Studies of coexistence mechanism between Aedes albopictus and Aedes flavopictus

Dissertation

Studies of coexistence mechanism between *Aedes albopictus* and *Aedes flavopictus*
ヒトスジシマカとヤマダシマカの２種の共存機構の研究

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DEDICATION

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Abstract

Aedes (*Stegomyia*) *albopictus* and *Aedes* (*Stegomyia*) *flavopictus* are sibling species that have overlapped distributions from southern to central Japan. *Ae. albopictus* occurs from the Ryukyu islands in Tohoku district, while *Ae. flavopictus* is dispersed throughout Japan including, Hokkaido region. Globally, *Ae*. *albopictus* causes reproductive interference with native species, sometimes caused even exclusion. It is still unrecognized, where the *Ae*. *albopictus* originated in Asia, but both species are native in Japan. Experimental tests of reproductive interference are needed to know the causes of coexistence between them in nature, that is likely to might limit their distributions.

We executed the reproductive interference study between *Ae*. *albopictus* and *Ae*. *flavopictus*, and particularly focused on the body size difference between the mating pair. Here, we examined the effects of body size on copulation duration, fecundity and hatchability of eggs in different mating, including, conspecific, heterospecific and double-mating (i.e. heterospecific mating followed by conspecific mating). Females mated only with heterospecific males produced few viable eggs, indicating that post-mating isolation is almost complete. When mated with heterospecific males before conspecific mating, the production of viable eggs was lower than when mated only with conspecific males, revealing the occurrence of reproductive interference. The degree of reproductive interference was larger in *Ae. flavopictus* than in *Ae. albopictus* when the female size was small but did not differ between them when the size was large. *Aedes albopictus* females appear to be able to distinguish *Ae. flavopictus* males from conspecific males and larger females are more successful in the rejection of heterospecific males. On the other hand, *Ae. flavopictus* were not able to discriminate between conspecific and heterospecific males. Considerably, body size of *Ae.*

flavopictus might have a significant effect on reproductive interference caused the dominance on *Ae. albopictus* during heterospecific mating. Here, we suggested that *Ae. flavopictus* did not replace by *Ae. albopictus* in an area, leads to cohabit to establish their ecological niche.

Our study aimed to observe the effects of vegetation, including, bamboo (*Phyllostachys pubescens*), cherry (*Prunus* × *yedoensis*) and beech (*Castanopsis sieboldii*) on the growth of *Ae. albopictus*. The highest larval mortality was observed in cherry and beech, conversely, the lowest in bamboo. Larval stages feed upon cherry and beech showed delay in development and adult emergence than that of bamboo. Female body size was larger when larvae raised with the bamboo compared to cherry plant. *Ae. albopictus* females oviposited more eggs in bamboo vegetation, however, adult reared by cherry plants laid less amount of eggs. Per capita performance of *Ae. albopictus* on bamboo plants was higher for the population growth compared to cherry and beech. Thus, *Ae. albopictus* larvae were affected by bamboo vegetation that might have influenced on the larval and adult growth. Our findings suggested that bamboo plants should avoid in future plantation program near the urban areas, as it might harbor a potential habitat for mosquitoes.

Body size has a strong linked to the fecundity and longevity that assists to predict the prevalence of mosquito-borne disease. Here, the relation between body size and fitness traits (fecundity and longevity) was observed through diet (low and high) and temperature treatments (25°C and 28°C) of *Ae. albopictus* and *Ae. flavopictus*. Large *Ae. albopictus* developed 59.6 % to 81.1% of follicles to eggs, whereas, small females developed 47.8% to 56.6% of follicles. In addition, *Ae. flavopictus* large and small females develop only 55.6% to 57.4% and 49.5% and 47% of follicles to eggs, respectively. In both species, body size showed a positive correlation with the number follicles. Fecundity increased with the body size, however, *Ae. albopictus* laid more eggs than *Ae. flavopictus*. Conversely, *Ae. flavopictus* retained more eggs compared to *Ae. albopictus*. Body size and egg retention were negatively correlated in both species. Notably, there was no significant relation (P>0.05) between the body size and longevity. Thus, the impact of body size on fecundity might be considered to control vector.

The global climatic variation might affect the distribution pattern of *Ae. albopictus* and *Ae. flavopictus*. Here, we have combined temperatures (25ºC and 28ºC) and humidity (52% and 47% RH) to check the impact on the fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*. *Ae. albopictus* laid large amount of eggs than *Ae. flavopictus*, regardless exposed to higher temperature (28ºC) with lower humidity (47% RH). Oviposition time of both species extended as relative as humidity increased (52% RH) at low temperature (25ºC). Conversely, higher temperature and lower humidity reduced the hatching of both species. Higher temperature (28ºC) and lower humidity (47% RH) hindered the longevity of *Ae. flavopictus*. However, *Ae. albopictus* exposed to those atmospheric conditions showed the deleterious effect on longevity Thus, our study enabled to determine suitable environmental condition for vector mosquito that might help to make reliable predictions of disease transmission.

Ae. albopictus is now widely distributed throughout the world, causing the elimination of related *Aedes* species, however, it has long been coexisting with *Ae. flavopictus* in Japan. Overall, varying body size of females exhibited difference in mating biology, fecundity and longevity that facilitated the coexistence of *Ae. albopictus* and *Ae. flavopictus*.

CHAPTER 1

General Introduction

1.1. Organization of the study

The organization of the dissertation is as follows:

In **Chapter 1**, specifies with a brief introduction reflecting the background, aim, and scope of the study.

In **Chapter 2**, provides a detailed description of the influence of body size on the reproductive interference of *Ae. albopictus* and *Ae. flavopictus*. Here, our study elucidates how body size may influence reproductive interference. In addition, how body size related different mating affects the copulation duration, fecundity and hatchability were examined as well as a detail on the introduction, materials and methods, results with the discussion and conclusion are presented here.

In **Chapter 3**, discusses about the effects of vegetation on the larval and growth performance of *Ae. albopictus*. How vegetation change influences the population performances of *Ae. albopictus*, were raised with three types of vegetation, including bamboo, cherry and beech leaves in the laboratory and describe the background, materials and methods, results with the discussion.

In **Chapter 4**, studies the influence of body size on the fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*. How the body size effects on fecundity by comparing the ovarian follicle and egg retention capacity as well as longevity and illustrates the background, materials and methods, results with the discussion.

In **Chapter 5**, investigates the temperature and humidity influence on the fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*. How the temperature and humidity fluctuations would affect the fecundity and longevity and depicts the background, materials and methods, results along with discussion.

In **Chapter 6**, I summarize the results of my research, draw a concluding statement.

1.2. Background of the study

Aedes albopictus is an aggressive and anthropophilic mosquito belonging to *Stegomyia* species. It has invaded in tropical and temperate areas of Southeast Asia, China and Japan (Bonizzoni et al. 2013), but lately, expanded its native range on all continents, except Antarctica (Benedict et al. 2007, Caminade et al. 2012, Kraemer et al. 2015). However, other *Stegomyia* species *Ae. flavopictus* constrains their distribution only in Japan and South Korea. Unlike *Ae. albopictus*, *Ae. flavopictus* does not limit its distribution range globally. The rapid expansion of *Ae. albopictus* causes tremendous health threats and associated with emerging and re-emerging diseases, including dengue, Chikungunya, and Zika (Weaver and Reisen 2010, Gardner et al. 2016, Wilder-Smith et al. 2017). In comparison with *Ae. albopictus*, *Ae. flavopictus* has no evidence as a vector in nature (Eshita et al. 1982) but they can transmit the dengue virus. Overall, global climate changes resulting increased temperature, rainfall, precipitation, along with urbanization might influence the disease outbreak.

Climate change seems to be the driving factors may allow the distribution of *Ae. albopictus* and *Ae. flavopictus*. *Ae. albopictus* has expanded its distribution and modulated by temperature (Lounibos 2002, Reinhold et al. 2018). Recent studies reveal that an increased temperature, influences the displacement of *Ae. japonicus* and *Ae. albopictus* in their native ranges in Nagasaki, Japan (Chaves 2016). *Ae. albopictus* is mainly dispersed in southern and central Japan and now expanding to northern areas (e.g. Tohoku district). Conversely, *Ae. flavopictus* occurs throughout Japan, including Hokkaido (Tanaka et al. 1979, Toma et al. 2002) and comprises three subspecies namely, *Ae. flavopictus* distributed in Palearctic region, *Ae. flavopictus downsi* in Amami and Okinawa islands, besides *Ae. flavopictus miyaraifrom* from Ishigaki Island and Iriomote Island of the Ryukyu archipelago (Toma et al. 2002). *Ae. albopictus* and *Ae. flavopictus* are susceptible to temperature changes (Alam and Tuno 2020), however, *Ae. albopictus* is more adapted to higher or variable temperatures compared to *Ae. flavopictus*. In variable temperature, their tolerance limit may clarify the different habitat use. Throughout their distribution ranges, *Ae. albopictus* is well adapted in urban areas, whereas, *Ae. flavopictus* is particularly located in forest zone as well as oviposit in bamboo stumps and tree holes (Tanaka et al. 1979, Sota et al. 1992, Chaves 2016). In Japan, *Ae. albopictus* and *Ae. flavopictus* widely overlapped their geographic distributions. However, they are expected to encounter in breeding surroundings, because their habitat selection is not rigid (Sunahara et al. 2002). Despite habitat choice, it is crucial to know what other factors influence the coexistence of these two species? Therefore, we consider reproductive interference may be the most plausible reason of the co-occurrence between this species in Kanazawa, Ishikawa prefecture, Japan.

Reproductive interference is an interspecific sexual interaction that can decrease the population growth, thus impeding the species co-occurrence in breeding habitats (Ribeiro and Spielman 1986). The misleading coupling of males during heterospecific mating with females initiates negatively, therefore, reduced the number of eggs (Bargielowski et al. 2011, Tripet et al. 2011). In addition, if two or more species are sexually interacted (McHugh 1972) with similar mating signals, the chances of interspecific interactions increase. Since the last two decades, the concept of reproductive interference has been an interest to explain the reasons of species displacement (Kyogoku and Sota 2015). In mosquitoes, it confirms that reproductive interference acts as a driver of the elimination of the other native *Aedes* species by *Ae. albopictus* (Lounibos 2002, 2007, Juliano and Lounibos 2005, Tripet et al. 2011, Bargielowski et al. 2013, 2015a, 2015b, Bargielowski and Lounibos 2014, Lounibos et al. 2016, Lounibos and Juliano 2018). As example, *Ae. albopictus* mated frequently with *Ae. polynesiensis* and *Ae. aegypti* (Bargielowski et al. 2013) resulting in displacement of mosquito species. However, it is interesting to know why *Ae. albopictus* did not able to displace *Ae. flavopictus*, while coexisting in a particular region.

In our study sites, Kanazawa City, Japan, both species coexist, where the encounter occurs rarely in breeding premises. So, there is a growing interest on the ecological significance of reproductive interference between two species. The recent studies Alam and Tuno (2019 unpublished data), hypothesized that heterospecific mating may occur seasonally, especially during summer. It may be expected that *Ae. albopictus* shifts from warmer urbanized areas to forest areas, where *Ae. flavopictus* already exists. Consequently, heterospecific mating occurs, due to fail to discriminate between conspecific and interspecific males. However, this trend is different in Nagasaki Prefecture, Japan. Although they overlap their distribution, can avoid heterospecific mating because resulting from the ability to distinguish conspecific and heterospecific species. Thus, the absence of reproductive interference may lead to long term coexistence of both species. Therefore, we are keen to understand whether heterospecific mating reduces re-mating with conspecific species or not. Apart from this, it is rarely studied that body size that may influence on reproductive interference. Recent findings demonstrate that *Ae. flavopictus* is larger in size compared to *Ae. albopictus* (Alam and Tuno 2020). Therefore, it is also our interest to evaluate whether body size influenced reproductive interference or not.

