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Original Research
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Effects of Caffeine on the Fertility of *Hermetia Illucens* (Stratiomyidae), fed with Coffee Pulp

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Abstract

Coffee Pulp is a residue with high concentrations of caffeine, which is a harmful alkaloid that affects fertility in Diptera. The main objective of this study is to define how the caffeine present in the Coffee Pulp affects the fertility of *H. illucens,* seeking to establish if the bioconversion of Coffee Pulp affects the long-term reproduction of adults. For this purpose, larvae were fed with fresh Coffee Pulp and solid vegetable waste. The pre-pupae obtained were weighed and the internal genitalia of 90 females from each diet were dissected. Afterwards, crosses were conducted between the adults from each diet, the egg clutches were weighed and the emerged larvae were counted. We encountered that the Coffee Pulp does not affect the adults' survival or the weight of the pre-pupae, but it does affect the developmental duration of the immature state. No significant differences were found in the oocyte production between diets, but their size varied according to the females' age. We found that the hatching rate varied significantly between the crosses and the control. With this study we can affirm that caffeine does not affect the fertility of *H. illucens*, but we cannot affirm that it affects the hatching rate. The economic and environmental impact of our results is significant, due to the potential that *H. illucens* has in the bioconversion of Coffee Pulp and the commercial use of the byproducts.

Keywords: Black Soldier Fly, Caffeine, Coffee Pulp

1. Introduction

The coffee industry is an important economic activity worldwide. In Colombia alone there are 941 hectares of coffee cultivated, which currently produce 14.2 million 60-kilo sacks[1]. From the coffee berry, exocarp (peel) and mesocarp (pulp) separated from the coffee bean, are denominated as Coffee Pulp (CP)[2] and this separation process requires a large amount of water[3], which represents a considerable discharge of contaminated wastewater to natural water sources[4]. The CP represents 60% of the mass in dry fruit and due to the high volume produced; we seek to achieve better management and/or exploitation. On the one hand, waste is used as fertilizer on coffee farms. However, the husk is very voluminous, so storage, handling and incorporation into the soil represent a significant challeng[2]. Moreover, several studies have concluded that these residues can be useful in animal feed, but there are serious limitations in their use, related to high concentrations of tannins and caffeine. Tannins are anti-nutritional and limit the absorption of nutrients such as nitrogen, and caffeine affects the palatability of animals, causing physiological effects on the central nervous system[2].

Hermetia illucens (Stratiomyidae), commonly known as the "black soldier fly" is distributed in tropical and subtropical regions with its external genital form being the only character to differentiate the sexes[5]. Mating usually occurs two days after the adults emerge and four days later, the

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female oviposits 320 to 630 eggs[6]. The larvae are voracious and feed on a wide variety of organic waste, converting them into by-products, such as nutrients, proteins, fatty acidsand vitamins, which are used in the feeding of farm animals (aquaculture, poultry, etc.)[5]. Adults do not feed, obtaining what is necessary for their development from fats stored during larval development[7] and their life cycle can last approximately 40 days, depending on diet and temperature[4]. Despite its major features as an organic matter reducer, no in-depth studies have been performed on the effect that feeding with CP can have on the fertility of *H. illucens,* however, works in diptera, demonstrate that caffeine can influence reproductive processes and have mutagenic effects, since high concentrations of it results in a decrease in the total egg production, abnormal development in ovaries, delayed oocyte maturation and can even be lethal in some larvae, as indicated by Srinivasan and Kesavan[8], Itoyama and Bicudo[9] and Laranja et al.[10-11]. Presently, the works performed by Larde[12-13] are known, in which they evaluate the physicochemical changes of the pulp and relate the larvae size with its weight and the biological cycle of CP-fed *H. illucens*[14].

Due to the fact that, to this date, no work has been performed to evaluate the effect of caffeine on the fertility of *H. illucens,* the present study is pioneering and its main objective to define the effects of caffeine on the fertility of *H. illucens,* seeking to establish whether CP bioconversion programs affect adult reproduction in the long term.

