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The Influence of Relative Humidity on the Frequency of Clutches of 3 Forensically Important Blow Flies (Diptera: Calliphoridae) †

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Abstract: Gravid females have numerous factors to take into consideration when choosing to lay eggs. The number of eggs laid per clutch compared to the number of clutches may impact the survival and fitness of her future offspring under conditions of varying temperature and humidity. Temperature and humidity interact to influence conditions of desiccation that may prevent eggs from successfully eclosing. The trade-off between clutch size and the number of clutches per female would be expected to vary under these conditions such that species more susceptible to egg desiccation should choose fewer and larger clutches, compared to species that are less susceptible. *Calliphora vicina* Robineau-Desvoidy eggs are least susceptible to desiccation, followed by *Phormia regina* (Meigen), with *Lucilia sericata* (Meigen) eggs as the most susceptible to desiccation, thus we expect *L. sericata* females to lay the fewest, largest clutches compared to *P. regina* and *C. vicina*. Individual females were released into an arena on a temperature gradient ranging from 10-40 °C under high (75-85%) and low (24-35%) relative humidity. *Lucilia sericata* laid either 1 clutch with 107 ± 10 (mean ± SE) eggs or 2 clutches of 50 ± 17 eggs. Both *C. vicina* and *P. regina* oviposited up to 3 clutches. *Calliphora vicina* laid 98 ± 24 eggs within 1 clutch, 55 ± 13 eggs when 2 clutches were oviposited, and 43 ± 12 when 3 clutches were laid. Lastly, *P. regina* laid 1 clutch of 86 ± 26 eggs, 42 ± 13 eggs when 2 clutches were oviposited, and 33 ± 4 eggs when 3 clutches were laid. Across all 3 species, clutch sizes ranged from 2-216 eggs per clutch. Despite our predictions, humidity did not affect clutch size ($F_{1,84}$=0.145, p=0.704), or the number of clutches laid ($F_{1,84}$=0.235, p=0.629). This suggests that humidity does not play a role in species-specific clutch size decisions, but each species varies in their oviposition strategies that impact their egg laying behaviour.

Keywords: humidity; oviposition; number of eggs; clutches

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1. Introduction

For female blow flies on an ephemeral, limited resource, energy towards reproduction used towards reproduction to maximize the survival and fitness of future offspring must be traded with energetic requirements for mating, escaping predators or foraging for nutrition or oviposition resources within their short lifespan **[1,2].** The decisions they face include egg deposition patterns of where the eggs are laid, when they are laid, and clutch size, defined as the number of eggs deposited within a single reproductive act **[3-5**]. Individual females will vary their clutch size according to their circumstances and the range of phenotypic plasticity within the species **[6-8**].

Abiotic factors such as temperature and relative humidity influence these egg deposition decisions. Temperature is a predominant factor as it limits many insects' development and survival. With egg laying behaviour, temperature impacts the number of eggs deposited and egg load **[9]**. Humidity also influences egg-laying behavior as the amount of moisture saturation in the air can affect egg eclosion and egg production **[10-13]**. Under lower humidity, eggs can desiccate with larvae unable to escape the chorion, whereas higher humidity can cause oversaturation, where individuals experience lack of oxygen and drowning **[14].** At relative humidities below 35%, *Lucilia sericata* (Meigen) eggs have a successful eclosion rate of 15-25%, whereas *Phormia regina* (Meigen) eggs have an eclosion rate of 30-50% and *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoraidae) eggs successfully eclose at a rate of 65-75% **[12, 13]**. At 80% relative humidity, all three species had an egg eclosion rate of 75-90%

In this study, we manipulated relative humidity to test the impact on egg-laying behaviour of individual females of *C. vicina*, *L. sericata* and *P. regina*. Under low humidity conditions, it is expected that clutch size and number of clutches would vary, such that species that are more susceptible to egg desiccation would choose fewer, larger clutches to increase survival of eggs laid interior in the clutch, compared to species that are less susceptible. We predict that the fewest, largest clutches will be laid by *L. sericata*, then *P. regina*, with more smaller clutches laid by *C. vicina*, due to their respective egg eclosion success rates in low humidity. At high humidity, we expect all three blow fly species to lay the same number of similar-sized clutches.

2. Materials and Methods

2.1. Colony Maintenance

Laboratory colonies of *L. sericata*, *P. regina*, and *C. vicina* maintained at the University of Windsor were used in these experiments. Colonies originated from wild-caught adults around the Windsor, Ontario, Canada area using King Wasp traps baited with pork liver. New adults were added to colonies every year. For *P. regina*, laboratories from Simon Fraser (Burnaby, British Columbia) and Ontario Tech University (Oshawa, Ontario) also provided *P. regina* to maintain colony diversity. Adults were held in 46 x 46 x 46 cm aluminum cages (Bioquip 1450C collapsible cage) containing sugar, water in an Erlenmeyer flask with dental wicks to prevent drowning, and skim milk powder paste *ad libitum*. Colonies are held at 12:12 (L:D), 20-30°C and 40-60% RH. Pork liver was used as an oviposition and larval rearing substrate. Egg masses and fresh 40 g of liver were placed in 1L mason jars with sawdust as a pupation substrate and landscape tarp lids until adult emergence. Multiple generations were used for the experiments.

