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Siriporn Phasomkusolsil
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Orawan Wongnet
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Kanchana Pantuwatana
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Jaruwan Tawong
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Nantaporn Monkanna
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Tanaporn Kornkan
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Silas A Davidson
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Betty K Poole-Smith
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Patrick W Mccardle
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Corresponding Author:
Siriporn Phasomkusolsil
Department of Entomology, US Army Medical Directorate, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

Comparison of *Anopheles cracens* (Stenogamous) and *Anopheles dirus* (Eurygamous) blood-feeding behaviors, survival rates and fecundity after first and second blood meals

Siriporn Phasomkusolsil, Orawan Wongnet, Kanchana Pantuwatana, Jaruwan Tawong, Nantaporn Monkanna, Tanaporn Kornkan, Silas A Davidson, Betty K Poole-Smith and Patrick W Mccardle

Abstract
Malaria is a serious health problem in Southeast Asia. The malaria vectors *Anopheles cracens* (Stenogamous) and *Anopheles dirus* (Eurygamous) were used to evaluate the impact of first and second blood meals on feeding, survival and hatching rates, and fecundity. Females were divided as follows: mated with oviposition, mated without oviposition, and virgin. The blood-feeding rates of *An. cracens* groups were significantly lower for the second blood meal than for the first. Additionally, for *An. dirus* mated without oviposition and virgin groups, blood-feeding rates were significantly lower for the second blood meal compared with the first blood meal. Survivorship and the number of eggs laid increased for *An. cracens* after the second blood meal, but the opposite was true for *An. dirus*. There were no differences in hatching rates. Thus, blood meals affected survivorship and fecundity in naturally mating *An. cracens* and induced-mating *An. dirus*.

Keywords: Stenogamous, Eurygamous, *Anopheles*, blood meal, feeding behavior, malaria

1. Introduction
Malaria is still a serious problem on the borders of Thailand, where frequently infections at times resulting in death occur. The fatal disease is endemic in a variety rural habitats with high rates of infection found both in hilly, forested areas and some coastal foci [1]. In 2015, 14,755 cases and 33 deaths were reported in the relatively undeveloped borders and hilly regions of Thailand [2]. *Anopheles dirus* sensu lato (Dirus complex) is widely distributed as the primary malaria vector in Thailand and Southeast Asia [3, 4]. The Dirus complex contains five species in Thailand, *Anopheles dirus*, *Anopheles cracens*, *Anopheles scanloni*, *Anopheles baimaii* and *Anopheles nemophilous* [5]. These sibling species vary in behavior and distribution, as well as in their susceptibility to *Plasmodium* [6]. *An. dirus* sensu stricto (formerly *An. dirus* species A) is the most important malaria vector in forests of several Thai provinces, especially along the mountainous border with Cambodia [7]. *An. dirus* has also been proven to be an efficient laboratory vector for both *Plasmodium falciparum* and *Plasmodium vivax* [8]. *An. cracens* (formerly *An. dirus* species B) is the predominant mosquito that bites both monkeys and humans [9]. It is found in southern Thailand, Perlis State Park, northern peninsular Malaysia and Sumatra, Indonesia [10]. *An. cracens* is the main vector for *Plasmodium knowlesi* in Kuala Lipis of peninsular Malaysia [11]. By definition, *An. cracens* and *An. dirus* are closely related sympatric species, and they are difficult to accurately differentiate from each other using morphological characteristics [12, 13]. Due to this difficulty, species were identified using both polytene chromosomal banding patterns [14] and the illustrated morphological keys for the adult *Anopheles* of Thailand [15]. More recently, molecular assays based on PCR were developed to identify these species [16, 17]. Additionally *An. cracens* and *An. dirus* can be distinguished according to mating behavior. *An. cracens* is a stenogamic species (mates in small tight spaces) in the laboratory [18]. Meanwhile, *An. dirus* is Eurygamic (mates in open spaces above ground) and does not mate in the confined space of laboratory cages because it needs

sufficient swarming space to induce its mating behavior [19]. In the laboratory, when mating is required, mosquito copulation can be artificially induced after the male's head is cut off [20]. The correlation between mating and blood-feeding behavior is poorly understood, with limited available information. Mating is not needed for egg development and maturation, but eggs can be deposited when insemination has occurred [21]. Mating may occur either before or after blood-feeding. In general, female mosquitoes always mate before taking a first-blood meal, but in *Anopheles gambiae*, a large proportion of virgins may blood-feed prior to mating [22].