2. Material and Methods

2.1 Study Site

The trials were conducted in a greenhouse at the Universidad del Valle, Cali - Colombia (3o 22' 37, 18"N, 76o 31' 48.6"W) from March to December 2016, at room temperature. The individuals used were selected from the colony of *Hermetia illucens,* maintained since 2012, in the technical storage unit of solid waste at the Universidad del Valle. The fresh CP used, came from crops of *Coffea arabiga,* Castilla variety, located in the Vereda El Faro, corregimiento Los Andes, Marandúa farm, in Valle del Cauca, Colombia (3° 25' 26.3"N 76° 35' 34.6"W) while vegetable waste was collected from the commercial fruit sales areas of the Universidad del Valle. The caffeine present in the fresh pulp was measured with HPLC.

2.2 Experiment Design

500 larvae of *H. illucens* at six days of age were arranged in 667 cm^2 bioconverters, fed with fresh CP and Solid Vegetable Waste (SVW) as control (Table 1). Daily pH measurements were collected (pH Meter FieldScout SoilStik) and temperature at three points of each bioconverter, relative humidity and ambient temperature, were measured with a data logger (USB Dickson TP125). Four replicates were made for each diet.

Table 1. Humidity of the fruit used[15-16], percentage in dry base and its equivalent in mg/larvae/day added every two days

Fruit	Humidity percentage	Added quantity (gr)	Dry base (gr)	mg/larvae/day
Papaya	90.86	240	21.9	21.9
Banana	87.88	200	24.24	24.24
Pineapple	84.00	200	31.8	31.8
Melon	91.42	240	20.6	20.6
Coffee pulp	80.00	750	150	150

2.3 Effect of Diet on Pre-pupal Weight and Sex

The percentage of pre-pupae that reached the adult state was recorded and each emerged adult was marked on the pterothorax, using Printmark and Sharpie, with a combination of different symbols depending on the emergence day and color according to sex[17]. Subsequently, they were released into 1 m^3 cages and supplied with fresh water daily.

2.4 Effect of the Diet on Female Fertility

Fifteen six-day-old female *H. illucens* were dissected daily with the aid of a Leica 2000 Zoom TM stereomicroscope, the number of oocytes/ovariole/female was counted, following the methodology by Tomberlin et al.[18], measuring the oocytes and spermatheca lengths, where the distal ejector duct, capsular duct and capsule width were taken

into account, the proximal ejector duct was discarded, given that the size can be overestimated, as it is more lax than the other segments.

2.5 Caffeine Effect on Male Fertility

To measure the effect of caffeine on male *H. illucens* and given the impossibility of measuring the testicles and doing a sperm count, the effect of caffeine on the larval survival of a generation previously fed with CP was evaluated. Females originating from CP-fed larvae and males originating from SVW-fed larvae and vice versa, were deposited separately in 1 m^3 cages. For each combination, the number of hatched larvae and unhatched eggs were counted to determine larval survival. The first half of adults hatched in SVW was used as control, while the second half were used for the crosses. However, in CP the opposite happened, the first adults were used for the crosses with the second half being used as control. Thirty egg clutches from each diet were weighed and deposited in 70% alcohol and the number of oocytes was counted to know the ratio between the weight of the clutches and the egg count[18].

2.6 Statistical Analysis

A non-parametric Mann Whitney test was used to evaluate the difference between larval development, pH and temperature for both diets, in order to define if the difference in larval and pupal development duration is related to the diets and to evaluate the relationship between sex and the day of adult emergence. On the basis of a negative binomial test, supported by the review of confidence intervals, the number of oocytes per diet, the number of eggs oviposited between crosses and the total number of larvae hatched between combinations were compared. To evaluate the correlation between larval weight and sex, a contingency table was made along with a χ^2 test (with Yates correction). Finally, through an ANOVA, the difference in pre-pupal weight and oocyte length between diets was evaluated. The relationship between the number of eggs and the weight of each clutch was evaluated by means of a linear regression. All the analyses and graphs were made in R[19].

3. Results

The pH (Figure 1a, Mann Whitney, df = 40, F = 319, p = 0.0142) and temperature (Figure 1b, Mann Whitney, df = 40, F = 176, p = 0.272) value had no negative effect on the larval development of *H. illucens*. The temperature ranged between 26.49 and 36.63°C. Initially, larvae fed with SVW presented more acidic values (3.33) than larvae fed with CP (6.76). This was due to the fact that the diet with SVW consisted of acidic elements such as pineapple, which has a pH of 3.6[20], however, the pH in both diets shifted from acid to basic. Furthermore, there were peaks of acidity after adding the daily vegetable ration in both diets, these peaks are observed in Figure 1a.