Freshly eclosed (24h) adults were placed into 3 experimental cages (one cage per species for *L. sericata*, *C. vicina* and *P. regina*), each cage consisting of 40 females and 20 males. Adults were provided with 50 g of fresh pork liver for 48 h, followed by exposure to pork liver for 1 h per day during days 3-5 until females were gravid. This provided a protein source for egg maturation but not as an oviposition substrate to ensure all females had not yet laid a clutch of eggs prior to the experiment.

2.2. Experimental Design

Experiments were performed inside a waterproof environmental box made from 6.35 mm thick plywood with a plexiglass top fully insulated with styrofoam to create and maintain two humidity levels: low (25-35% RH) from the room temperature with a dehumidifier and high (75-85% RH) using a timed humidifier. Access to the box is via a door on the front that is held closed with latches. Inside the box is a plexiglass and mesh arena on top of an aluminum temperature gradient with a cold end created using a styrofoam container filled with ice and a hot end created by heating wire, producing a temperature range from 10-40°C (Figure A1). Thermocouples (Sper Scientific 800023 4-

Channel Thermometer; ±0.2% reading +1°C) were connected underneath the aluminum plate to measure the temperature along the gradient and a datalogger (HOBO U12-012, Onset, Pocasset, MA) was placed inside the environmental box to record the humidity levels every 30 minutes. An oviposition media, made of 500 g of aged liver **[15]**, blended with 500 ml of boiling water with 10 g of agar (BD Diagnosis Bacto™ Agar Solidifying Agent), was placed on top of the gradient. Individual gravid females from each species were released inside the arena and observations were made hourly until either eggs were laid and females were removed at that hourly check, or a maximum of 8 h, whichever was earliest. When females oviposited, the number of clutches laid were recorded. Egg masses within each clutch were measured, photographed and analyzed using ImageJ **[9,16]**.

2.3. Statistical Analyses

All statistical analyses were performed on RStudio 1.3.1073. To test the probability between *L. sericata*, *P. regina* and *C. vicina* ovipositing more than 1 clutch within each humidity level, a logistic regression (GLM function, family=binomial, link="logit") was used, where references were assigned as humidity and species were treated as categorical variables.

For the estimated number of eggs within a clutch, a two-way ANOVA (aov function) was used to analyze the effect of humidity, species, and the interaction between humidity and species. For the number of clutches laid, a two-way analysis of variance (ANOVA) (aov function) was also used to analyze the effect of humidity, species, and the interaction between humidity and species, and where applicable, a Tukey's honest significant difference post-hoc analysis was performed (HSD.test function).

3. Results

Overall, humidity did not have an effect on the probability of each species ovipositing more than 1 clutch (GLM: $z=0.733$, $p = 0.463$). However, there was a difference between species ($F_{2,84}=18.1$, $p=<0.001$) with the odds of *C. vicina* ovipositing more than 1 clutch is 11 times greater than that of *L. sericata*, and the odds of *P. regina* ovipositing more than 1 clutch is 14 times greater than that of *L. sericata* (Fig. 1). Both *C. vicina* and *P. regina* laid up to 3 clutches per females, whereas *L. sericata* females laid a maximum of 2 clutches. Post-hoc comparisons using the Tukey HSD test indicated significant differences between *L. sericata* and *C. vicina* ($p=<0.001$) and *P. regina* and *L. sericata* ($p=<0.001$).

The number of eggs within each clutch did not depend on humidity ($F_{1,84} = 0.145$, $p=0.704$), where each species laid the most eggs within the first clutch laid, followed with decreasing number of eggs as each species laid more than 2 clutches, regardless of humidity (Fig. 2). Post-hoc comparisons using the Tukey HSD test indicated significant differences between *P. regina* and *L. sericata* ($p=0.005$), with *P. regina* ovipositing 46 eggs less than *L. sericata*