Colonies of *An. cracens* and *An. dirus* are bred in our insectary at the Armed Forces Research Institute of Medical Sciences (USAMD-AFRIMS) in Bangkok, Thailand. These mosquitoes are reared to support research on malaria and dengue fever, as well as to support repellent testing and pesticide resistance evaluations. We conducted experiments to evaluate feeding responses and survival rates of three groups of females, mated with oviposition, mated without oviposition and virgin, after blood-feeding. The objectives of this study were to assess the effects of blood-feeding behaviors during the first and second blood meals of each sibling species between *An. dirus* and *An. cracens* on the feeding response, survival rate and fecundity.

2. Materials and methods

2.1 Mosquito colonies

Anopheles (Cellia) cracens Sallum and Peyton were originally collected from Sabah, Malaysia and *Anopheles (Cellia) dirus* Peyton and Harrison were originally collected from Ban Phluang, Chanthaburi, Thailand. Laboratory colonies were established at the Department of Entomology, AFRIMS, Bangkok, Thailand. Both species were reared and maintained based on the standard operating procedures of the laboratory [23].

2.2 Mosquito rearing and conditioning

The insectary used regulated temperature and humidity control systems for the optimal growth of Anopheline mosquitoes**.** Mosquito maintenance and all of the experiments conducted were carried out at 25 ± 2 °C and 70%–90% relative humidity with a 12-h light/ 12-h dark cycle. Anopheline larvae were provided a diet of finely ground (42-mesh sieve; 355 µm US standard) OPTIMUM® (Perfect Companion Group Co., Samutprakarn, Thailand) to produce synchronized emergence and uniformly large adults until they all pupated**.** Pupae were transferred into plastic containers with water and placed in screen cages (40 × 30 × 30 cm) for adult eclosion. Adults were continuously supplied saturated cotton) soaked with 5*% (v/v)* multivitamin syrup solution (Haemo-Vit®, Boss Pharmacare Co., LTD., Samut Sakhon, Thailand) mixed with 5*% (w/v)* sucrose solution (Lin®, Thai Roong Ruang Industry Co. Ltd., Kanchanaburi, Thailand) [19]. Afterwards, females were separated and placed in separate containers for blood meals.

2.3 Establishing groups

The females were then collected and maintained as three treatment groups.

Group 1: Mated females permitted to lay eggs (mated females with oviposition)

In the first group, females were mated with males and permitted to lay eggs. *An. cracens* (stenogamous) can copulate without male swarms and mates easily in small spaces. After emergence, *An. cracens* females (*n* = 100) and males (*n* = 200) were introduced into an insect cage (40 × 30 × 30 cm) and allowed mate for 1 week with males (low female: high male ratio). Mating was confirmed as described previously [24]. *An. dirus* females are eurygamous and require swarming to induce mating behavior [25]. They cannot mate in the confined spaces of laboratory cages, and copulation must be artificially induced. In this study, 5–7-day-old *An. dirus* females (*n* = 100) were force-mated with males using standard procedures [26]. Then, females received the first blood meal through the membrane. After blood-feeding, 50 ml of filtered water was added to a plastic oviposition cup (9-cm diameter × 5.5-cm height) and placed in a cage. Females in the mated treatment group were allowed to oviposit overnight.

Group 2: Mated females not permitted to lay eggs (mated females without oviposition)

In the second group, both *An. cracens* (*n* = 100) and *An. dirus* (*n* =100) females were allowed to mate using the same method as described for the first group. However, mated females were not permitted to lay eggs. After the blood meal, an oviposition cup was not placed in the cage.

Group 3: Virgin females

For the third group, pupae were kept in plastic cups and transferred to screen cages (40 × 30 × 30 cm) until adults emerged. Virgin females (*n* = 100) and males were separated every 12 h, which prevented insemination because there was not enough time for the males' genitalia to rotate into the mating position [27].