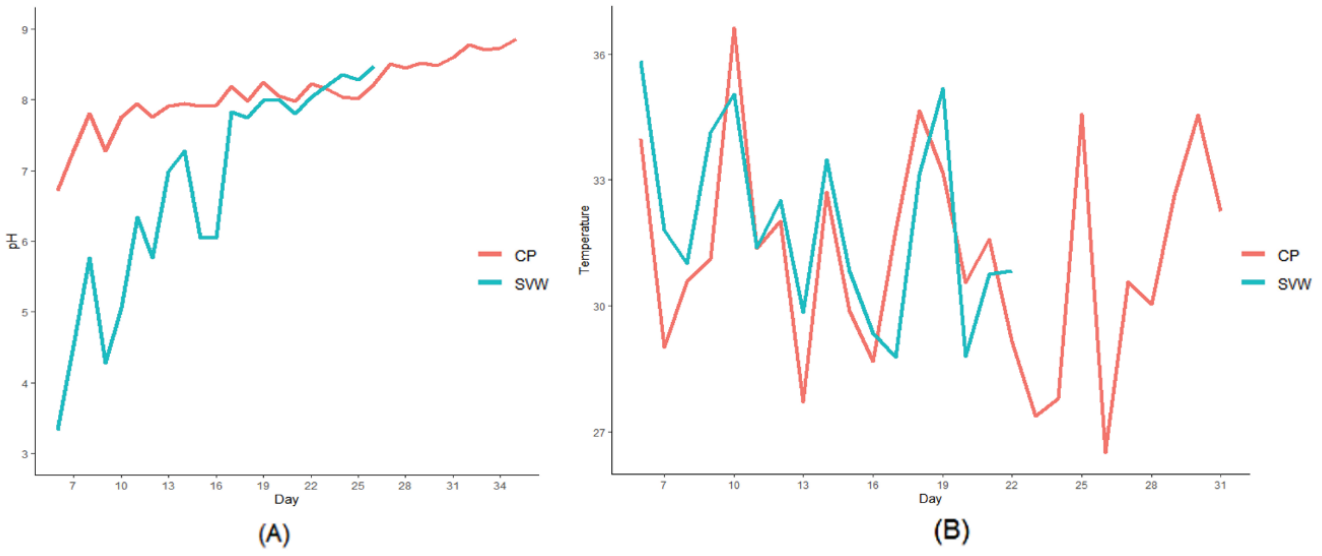


Figure 1. Variations in *H. illucens* larval development, in relation to pH and temperature, fed with CP and SVW.

3.1 Effect of Diet on Immature States

At an average environmental temperature of 31.74 ± 2oC, Relative Humidity (RH) of 51.85 ± 5 and 0.5% of caffeine contained in CP, it was found that the larval development time of *H. illucens* is longer in CP than in SVW (Mann Whitney, df = 2732, F = 2002100, $p < 2.2e-16$) and although it was found that significant differences existed between the diets and the duration of the pupal development (Figure 2) (Table 2, Mann Whitney, df = 1366, F = 793880, $p = 1.44e-11$), it was observed in both diets that the majority of individuals (98% in SVW and 93% in CP), will take between 14 and 24 days to complete the pupal stage. The duration from egg to adult in *H. illucens,* took on average 40 days in SVW and 53 days in CP.



Figure 2. Number of migrated *H. illucens* pre-pupae from larvae fed with CP and SVW, aged 19 to 43 days, respectively.

Table 2. Survival rates and duration in days, of the different stages of development of *H. illucens,* fed with SVW and CP

	Larva to pre-pupa development		Pre-pupa to adult development	
	Survival (%)	**Stage duration (days)**	**Survival (%)**	**Stage duration (days)**
CP n = 2000	75.7	35 ± 3.6	93.6	18 ± 3.7
SVW n = 2000	78.6	24 ± 3	83.7	17 ± 2.1