Reproductive interference with the larval resource competition may permit cohabitation and distribution of species. The confront of both species may be mediated by the choice of breeding habitats. Moreover, in breeding ground, larvae feed on organic debris from the nature, particularly microorganism as well as plant debris. Vegetation inputs into breeding habitats can alter the mosquito community that provides the energy source for the larvae, consequently, making the radical changes of ecosystems (Sax et al. 2005). Particularly, mosquito larvae are profoundly affected by the terrestrial plants with different nutrients

(Yanoviak 1999) resulted in an increased larval growth and attracted adult mosquitoes for oviposition (Shaalan and Canyon 2018). However, few plant species comprise of lethal products (e.g., tannins and lignins) that hinders the growth and development of larvae (Rey et al. 1999, David et al. 2000c). Japan has a great variety of terrestrial plant species, including bamboo, deciduous and evergreen forests. It grasps an attention to researcher because *Ae. albopictus* have preference sub-urban and urban land with dense vegetation (Rey et al. 2006), in particular, tree holes and bamboo stumps. In recent, it is predicted that *Ae. albopictus* is going to expand in their northern distribution because of expansion of unmanaged bamboo groves. The bamboo expansion is radically changing due to inappropriate forest management strategies (Suzuki and Nakagoshi 2011). Few studies have been conducted on vegetation as a contributing factor of *Ae. albopictus* distribution. To address this, there is an urge to elucidate the role of different vegetation that interact with the larval and adult development of *Ae. albopictus*.

Global climate has been linked to increase the frequency of vector-borne disease. This disease is related with biotic and abiotic factors that affect the vector abundance. The vector abundance might be influenced by temperature and food. Diet exerts the larval development rate (Kivuyo et al. 2014), fecundity (Reiskind and Lounibo 2009, Alto et al. 2012, Takken et al. 2013) and survival (Aznar et al. 2018). Particularly, high diet, low density and lower temperature corresponds to larger adults (Zeller and Koella 2016), which exhibits more fecund (Briegel 1990, Renshaw et al. 1994) and longer survival rate (Sultana and Tuno 2020, unpublished data). However, low diet produces smaller adult (Lehmann et al. 2006), which require more blood meals for oviposition and vectorial capacity (Muturi et al. 2011). The female's vectorial capacity is linked to fecundity and longevity that influences the disease transmission (Nasci 1986, Briegel 1990, Takken et al. 1998, Alto et al. 2008, Helinski and Harrington 2011, Takken et al. 2013, Barreaux et al. 2018). Overall, body size can determinant because it affects the survival and longevity of mosquito (Nasci 1986). Furthermore, both biotic and abiotic factors impacting *Ae. albopictus* life history traits, including developmental duration, biting tendency, gonotrophic cycle, body size, oviposition, and survival (Briegel 1990, Rueda et al. 1990, Tun-lin et al. 2000, Delatte et al. 2009). However, it is still unknown how environmental factors may influence the life traits of *Ae. flavopictus*. Our present study aims to focus how environmental factors (e.g. temperature, humidity and diet) affects the life history of *Ae. albopictus* and *Ae. flavopictus* by means of egg size, development period, fecundity, egg retention capacity, ovariole size, body size as well as longevity.

1.3. Objective of the study

The most invasive *Aedes albopictus* species distributed globally, whereas *Aedes flavopictus* limit their distribution. Around the world, *Ae. albopictus* causes reproductive interference with native *Aedes* species resulting in exclusion of species. In Japan, it would be remarkable that *Ae. albopictus* coexist with *Ae. flavopictus* and did not replace by *Ae. albopictus*? Furthermore, a little study has been directed to body size difference with mating partners affects reproductive interference. So, the purpose of this research is to test the effect of body size on copulation duration, fecundity and hatchability after conspecific, heterospecific and double-mating between *Ae. albopictus* and *Ae. flavopictus*.

In the next century, many countries are susceptible to disease transmission by *Aedes* spp. Therefore, it is crucial to understand the vector ecology with environmental variables to control disease. Here, we investigate the body size, fecundity as well as longevity of two vector species to prevent the epidemic or distribution of disease. **The precise objectives of this study are as follows**:

1. To determine the impact of body size on the reproductive interference of *Ae. albopictus* and *Ae. flavopictus*.
2. The effects of vegetation on the larval and adult growth performance of *Ae. albopictus*.
3. To determine the body size effect on the fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*.
4. To estimate the influence of temperature and humidity on the fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*.

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CHAPTER 2

Reproductive interference between *Aedes albopictus* and *Aedes flavopictus* at a place of their origin

2.1. Introduction

Reproductive interference, a phenomenon where the production of offspring through conspecific mating is reduced by the preceding or following heterospecific mating, can be an important key in considering speciation and species interactions. The idea of reproductive interference was proposed by a theoretical research of Levin and Anderson (1970), and its utility in explaining a wide range of biological phenomena has been revealed since early 1980s. For example, reproductive interference is assumed to cause extinction of either of the sibling species that coexist (Ribeiro and Spielman 1986, Kuno 1992, Yoshimura and Clark 1994, Feng et al. 1997, Kishi and Nakazawa 2013). Contrarily, it may interfere the population growth of them and thereby reduce their competition and promote their coexistence.

In mosquitoes, reproductive interference has been extensively studied in *Aedes* species, particularly *Ae. aegypti* and *Ae. albopictus* (Gubler 1970a, b, Nasci et al. 1989, Tripet et al. 2011, Bargielowski et al. 2013, 2015a, b, Giatropoulos et al. 2015). In southeastern USA, *Ae. aegypti* has decreased the number after the arrival of *Ae. albopictus* which is native to Southeast Asia and recently expanding its distribution to not only the Americas but also Africa, Europe, Australia and Middle East (Gratz 2004, Benedict et al. 2007). It has been suggested that the decrease of *Ae. aegypti* is likely attributable to satyrization, a form of asymmetric reproductive interference, where insemination by *Ae. albopictus* males induces refractoriness to further mating in *Ae. aegypti* females, whereas insemination by *Ae. aegypti* does not affect mating behaviors of *Ae. albopictus* females (Nasci et al. 1989, Tripet et al. 2011, Bargielowski et al. 2013, 2015a, b). However, *Ae. albopictus* failed to establish its dominance over *Ae. aegypti* in Miami (O'Meara et al. 1995, Lounibos et al. 2010). On the other hand, reproductive interference incurs costs to females (Leahy and Craig 1967, Lima-Camara et al. 2013, Kyogoku and Sota 2015) and therefore, leads the evolution of resistance to reproductive interference, abilities to discriminate between conspecific and heterospecific males or habitat segregation (Lounibos et al. 2010, Bargielowski et al. 2013, 2015a, b). However, reproductive interference is not the only mechanism of the replacement or distributional changes of *Ae. aegypti* and *Ae. albopictus*, but larval competition, climatic regimes and habitat changes such as urbanization have also been suggested to play important roles in their replacement (Chan et al. 1971, Hawley 1988, Kaplan et al. 2010, Lounibos et al. 2010 Bargielowski et al. 2013, 2015a, b, Lounibos and Juliano 2018).

In Japan, *Ae. albopictus* and *Ae. flavopictus* (Yamada) are most common among *Aedes* species and show widely overlapped geographic distributions; the former is mainly distributed in southern and central Japan and now expanding its distribution to northern areas (e.g. Tohoku district) probably attributable to global warming (Kobayashi et al. 2002, Mogi and Tuno 2014), while the latter (with two regional subspecies) occurs throughout Japan including Hokkaido, the northernmost district, and also in Korea (Tanaka et al. 1979, Toma et al. 2002). Throughout their distribution ranges, the former mainly inhabits residential areas, whereas the latter does in natural environments such as bamboo groves and forests (Tanaka et al. 1979, Sota et al. 1992). However, they are assumed to encounter with each other, because their habitat selection is not rigid (Sunahara et al. 2002). It is therefore possible that either of them eliminates the other through reproductive interference, as has been observed in the case of *Ae. aegypti* and *Ae. albopictus* in southern USA.

Here, we investigated whether reproductive interference occurs between *Ae. albopictus* and *Ae. flavopictus*, particularly focusing on the body-size difference between the copulating pair. In these species, females are usually larger than males and *Ae. flavopictus* is larger than *Ae. albopictus* in both sexes when collected outdoors in the same seasons or when reared at the same temperatures in the laboratory (Alam and Tuno 2020). Therefore, the size difference between *Ae. flavopictus* females and *Ae. albopictus* males is very large, while the difference between *Ae. albopictus* females and *Ae. flavopictus* males is small. Such body-size difference between the copulating pair has been known to influence mating success in many insects including mosquitoes (Okanda et al. 2002, Ponlawat and Harrington 2007), and therefore it could also affect the outcome of reproductive interference. In the present experiment, *Ae. albopictus* and *Ae. flavopictus* individuals that varied in the body size were produced by rearing larvae at different temperatures (22°C and 28°C); individuals reared at 22°C were larger than those reared at 28°C.

2.2. Materials and Methods

2.2.1. *Experimental designs*

To examine the effects of heterospecific mating on copulation duration and the production of offspring, we conducted three kinds of experiments 1) conspecific mating (control), 2) heterospecific mating and 3) double-mating (i.e. heterospecific mating followed by conspecific mating). In addition, the effects of body size difference between copulating pairs on copulation duration and the production of offspring were investigated using small and large adults that are produced by rearing larvae at high and low temperatures, respectively. In conspecific and heterospecific mating, a small/large virgin female was mated with a large/small male to increase the variation of the size difference of mating pairs. In double-mating, a small/large virgin female was first mated with a large/small male of different species and immediately mated with a conspecific large/small male. The effects of heterospecific mating and the body size difference between copulating pairs on copulation duration, the frequency of females that laid eggs, the number of eggs laid, and the number of viable eggs (i.e. those that hatched) were analyzed. The details of experiments are described below.

2.2.2. *Mosquito colony and rearing of larvae*

A laboratory colony of *Ae. albopictus* was established from adults and larvae in a residential area (36°31'N, 136°41'E, 50 m in altitude) and a colony of *Ae. flavopictus* was from larvae collected in a bamboo stump (36°33'N, 136°42'E, 100 m in altitude) in Kanazawa city, Ishikawa Prefecture in June 2018. Both colonies were maintained at 25 ± 1 °C under 70–90% relative humidity and 14L/10D (14 h light–10 h dark) photoperiod conditions in incubators (140 cm in width, 140 cm in depth and 190 cm height; AFC19-3.47KH, Amefrec Co., Ltd. Osaka, Japan). Adult mosquitoes were reared in rearing cages (20 cm width × 20 cm height× 30 cm length). To allow mosquito females to lay eggs, blood meal was provided and then placed in a cage with a cup containing dechlorinated water and paper strip. The strips with eggs were kept wet for three days at 25°C for embryonic development and then dried. At the start of experimental rearing, stripes with eggs were submerged in dechlorinated water in plastic trays (25 cm width × 34 cm length × 6 cm height). Hatched larvae were fed with fish food (TetraMin, Spectrum Brands Japan Co. Ltd., Yokohama, Japan) until pupation. Pupae were transferred into an above-mentioned rearing cage, and adults that emerged were provided with 3% sucrose solution on a piece of cotton. In both species, third generation offspring were used in the experiments.

