The effect of anticoagulants in artificial blood meals on the mortality, fecundity, and fertility of *Culex quinquefasciatus* and *Aedes aegypti* (Culicidae)

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ABSTRACT: Blood sources used for insect colonies and their effects on fecundity and fertility have been studied in multiple mosquito species, but the effect of anticoagulants that prevent clotting of blood has received minimal attention. Here, we identify the effect two anticoagulants have on the mortality, fecundity, and fertility of *Culex quinquefasciatus* (Sebring and BCS strains) and *Aedes aegypti* Liverpool. Each mosquito species was provided with one of three treatments: direct feeding on live chicken (LC), blood from freshly exsanguinated chicken treated with heparin (EXS) or commercially purchased chicken blood treated with Alsever's solution (ART). No significant effect of treatment on mortality was observed. Both *Cx. quinquefasciatus* Sebring and BCS strains demonstrated a significant effect of treatment type on fecundity with the number of eggs laid for LC being 1.40-fold higher than EXS and 2.14-fold higher than ART for Sebring. For BCS strain mosquitoes, LC was 1.55-fold higher than ART, and EXS was 1.57-fold higher than ART, but there was no significant difference between LC and EXS. For *Ae. aegypti* mosquitoes, only a significant difference in mean egg counts was observed between LC and ART treatments, with LC laying 1.46-fold more eggs. No significant effect on fertility was observed among any mosquitoes for any treatment. These results demonstrate the negative effect of anticoagulants on the fecundity for multiple mosquito taxa. This may affect the ability of labs to produce large numbers of mosquitoes or colonize wild mosquito populations and should be taken into account when considering colony maintenance or vector biology research. ***Journal of Vector Ecology* 46 (2): 137-142. 2021.**

Keyword Index: Mosquito, colony, bloodmeal anticoagulant, mortality, fertility, fecundity.

INTRODUCTION

Mosquito colonies are maintained and mass-reared all over the world in the context of research and mass-release control programs. Given that medically important mosquito species require a blood meal to lay eggs (Foster 1995), the initial mosquito colonies were maintained by direct feeding on vertebrates (Boyd et al. 1935, Rozeboom 1936, Munstermann 1997). As time progressed and concerns about the ethics of live animal feeding and costs arose (Bailey et al. 1978), studies have attempted to find methods of artificial feeding to replace live host feeding (Wetzel 1979, Deng et al. 2012, Luo 2014). Additionally, the advent of mass-rear and release control programs necessitated efficient methods to reduce reliance on live vertebrates (Gonzales et al. 2018). Currently, most labs utilize an artificial method of feeding by allowing mosquitoes to obtain warmed blood through a membrane. While this approach is convenient, the substitute for direct feeding on live animals could affect mosquito biology or behavior.

Artificial feeding of mosquitoes involves a blood storage apparatus (glass, metal, or plastic) that is heated and covered with a membrane for mosquitoes to feed through. Many versions of these membrane feeders have evolved over the years, including water-jacketed glass membrane feeders (Rutledge et al. 1964) and more recent versions using 3D printing technology (Witmer et al. 2018, Graumans et al. 2020). Commercially available units relying on electrical power to warm the blood, instead of water, include Hemotek units (Hemotek, Ltd., Blackburn, UK) and the Apex Blood-Feeding System (Apex Bait Technologies, Inc., Santa Clara, CA). Blood provided to mosquitoes using an artificial system needs to be defibrinated or treated with an anticoagulant such as citrate or Alsever's solution to prevent clotting during the feeding process (Richards et al. 2012, Phasomkusolsil et al. 2013). While the artificial feeding systems eliminate the need for feeding directly on a live animal, they could introduce opportunities for modifying mosquito physiology or behavior which could confound research results. For example, studies gathering life-table data, such as survivorship following feeding on a membrane feeder with blood containing an anticoagulant, could have different results than if feeding directly on a live animal (Lima et al. 2003). Additionally, vector competence studies of mosquitoes routinely measure the transmission rate, or the proportion of exposed mosquitoes with virus in saliva, which could be influenced by biological changes induced by artificial blood meals containing an anticoagulant (Goddard et al. 2002).

