and Pascual 2006). Problems occur when biofilms cover major portions of the outer surface of insect eggs used in MDT, so discovering new ways for surface sterilization is extremely important. It can be inferred that agitation might disrupt the structure of the biofilm covering the insect eggs, creating openings that allow applied disinfectants to thoroughly kill any bacteria present.

In a previous study, researchers reported on the significantly lower survival rates of fly eggs immersed in large volumes of liquids for extended periods of time then on ones immersed in lower volumes (Charabidze et al. 2015). However, no known study has been done on the effect of agitation on the hatch rate of *L. cuprina* eggs. Information gathered on the mortality of insect eggs from agitation can be applied to improve the process of MDT and other procedures through possible new methods of sterilization. This study was preformed to understand if *L. cuprina* eggs can tolerate being agitated within a liquid.

Materials and Methods

L. cuprina were collected from carrion found alongside FM 2818 that runs through Bryan/College Station, TX. These maggots were reared on 50 g of food grade beef liver (Foster Farms, Livingston, CA) until they pupated. After pupation, adult flies emerged in colonies made inside of 12 x 12 x 12” Collapsible fly cages (Bioquip, Rancho Dominguez, CA) and were maintained under a 12:12 (L:D) cycle. The adults were given dry sugar (C&H Sugar, Crockett, CA) in plastic petri dishes and water (H2O) on cotton balls (Curad, Mundelein, IL) *ad libitum* until two days after eclosion from their pupae, where they were given 50 g of beef liver as a protein meal. Fly eggs from within these colonies were collected for deagglutination by placing 10 g of fresh cow liver as an oviposition medium into each cage for three hours.

Egg clutches were removed from each liver using a hobby size 2 paint brush (Dixon Ticonderoga Company, Heathrow, FL). The eggs were then placed on paper towels moistened with RO (reverse osmosis) water, where they were covered for approximately five minutes with a moist black cloth (Baum Textiles Inc., Jersey City, NJ). Immediately after removing the cloth cover, the egg clutches were gently stirred using the paint brush until they were broken apart and thoroughly mixed together. Approximately 30 eggs were placed on each 13 mm GE Magna nylon membrane filter (20.0 um) (Maine Manufacturing LLC, Sanford, ME).

The filters were placed in sterilized 13mm poly carbonate luer-lock filter holders (Cole-Parmer, Vernon Hills, IL), where 20 ml Luer-lock plastic syringes (Nucmedor, San Francisco, CA) were used to force 2 ml (2 cc) of Pringles Saline Solution (9.0 g NaCl, 0.2 g CaCl, 0.2g KCl, 4.0g Glucose—dissolve in DI water and dilute to 1L) through the filter to completely submerge the eggs. These steps were used for both the control and the agitation treatment respectively. For the control, the insect saline solution was left soaking the eggs for five minutes. For the treatment, the filter was attached to an EMD Millipore Rotary Agitator to agitate the eggs at 5 rpm for five minutes. Afterwards in both treatments, the remainder of the insect saline was forced through the filter.