2.2.3. *Generation of large and small mosquitoes*

To produce large and small adult mosquitoes, larvae were reared at two different temperatures, 22°C and 28°C, under 14L/10D photoperiod conditions with a sufficient amount of food, because the body size decreases with the increase of rearing temperature (Farjana and Tuno 2013, Alam and Tuno 2020). When they pupated, they were individually kept in vials with mesh cover till emergence at 25°C in incubators. When adults emerged, they were sexed, and females and males were separately kept in rearing cages with free access to 3% sucrose solution. Body sizes of experimental individuals were shown in Table 1.

Mating experiments

2.2.4.(a). **Conspecific mating**

All the virgin females used in the experiment were allowed to suck blood from human hands one day before mating experiments. Mating experiments were performed by releasing engorged virgin females (2–5 days old) in a cage in which 10 virgin males (2–5 days old) were released in advance. In the present experiment, large females were released in cages with small males and small females were released in cages with large males to increase the variation of the size difference between mating pairs. This experimental setup is based on our field observations. In the field, males of these *Aedes* species are attracted to humans as well as females; they are aiming to mate with females that come to suck blood. According to our observations, the number of males collected around us at a time was usually less than 10.

In the experiment, females were released by turns; i.e. one female was released at once. When copulation occurred, the duration of copulation was measured. Then, the female was collected and individually placed in vials for fecundity check, and another female was released in the cage. In this experiment, it was not determined with which one of 10 males the female mated. In this way, mating experiment was repeated 20 times with a cage containing 10 males. When 20 mated females were obtained, males were killed and measured for their wing length under a stereoscopic microscope. The variation in wing length among 10 males was small; i.e., the standard deviation was less than 5% of the mean (data are not shown). The experiments were continued with a new cage with another 10 virgin males. In total, 40 large and 40 small mated females were obtained for each species.

2.2.4.(b). **Heterospecific mating**

Experiments were conducted in the same way as above except a heterospecific female was released in the cage. In total, 40 large and 40 small mated females were also obtained for each species.

2.2.4.(c). **Double-mating (heterospecific mating followed by conspecific mating)**

First, heterospecific mating experiments were conducted as above. When copulation ended, the mated female was immediately transferred to another cage with 10 conspecific males. When copulation occurred, copulation duration was measured. As well as the above experiments, a large female was released in a cage with small males, and a small female was released in a cage with large males. In total, 40 large and 40 small mated females were obtained for each species.

2.2.5. ***Measurements***

To examine egg production, every mated female was isolated in a plastic vial (3 cm diameter × 6 cm height) with a piece of wet filter paper on the bottom and a cotton ball soaked with sugar solution on mesh sheet covering the vial mouth. The vials were left for five to seven days at 25°C and then checked for the presence/absence of eggs on the filter paper. On the seventh day, the females were killed and measured for wing length. If eggs were laid, they were counted and immersed in dechlorinated water for 15 days to determine hatchability. Eggs that did not hatch were bleached and examined for embryogenesis under a microscope according to the procedure of Trips (1970).

2.2.6. ***Data analysis***

The effects of the mating type (i.e. conspecific, heterospecific or double-mating), species, the difference of body size between copulating pairs on the number of eggs laid, the number of eggs hatched, and the hatching rate of eggs were analyzed by generalized linear model (GLM). As referred previously, it was not determined with which one of 10 males in the cage the experimental females mated. Accordingly, the body size difference between the copulating pair was obtained by subtracting the average wing length of 10 males from the female wing length. The analysis on the number of eggs hatched or laid was performed with the identity link function and the normal distribution error, and the analysis on the hatching rate was with the logit link function and the binomial distribution error. For explanatory variables, the mating type, species, the body size difference and the interaction terms of these parameters were used. Based on the results of the analysis by GLM, the number of hatched eggs was further compared between *Ae. albopictus* and *Ae. flavopictus* by Mann-Whitney test. Copulation duration was compared between conspecific and heterospecific mating by ANOVA. In addition, regression analysis was performed to examine the effect of the female size on the hatching rate of eggs, the effect of the body size difference of copulating pair on copulation duration, and the effect of copulation duration on the hatching rate of eggs. The statistical analysis was performed using JMP 11.2.1 (SAS Institute Inc., Cary, USA).

2.3. Results

The wing length of females (FWL) and males (MWL), copulation duration, the number of eggs laid per female, and the number of viable (i.e. hatched) eggs produced per female and the hatching rates of eggs in the conspecific, heterospecific and double- mating (heterospecific mating followed by conspecific mating) experiments with *Ae. albopictus* and *Ae. flavopictus* (Table 1). In general, females were larger than males in both species and *Ae. flavopictus* was larger than *Ae. albopictus* when reared under the same conditions (Table 1). Therefore, the wing size differences of copulating pairs (defined as “FWL minus MWL” in this study) were usually positive except for some combinations of *Ae. albopictus* females and *Ae. flavopictus* males. There were substantial variations in copulation duration, the number of eggs laid per female, and the number of viable eggs produced per female and the hatching rates of eggs.

The results of the GLM analysis are shown in (Table 2). Mating type, female species and the interaction terms have significant effects on the number of eggs laid and the number of viable eggs produced. Furthermore, not only these two factors, but also female size and their interactions have significant effects on the hatching rate. In the analyses on the effect of each factor, the number of viable eggs produced was significantly (Mann-Whitney test, $P < 0.001$) larger in *Ae. albopictus* than in *Ae. flavopictus* in the conspecific and double-mating experiments (Fig. 1). In the heterospecific mating experiments, viable eggs were not produced except for three eggs that were produced by a pair of *Ae. albopictus* female and *Ae. flavopictus* male (Table 1). These eggs grew into males, but their fertility was not determined.

Figure 2 shows the relationship between the hatching rate and the size difference of copulating pairs (i.e. "FWL minus MWL"). The hatching rate of eggs oviposited by *Ae. flavopictus* females in the double-mating experiments was relatively low when the size difference in the heterospecific mating was small but significantly increased with the increase of the size difference (GLM, $P = 0.0004$). In addition, the hatching rate was only 0.24 in eggs produced by small *Ae. flavopictus* females that experienced double-mating with large *Ae. albopictus* and *Ae. flavopictus* males, whereas the rate was 0.69 in those produced by large *Ae. flavopictus* females that experienced double-mating with small males (Table 1). In the other cases, the hatching rate was relatively high (Fig. 2) and no significant effects of the size difference was observed (GLM, $P > 0.2$).

Copulation duration of *Ae. albopictus* females was significantly longer when copulated with conspecific males than when copulated with heterospecific males (ANOVA, $P < 0.001$), but that of *Ae. flavopictus* females did not significantly differ whether they copulated with conspecific or heterospecific males (ANOVA, $P = 0.228$). When copulation occurred between conspecific pairs, the duration significantly increased with increase of the size difference in *Ae. flavopictus* (Regression analysis, $P <0.001$), but not so distinctly in *Ae.*

albopictus (Fig. 3A). When copulation occurred between *Ae. albopictus* females and *Ae. flavopictus* males, the duration significantly decreased with the increase of the size difference (Regression analysis, *P*<0.001; Fig. 3B). In contrast, the duration significantly increased with the increase of the size difference in copulation between *Ae. flavopictus* females and *Ae. albopictus* males (Regression analysis, *P*<0.05, Fig. 3B). Larger males of *Ae. albopictus* are having the shortest copulation duration, and that may impact the further hatching rate.

Figure 4 shows the relation between the copulation duration and the hatching rate. In the conspecific mating experiments, the copulation duration did not have significant effects on the hatching rate (GLM, *P*>0.1). As well, the duration of conspecific copulation did not significantly affect the hatching rate of *Ae. albopictus* in the double-mating experiments (GLM, *P* > 0.05). However, in *Ae. flavopictus* females, the conspecific copulation duration in the double-mating experiments showed marginally significant effects on the hatching rate (GLM, *P* =0.0554 Fig. 4).

In summary, reproductive interference occurred between *Ae. albopictus* and *Ae. flavopictus* and its degree was asymmetrical; i.e. the production of viable eggs was reduced in females of both species when heterospecific mating occurred precedingly to conspecific mating and the degree of the reduction was larger in *Ae. flavopictus* (Table 1). In addition, the degree of interference was greater when the size difference of mating pairs was smaller in *Ae. flavopictus* (Table 1). In *Ae. flavopictus* females, the hatching rate of their eggs was reduced with the decrease of the duration of copulation with heterospecific males (Fig. 4), and the duration was reduced when the size difference was smaller (Fig. 3). In *Ae. albopictus* females, however, the hatching rate of their eggs was scarcely affected by the duration of copulation with heterospecific males (Fig. 4). On the other hand, *Ae. albopictus* females are assumed to be able to shorten the duration of copulation with heterospecific males when the body size difference was larger (i.e., copulating males were smaller).

2.4. Discussion and Conclusions

Ae. albopictus shows a more anthropophilic distribution than *Ae. flavopictus* that mainly occurs in forests away from populated towns. However, their habitat selection is not rigid (Sunahara et al. 2002), and *Aedes* males, as well as females, are attracted to host animals including humans to find mates (Nelson 1986, Yuval 2006), suggesting that *Ae. albopictus* and *Ae. flavopictus* could encounter around their hosts. On the other hand, the body-size difference between *Ae. flavopictus* and *Ae. albopictus* may affect their mating performance and the production of offspring through reproductive interference. To address these issues, we examined the effects of heterospecific mating on offspring production in relation to the body size difference between mating pairs.

We found that reproductive interference occurred between *Ae. albopictus* and *Ae. flavopictus* under the experimental setting; i.e. the production of eggs and the hatchability were reduced when heterospecific mating occurred before conspecific mating. Such reproductive interference has also been reported between *Ae. albopictus* and *Ae. aegypti* and revealed to play an important role in their population dynamics (Nasci et al. 1989, O'Meara et al. 1995, Kaplan et al. 2010, Tripet et al. 2011, Bargielowski et al. 2013, 2015a, b). The present study further found that the body size difference between the copulating pair had a large effect on the production of viable eggs (i.e. those that have a capacity to hatch) in *Ae. flavopictus* females; i.e. in comparison with a case where mating occurs only with conspecific males, the production of viable eggs was approximately 1/4 and 1/16 in large and small *Ae. flavopictus* females that mated with *Ae. albopictus* males before conspecific males, respectively. On the other hand, such effect of the body size difference was not observed in *Ae. albopictus*; the production of viable eggs did not differ between large and small *Ae. albopictus* females that mated with *Ae. flavopictus* males before conspecific males (i.e., 1/4 for both).

Remarkably, duration of heterospecific copulation of *Ae. albopictus* females decreased with the decrease of the size of copulating males relative to own size, while the duration of conspecific copulation was prolonged to some extent with the decrease of the size of copulating males. This suggests that *Ae. albopictus* females can distinguish conspecific males from *Ae. flavopictus* males and relatively larger females are more successful in rejection of heterospecific males. In the double-mating experiments, however, the hatching rate of *Ae. albopictus* eggs was not so distinctly higher even when the copulation duration with heterospecific males was shorter. On the contrary, in *Ae. flavopictus*, heterospecific copulation, as well as conspecific copulation, was prolonged with the increase of female size relative to copulating males, and the hatching rate of eggs tended to increase with the increase of duration of conspecific (and heterospecific) copulation in the double-mating experiments. Consequently, the productivity of larger females is less affected by heterospecific mating in *Ae. flavopictus*.