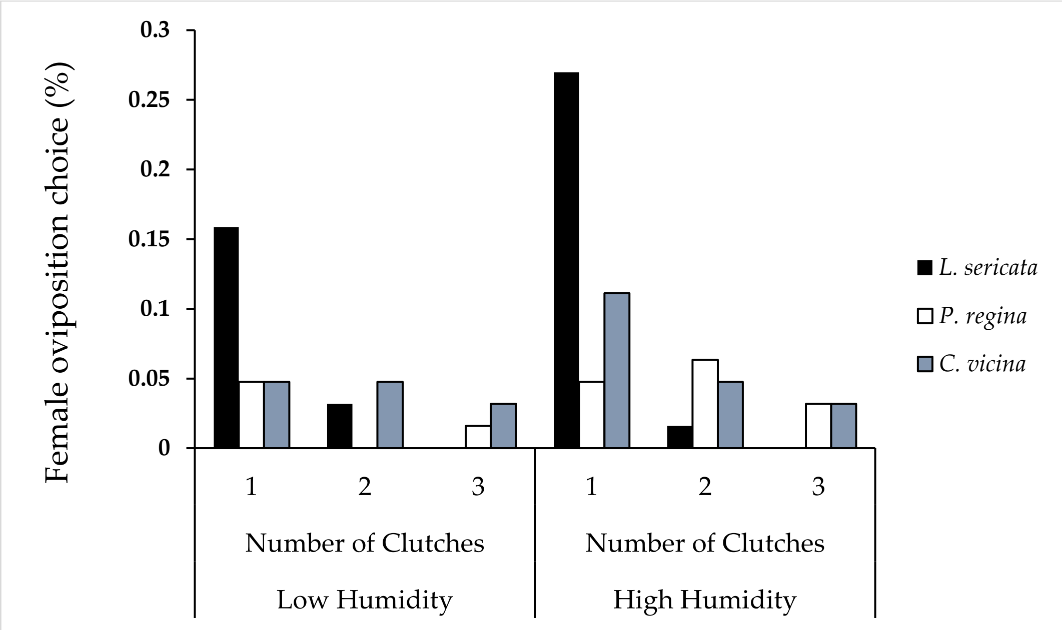

Figure 1. Total percentage of females of *Lucilia sericata* (Meigen) (n=30), *Phormia regina* (Meigen) (n=13), and *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoraidae) (n=20) choosing to lay 1, 2, or 3 clutches over a maximum of 1 hour upon initiation of oviposition within high (75-85% RH) and low (25-35% RH) relative humidity treatments.

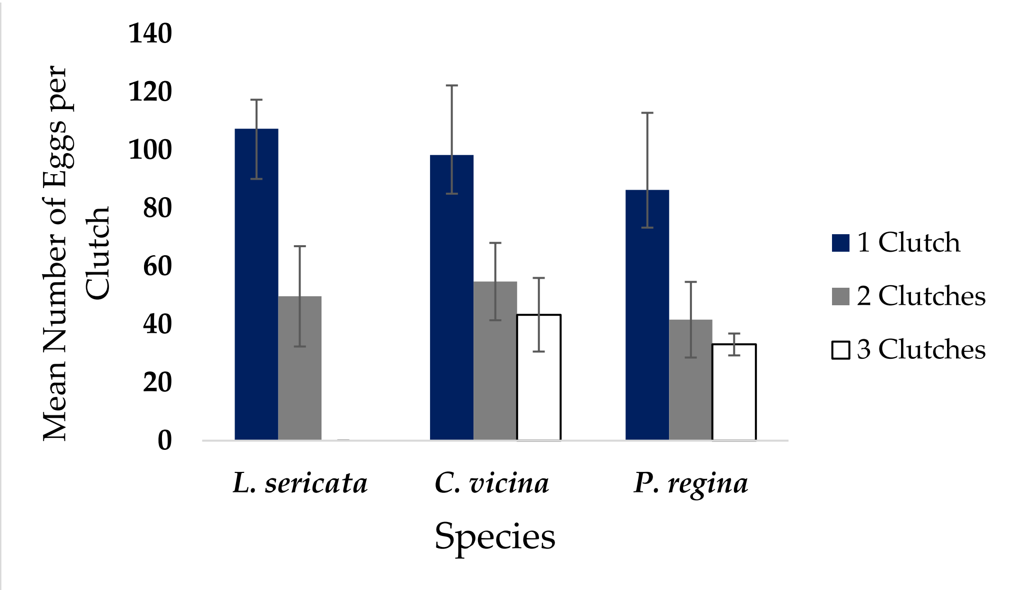

Figure 2. Mean (± SE) number of eggs laid within each clutch over a maximum of 1 hour upon initiation of oviposition by *Lucilia sericata* (Meigen) (n=30), *Phormia regina* (Meigen) (n=13), and *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoraidae) (n=20) females pooled across humidity treatments ($p= 0.704$).

4. Discussion

Egg clustering is a strategy used by females to protect against desiccation and has been observed in numerous insects. For example, in Black soldier flies, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), they lay their eggs in clusters composed of multiple layers to reduce egg desiccation, as eggs subjected to 25% RH took longer to eclose and had a low eclosion success of 7%, with eggs desiccated on the surface of their egg clusters, compared to 70% RH where eggs experienced faster eclosion and higher eclosion success of 75% **[10]**.