There have been numerous studies on the effects of different vertebrate species as blood meal sources on the fecundity and fertility of laboratory mosquitoes due to the importance of mosquito colonization (Richards et al. 2012, Phasomkusolsil et al. 2013). There have also been multiple studies that attempt to look at the effect of artificial feeding when compared to live host-feeding. Richards et al. (2012) found that indeed there was a significant effect on the fecundity and fertility of *Culex quinquefasciatus* when fed on

live animals. However, this study and others fail to directly compare the effect of anticoagulants on the fecundity of these mosquitoes, especially on the same host blood source. This study aims to identify the effect of these anticoagulants on the fecundity, fertility, and mortality of *Cx. quinquefasciatus* and *Aedes aegypti*.

MATERIALS AND METHODS

Mosquito colony maintenance

Experiments were carried out with one-week-old female *Cx. quinquefasciatus* and *Ae. aegypti* Liverpool (AEG) mosquitoes. Two strains of *Cx. quinquefasciatus* were used: Sebring strain (SEB) and a locally colonized strain from Bryan-College Station, TX (BCS). *Culex quinquefasciatus* (BCS) used for this study were collected as egg rafts in 2018 and were between F12 and F15 generations removed from the wild during the current study. The *Cx. quinquefasciatus* (SEB) was colonized in 1988 (Sbrana et al. 2005) and *Ae. aegypti* Liverpool was colonized in 1936 (Macdonald 1962). Mosquitoes were maintained on a natural night and day light cycle with a constant 50% humidity with a 10% sucrose solution provided *ad libitum*.

Experimental feeding

All mosquitoes were sucrose-starved 24 h prior to feeding to increase feeding success, with only access to water. Mosquitoes had not been previously offered a blood meal and were all nulliparous. All artificial blood-feeding was carried out using an artificial feeder (Hemotek, Ltd., Blackburn, UK) and parafilm membranes. Male chickens within two days of hatching were used for the experiment since they are routinely culled by poultry facilities and were available for our research. Each group of mosquitoes was allowed to feed for one h from one of three feeding treatments: direct feeding on a live chicken (LC), blood from an exsanguinated chicken that was treated with heparin (Sagent Pharmaceuticals, Schaumberg, IL, U.S.A.) and fed artificially (EXS), or commercially purchased chicken blood (HemoStat Laboratories, Dixon, CA, U.S.A.) treated with Alsever's solution (ART) and fed artificially. The group fed on live chickens was allowed to feed on one individual for 30 min and then a new chicken was swapped out for the next 30 min to avoid prolonged restraint of the same individual chicken. The blood from HemoStat is whole chicken blood that came from multiple chickens and was pooled together to create a large enough volume. This whole chicken blood was then treated with Alsever's solution so that 50% of the solution was the anticoagulant and the remainder whole chicken blood. The Hemotek feeder heated artificial treatments to 37° C, and a newborn chicken has a natural body temperature of about 39.7° C. Heparin was drawn into the syringe and then expelled back into the container to coat the syringe surface prior to exsanguination of the chicken. The two artificial feeding treatments used the same Hemotek membrane feeder as colony maintenance. After blood-feeding, all fully engorged mosquitoes were separated and placed individually in their own containers to keep track of fecundity. Mosquitoes considered fully engorged had abdomens containing a complete blood meal and a Sella score of two (Sella 1920), while those that were partial blood meals were removed from the study. Each mosquito was provided with 10% sucrose and housed at 27° C, and *Culex* mosquitoes were provided a cup of water for oviposition. *Aedes aegypti* mosquitoes were provided a container with filter paper for oviposition. Only mosquitoes that took a blood meal were included in the analysis. All work with chickens was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC AUP 2018-0175).