It appeared in the present study that *Ae. albopictus* females have evolved an ability to discriminate between conspecific and heterospecific males. In contrast to *Ae. albopictus* females, *Ae. flavopictus* females have not evolved such ability. In Okinawa island, where *Ae. albopictus* and a subspecies of *Ae. flavopictus*, *Ae. f. downsi* Bohart and Ingram, are distributed, males of both species have been reported to discriminate between conspecific and heterospecific females, although if females were able to discriminate was not studied (Miyagi and Toma 1989). The studies of *Ae. aegypti* and *Ae. albopictus* in USA have suggested that reproductive interference drives rapid evolution of such mechanisms (Lounibos et al. 2010, Bargielowski et al. 2013, 2015a, b). *Aedes albopictus* has been expanding its distribution range to the north in Honshu, the mainland of Japan. The two species may encounter for the first time in northern parts. We demonstrated that even in their origin *Ae. albopictus* may cause reproductive interference with its sibling species, *Ae. flavopictus*, and the outcome can be affected by their body size difference.

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CHAPTER 3

Effect of vegetation on the larval and adult growth performance of *Aedes albopictus* (Diptera: Culicidae)

3.1. Introduction

A growing source of environmental change is global climatic variation (Walther et al. 2002). Increasing temperatures and shifting precipitation patterns may have profound impacts on ecology and human health (Patz et al. 2005). The effect of global climate change on the geographical distribution and abundance of vectors and vector-borne infectious diseases is an important issue for human health. The Asian tiger mosquito, *Ae. albopictus* (Skuse) is an emerging problem in the world and accountable for the transmission of diseases, including dengue, Zika, Chikungunya and West Nile virus (Cupp et al. 2007, Wilder-Smith et al. 2017). However, the impact of climate variability on the distribution of *Ae. albopictus* has been anticipated on a global scale (Sutherst 1993), for Japan (Kobayashi et al. 2008a), for Europe (European Centre for Disease Prevention and Control [ECDC] 2009), (Caminade et al. 2012) and for North America (Erickson et al. 2012). All these studies projected a significant influence of variations in climate on the distribution pattern of *Ae. albopictus*. In Japan such phenomena have been studied on the native range of *Ae. albopictus* (Kobayashi et al. 2008b, Mogi and Tuno 2014). In northern Japan, thermal conditions have become increasingly suitable for *Ae. albopictus*, and the estimated expansion rate to the north was ca. 26 km per decade (Mogi and Tuno 2014).

In these studies, only thermal condition was subjected. However, in Japan, land use and vegetation changes are taking place simultaneously with global warming. Most serious and rapid changes is the bamboo expansion due to abandonment of forest management following depopulation and ageing in local communities (Suzuki and Nakagoshi 2011). Although bamboo is an economically important plant, but some species are known to be invasive. Since the 1980s, the bamboo business has established rapidly in Asia, Africa and the Americas (FAO, 2007). In Japan, we historically used two exotic bamboo species (Poaceae), moso (*Phyllostachys edulis* (Carrière) (Houzeau de Lehaie) and madake (*P. bambusoides* Siebold et Zuccarini) in managed forestations (Okutomi 2005b). However, the Japanese bamboo trade declined in the 1970s and many bamboo plantations are left unmanaged, invading the adjacent native vegetation (Suzuki 2015). In recent, southern and central Japan are affected by bamboos and predicted that global climatic change may cause the problem to spread north in the coming decades. Potentially risks also occur in other Asian, African, and Latin American countries with increasing bamboo productions. Since 1970, when bamboo industry was declined in Japan, the area of bamboo groves has been expanding, and is predicted to spread to northern and higher altitude areas in future with global warming scenarios (Takano et al. 2017). From this extent, we focused the importance of leaf litter as an influence on the growth performance of *Ae. albopictus*.

Bamboo groves are typical mosquitoes breeding site in Asian countries. In breeding grounds, larvae exploit the leaf debris by filtering, browsing microbes on the surface (Cummins and Klug 1979, Fish and Carpenter 1982, Walker and Merritt 1991, Kaufman et al. 2001, Kaufman and Walker 2006, Murrell and Juliano 2008, Walker et al. 2010). The larval growth rate depends on leaf decomposition, chemical properties and microbial contents of leaf litter (Cummins and Klug 1979, Dieng et al. 2002). We anticipate that *Ae. albopictus* is going to expand in their northern distribution and increase more in number with the expansion of unmanaged bamboo groves. We did not have any comparative data on whether bamboo forests are suitable for mosquito growth. Therefore, in this study we compared the growth rate of aquatic stage of *Ae. albopictus* using three different types of leaves: bamboo (*Phyllostachys pubescens*), cherry (*Prunus × yedoensis*) (Rosaceae) as popular deciduous broad leave tree in town, and beech (*Castanopsis sieboldii*) (Fagaceae), evergreen broad-leaved tree, a dominant tree species in natural vegetation in the warm temperate zone of Japan. This study provides new insights into the larval growth among the three leaf species in the laboratory condition.

3.2. Materials and Methods

3.2.1. ***Experimental designs***

To assess the effects of vegetation on the larval growth, we conducted an experiment in 500ml food-grade cups, measuring (size diameter 12cm by height 5cm). Bamboo, cherry and beech leaves were used by 0.2 g of each species in 200 ml of dechlorinated water at two temperatures 22°C and 28°C. In addition, leaf treatments were made by fermenting 0.2 g leaves in 200 ml of water. We kept the microcosms for seven days while the leaves were soaked, and microbes grew. Then the water was filtered using mesh cloth to eliminate leaf debris preceding to adding the first instar larvae. Leaf litter water offered as a source of food and first instars larvae were introduced in microcosms. To determine the impact of leaf litter, we observed the larval development time in each instar, mortality rate, male and female body size as well as fecundity. The larval instars were checked regularly and confirmed by the appearance of exuviates at every stage. We observed female fecundity in each vegetation treatment. After laying eggs, they were killed and measured the wing length.

3.2.2. ***Source of vegetation***

Experiments were conducted by using three plant species, including bamboo (*Phyllostachys pubescens*), cherry (*Prunus × yedoensis*) and beech (*Castanopsis sieboldii*). Among them (*Phyllostachys pubescens*) and (*Prunus × yedoensis*) is distributed in forest areas of Kakuma campus (36°33'N, 136°42'E) at Kanazawa University, Ishikawa prefecture. Beech (*Castanopsis sieboldii*) leaves were allocated in forests and park areas and collected from the Noto peninsula (37° 21′ N, 136° 56′ E). Only fresh leaves were collected being oven-dried before the experimental trial.

3.2.3. *Collection of Aedes albopictus mosquito*

The adults and larvae were collected from Okuwa residential area (36°31'N, 136°41'E, 50 m in altitude) to establish a laboratory colony in June 2019. They were maintained at 25 ± 1 °C under 70–90% relative humidity and 14L/10D photoperiod conditions in incubators (one cell is 140 cm width, 140 cm depth, 190 cm height; AFC19-3.47KH, Amefrec Co., Ltd. Osaka). Larvae were raised to adulthood on fish food (TetraMin; Tetra, Germany) and adult mosquitoes were kept in cages (20 cm width × 20 cm height× 30 cm length). They offered blood meal for oviposition. The experiments were conducted using F3 generation of *Ae. albopictus*.

3.2.4. *Microcosms experiments*

In our study, each 500 ml microcosm consisted (size diameter 12cm by height 5cm) 0.2 g of dry leaf litter filled with 200 ml of tap water prior to the experiment (Alam and Tuno 2019). For each plant species, 50 first instar larvae were raised in 500 ml microcosm with 200 ml of water. Experiments were conducted at 22° C and 28° C with a 14:10 light: dark cycle and checked each microcosm for larval mortality, development time of each instar. During the study, dead larvae were removed daily and recorded by the date of death. Larval mortality was analyzed by dividing the total number of dead larvae by the initial number of larvae. The microcosms were observed until pupation, and they were placed individually. The adult development time computed to the number of days from larval introduction until the day of adult emergence. After emerging male and female from each experimental group was kept in cages. Female mosquitoes were offered with a blood meal from human hand and kept individually in a plastic vial and for checking fecundity with 3% sugar solution. In each experimental trial, 10 blood-fed females were allowable to oviposit for five consecutive days. The total number of eggs laid by per female for each leaf species was documented and dissected out after oviposition to measure the wing size. After mating the wing length of males was also determined for body size. All the experimental trials were replicated two times during the study.

3.2.5. *Data analysis*

We evaluated the population performance parameter *I* of Livdahl and Sugihara (1984), with few modifications:

$$I = \frac{\ln\{(\frac{1}{N_0})\Sigma f(\mathrm{m_x})\}}{\sum wx3{+}\Sigma\overline{x}}$$

Where N_0 denotes the initial number of larvae, *f* (mx) predicts the numbers of eggs laid by females in each leaf litter treatment, the cubic value of the female wing length w_{x3}denotes the average wing length, $\overline{x}$ means the development days of adult. Here, we calculated *I* for each rearing microcosm under three leaf litters at two temperatures. The performance index *I* value were compared among the all experimental treatments of *Ae. albopictus*.

Percentage of larval mortality, length of larval development, adult development time, body size and total female fecundity were calculated. Two-way analyses of variance (ANOVA) were used to compare the larval mortality, larval and adult development in different leaf litter. The effect of treatment on the adult development time, body size and fecundity size were determined by using analysis of variance (ANOVA) followed by the Tukey–Kramer honestly significant difference (HSD) test. Data analyses were executed using SPSS version 23 (SPSS Inc. IBM).

3.3. Results

3.3.1. *Larval mortality*

The mortality rate was steadily lower in the first and second instar moreover; 21% and 44% of total mortality occurred in the third and fourth instar at 22°C and 18% and 31% of larval death followed by third and fourth instar at 28°C, respectively in three vegetation treatments. Leaf litter and the larval instar as well as their interaction did not significantly affect the mortality (Two-way ANOVA: leaf litter x larval instar: $P>0.005$ in all cases) (Table 3). The mortality rate of *Ae. albopictus* was lower in bamboo leaf microcosms. At temperature 22°C larvae exposed to cherry resulted in faster rates of mortality in contrast, mortality is affected by beech leaf treatment at 28°C.

3.3.2. *Development time*

The effect of vegetation on the larval development time was significant (Two-way ANOVA: leaf litter x larval instar: F= 112.0, *P*<0.0001; leaf litter x larval instar: F= 11.7, *P*<0.0001) at 22°C and 28°C, respectively. The first and second instar took smaller time to develop notably; the fourth instar larvae had the longest developmental duration than another instar. Contrastingly, the larval development time was remarkably longer in cherry and beech, while shorter in bamboo leaf litter treatments. In addition, vegetation significantly affected the adult development time with temperature interaction (for leaf litter: F= 171.4, *P*<0.0001; for temperature: F= 1851, *P*<0.0001; interaction: F= 90.0, *P*<0.0001). Bamboo treatment resulted faster emergence of the adult, therefore, cherry and beech leaf took longer time to develop adult at 22°C and 28°C, respectively (one-way ANOVA: *P*<0.05) (Table 4).