2.4 Artificial blood-feeding technique

For the artificial membrane feeding system, blood meal was offered to both *An. cracens* and *An. dirus* that was performed according to the technique described by Rutledge *et al* [28] and Dias *et al* [29]. At 12 h prior to blood-feeding the vitamin-saturated cotton balls were removed from the holding cages and replaced with water-saturated cotton balls. Then, the water-soaked cotton balls were removed 6 h prior to blood-feeding. Water was filled and circulated in a circulating LAUDA® water bath (LAUDA DR. R. WOBSER GMBH & CO. KG) at 37 °C through the blown glass feeder cups (surface area: 2.54 cm^2). Sausage casing membranes were stretched over feeder cups and placed in contact with the mesh netting top of the plastic container holding the female mosquitoes. In total, 1.5 ml human blood containing citrate as an anticoagulant (obtained from the Thai Red Cross Society) was added to the glass feeder well, and mosquitoes were allowed to blood-feed for 30 min. The numbers of fully engorged females were recorded. The cotton balls saturated with 5% multivitamin syrup and sugar solution were provided and changed daily to reduce the formation of bacterial and fungal growth. The numbers of unfed females were recorded and discarded.

2.5 Percentages of fed females and survival rates after the first and second blood meals

Using an artificial membrane-based feeding method, 5–7-d-old mosquitoes were placed in a cage and provided the first blood meal. After 30 min of feeding, the numbers of fully blood-fed mosquitoes were recorded, and mosquitoes that did

not feed or only partially fed were discarded. A second blood meal was given 8 d after the first blood meal, and the fully blood-fed females (13–15-d-old mosquitoes) were separated from those that did not blood-feed.

The blood-feeding rate was determined as the number of blood-fed mosquitoes/number of mosquitoes tested × 100 [30]. Mosquitoes were provided access to 5% multivitamin syrup and sugar solution immediately after blood-feeding. Survival rates of engorged females were recorded on d 7 after being fed the first/second blood meals. Ten replicates were performed for each group.

2.6 Fecundity and hatching rate

In females from the first group (mated females with oviposition), the fecundity and hatching rate were observed. Three days after the first blood-feeding (8–10-d-old mosquitoes), 30 engorged females of each species were randomly selected (3 females from each replicate). Each blood-fed female was held in an individual glass vial containing a piece of moist filter paper as an oviposition substrate. The number of eggs each female laid by 48-h post-feeding was counted using a dissecting microscope and recorded. All the eggs per species were immersed in plastic rearing trays with water to observe hatching. The numbers of newly hatched larvae were recorded after 2 d. At 3 d after the second blood-feeding (16–18-d-old mosquitoes), the oviposition and hatching processes were repeated for the first group.

The fecundity rate was determined as the number of eggs laid per female. The hatchability rate was determined as the total number of hatched larvae/total number of eggs × 100 [31].

2.7 Statistical analyses

Mean percentage feeding and survival at 7-d post-treatment were calculated. Results were analyzed using a one-way analysis of variance (IBM SPSS Statistics for Windows, Version 25.0; IBM Corp). If significant differences in blood-feeding and survival rate within three treatment groups, mated females with oviposition, mated females without oviposition and virgin females, were detected, multiple comparisons were made using Duncan's Multiple Range test ($P< 0.05$). Paired t-tests were used to analyze the feeding rates, fecundity levels and hatching rates after the first and second blood meals, with statistically significant differences determined at $P = 0.05$.

3. Results

3.1 Percentage of fed females after first and second blood meals

Mean first and second blood-feeding rates of both *An. cracens* and *An. dirus* females are presented in Figure 1. In a comparison between first and second blood-feeding rates, all the groups of *An. cracens*, mated with oviposition (73.3%, $P = 0.01$), without oviposition (74.5%, $P< 0.01$) and virgin (63.8%, $P = 0.04$), were significantly lower after the second blood meal (Fig. 1A). For the *An. dirus* females, two groups, mated females without oviposition (94.0%, $P = 0.04$) and virgin females (93.6%, $P = 0.01$) had significantly lower blood-feeding rates after the second blood meal compared with after the first blood meal (Fig. 1B).

3.2 Female survival rates after first and second blood meals

The effects of the first and second blood meals on the survival of *An. cracens* and *An. dirus* females at 7 d after each blood meal were studied (Table 1). For *An. cracens*, the first blood-feeding had no significant effect on the survival rates among the three groups ($P = 0.14$). However, there were significant differences in the post-feeding survival rates after the second blood meal among the three treatment groups ($P = 0.03$). The overall survival rates of *An. cracens* mated females with oviposition (95.6%), mated females without oviposition (92.2%) and virgin females (95.7%) after the second blood meal were higher than after the first blood meal (91.0%, 84.6% and 91.6%, respectively). For *An. dirus*, the overall survival post-feeding survival rates after the second blood meal were lower than after the first blood meal. In addition, the mean survival rates of *An. dirus* mated females with oviposition and virgin females after the second blood meal experienced reduced longevity (93.0%, $P=0.05$ and 91.9%, $P=0.01$, respectively) compared with after the first blood meal (95.3% and 96.8%, respectively). However, the longevity of *An. dirus* females after the first and second blood meals was not significantly different among the three treatment groups ($P > 0.05$).