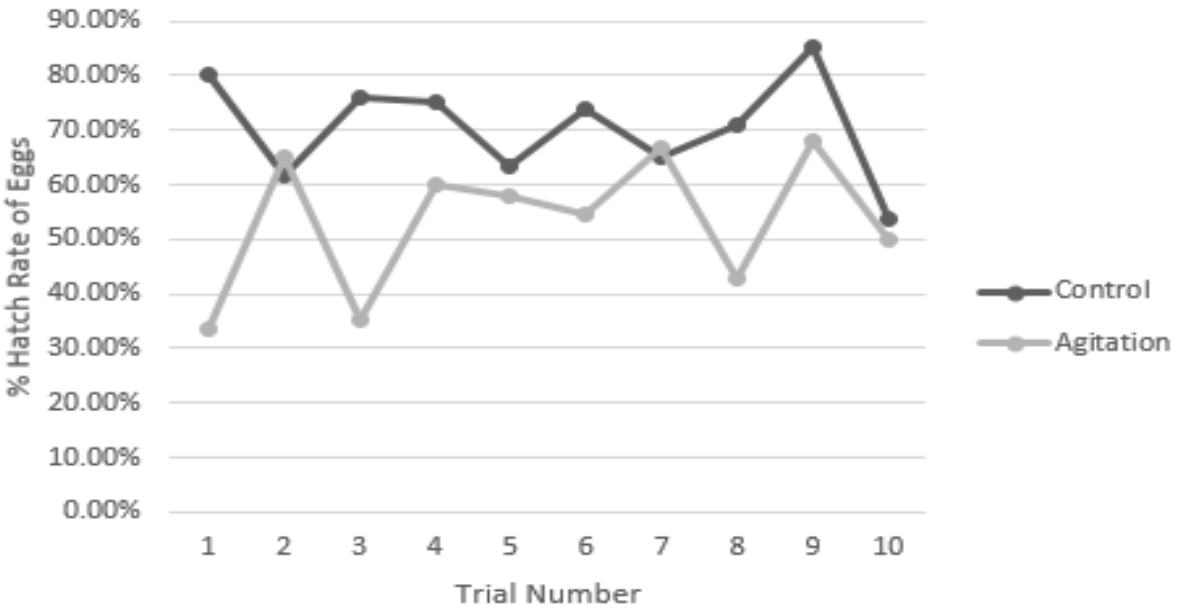

Fig. 1. Comparison of All Hatch Rates of *L. cuprina* Eggs in Liquid

Nutrient agar (Thermo-Fisher Scientific, Waltham, MA) was autoclaved and prepared according to directions. Approximately 5 ml of liquid nutrient agar was poured into 2 oz. Diamond Multi-Purpose Mini Cups with Lids (50ct) (Diamond Foods, Stockton, CA). The lids were placed on each cup to allow to agar to solidify. After the eggs were treated, they were removed from the filters using the paint brush and placed on agar in cups to observe for hatching. Data were analyzed using a simple T-test in IBM SPSS Statistics (2015).

Results

A comparison of the treatments showed significantly ($P = 0.012$) lower hatch rates for the eggs when agitated (Fig. 1).

The mean successful hatch rate of the control treatment was 70.46% with a standard deviation of 0.094793, while the mean successful hatch rate of the agitation treatment was 53.38% with a standard deviation of 0.040098 (Fig. 2). This was a decrease of 17.08% in survival for the fly eggs being tested.

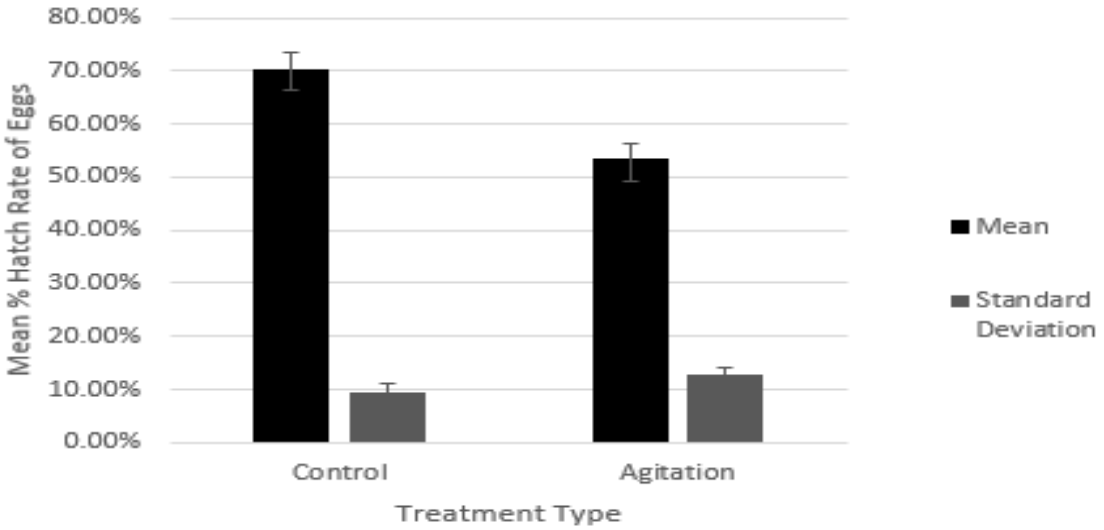

Fig. 2. Comparison of Mean Hatch Rates of *L. cuprina* Eggs in Liquid + SD and SE

Discussion

In this study, we found that agitation significantly (P=0.012) lowered the hatch rate for *L. cuprina* eggs compared to the non-agitated control.

Dupree and Morgan (1902) noted that agitation seems to be associated with hatching while working with several *Aedes spp.* eggs. Eggs of certain species of flies that lay on the surface of the water or upon the bottom of the breeding vessel for days, will hatch if the vial is shaken or the egg removed, but will remain unhatched if left undisturbed (Christophers 2009). A previous study confirms this theory, with only five minutes of water agitation resulting in an almost immediate hatch rate of 63.3% versus 0% for non-agitated controls (plus another 3.9% versus 0.3% respectively) in *Anopheles gambiae* (G.) (Diptera: Culicidae) (Ebrahimi et al. 2014). These data seem to be the opposite of what was observed in the fly eggs tested in this study. It can be assumed that the reason behind the unaffected mortality for mosquitos involves something physiologically or morphologically different in the eggs themselves.

One major limitation for this study was not testing if agitation adversely affected the bacterial growth rate. A study on the influence of agitation and aeration on the bacteria *Xenorhabdus nematophila* (Thomas and Poinar) (Enterobacteriales: Enterobacteriaceae) showed that agitation increases its biomass in batch cultures (Wang and Zhang 2007). Agitation causes the bacteria to move around in its biofilm and spread over the surface it inhabits allowing for better aeration (oxygen absorption).

While it improved the life of *X. nematophila*, without further testing it is unknown what effects agitation might have on the bacteria found on *L. cuprina* eggs.

Properly understanding sterilization techniques is important for MDT to reduce chance of infection of patients' wounds from the maggots themselves. Our findings demonstrate that agitation is currently not a good addition to the process of sterilizing fly eggs for MDT because of the significantly lowered hatch rate compared to the non-agitated. Any future studies should test if the possible increase in mortality of bacteria caused by disruption in the biofilm from agitation balances out the reduced hatch rate of the eggs from the process.

Acknowledgement

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