3.3.3. *Body size and fecundity*

Vegetation significantly affected the female body size (One-way ANOVA: *P*<0.0001) (Table 4). Body size was greater (Mean ± SD) (3.10 ± 0.08 mm, 2.50 ± 0.07 mm) in the bamboo leaf treatment at 22°C and 28°C, respectively, however, cherry leaf resulted in the smallest size adult (Table 4). There were no significant variances in the number of eggs laid with different vegetation treatment (One-way ANOVA: P=0.06, P=0.33) at 22°C and 28°C, respectively (Table 4). The highest number of eggs (Mean ± SD) (21.3 ± 11.7, 26.1 ± 14.2) were laid by mosquitoes exposed to bamboo at 22°C and 28°C, respectively (Table 4). Moreover, female fecundity decreased in cherry leaf compared to others. Markedly, we observed that a smaller number of eggs were laid by *Ae. albopictus* raised in cherry plants, where female size was too small. *Ae. albopictus* females favorably laid more eggs in bamboo while avoiding cherry vegetation. Clearly, *Ae. albopictus* favor bamboo, for faster larval growth, increase body size and laid a larger amount of eggs.

The values of *I* were calculated to combine the effects of vegetation and temperature on *Ae. albopictus* (Figure 5). The *I* value was the highest in bamboo therefore, the lowest in cherry and beech at temperature 22_{o}C and 28_{o}C, respectively. Thus, the *I* value favored *Ae. albopictus* in bamboo for their growth, whereas, population performance reduced drastically in cherry and beech leaf.

3.4. Discussion and Conclusions

In the study, larval mortality revealed that *Ae. albopictus* were less tolerant of cherry and beech leaf, however, lower in the bamboo treatment. This finding suggests that beech (Mau et al. 2001, Dieng et al. 2002) and cherry (Kelly et al. 2010, Kim and Muturi 2012) contain lethal components that inhibit microbial production resulting in low levels of larval growth (Rey et al. 1999, David et al. 2000c). These findings are inconsistent with previous findings that the effects of sugar maple (deciduous) act as an excellent food source for *Ae. triseriatus* larvae (Kafuman et al. 2008). Previous research has established that leaf litter from few plant species contains toxin materials, affect nutrient availability resulting in larval mortality (David et al. 2000a, 2002, Tilquin et al. 2002, Dieng et al. 2002, Ansari et al. 2005).

Ae. albopictus larval performance varies among vegetation, whereas bamboo leaf litter elevated the larval and adult development. These results propose that bamboo vegetation enhanced diversity of microbes in the breeding ground that may allow faster growth of larvae. These results are most likely related to Alam and Tuno (2019 unpublished) who showed that bamboo is more convenient for the development of *Ae. albopictus* and *Ae. flavopictus*. We speculated that disorderly bamboo plant incursions into the forest and urban areas enhance breeding habitats, could promote the larval abundance. Therefore, in our study, cherry and beech leaves prolonged the development period. These results agree with the observations of

Kim and Muturi (2012) where pine and maple leaf (deciduous) treatments took longer time to develop *Aedes* species. Nevertheless, vegetation might enhance the growth, but toxic compounds from plants can have lethal effects on the larval growth may be extended the development time (Sunahara and Mogi 2002, Shaalan and Canyon 2018). The longer development time might increase the larval acquaintance to mortality factors, including predation, parasites and desiccation prior to adult development. However, the available literature also includes variable findings regarding maple leaf litters showed the suitable substrate for *Ae. triseriatus* (Fish and Carpenter 1982) and *Ae. albopictus* growth (Dieng et al. 2002). The prolonged development time may cause the biological changes of larvae, foster to the smaller size of population.

Our demonstrated that larger mosquitoes from bamboo plants had the greatest body size conversely, cherry leaf treatments produce the smallest size adult as well as showing lower fecundity. The toxic products of cherry that could function as nutrients inhibitors, thus, negatively effect on body size (Murrell and Juliano 2008) as well as beech also limit the growth of *Ae. albopictus*. Carpenter (1983) noted the similar results that beech influences *Ae. triseriatus* growth and survivorship. For adult's mosquito species, body size is also related to reproductive success (Armbruster and Hutchinson 2002, Lounibos et al. 2002, Leisnham et al. 2009, Leisnham and Juliano 2010). The fecundity of adult females was influenced by bamboo, but cherry vegetation limited the rate. It might be concluded that larger adults emerged from bamboo were likely exposed to the better food sources and to be more potent for oviposition (Ponnusamy et al. 2008). Sultana and Tuno (2020 unpublished) observed larger females live longer than smaller females and were more fecund. Few studies have shown small females, produced a few numbers of eggs (Jalil 1974). In addition, large females have a greater vector potential which influence to search a second blood meal (Nasci 1986). Wachira et al. (2014) noted that the nutritional value and chemical compounds of different leaf species, may affect the fecundity. The population performances of *Ae. albopictus* was the utmost in bamboo, but it was completely low in cherry and beech. We predict that *Ae. albopictus* will be more efficient as a competent vector in a bamboo forest area, while, cherry and beech produce the least. Thus, variations in vegetation have the potential to affect the risk of disease transmission.

In summary, our results provide interesting insights into the importance of vegetation on *Ae. albopictus* growth. In bamboo, *Ae. albopictus* performed better, but cherry, had detrimental effects on the body size and fecundity. The bamboo plantation facilitates a risk of invading surrounding ecosystem (Suzuki 2015). Thus, it is vital to recognize the vegetation close to the urban areas to control this vector species. We assume that bamboo plant might be initiated mosquito problems by the proliferations of larvae in bamboo stumps. Indeed, the availability of cherry and beech plant in an area may play a key role to reduce the risk of disease transmission. These results might be vital to control of *Ae. albopictus* to restrict bamboo invasion.

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CHAPTER 4

The influence of body size on the fecundity and longevity of *Aedes albopictus* and *Aedes flavopictus*

4.1. Introduction

Mosquito-borne disease increases prominently due to the climatic change (Patz et al. 1998, Messina et al. 2015). In a changing climate, differences in the temperature and food availability are likely to have different body sizes of adult that effects on the fecundity and longevity. The transmission of vector-borne disease is strongly linked to body size that influences the female's vectorial capacity, including, fecundity and longevity (Nasci 1986, Briegel 1990, Takken et al. 1998, Alto et al. 2008, Helinski and Harrington 2011, Takken et al. 2013, Christiansen-Jucht et al. 2015, Barreaux et al. 2018). Few studies have provided information that the large size of females increased the probability of survival, acquiring blood meal, the parasite infectivity (Lyimo and Koella 1992), as well as the vectorial capacity (Kitthawee et al. 1992). Recently, considerable attention has been given to fecundity and longevity of female, in order to decrease or eliminate the disease transmission.

Environment can influence the larval development which affects the adult mosquito (Moller-Jacobs et al. 2014). The temperature and diet could play a putative role to differ in adult body size. For instance, high nutrients with lower temperature at the larval stage lead to larger adults, in contrast, low diet and higher temperature produce smaller bodied adult (Sibly and Atkinson 1994, Kingsolver and Huey 2008). Thus, body size affects the life history traits of adult mosquito (Rueda et al. 1990, Tun-Lin et al. 2000, Delatte et al. 2009, Farjana et al. 2012, Zeller and Koella 2016, Sasmita et al. 2019) that affects the survival and longevity (Nasci 1986).

In Japan, *Ae. albopictus* and *Ae. flavopictus* are well-known vectors species. Globally, population is at threat from mosquito-borne diseases, including dengue, yellow fever, Chikungunya and Zika due to expand of *Ae. albopictus* (Lambrechts et al. 2010, Medlock et al. 2012, Weaver and Lecuit 2015, Maurice et al. 2010, Faria et al. 2017). However, there is a little confirmation of *Ae. flavopictus* as a vector (Eshita et al. 1982). Both species coexist in nature, therefore, *Ae. flavopictus* did not replace by *Ae. albopictus* due to the body size (Sultana et al. 2020, unpublished). *Ae. flavopictus* act as a new leading vector and tends to be larger than *Ae. albopictus* (Alam and Tuno 2020). Therefore, it is important to figure out where both species are different in body size, that could become at risk for diseases transmission. So, it is an urgent need to address the body size linked to fecundity and longevity of both species to control the vector species. Herein, we focus the body size effects on fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*.

In our study, we varied body size of females at two temperatures (25 °C and 28 °C) and two diets (low and high) treatments. Consequently, we investigated the effect of body size on the fecundity, by comparing the ovarian follicle, egg retention capacity as well as their longevity. The broaden knowledge on fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus* might be useful for the future predictions of disease transmission resulting in public health impact.

4.2. **Materials and Methods**

4.2.1. ***Mosquito strain***

Larvae and adult of *Ae. albopictus* were obtained from Okuwa residential area (36 ° 31'N, 136 ° 41'E, 50m altitude) and *Ae. flavopictus* were collected from Kakuma bamboo forests (36 ° 33'N, 136 ° 42'E, 100m altitude) in June 2019 to establish a laboratory colony. Mosquitoes were reared in insect rearing chambers at 25°C, 70–90% RH and 14:10 h L:D. Female mosquitoes were offered blood meal and induced to oviposition in rearing cage (20 cm width × 20 cm height× 30 cm length). The eggs were kept in the plastic tray (25 cm width ×

34 cm length × 6 cm height) for hatching. Early hatched first instar larvae were supplied finely grinded fish food (TetraMin; Tetra, Germany). The rearing tray was inspected daily until pupation. After emerging adults kept in cages with 3% cotton-soaked sucrose solution. Both colonies were maintained up to F3 generations.

4.2.2. ***Rearing larger and smaller size mosquitoes***

The first instar larvae were divided into four experimental groups by using low and high diet at 25°C or 28°C temperature. The treatment combinations were: 25°C x low diet, 25°C x high diet, 28°C x low diet and 28 °C x high diet. The dietary treatment was imposed at each temperature and offered fish food (TetraMin; Tetra, Germany). The low and high diet combination was: first to fourth instar: 0.05 and 0.1 mg/larvae/day, and 0.4 and 0.8 mg /larvae/day, respectively (Farjana and Tuno 2012). We reared 200 larvae per tray for each experimental trial. Prior to the daily addition of diet (low and high), the water was replaced. The larval development, molting and pupation were checked regularly. Pupae were kept individually in plastic vials until their emergence. After emerging, adults from different temperature and diet condition were placed in rearing cages with 7% sucrose solution.

4.2.3. ***Development days, oviposition and counting retained eggs***

Development time was recorded until adulthood was reached. After emergence from each diet treatment adult females were offered the blood meal. A total of 50 females was used from each species for checking fecundity. Adults were kept with the male for mating and placed individually into a plastic vial (3 cm diameter × 6 cm height) covered by mesh with 7% sugar solution. After 5 days, every female was checked for oviposition and if they did not lay eggs, they were kept 2 more days for further investigations. To check the retained eggs in the ovaries, oviposited female was dissected under microscope (Farjana and Tuno 2012). The total number of eggs was counted by adding the number of eggs laid and retained eggs in the ovaries. For all experiments, body size was estimated based on wing size and measured by using a micrometer under microscope.

4.2.4. ***Measurement of ovarian follicle***

A total of 30 unfed females from each species (2 temperature x 2 diet) mosquitoes were anesthetized for 5 to 10 minutes in the refrigerator before dissection. We dissected female reproductive tracts, by following Meadows (1968) method and ovarioles rinsed in PBS pH 7.2 buffer solution. We counted primary follicles (Christophers stage II) which is about 100 micrometers long, and it persists in previtellogenic resting stage (Clements 1992). The number of follicles in every ovary were doubled to denote the total number of follicles. We have counted the ovarioles and the wing length was measured to check their body size. We also compared the number of ovarioles with the body size.