3.3 Fecundity and hatching rate

After engorged females from the first group (mated females with oviposition) were transferred individually into glass vials, the numbers of eggs laid per female were recorded. The eggs were submerged in the plastic tray, and the number of first larval stage hatchings were also recorded. For *An. cracens*, the mean number of eggs laid after feeding on the first blood meal (129 eggs ± 41.2) was significantly lower than after the second blood meal (180 eggs ± 49.1; $P = 0.01$). Conversely, the number of eggs laid by *An. dirus* after feeding on the first blood meal (199 eggs ± 26.9) was significantly greater than after the second blood meal (161 eggs ± 41.9; $P=0.05$). Additionally, for *An. cracens* and *An. dirus*, there were no significant differences between the egg hatching rates after the first (78% and 91%, respectively) and second (79% and 86%, respectively) blood meals.

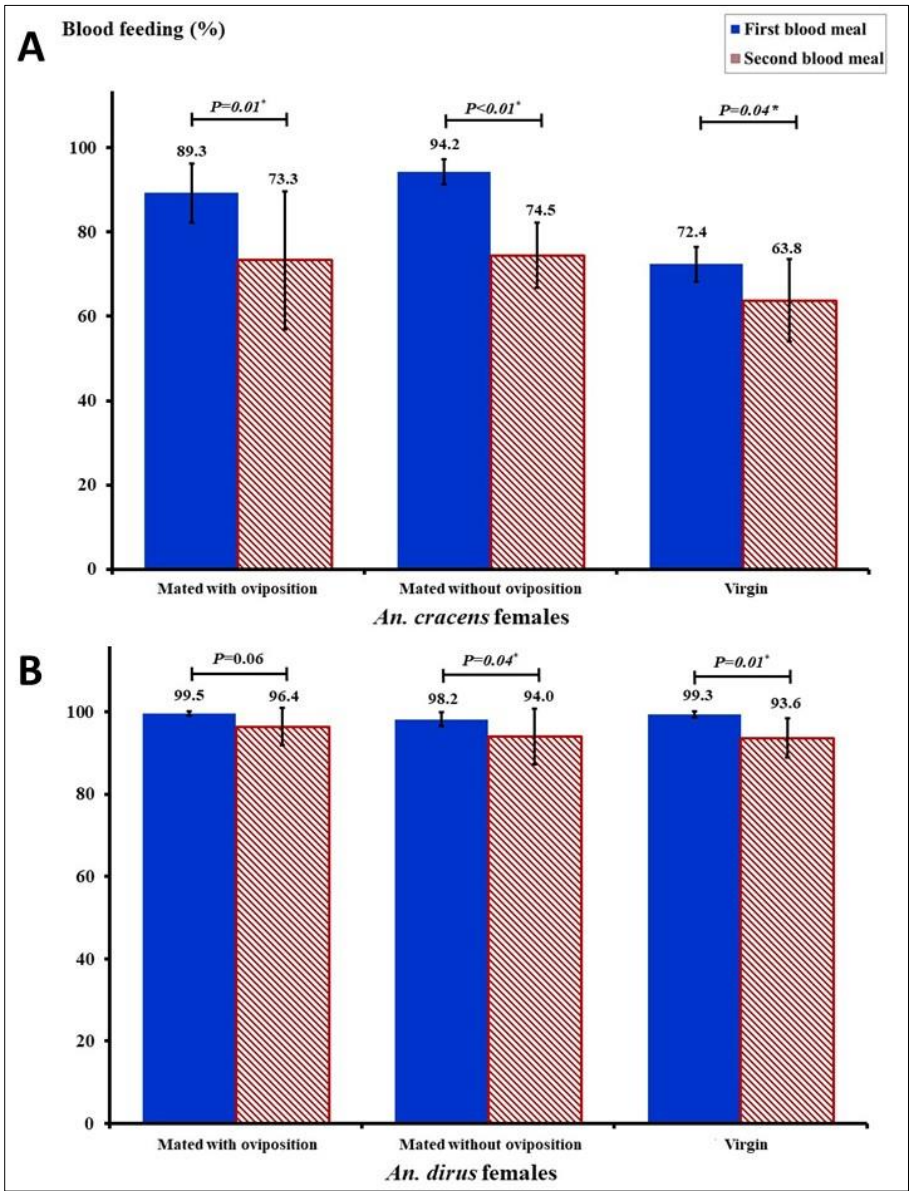

Fig 1: Mean percentage first and second blood-feedings of mated females with oviposition, mated females without oviposition and virgin females of *An. cracens* (A) and *An. dirus* (B). After the first blood meal and investigation of the female survival rates per cage, a second blood meal was offered. *Significant *P*-values in each row between first and second blood meals as assessed by t-tests ($P< 0.05$). Bars depict means, and error bars indicate standard deviations. (Y axis=blood-feeding %; X axis=establishing groups).

Table 1: Effects of first and second blood meals on the survival of female *An. cracens* and *An. dirus* at 7 days after each blood meal.

Mosquito sp.	**An. cracens**			**An. dirus**		
Treatment group	**Survival (%) at day 7 after first blood meal ± SD**	**Survival (%) at day 7 after second blood meal ± SD**	***P* value****	**Survival (%) at day 7 after first blood meal ± SD**	**Survival (%) at day 7 after second blood meal ± SD**	***P* value****
	(A/B)[1]	(C/D)[2]		(A/B)	(C/D)	
Mated females with oviposition	91.0±5.3a[3]	95.6±2.1a	0.04	95.3±3.1a	93.0±3.1a	0.05
	(81/89)	(57/60)		(95/100)	(85/91)	
Mated females without oviposition	84.6±8.0a	92.2±4.4b	<0.01	95.0±4.0a	94.7±2.6a	0.83
	(80/94)	(55/60)		(93/98)	(83/88)	
Virgin females	91.6±11.1a	95.7±2.8a	0.29	96.8±3.2a	91.9±4.4a	0.01
	(67/72)	(40/42)		(96/99)	(83/90)	
P value*	0.14	***0.03***		0.52	0.19	

1A/B, average number of live females at d 7 after first blood meal/Total number of engorged females that fed on the first blood meal.