Mortality, fecundity and fertility

Mosquitoes that died before laying eggs were removed from the experiment and recorded in order to determine the differences in mortality among treatments. In this study, we defined fecundity as the number of eggs laid by mosquitoes that survived throughout the duration of the study. We defined fertility as the number of eggs that hatched into larvae within the given period. Mosquitoes were given two weeks to lay eggs, and eggs were removed daily. This time period was provided to ensure enough time for egg development and laying. *Culex* egg rafts were photographed and the number of eggs was counted digitally and recorded. After being photographed, the eggs were moved into a larger container for hatching. Eggs were provided with larval food and allowed to hatch. After three days, any unhatched eggs were considered unviable and larvae that hatched were then counted. For *Ae. aegypti,* the number of eggs was counted immediately under a dissecting scope. Eggs not laid on the substrate (i.e., on the ovicup container) were carefully removed and counted. Eggs were then dried for a week before hatching in the same way. Incubator conditions for eggs were the same as adult mosquitoes (27° C, 50% humidity). Experiments were repeated multiple times on different days to achieve a sufficient sample size for analysis, with SEB mosquitoes having three trials, BCS mosquitoes having two trials, and AEG mosquitoes having five trials. The conditions for each of these experiments were consistent, with differences only in the age of the mosquito and the chicks utilized.

Analysis

Chi-square test of independence was done to determine if treatment had a significant effect on the mortality and the egg-laying ability of mosquitoes. Egg count data were non-normal and thus transformed by a degree of 0.825 ($x^{0.825}$) to create a normalized distribution for a one-way analysis of variance (ANOVA). This transformation number was obtained using Tukey's transformation (rcompanion package) and was used only for the analysis to create a normal distribution. The ANOVA was used to compare the overall mean egg quantities laid by each mosquito. If the ANOVA was significant, Tukey's HSD post-hoc test was then done to analyze between treatment type from the ANOVA. Non-egg-laying mosquitoes were included in the fecundity analysis as zeros. Egg hatch rates did not have a normalized distribution, including attempts at transformations, so we evaluated differences among treatments for each species using the non-parametric Kruskal-Wallis test. Hatch rates were calculated as

the number of larvae hatched divided by the number of eggs laid x100. Only mosquitoes that laid eggs were included in the analysis. Analyses were performed using the R statistical software v3.5.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Influence of blood source on mortality

When mortality was examined using the chi-square test of independence, there were no significant effects of any treatment on the mortality of mosquitoes in this study (SEB, df = 2, χ^2 = 1.514, p = 0.4691; BCS, df = 2, χ^2 = .1741, p = 0.9166; AEG, df =2, χ^2 = 3.236, p = 0.1983) (Table 1).

Influence of blood source on egg-laying ability and fecundity

When examining the effect of treatment type on whether mosquitoes laid eggs or not (chi-square test of independence), there was a significant effect of ART treatment type on egg-laying for both SEB and BCS, but not AEG mosquitoes (SEB, df = 2, χ^2 = 6.643, p = 0.0361; BCS, df = 2, χ^2 = 12.784, p = 0.0017; AEG, df =2, χ^2 = 3.120, p = 0.2019)(Table 2). When fecundity was examined among mosquito species, the effect of the different feeding treatments was significant for all mosquito species (SEB, ANOVA, df = 2, F = 20.95, p < 0.001; BCS, ANOVA, df = 2, F = 7.604, p < 0.001; AEG, ANOVA, df = 2, F = 3.146, p = 0.045). SEB mosquitoes that fed on live chickens (LC) laid significantly more eggs when compared to the other treatments (Tukey's HSD, LC-EXS, p = 0.002; LC-ART, p < 0.001; Table 3). Additionally, SEB that fed on the exsanguinated chicken (EXS) treatment laid significantly more eggs than those that were fed commercially purchased chicken blood (ART) (Tukey's HSD, EXS-ART, p = 0.006). SEB that fed on LC laid 1.40 times more eggs on average than mosquitoes that fed on EXS treatments, and 2.14 times more eggs than ART treatment mosquitoes. Additionally, EXS mosquitoes laid 1.53 times more eggs than ART-treatments for SEB mosquitoes (Table 3). For BCS mosquitoes, both LC and EXS laid significantly more eggs than ART (Tukey's HSD, LC-ART, p = 0.0036; EXS-ART, p = 0.0022), but no significant effect was observed between LC and EXS treatments. BCS that fed on LC laid 1.55-fold more eggs than ART-treatment mosquitoes, and those that fed on EXS laid 1.57-fold more eggs than ART treatments (Table 3). For AEG mosquitoes, a significant effect was only observed between LC and ART treatments (Tukey's HSD, LC-ART, p = 0.036). AEG that fed on LC laid an average of 1.46 fold more eggs than ART-treatment mosquitoes (Table 3).