4.2.5. ***Longevity and female body size***

Longevity was measured as the time between the female adult emergence and death, which was observed every 24 hours. A total of 30 females that emerged from each diet combination were placed in a separate film vial (3 cm diameter × 6 cm height) with cotton soaked distilled water. The distilled water tested how long a female mosquito could be sustained in the environment. The survival rate was recorded daily till death. Wing of each mosquito was fixed on slide and measured by using microscope.

4.2.6. ***Data analysis***

We analyzed development time of adult mosquitoes depending on the temperature and diet interaction by using generalized linear models (GLM). In addition, Two-way ANOVA was applied to analyze the effects of temperature and diet influence on body size (wing length). Linear regression was used to analyze the effect of body size on the number of eggs and number of follicles. To test the influence of body size on egg retention logistic regression analysis was performed. Since we are interested to know how temperature, diet and body size combinedly influence the fecundity and longevity, we used generalized linear model (GLM) for fecundity and Cox proportional hazards for longevity. We also used the Mantel-Cox Log-Rank test in longevity across the adult raised by different temperature and diet treatment. Statistical analyses were performed using SPSS version 23 (SPSS Inc. IBM).

4.3. **Results**

4.3.1. ***The effect of temperature and diet on adult development time and body size***

Interactions between the temperature and diet treatment had a significant effect on adult development of *Ae. albopictus* and *Ae. flavopictus* (GLM: $\chi2$ = 41.78, df = 1, $P < 0.0001$, $\chi2$ = 246.1, df = 1, $P<0.0001$), respectively (Fig.6). The shortest adult development time was observed in *Ae. albopictus* reared at 28°C with high diet treatment (Mean ± SD) (8.27 ± 0.67 days) and the longest was (11.27 ± 1.16 days) at 25°C with low diet. Moreover, in *Ae. flavopictus* followed the longest development time, by nearly 12.95 days between 28°C and low diet treatment; shortest development time was (9.06 ± 1.19 days) under higher temperature and high diet treatment (Fig. 6).

Ae. albopictus and *Ae. flavopictus* reared at high diet had large wing size (Mean ± SD) (2.91 ± 0.15 mm at 25°C and 2.86 ± 0.10mm at 28°C), (3.40 ± 0.10mm at 25°C and 3.0 ± 0.09mm at 28°C), respectively. In low diet, *Ae. albopictus* wing size was (2.50 ± 0.07mm, 2.31 ± 0.10mm) and *Ae. flavopictus* was (2.89 ± 0.10mm, 2.64 ± 0.11mm), at 25 °C and 28°C, respectively (Table 5). *Ae. flavopictus* was significantly larger than *Ae. albopictus* ($P<0.0001$) in all temperature and diet treatment. However, body size, decreased with the increasing of temperature with low diet treatment in both species. The effect of temperature and diet significantly interacted with the body size of both species (Two-way ANOVA: F= 43.86, $P<$ 0.0001).

4.3.2. ***Relationship between body size and fecundity***

The number of eggs laid was significantly influenced by the body size ($P<0.001$) in both species. We found the significant effects of temperature and diet interaction on fecundity of *Ae. albopictus* ($P<0.05$) therefore, no effects on *Ae. flavopictus* (P=0.2) (Table 6). In addition, body size, temperature and diet significantly influenced the number of eggs ($P<0.001$) (Table 6). There was a positive relationship observed between the number of laid eggs without retention and body size (wing) in *Ae. albopictus* and *Ae. flavopictus* ovaries (R_2= 0.48, R_2= 0.21), respectively (Fig. 7). However, *Ae. flavopictus* retained more eggs in their ovaries than *Ae. albopictus*, whereas body size and the retained eggs were not positively correlated (R_2= 0.00, R_2= 0.01), respectively (Fig. 7). Overall, *Ae. albopictus* laid 51.5% of eggs and 48.5% retained, however, *Ae. flavopictus* laid only 29% of eggs, and the remaining 70% eggs retained in their ovaries after oviposition. The body size of *Ae. albopictus* (logistic regression, r_2= 0.000, $\chi 2$= 0.013, P=0.908) did not influence the egg retention, whereas *Ae. flavopictus* laid only small number of eggs (logistic regression, r_2= 0.045, $\chi 2$= 8.92, $P<0.003$). The body size was positively related to the number of follicles in *Ae. albopictus* and *Ae. flavopictus* (R_2= 0.39, R_2= 0.31), respectively (Fig. 8). The large females having more follicles compared to small females. In *Ae. albopictus* large females developed (59.6 % - 81.1%) of follicles to eggs under high diet, whereas, small females in low-diet conditions developed (47.8%- 56.6%). In addition, under high diet, *Ae. flavopictus* large females developed (55.6%-57.4%) of follicles to mature eggs and low diet treated small females developed only (49.5%-47%) of follicles to eggs (Table 5). Large *Ae. albopictus* from higher temperature with high diet laid the highest number of eggs therefore, *Ae. flavopictus* female fecundity decreased in higher temperature laid the fewest number of eggs (Table 5).

4.3.3. ***Relationship between body size and longevity***

Neither longevity was influenced by the body size nor the temperature and diet interaction (Cox proportional hazards: P>0.05) in both species (Table 6). Therefore, *Ae.*

albopictus and *Ae. flavopictus* female's longevity was not strongly interacted with the conditions of body size, temperature and diet ($P>0.05$) (Table 6). Results from survival analysis showed that large *Ae. albopictus* females survived longer (9 days) at 25°C with high diet, than any other treated females (Log-rank tests: $n = 30$, $P = 0.12$) (Fig. 9). Likewise, large *Ae. flavopictus* females survived significantly longer (11.93 days) at 25°C with high diet compared to the other female's group (Log-rank tests: $n = 30$, $P< 0.01$) (Fig. 9). Moreover, *Ae. flavopictus* small females died faster (3.93 days) at 28°C with low diet. Overall, *Ae. flavopictus* survived longer in lower temperature than *Ae. albopictus* (Log-rank test: $P<0.004$ in both species comparisons). Thus, no significant relationship was apparent between longevity and body size in *Ae. albopictus* and in *Ae. flavopictus*, excluding reared at 28°C with low diet treatment ($R_2=0.13$, $P<0.04$) (Fig. 10).

4.4. **Discussion and Conclusions**

Our study represents the relations of temperature and diet determined the body size of adult which affects the fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*. We compared the body size variation between the two species to clarify the relationship between fecundity and longevity. In concordance with previous studies, results here demonstrated a relation between temperature and diet on development time (Telang et al. 2007, Farjana et al. 2012, Kivuyo et al. 2014), indicating that high diet accelerates development time whereas low diet took longer time to develop. Other studies have shown that low diet might be associated with longer development (Vantaux et al. 2016, Aznar et al. 2018). So, we speculate that energy was depleted of insufficient food, resulting prolonged development period. The current study revealed that, at 25 °C with high diet treatment mosquitoes had the largest body size, although the body size decreased at 28 °C with low diet. *Ae. albopictus* body size was (2.31-2.91mm) were consistent with data obtained by Farjana and Tuno (2012) who manipulated temperature and diet to yield adult with body size of (2.14-2.83 mm). However, *Ae. flavopictus* body size was (2.64 -3.40 mm) comparatively larger than *Ae. albopictus* (Alam and Tuno 2020). In nature, low diet reduces the body size (Zeller and Koella 2016) thus, it can be suggested that temperature and diet factors may influence the adult size.

Our results clearly showed that large females become more fecund due to their body size than small females which is concordance the study of Nasci (1986) and Briegel (1990). Though, *Ae. flavopictus* was larger in size but less fecund than *Ae. albopictus*. Alam and Tuno (2020) exposed the similar findings that *Ae. flavopictus* females would be larger in size, therefore, *Ae. albopictus* oviposited more. Egg retention occurred in an environment (Packer and Corbet 1989) and female mosquito retained few mature eggs in their ovaries (Magnarelli 1975). In our study, the egg retention capacity varied between *Ae. albopictus* and *Ae. flavopictus* owing to their body size, whereas *Ae. flavopictus* retained 70% of eggs. However, *Ae. flavopictus* female's fecundity revealed that they skip oviposition and releasing only fully developed eggs. From this evidence, it may imply that *Ae. flavopictus* has more adaptive tolerance in environment and therefore, may have the tendency to retain more eggs. Likewise, *Ae. albopictus* retained 48.5% of eggs in their ovaries, a similar phenomenon that has been observed previously for this species, and 66.5% retained eggs (Farjana and Tuno 2012). Moreover, small females have retained more eggs in their ovaries (Farjana and Tuno 2012) and it might be used for dispersal along with survival for future. Large females of both species retained fewers amounts of eggs, on the contrary, to previous studies Suzuki et al. (1993) suggested that egg retention was more in large females. In our study, the relationship between body size and ovarian follicle was positive (Farjana and Tuno 2012) and large *Ae. albopictus* contains more follicles. Taken together, follicle development was depending on body size, whereas small females may need a blood meal to developed follicles (Mori 1979, Nasci 1986, 1990, Briegel 1990, Chambers and Klowden 1990, Farjana and Tuno 2012).

Body size and longevity relation in mosquitoes are important and large females live longer than small females (Nasci1986, Maciel-de-Freitas 2007). In the study, *Ae. flavopictus* females survived longer than *Ae. albopictus*. These findings suggest that larger mosquitoes reserve more energy (Briegel 1990), increases the resistance ability in desiccation (Aboagye-Antwi and Tripet 2010), resulting in higher probability of survival (Hawley 1985, Nasci 1986, Suzuki et al. 1993). Thus, longer lifespan of *Ae. flavopictus* might help to adjust to extreme temperatures, starvation conditions, which help to stop reproduction and retained more eggs than *Ae. albopictus*. A remarkable result in our study was that body size had a little effect on longevity. While many studies corroborate that larger mosquitoes had the higher survival than smaller (Tun-Lin et al. 2000, Barreaux et al. 2018, Ezeakacha and Yee 2019). Mogi et al. (1996) noted the relation between body size and longevity whereas, *Ae. aegypti* longevity was greater than *Ae. albopictus*. In addition, it appears that large mosquito may boost up the infection rate, whereas smaller mosquitoes reduced (Telang et al. 2012). During the course of the study, the difference in longevity of these two vector species may have an impact on vector control.

To conclude, the relation between body size with fecundity and longevity differed in both species. Though, *Ae. flavopictus* were larger in body size but less fecund and retained more eggs in their ovaries than *Ae. albopictus*. This confirmed that *Ae. flavopictus* reserved more energy which may reduce their fecundity in adverse natural conditions. In addition, *Ae. flavopictus* females survived longer in lower temperature compared to *Ae. albopictus* which may affect vectorial capacity in disease transmission. This body size difference has an important implication for the predictions of vector borne diseases that how two species respond in pathogen transfer. Consequently, it might be helpful to enable more robust and reliable forecast of vector population.

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CHAPTER 5

Temperature and humidity influence on the fecundity and longevity of *Aedes albopictus* and *Aedes flavopictus*

5.1. **Introduction**

The atmospheric conditions, particularly, temperature and humidity constrain the geographical distribution and expansion of mosquitoes. Over the past decades, the atmospheric temperature has been increased at 0.8°C, at a rate of approximately 0.15–0.20°C per decade (NASA). In recent decades, climate-change focus on temperature (Stocker 2014), water vapor levels (Wang et al. 2016) as well as relative humidity (Hales et al. 2002), which promotes mosquito borne infectious diseases (Hales et al. 2002, Liu- Helmersson et al. 2014, Mc Michael 2013, Murray et al. 2013, World Health Organization (WHO 2015). Therefore, the expansion of dengue transmission occurred due to temperature and humidity (Hales et al. 2002, Eisen et al. 2014).