2C/D, average number of live females at d 7 after second blood meal/Total number of engorged females that fed on the second blood meal.
[3]Means in each column followed by different lowercase letters are significantly different among treatment groups
*Significant *P*-values $P< 0.05$ in each column as assessed by one-way ANOVA and Duncan's Multiple Range tests are indicated in bold italics.
** Significant *P*-values between first and second blood meal as assessed by paired t-tests ($P< 0.05$) are indicated in bold italics in each row.

4. Discussion

The intensity of malaria transmission is highly dependent on vectorial capacity, which is affected by population density [32], oviposition interval (blood-feeding frequency) [33], survival rate [34] and anthropophilic behavior [35]. Both *An. cracens* and *An. dirus* are anthropophilic [10, 36] and anautogenous insects that require a blood meal for egg development [37]. However, there are some stenogamous mosquito species that are autogenous (not requiring a blood meal to produce eggs) such as *Anopheles petragnani* [38], *Culex pipiens* [39], *Aedes taeniorhynchus* [40] and *Aedes detritus* [41]. Our results showed that the first and second blood meals affect feeding rates, survivorship and reproduction in colonies of both the natural mating *An. cracens* (stenogamic species) and the induced-mating *An. dirus* (Eurygamic species) under the conditions of this test.

In most mosquitoes, flight is an essential component of mating behavior and it is associated with swarming [42]. In *Anopheles*, males initiate mating in swarms and single females fly into the swarm. They apparently copulate near the blood meal host [43]. Thus, males must be able to locate females in search of a blood meal. In the AFRIMS laboratory, the eurygamic behavior of *An. dirus* does not allow it to mate in the limited space and small cages inhibit or reduce the formation of dancing male swarms. Therefore, artificial mating techniques have been developed to maintain laboratory *An. dirus* colonies. However, *An. cracens* can successfully copulate in small cages. The *Anopheles* Y chromosome is critical to their sexual dimorphism and also controls the stenogamy–eurygamy mating behavior [44]. Additionally, there is a difference in the size of the male genitals, with the genitalia of *An. cracens* being larger than those of *An. dirus* [24]. However, the frequency of the clasper movement during induced copulation in *An. cracens* was lower and the copulation period was than in *An. dirus* [45].