Influence of blood source on fertility

When fertility was examined among the feeding treatments, there were no significant effects for any of the mosquito species (SEB, df = 2, χ^2= 2.417, p = 0.2986; BCS, df = 2, χ^2= 0.1978, p = 0.9058; AEG, df =2, χ^2= 1.90, p = 0.3868) (Table 4). Because of issues preventing AEG eggs from hatching in trials one and two, no hatch data is available for these trials, so these trials were not included in the analysis. Combining treatment groups, the mean fertility rate for SEB was 75.5% (±2.7), for BCS was 84.4% (±2.1), and for AEG was 78.9% (±2.5).

Table 1. The number of dead mosquitoes during the study by mosquito species, treatment, and trial. Percent of total mosquitoes is represented in parentheses. Percent mortality was calculated as the number of dead mosquitoes divided by the total number of mosquitoes that obtained a full blood meal (n). Species identifications are Sebring strain *Culex quinquefasciatus* (SEB), Bryan-College Station strain *Culex quinquefasciatus* (BCS), and Liverpool strain *Aedes aegypti* (AEG).

Trial	Species	Mortality (%)					
		ART	n	EXS	n	LC	n
1	SEB	3 (7.3)	41	2 (11.8)	17	2 (20.0)	20
2		1 (6.3)	16	2 (16.7)	12	1 (8.3)	12
3		0 (0)	9	2 (7.1)	28	1 (6.7)	15
Total		4 (6.1)	66	6 (10.5)	57	4 (8.5)	47
1	BCS	2 (4.0)	50	2 (5.1)	39	2 (4.4)	45
2		3 (6.7)	45	3 (12.5)	24	1 (2.2)	35
Total		5 (5.3)	95	5 (7.9)	63	3 (3.8)	80
1	AEG	7 (17.5)	40	1 (4.8)	21	3 (12.5)	24
2		1 (3.6)	28	3 (10.7)	28	3 (16.7)	18
3		0 (0)	2	1 (5.0)	20	0 (0)	24
4		4 (28.6)	14	3 (11.5)	26	3 (15.0)	20
5		7 (35.0)	20	4 (25.0)	16	3 (23.1)	13
Total		19 (18.3)	104	12 (10.8)	111	12 (12.1)	99

Table 2. The number of egg-laying mosquitoes during the study by mosquito species, treatment, and trial. The percent of egg laying mosquitoes was calculated as the number of egg-laying mosquitoes divided by the total number of mosquitoes that remained alive at the end of the seven days (n). Species abbreviations are Sebring strain *Culex quinquefasciatus* (SEB), Bryan-College Station strain *Culex quinquefasciatus* (BCS), and Liverpool strain *Aedes aegypti* (AEG).

Trial	Species	Egg-Laying (%)					
		ART	n	EXS	n	LC	n
1	SEB	30 (79.0)	38	14 (93.3)	15	18 (100)	18
2		12 (80.0)	15	10 (100)	10	10 (90.9)	11
3		8 (87.5)	9	23(88.5)	26	12 (86.7)	14
Total		50 (85.3)	62	47 (90.2)	51	40 (93.0)	43
1	BCS	28 (58.3)	48	30(81.1)	37	37 (86.6)	43
2		34 (81.0)	42	19(90.5)	21	28 (82.4)	34
Total		62 (69.9)	90	49 (85.5)	58	65 (84.4)	77
1	AEG	23(69.7)	33	16 (80.0)	20	17 (81.0)	21
2		22 (81.5)	27	22 (88.0)	25	14 (93.3)	15
3		2 (100)	2	17 (89.5)	19	22 (91.7)	24
4		8 (80.0)	10	15 (65.2)	23	13 (76.5)	17
5		10 (76.9)	13	8 (66.7)	12	7 (70.0)	10
Total		65 (74.0)	85	78 (73.4)	99	73 (83.9)	87