3.1. Relationship between Pre-pupal Weight, Sex and Diet

In Figure 3 the correlations between larval weight, sex and diet are presented. No significant differences were found between diet and pre-pupal weight (Anova, df = 30, F = 0.241, $p = 0.626$), we did encounter significant differences between the weights, between the diets and the migration days (Anova, df = 33, F = 154.4, $p < 2.3e-13$). Moreover, a relationship between pre-pupal weight and sex was found in CP ($\chi 2$, F = 563.05, $p < 2.2e-16$) and SVW ($\chi 2$, F = 1124, $p < 2.2e-16$). Pre-pupae weighing less than 120 mg have an 82% probability of being male when the larvae were fed with CP and 61% with SVW; pre-pupae weighing more than 120 mg have a 72% probability of being female when the larvae were fed with CP and 81% in SVW. In Figure 4, the asynchrony in the emergence of both sexes is presented, the males emerge earlier than the females, with significant differences being found between the emergence day and the sex, in SVW (Mann Whitney df = 763, F = 89562, $p = 5.331e-08$) and CP (Mann Whitney, df = 1423, F = 372090, $p < 2.2e-16$).

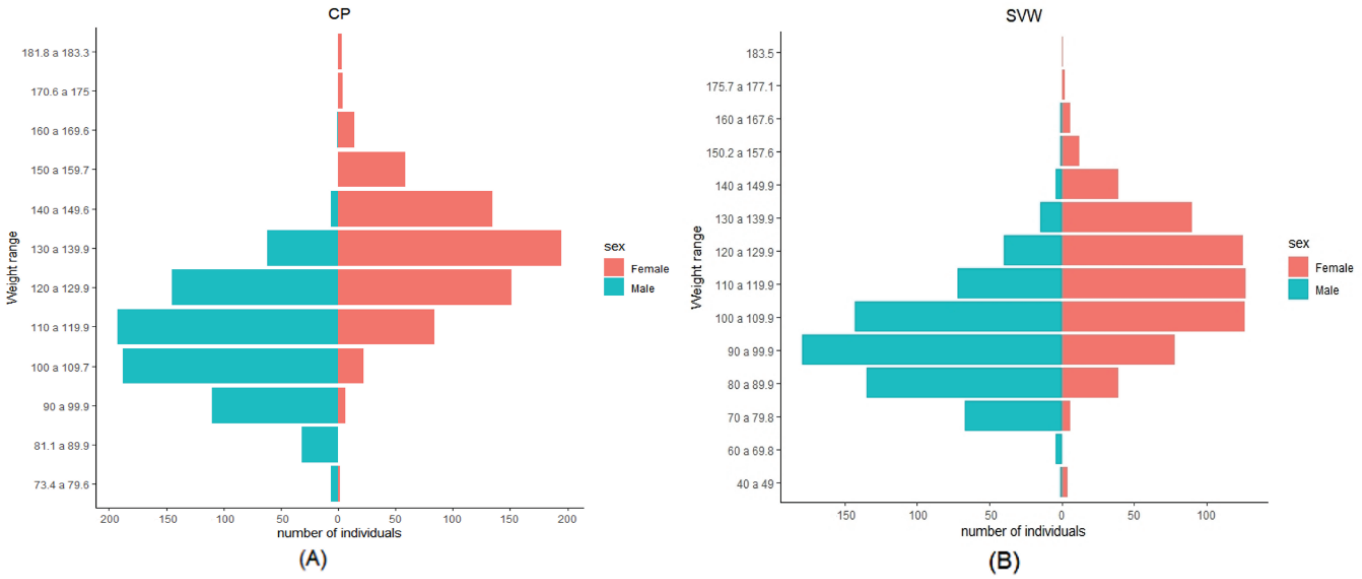


Figure 3. Relationship between pre-pupal weight and sex of *H. illucens,* fed with CP (A) and SVW (B).