In general, female mosquitoes mate with males after their emergence and start looking for a blood meal for egg maturation, and is digested within a few days [46]. The female mosquito reproductive (gonotrophic) cycle starts at the blood meal and ends with egg-laying, and it is continuous throughout the female's life [47]. The females oviposit in a suitable breeding-site and look for another blood meal. In anophelines, the first blood meal might promote the ovarian follicles into the late resting stage, and a second meal is required for complete maturation [48, 49]. Richards *et al.* [50] reported that a second blood-feed was necessary to start a new cycle of digestion and oviposition. The mosquito requirement for more than one blood meal to successfully complete a gonotrophic cycle has been correlated with the nutritional content of the first ingested meal [51], mosquito parasites, female body size [52], copulation status [53] and the quality and quantity of the blood meal [54]. The timeframe for female mosquitoes to complete a single gonotrophic cycle (from blood-feeding, to oviposition, to blood-feeding again) is usually a few days [55].

In this study, feeding rates were determined during the first and second blood meals. The blood-feeding rates of *An. cracens* and *An. dirus* (ranges = 94.2%–72.4% and 99.5%–98.2%, respectively) for the first feeding varied among treatment groups (with and without oviposition and virgin). We observed a reduction in feeding rates (ranges = 74.5%–63.8% and 96.4%–93.6%, respectively) for the second blood-feeding. Furthermore, we found a feeding response in which mated *An. cracens* females fed more than virgin females. This observation agrees with those of previous studies [56, 57] in which mosquito females were always mated before blood-feeding. In addition, the survival rates of mated females with and without oviposition, as well as virgin females, were compared after the two blood meals. There were no significant differences among treatment groups, except in *An. cracens* after the second blood meal ($P < 0.03$). Villarreal *et al.* [58] reported that when *Aedes aegypti* females were allowed multiple feedings, the number of blood meals they consumed did not significantly affect survival. Contrary to the interpretation of Dao *et al.* [59] in which mated *An. gambiae* females showed reduced survival rates compared with unmated females. However, our results showed that female *An. cracens* had a greater survivorship after the second blood meal than after a single meal, and they produced significantly more eggs. Our results are generally in agreement with those of Briegel and Horler [60] in which *Anopheles* mosquitoes having more than one blood meal had increased fecundity. Meanwhile, our results showed that the survival rate of *An. dirus* was reduced after the second blood meal, and we also observed a significant decrease in fecundity from the first to the second blood-feeding. Nevertheless, the blood meals did not influence fertility. This result supports previous studies in which at least two blood meals were necessary for survivorship [61, 62] and fecundity [60, 63, 64]. Previous studies on various mosquito species have demonstrated that female mosquitoes require two meals to be of epidemiological significance for disease transmission, and it is highly likely that, during their lifetimes, females will consume multiple blood meals [65]. However, females of most mosquito species require both blood and sugar for reproduction and survival [66]. A diet combining both blood and sugar results in the highest survival rate in *An. gambiae* [67]. It is also important to determine the effects of multiple blood-feeding on mosquito fitness. *Anopheles* females often require two or more blood meals for their first oviposition [60], which enhances the rate of pathogen transmission by increasing the frequency of host contact [68].

Comparisons of stenogamous and eurygamous mating behaviors in *Anopheles* mosquitoes have suggested correlations between mating behavior and insemination rate [24, 69]. Taii *et al.* [70] reported the highest insemination rates in *Anopheles peditaeniatus* (stenogamous), while the *Anopheles crawfordi* (eurygamous) had the lowest rate in cages. Additionally, researchers have attempted to develop techniques, such as a blue stroboscopic light [26], different sized cages [25] and thermo-periods [71], to successfully alter many anopheline colonies from a eurygamic to stenogamic status. This research is crucial for improving mating competitiveness, mating specificity and fitness studies, which are necessary to develop mosquito control measures.

5. Conclusions

Here, we determined that the first and second blood meals affected both the survivorship and fecundity of *An. cracens* (stenogamous) and *An. dirus* (eurygamous).

a) Survival rates of *An. cracens* increased after feeding on the second blood meal, while the opposite was true for *An. dirus*.
b) The number of eggs laid by *An. cracens* was higher after feeding on the second blood meal, while the opposite was true for *An. dirus*.

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