We expected the number and size of egg clutches to vary within species between the humidity treatments. Surprisingly, this was not the case, however we did observe overall differences between species tested. *Lucilia sericata*, predominately oviposited 1 clutch with more eggs compared to *C. vicina* and *P. regina*. Given the low egg eclosion success of *L. sericata* at low humidity **[12-13,17]** we expected *L. serciata* to oviposit fewer, larger clutches. They never oviposited more than 2 clutches even at high humidity, which could be an oviposition strategy for *L. sericata*, where females maximize their reproductive fitness by ovipositing more eggs in fewer clutches as a response to unpredictable future humidity conditions.

Both *C. vicina* and *P. regina* oviposited up to 3 clutches across low and high humidity treatments. We expected *P. regina* to be affected by low humidity due to low egg eclosion success **[12,13]** but clutch size did not vary with humidity. In contrast, we did not expect *C. vicina* to vary its clutch size or number of clutches per females due to humidity, as humidity doesn't appear to impact egg eclosion success **[12,13]**. Indeed, there was no difference in number of clutches or clutch size between high and low humidity treatments for *C. vicina*. With multiple clutches laid by both *C. vicina* and *P. regina*, there were also fewer eggs oviposited within each clutch as more clutches were laid per female. This suggests that the predominate oviposition strategy for these two species is to lay more, smaller clutches compared with *L. sericata*.

There was a wide range of clutch sizes across all species, ranging from 2-216 eggs per clutch. We did not expect to see the overall number of eggs laid to be relatively low, however, females were removed and not allowed to continue oviposition longer than 1 hour once they started. It is possible that females had additional eggs available to lay when they were removed from our arena. Wall **[18]**, estimated a mean of 225 ± 7 eggs per clutch for *L. sericata*, which is higher than the eggs reported in this study. Variation in clutch size may be a response to the females' assessment of an oviposition resource, where her environment determines whether a resource is suitable for the offspring, and varies under different environmental circumstances **[4,19]**, such as temperatures **[9]**, or the interaction of temperature and humidity on clutch size decisions **[20,21]**.

5. Conclusion

This study examined the effects of relative humidity on the number and sizes of clutches laid by three blow fly species. Species varied in their oviposition choices; however, humidity did not appear to determine how many eggs per clutch or how many clutches each female chose to lay in their initial clutch size decisions. Additional studies are required to determine if other environmental factors on their own or interacting with humidity influence clutch size decisions in blow flies.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix B

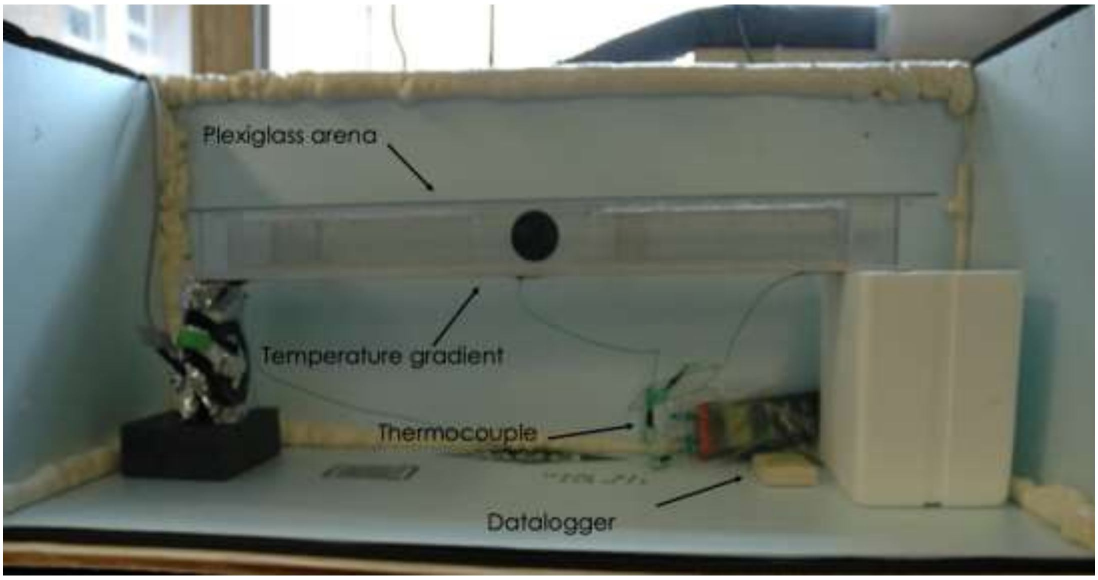

Figure A1: Experimental set up of the arena on top placed on top of the temperature gradient inside the "environmental box", temperatures ranging from 10-40°C.

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