To study climate crisis consequences, Japan is the perfect place to work on *Aedes albopictus* and *Aedes flavopictus* vectors. Both species are common and susceptible to temperature changes (Alam and Tuno 2020) whereas, *Ae. albopictus* is not merely restricted to tropical areas, but also occurs in temperate regions of eastern Asia (Nawrocki and Hawley 1987). For the last few decades, *Ae. albopictus* has expanded its distribution and modulated by temperature (Lounibos 2002, Juliano and Philip 2005, Reinhold et al. 2018), triggers vector-borne diseases such as Chikungunya, dengue, Yellow fever and Zika (Benedict et al. 2008, Waldock et al. 2013, Wilder-smith et al. 2017). Recent studies show that mosquito activity, development, mortality, reproduction and longevity affected by temperature, relative humidity and precipitation (Costa et al. 2010, Roiz et al. 2014, Danis-Lozano et al. 2015, Panackal 2016, Khan et al. 2018, Drakou et al. 2020). Mosquito are thermophilic in nature (Bonizzoni et al. 2013) can react to different ecological parameters, including temperature (Paupy et al. 2009) and humidity (Peel et al. 2007). For instance, in Japan, temperature regulated the northward extension of *Ae. albopictus* (Kobayashi et al. 2002, Mogi and Tuno 2014). Likewise, local thermal condition and the density also initiate, it may assume that global warming will allow for this distribution (Kobayashi et al. 2002, Mogi and Tuno 2014). Moreover, another competitive species *Ae. flavopictus* (Omori et al. 1952, Tsuda et al. 2003), dispersed across the altitudinal range of Mt. Konpira in 2014. It is predicted that atmosphere changes might drive their expansion (Chaves 2016). In Japan, average humidity ranging from 52% to 77% and temperatures from 0.9°C to 23°C in the year round (Japan Meteorological Agency 2019). This implies that the sensitivity of *Ae. albopictus* and *Ae. flavopictus* changes with the broader environmental temperature and humidity. We addressed the temperature and humidity fluctuations would affect mosquito fecundity and longevity.

Global warming is tending to influence the life-history and survival of *Ae. albopictus* and *Ae. flavopictus*. As both species continue to amplify their distribution, it appears more important to know their biology in context of climatic response. If much is noted about the effects of *Ae. albopictus*, reasonably a little data is available on *Ae. flavopictus*. Combination of the temperature and humidity effects on *Ae. albopictus* and *Ae. flavopictus* will help to improve forecasts of *Aedes* associated disease transmission. Consequently, more extensive information mosquito life-history parameters, will promote more robust and reliable predictions of vector.

5.2. Materials and Methods

5.2.1. ***Mosquito strain***

Adult and larvae of *Ae. albopictus* were collected from a residential area (36°31'N, 136°41'E, 50 m in altitude) and *Ae. flavopictus* from Kakuma bamboo forest (36°33'N, 136°42'E, 100 m in altitude). For colony maintenance, larvae were reared at 25±1°C under 70–90% relative humidity and 14L/10D photoperiod conditions in incubators (one cell is 140 cm width, 140 cm depth, 190 cm height; AFC19-3.47KH, Amefrec Co., Ltd. Osaka) and provide fish foods (TetraMin® fish food). The adult's females were maintained with a 3% sucrose solution and offer a blood survival od meal for egg production. The F2 and F3 generations were used in the experiments.

5.2.2. ***Control of temperature and relative humidity***

Adults were kept in different temperature and humidity setting. For manipulation of humidity condition plastic cover in mosquito net were removed from the rearing cage and adult mosquitoes were exposed at temperature 25°C and 28°C. Humidity readings were taken every day throughout the experiment by using humidifier Therm Pro (Model No. TP-50). The average of humidity percentage was used in the study.

5.2.3. ***Fecundity***

We reared 200 larvae per tray in each trial of an experiment and add (0.04 mg/larvae) fish foods (TetraMin®). After emerging, adults were kept in different temperature and humidity condition. Four experimental groups were performed, in the following conditions: females kept at temperatures 25°C with 52% relative humidity; 28°C with 47% relative humidity for both species. A total of 50 females was used for checking fecundity and were placed individually into a plastic vial (3 cm diameter × 6 cm height) with 7% sugar solution. Fecundity was assessed based on the amount of eggs laid by females. The egg production was checked daily until oviposition, and the times were counted. The number of eggs per female was recorded, and paper strips with eggs were dried for embryonic development.

5.2.4. ***Egg hatchability***

The effect on egg hatchability was inspected under different relative humidity levels (47% and 52%RH) at two temperatures at 28°C and 25°C. Newly laid eggs were obtained and placed in plastic trays (25 cm width × 34 cm length × 6 cm height) with tap water. The number of larvae was counted for hatching ratio.

5.2.5. ***Longevity***

Longevity of females was measured under the different temperature and humidity. A total of 50 freshly emerged females from each treatment were placed in a film vial (3 cm diameter × 6 cm height) with cotton-soaked water. Longevity was calculated as the period between the emergence and death. It was recorded everyday up to their death.

5.2.6. ***Data analysis***

The effects of temperature and humidity on oviposition time, number of eggs and hatching were evaluated with Multivariate analysis of variance (MANOVA). To check the same effects on longevity, we used Cox proportional hazards for both species. In addition, we used the Mantel-Cox Log-Rank test in longevity of the adult fostered by the different temperature and humidity treatment. The impact of temperature and humidity effects on the average oviposition time, number of eggs, variation in ovipositing females and percentage of hatching rate were compared by using the Student's t-test. Statistical analysis SPSS version 23 (SPSS Inc. IBM) was used for performing analysis.

5.3. **Results**

5.3.1. ***Effect on fecundity***

The temperature and humidity effects were observed on the fecundity of *Ae. albopictus* and *Ae. flavopictus* (Table 7). The highest amount of eggs (19.4 ± 9.98) were laid by *Ae. albopictus* at temperature (28°C) with lower humidity (47% RH) (Table 7). The multivariate test, Wilk's Lambda was significant (P <0.0001) for all trials in fecundity of both species. Both temperature and humidity had no influence on the egg production of *Ae. albopictus*

(MANOVA: F= 2.97, P=0.08) (Table 8). A significant reduction in fecundity (6.12 ± 8.31) occurred at 28°C with 47% humidity setting in *Ae. flavopictus* (MANOVA: F= 11.9, *P*< 0.01) (Table 8). Temperature and humidity variations also affected the number of ovipositing females, specifically, at higher temperature and lower humidity. Overall, temperature and humidity interaction did not initiate the ovipositing female of *Ae. albopictus* (Logistic regression: $\chi 2 = 0.68$, *P*= 0.40) and laid 92% and 96% eggs at 25°C and 28°C with humidity settings (52% RH and 47% RH), respectively. Therefore, the interaction between the higher temperature and lower humidity inhibits the oviposition rate 54% (27/50) of *Ae. flavopictus* whereas, 90% (45/50) of females lay eggs at 25°C with 52% RH (Logistic regression: $\chi 2 = 13.70$, *P*<0.0001) (Table 8).

Table 7 shows the oviposition time variation in two temperatures along with humidity. The longest oviposition period was recorded at 25°C/52% with a mean of (5.14 ± 0.63) days. However, the shortest oviposition period was observed at 28ºC and 47% humidity in *Ae. albopictus* and *Ae. flavopictus* as well as varied up to 4.3 to 4.9 days, respectively. The oviposition days significantly influenced by temperature and humidity for *Ae. albopictus* and *Ae. flavopictus* (MANOVA: F= 47.3, *P*< 0.0001; F= 6.03, *P*<0.01), respectively (Table 8).

5.3.2. ***Effect on egg hatching***

Under the different temperature and humidity settings, hatching percentage is showed in (Table 7 and Fig. 11). At the highest relative humidity (52%RH) with lower temperature (25ºC), eggs from *Ae. albopictus* and *Ae. flavopictus* had the utmost percent of hatching (89.8% and 63.6%), respectively. Both species, egg hatching was affected by the lowest humidity and the highest temperature, caused a reduction in egg hatchability (81.1% and 29.4%), respectively. Therefore, the temperature and humidity interaction had a substantial effect on hatching of *Ae. flavopictus* (F= 23.7, *P* < 0.0001) but not of *Ae. albopictus* (F= 2.14, P> 0.14) (Table 8).

5.3.3. ***Effect on longevity***

The lifespan varied under the different temperature and humidity treatment. The survival of *Ae. albopictus* and *Ae. flavopictus* under different temperature and humidity condition was not significantly influenced (Log-rank tests: $\chi2$ =5.88, df= 3, *P* = 0.11 in all cases) (Fig. 12). Therefore, *Ae. flavopictus* lifespan was prolonged for up to 7 days at (25°C/52% RH) therefore, the lowest survival period was 3 days when the females were kept in (28°C/47% RH) temperature and humidity condition. Particularly, *Ae. albopictus* at (25°C/52% RH) and at (28°C/47% RH) temperature and humidity condition longevity varied 5.2 days and 4.7 days, respectively. Results from survival analysis revealed that longevity was not significantly influenced by the temperature and humidity interaction (for *Ae. albopictus*: $\chi2$ = 2.94, *P* = 0.86) and (for *Ae. flavopictus*: $\chi2$ = 0.11, *P* = 0.73) (Table 8).

5.4. **Discussion and Conclusions**

To our knowledge, our study represents the associations of temperature and humidity effects on fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*. Both species proved that they are sensitive to temperature and humidity, varying oviposition patterns, fecundity as well as survival. Our results support the evidence from previous observations with the role of temperature and humidity on mosquito (Glasser and Gomes 2002, Miciele and Campos 2003, Beserra et al. 2006). *Ae. albopictus* showed the utmost fecundity at higher temperature (28°C) with lower humidity (47% RH), however, *Ae. flavopictus* at lower temperature and higher humidity condition (25°C/52% RH). The similar results reported by (Costa et al. 2010, Khan et al. 2018) who showed that high humidity increases fecundity. We can suggest that *Ae. flavopictus* females may have a particular hygro preference on fecundity and ovipositional period prolonged with increasing relative humidity. In another study, Alam and Tuno (2020) explained that the temperature had effects on *Ae. albopictus* and *Ae. flavopictus* population whereas, *Ae. albopictus* laid a larger amount of eggs than *Ae. flavopictus*. In the field collection, Alam and Tuno (2019) reported that wild *Ae. albopictus* stopped laying eggs in hot summer.

Our study revealed that higher temperature incorporates with lower humidity decreased the oviposition of *Ae. flavopictus* but not in *Ae. albopictus*. During this condition, it increases water loss from the chorion might help to resist in desiccation. Previously, such negative impact of the higher atmospheric condition with lower humidity on oviposition reported on mosquito, resulting in severe reductions of population (Reeves et al. 1994, Canyon et al. 1999, Alto and Juliano 2001). Interestingly, both species keep a few amounts of eggs in their ovaries instead of egg laying and *Ae. flavopictus* confirmed more compared to *Ae. albopictus* (Sultana and Tuno 2020, unpublished data).

At (25ºC/52% RH), the oviposition period can be prolonged up to 5 days, compared to conditions of high temperatures (28ºC/47% RH). These results suggest that both populations in higher temperature can take more periods in lower temperature. The highest oviposition rates documented in *Ae. aegypti* at the higher humidity corresponds with results described by Canyon et al. (1999). It appears that the weather conditions influence the oviposition substrates (Reiter et al. 1995), would help for the dispersion of the mosquito, thereby reducing fecundity.