Table 3. Mean egg (fecundity) counts for all mosquito species utilized by treatment type. Species abbreviations are Sebring strain *Culex quinquefasciatus* (SEB), Bryan-College Station strain *Culex quinquefasciatus* (BCS), and Liverpool strain *Aedes aegypti* (AEG).

Trial	Species	Mean Egg Counts (±SE)					
		ART	n	EXS	n	LC	n
1	SEB	55.23(±7.11)	30	83.64(±9.83)	14	132.67(±8.20)	18
2		34.0(±6.16)	12	84.10(±8.65)	10	69.70(±11.77)	10
3		71.78(±13.55)	8	77.13(±9.55)	23	119.25(±18.62)	12
Total		52.78(±5.29)^{A}	50	80.55(±5.83)^{B}	47	112.90(±8.36)^{C}	50
1	BCS	44.07(±13.47)	28	113.90(±12.69)	30	117.89(±10.45)	37
2		89.50(±9.84)	34	103.42(±10.78)	19	96.25(±11.56)	28
Total		69.98(±8.63)^{A}	62	109.84(±9.93)^{B}	49	108.57(±7.87)^{B}	65
1	AEG	13.65(±4.25)	23	36.81(±6.78)	16	51.47(±7.61)	17
2		42.78(±7.85)	22	53.04(±9.43)	22	50.86(±10.07)	14
3		93.50(±6.01)	2	48.53(±6.79)	17	48.50(±6.44)	22
4		30.88(±6.89)	8	21.60(±6.58)	15	36.07(±6.97)	13
5		30.40(±7.0)	10	21.25(±8.79)	8	21.0(±7.37)	7
Total		30.66(±3.93)^{A}	65	39.42(±3.97)	78	44.79(±3.73)^{B}	73

Letters denote significant differences among treatments using Tukey's HSD.

Table 4. Mean hatch (fertility) rates for all mosquitoes species utilized by treatment types. No hatch data are available for AEG trials 1 and 2, so they were not included in the analysis. Only mosquitoes that laid eggs were included in the analysis. Species abbreviations are Sebring strain *Culex quinquefasciatus* (SEB), Bryan-College Station strain *Culex quinquefasciatus* (BCS), and Liverpool strain *Aedes aegypti* (AEG).

Trial	Species	Mean Hatch Rates (±SE)					
		ART	n	EXS	n	LC	n
1	SEB	76.9(±5.6)	20	75.29(±9.7)	11	79.2(±6.1)	18
2		68.3(±10.6)	9	79.7(±7.2)	10	82.4(±7.7)	9
3		49.9(±15.4)	7	80.5(±5.6)	19	70.3(±12.1)	10
Total		69.5(±5.2)	36	78.9(±4.1)	40	77.6(±4.7)	37
1	BCS	78.2(±11.7)	8	80.3(±4.8)	23	84.6(±4.3)	31
2		87.1(±4.08)	26	84.4(±6.59)	17	87.8(±4.54)	22
Total		85(±6.2)	34	82(±3.9)	40	85.9(±3.1)	53
1	AEG	N/A	0	N/A	0	N/A	0
2		N/A	0	N/A	0	N/A	0
3		77.6(±4.7)	2	79(±2.62)	15	70.4(±4.5)	20
4		60.9(±1.3)	6	91.8(±3.4)	7	95(±13.6)	9
5		75.1(±13.2)	7	80.6(±15.3)	4	95.5(±1.8)	4
Total		69.8(±8.1)	15	82.7(±2.9)	26	80.1(±3.5)	33