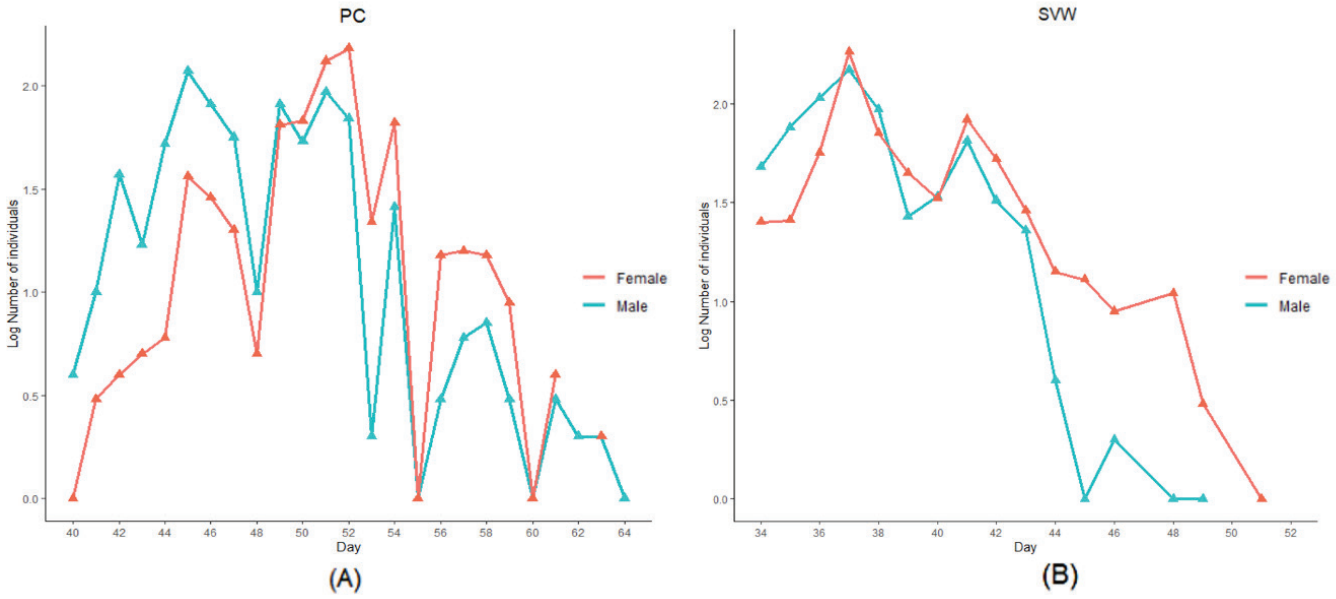


Figure 4. Relationship between emergence age and sex, in *H. illucens* adults, fed with CP (A) and SVW (B).

3.2 Effect of Diet on Female Fertility

Of the 90 females dissected in each diet, no significant differences were found in length (Anova, df = 176, F = 0.056, $p = 0.814$) and number of oocytes (Binomial negative GLM, $p = 0.049$) (Table 3). Those females that did not copulate had reduced oocytes with sizes below 0.4 mm, whereas females with oocytes above this size were pregnant. (Figure 5), for a total of 66 and 57 pregnant females, in CP and SVW respectively. No variations or malformations were found in the spermatheca of the females originating from larvae fed with CP and SVW. High concentrations of caffeine did not affect fertility or larval weight.