DISCUSSION

This study documents that anticoagulants can have a significant effect on fecundity of colonized *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes. Direct feeding on live chickens resulted in significantly more eggs laid by all mosquitoes, especially when compared to the commercially purchased chicken blood that is frequently used in labs (Table 3). The SEB and BCS mosquito results corroborate the Richards et al. (2012) study that found *Cx. quinquefasciatus* fed on live chickens had a 1.21-fold higher mean number of eggs laid compared to chicken blood-treated Alsever's solution delivered using an artificial membrane feeder. These differences in egg-laying by different treatments would likely be of minor concern for routine colony maintenance. However, studies quantifying fecundity following artificial blood meals with anticoagulant might be underestimates due to the artifact of the anticoagulant. A study of female *Culex coronator* feeding on diverse vertebrate blood with anticoagulants through membrane feeders were monitored for the number of eggs in a raft, which were likely lower than if the females had fed directly on live animals (Alto et al. 2014). To our knowledge, we are the first to demonstrate this significant impact of anticoagulants on the fecundity of colony mosquitoes when comparing the same blood source (i.e., chicken blood). For both BCS and AEG mosquitoes, LC egg quantities were not significantly different from EXS, suggesting that this method of colonization may be a suitable alternative to live-animal feeding. However, commercial blood still requires animal use, which could hinder mass rearing operations.

This study suggests heparin as an anticoagulant reduced fecundity for one of three mosquito species evaluated. The commercial blood treated with Alsever's solution is difficult to compare to the other treatment, given that this blood was obtained from adult chickens on a diet which would have been different than the freshly hatched chicks. Because of this, there may have been significant differences in the blood chemistry between the adult and freshly hatched chickens. In addition, the heparinized blood was fresh and not refrigerated before feeding to mosquitoes, unlike the commercial chicken blood. Finally, the volume of the anticoagulant added to the blood may have affected our observed results. Heparin was used to coat the syringe when blood was obtained from chicks which resulted in very small volumes of anticoagulant. The commercial blood obtained from Hemostat had Alsever's solution added to whole blood reaching 50% of total volume. This would mean that mosquitoes feeding on the commercial blood with Alesever's was diluted and thus contained fewer red blood cells. These nutritional differences may be a possible explanation for the differences we observed. The commercial chicken blood from HemoStat Laboratories was included in this experiment because this is the primary blood source our lab and others have used for *Culex* mosquito colony maintenance, and it has been utilized in other studies for comparison (Richards et al. 2012). We also point out a limitation for interpreting the *Ae. aegypti* results since we only utilized the highly colonized Liverpool strain. Observing differences between the Sebring strain and newly colonized *Cx. quinquefasciatus* colony warrants future investigations of how recently colonized *Ae. aegypti* might respond differently to anticoagulants.

Our study demonstrated no effect of anticoagulants on the fertility of all mosquito groups utilized. Richards et al. (2012) found a significant effect of live host blood sources on *Cx. quinquefasciatus* fertility, which is in contrast with our

results. Additional studies looking at mosquito fertility are necessary to fully understand the effect of live host blood-feeding, ideally utilizing similar blood sources.

Although artificial feeding may be a viable alternative to direct feeding on a live host, there is a significant cost to fecundity of the female mosquitoes. The ART treatment frequently had mosquitoes that did not lay eggs during trials, suggesting that it may be a less nutritious blood source for laying eggs. This may not be ideal for labs attempting to mass-rear mosquitoes for release, or those in need of large quantities of mosquitoes frequently. Additionally, attempting to colonize mosquitoes from the wild may be more difficult when trying to use commercially purchased animal blood since the mosquitoes could be reluctant to feed on a membrane, and from reduced fecundity due to the anticoagulant. Although fecundity was reduced with the use of anticoagulants for all mosquitoes, the reduction might be of minimal importance for the maintenance of species that are already colonized. Future studies should be cautious with the use of artificial blood-feeding and anticoagulants that could influence insect physiology.

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