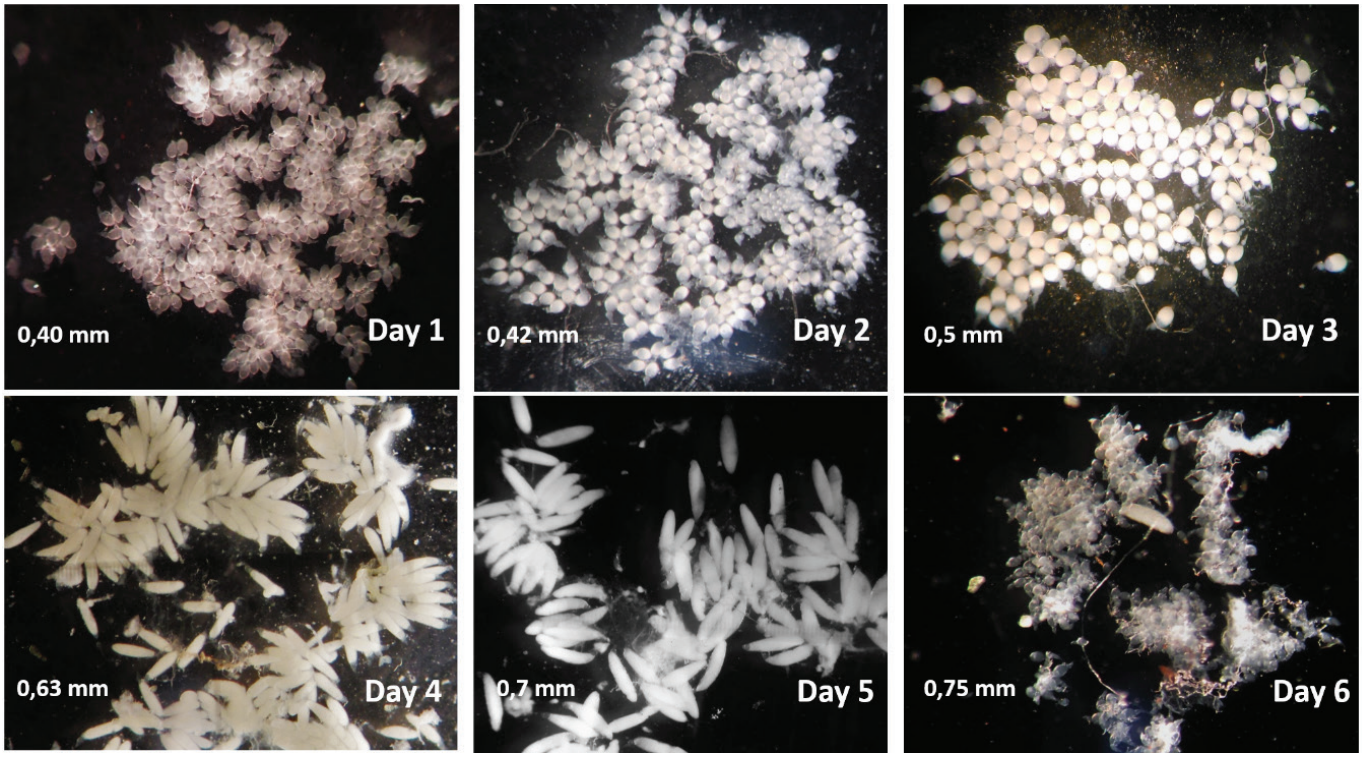


Figure 5. Variation in oocyte size (mm), in *H. illucens* females, up to six days of age.

Table 3. Variations in spermatheca size, length and total number of oocytes, depending on the age of the female (days) of *H. illucens* fed with CP and SVW

Age of the female (days)	CP diet			SVW diet		
	Number of oocytes	Length of oocytes (mm)	Size of spermatheca (mm	Number of oocytes	Length of oocytes (mm)	Size of spermatheca (mm)
1	704±92.7	0.39±0.06	11.492±1.0	693±99.3	0.397±0.04	10.58±1
2	682±105.1	0.42±0.12	11.52±0.9	622±103.2	0.455±0.046	10.95±1.7
3	640±117	0.48±0.08	10.85±0.84	618±99.7	0.496±0.058	10.67±0.99
4	574±141.4	0.54±0.1	11.15±0.82	567±122.4	0.625±0.23	10.44±1.51
5	636±134	0.71±0.21	10.6±0.83	556±110.3	0.714±0.23	9.5±0.89
6	547±119.1	0.77±0.18	10.22±0.91	557±104.4	0.745±0.22	9.8±1.85

3.3 Hatching Rate According to Diet

A positive link was found between the weight of the clutches and the number of eggs (Table 4). No significant differences were found on the number of eggs oviposited between only CP-fed females and only CP-fed males (GLM, Binomial negative, $p = 0.35$), however, differences were found in the number of larvae hatched between the crosses and the control (Table 5, GLM, Binomial negative, $p < 2e\text{-}16$). The low hatching rate of eggs originating from SVW-fed larvae and only males from PC-fed larvae may be due to the fact that females oviposited infertile eggs.

Table 4. Correlation coefficient between egg clutch weight (milligrams) and number of eggs, and their respectiveweight (milligrams) between diets

Treatment	Spearman correlation	Regression model	Weight ranges per egg clutch (mg)	Egg weight (mg)
SVW n = 30	Rho: 0.972	31.6 + 44.0 (p <0.05)	1.9 a 17.9	0.0204 ±0. 003
CP n = 30	Rho: 0.915	46.2 + 45.4 (p <0.05)	2.2 a 19.5	0.0199 ±0. 003

Table 5. Number of clutches and hatching rate, between combinations of *H. illucens* females and males, originating from larvae fed with CP and SVW

Number of egg clutches	Treatment	Hatched eggs	Unhatched eggs	Hatching rate
n = 56	CP	13.509	16.078	45.6
n = 42	SVW	05.567	14.054	28.4
n = 65	female(+)-male(-)	10.931	16.495	39.8
n = 59	male(+)-female(-)	08.161	20.821	28.1

(+) originating from larvae fed with CP
(-) originating from larvae fed with SVW

4. Discussion

Regarding the pH and the temperatures reached in the larval development units, they coincide with the results obtained by Alattar[21], when studying leachate-fed larvae of *H. illucens*. Likewise the shift of the pH from acid to basic by the larvae in the first days of development, coincides with the results obtained by Alattar[21] who reported that *H. illucens* larvae modify pH values, supporting ranges from 0.7 to 13.7.

As for the duration of the immature states, fed with the two diets, they correlated with the results obtained by Tomberlin et al.[18], who recorded a developmental period of 40 to 41 days, with a chicken manure diet at temperatures of 30oC and 60-70% relative humidity, additionally Gobbi[5], found that larvae fed with meat meal, their larval development was 52.4 days at 25oC and 60% relative humidity. Since the diet with CP has less nutrient supply, *H. illucens* larvae take longer to accumulate the energy necessary to accomplish their development, compared to larvae fed with SVW.

With regard to the link between the pre-pupae weight and sex, our results coincide with those obtained by Tomberlin et al.[22], who concluded that females weigh 13% more than males and spend more time as larvae. This result is justified by the fact that a long larval development will allow to accumulate more energy reserves, resulting in greater fertility. Furthermore, Gobbi[5], observed in all the evaluated diets, that the females were larger than the males, finding a positive relationship between the size of the female and, the size of the ovaries and the oocytes, demonstrating that females with greater size are more fertile.

As for the effect of the diet on female fertility, our results coincide with those reported by Tomberlin and Sheppard[6], who state that mating must occur for oocytes to mature and that the greatest number of mating events occur two days after emergence and that oocytes are fully formed two days after mating.

Unlike the results reported by Srinivasan and Kesavan[8], Laranja et al.[10] and Itoyama and Bicudo[9] who found that caffeine in larvae and adults of *Musca domestica, Aedes aegypti,* and *Drosophila prosaltans* decreases fertility and causes a significant reduction in larval size and longevity, in some cases becoming lethal; in our trials, no

malformations were found in the female spermatheca and high concentrations of caffeine did not affect fertility or larval weight. These results may be attributed to the fact that, unlike other Diptera, *H. illucens* does not feed in the adult state, since it is in the immature state when all the nutritional elements necessary for adult fertility are stored. For this reason, the food quality supplied to the larvae is important, since the energy stored will play a fundamental role in the formation and subsequent development of the adult. (Gobbi[5]), that is, *H. illucens* reaches the pre-pupal stage, with a reserve of 42% protein and 35% fat and fatty acids. In contrast, the *M. domestica* pupae contain 9 to 15% fat and adults require nutrition for successful reproduction and ovoposition[23].

The positive correlation found between egg clutch weight and number of eggs was also found in studies by Tomberlin et al.[22], with different diets.

The low hatching rate found in the crossing of females originating from larvae fed with CP and males originating from larvae fed with SVW (control), can be explained in the works done by Tomberlin et al.[22], and García and Iannacone[24], who found that *Anastrepha fraterculus,* without having copulated, produces infertile eggs the first two days, when confined in cages or experimental containers[25]. Additionally Brockerhoff et al.[26] observed several *Strobilomyia neanthracina* copulations in greenhouse conditions, but none of the oviposited eggs hatched, claiming that this could be due to the absence of sperm in the sperm capsules of all the females, or a lack of stimulus for mating. The fact that the larvae fed with CP take longer to reach pupa indicates that they can store more resources, which are fundamental for fertility[22].

5. Conclusions

Under experimental conditions, the amount of caffeine present in CP, does not affect either survival or accumulated larval biomass, however, the duration of larval development is affected.

A positive relationship between pre-pupal weight and sex was demonstrated, as well as a significant difference between emergence time and sex, with females weighing more and emerging first than males.

Our trials allow us to assert that caffeine does not affect the fertility of *H. illucens,* since there was no reduction in the production of oocytes, nor did the reproductive structures present malformations.

Additionally, in females of the same age, it was found that the size of the oocytes is related to the time elapsed since mating.

From the results obtained in this study we can affirm that *H. illucens* may be included in bioconversion programs of CP, since the caffeine ingested by the larvae does not affect the fertility of the adults.

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7. Conflict of Interest

Authors declare that they have no conflict of interest.

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