The decrease in hatching rate of *Ae. albopictus* and *Ae. flavopictus* were observed in high temperatures related to low humidity. With the escalation of temperature, a few numbers of larvae were produced at (28°C/47% RH). Therefore, the hatching rate was increased at (25°C / 52%RH) relative humidity. Juliano et al. (2002) compared egg mortality rates of two *Aedes* species under changed temperature (22°, 24° and 26°C) and humidity (25%, 55%, 75%, and 95% RH) whereas, *Ae. albopictus* showed higher mortality excluding the highest humidity. In accordance with our result, Dickerson (2007) also has demonstrated that *Ae. albopictus* hatching rate associated with humidity. Our study supports evidence from the observation of Alam and Tuno (2019) who reported that higher temperature reduced the hatching rate of *Ae. albopictus* (0.6 to 26%) and unhatched eggs were empty. It may be that under environmental stress reserved energy from eggs used for future survival. However, Ae. *flavopictus* hatching experience had a greater impact from humidity and temperature condition. These results support the idea that low humidity and higher temperature adversely effects on hatching.

The limited temperature and low humidity condition have adverse effects on longevity (Mayne 1930, Hylton 1969). When *Ae. flavopictus* kept at 25°C and 52% relative humidity, the duration was prolonged for up to 7 days. This day was shortened near to half when yielded at 28°C with lower humidity. This also accord with our earlier observations of Gao et al. (1984), who showed that longevity of *Ae. albopictus* shortened with declining humidity (from 97 to 0% RH) at 30°C and 35°C. In another study, Sota and Mogi (1992) measured the longevity under altered humidity conditions (42%, 68% and 88% RH) at 25ºC, whereas, *Ae. aegypti* lived longer than *Ae. albopictus*. In another research, there was little difference in adult survival observed, between the different humidity range of 60–90% (Alto and Juliano 2001, Lowenberg Neto and Navarro-Silva 2004, Delatte et al. 2009). Reiskind and Lounibos (2009) also compared the longevity of *Aedes* species and survive on an average 8 days at (85% RH) to 6 days at (35% RH). Mogi et al. (1996) noted that *Ae. albopictus* survival decreased from 2.1–4 days at 90% RH to 1.4–1.8 days at 70% RH. Thus, temperature and humidity differences contribute the desiccation of individuals, thus, it might help to survive the mosquitoes in urban areas with low humidity. Recent studies showed that low levels of relative humidity are dreadful to mosquito. Overall, temperatures and humidity influenced the longevity of *Ae. albopictus* and *Ae. flavopictus*. In a similar study, the impact of temperature and humidity is likely to be the most crucial feature which affecting longevity of mosquito (Reeves et al. 1994, Joshi 1996, Mogi et al. 1996, Delatte et al. 2009, Waldock et al. 2013, Alto et al. 2015, Lounibos et al. 2010, Carrington et al. 2013). *Ae. flavopictus* species longevity appears that at 25 °C with greater ranges relative humidity initiate to survive longer than *Ae. albopictus* (Sultana and Tuno 2020, unpublished data). Therefore, higher temperature with lower humidity is fatal for both species, particularly, *Ae. flavopictus* died within 3 days. At lower temperatures, increased longevity is important because adults have a greater chance of disease transmission. Thus, longer survival adults at lower temperatures adapt in desiccation resistance (Sawabe and Mogi 1999).

In summary, the relationship between temperature and humidity on *Ae. albopictus* and *Ae. flavopictus* may provide future insights into the invasion of disease transmission. *Ae. flavopictus* was found to be more sensitive to lay eggs, particularly at the higher temperature along with lower humidity than *Ae. albopictus*. Conversely, *Ae. albopictus* females produced more eggs at higher temperature 28ºC with 47% relative humidity. Oviposition time extended with lower temperature and increased of relative humidity. However, hatching rate reduced by the increasing of temperature and lower humidity. Female longevity period increased at lower temperature 25ºC with higher 52% relative humidity. The present study would help to control *Aedes*-borne viral transmission in response to temperature and humidity stress from climate change.

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CHAPTER 6

Summary and Conclusion

6.1. **Summary**

Aedes albopictus and *Aedes flavopictus* are important vector species in Japan. Both species are coexisting in different breeding habitats. To update our knowledge, in this regard, we assessed the reproductive interference of both species. We also determined the leaf litter effects on the larval growth of *Ae. albopictus*. Our study also estimated the body size as well as, temperature and humidity influence on the fecundity and longevity. The topics have been discussed in the individual chapters, here, only the summary of earlier chapters will be considered.

In our study, we evaluated the body size differences during mating may affect reproductive interference of *Ae. albopictus* and *Ae. flavopictus*. Both species produced sterile eggs in heterospecific mating. In heterospecific mating, *Ae. flavopictus* copulated longer which has adverse effect in re-mating with conspecific males. Consequently, female species can thereby reduce their fecundity and hatchability. Therefore, *Ae. albopictus* courtship by heterospecific males may not interfere further conspecific mating, it appears that they might be able to differ heterospecific and conspecific males. Thus, double-mating might not decrease the female fecundity and hatching. Reproductive interference has an antagonistic effect on *Ae. flavopictus* than *Ae. albopictus* females. However, *Ae. flavopictus* caused the dominance on *Ae. albopictus* during mating due to the large body size, potentially initiate species co-existence. These results supported that both *Ae. albopictus* and *Ae. flavopictus* would continue in breeding sites, where coexisting species were different in body size, favorable to *Ae. flavopictus* in terms of reproductive interference.

We studied the effect of leaf litter on the larval and adult growth of *Ae. albopictus*. Three different plant species, including bamboo (*Phyllostachys pubescens*), cherry (*Prunus* × *yedoensis*) and beech (*Castanopsis sieboldii*) were used in our study. *Ae. albopictus* exhibited better growth in bamboo, therefore, beech and cherry had detrimental effects. Bamboo leaf promotes large bodied females and laid more eggs, conversely, cherry leaf produced the smallest size adult and reduced the fecundity. Overall, bamboo leaf was suitable for the larval growth than the other leaf treatment. Therefore, our findings suggested that bamboo plants should avoid in future plantation program near the urban areas, as it might harbor a potential habitat for *Ae. albopictus*.

We estimated the body size influence on the fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*. Large bodied *Ae. albopictus* females laid more eggs than *Ae. flavopictus*. Though, *Ae. flavopictus* were larger in body size but reserved more eggs in their ovaries compared to *Ae. albopictus*. Remarkably, longevity was not influenced by body size whereas, large *Ae. flavopictus* survived longer than *Ae. albopictus*. Thus, body size has a critical role for the predictions of vectorial capacity to control mosquito species in an area.

We focused temperature and humidity effects on the fecundity and longevity of *Ae. albopictus* and *Ae. flavopictus*. Oviposition time of both species extended as relative humidity increased (52%) at low temperature (25ºC). Conversely, higher temperature and lower humidity reduced the hatching of both species. Higher temperature (28ºC) and lower humidity (47% RH) hindered the fecundity and longevity of *Ae. flavopictus*. However, *Ae. albopictus* exposed to those atmospheric conditions exhibited better fecundity with the deleterious effect on longevity. So, this study enabled to determine suitable environmental condition for vector mosquito that might help to make reliable predictions of disease transmission.

6.2. Concluding statements

Summering all the results it could be determined that:

1. The heterospecific mating between two species favor *Aedes flavopictus* due to the larger body size and reproductive interference could not be the cause of the elimination of *Aedes flavopictus* by *Aedes albopictus*. (**Chapter 2**)

2. *Ae. albopictus* larval and adult growth facilitated by bamboo leaf. (**Chapter 3**)

3. As fecundity increased with the body size, *Ae. albopictus* laid more eggs than *Ae. flavopictus*, however, longevity was not influenced by body size. (**Chapter 4**)

4. Higher temperature (28ºC) and lower humidity (47% RH) hindered the fecundity and longevity of *Ae. flavopictus* conversely, *Ae. albopictus* exposed to those atmospheric condition ensured better fecundity but had the negative effect on longevity. (**Chapter 5**)

Table 1. Wing length (mm) (Mean ± SD) of females and males, copulation duration (Sec) (Mean ± SD), numbers of eggs oviposited (Mean ± SD) and hatched per female and hatching rates of eggs in conspecific (C), heterospecific (I) and double-mating (D: heterospecific mating followed by conspecific mating) experiments with *Aedes albopictus* (A) and *Aedes flavopictus* (F). L: large individuals reared at 22°C, S: small individuals reared at 28°C.

				Male (first mating)			Male (second mating)			Copulation duration				
Mating	Female	N	Wing length		N	Wing length		N	Wing length	first mating	second mating	Number of of eggs oviposited	Number of eggs hatched	Hatching rate of eggs
C	A (L)	40	3.16 ± 0.08	A (S)	20	2.23 ± 0.09	-	-	-	155.6 ± 29.4	-	33.7 ± 30.1	33.7 ± 30.1	1
C	A (S)	40	2.87 ± 0.18	A (L)	20	2.58 ± 0.07	-	-	-	146.6 ± 31.2	-	35.4 ± 38.9	34.6 ± 38.1	0.98
C	F (L)	40	3.61 ± 0.14	F (S)	20	2.47 ± 0.10	-	-	-	175.8 ± 28.6	-	17.2 ± 10.8	16.0 ± 10	0.93
C	F (S)	40	3.24 ± 0.13	F (L)	20	2.78 ± 0.08	-	-	-	127.1 ± 22.5	-	14.7 ± 9.3	12.8 ± 9.2	0.87
I	A (L)	40	3.14 ± 0.09	F (S)	20	2.47 ± 0.09	-	-	-	45.1 ± 25.3	-	0.4 ± 1.1	0	0
I	A (S)	40	2.89 ± 0.15	F (L)	20	2.79 ± 0.11	-	-	-	147.0 ± 42.3	-	8.1 ± 14.2	0.1 ± 0.5	0.01
I	F (L)	40	3.60 ± 0.13	A (S)	20	2.29 ± 0.13	-	-	-	164.5 ± 68.7	-	0.8 ± 1.7	0	0
I	F (S)	40	3.29 ± 0.16	A (L)	20	2.59 ± 0.08	-	-	-	118.2 ± 43.5	-	2.6 ± 4.4	0	0
D	A (L)	40	3.17 ± 0.10	F (S)	20	2.48 ± 0.12	A (S)	20	2.27 ±0.09	55.3 ± 14.2	161.3 ± 54.2	11.2 ± 6.9	10.5 ± 6.8	0.94
D	A (S)	40	2.89 ± 0.15	F (L)	20	2.75 ± 0.14	A (L)	20	2.60 ± 0.10	144.1 ± 38.7	123.3 ± 45.4	10.0 ± 7.7	8.6 ± 7.5	0.86
D	F (L)	40	3.61 ± 0.11	A (S)	20	2.25 ± 0.14	F (S)	20	2.44 ± 0.10	150.4 ± 60.4	130.9 ± 46.4	5.1 ± 3.6	3.5 ± 3.3	0.69
D	F (S)	40	3.26 ± 0.16	A (L)	20	2.61 ± 0.15	F (L)	20	2.80 ± 0.10	113.9 ± 43.5	84.1 ± 40.4	3.4 ± 3.2	0.8 ± 1.4	0.24

Table 2. Summary of generalized linear model (GLM) analyses on number of eggs hatched, number of eggs laid and hatch rate of eggs.

Parameter	Number of eggs oviposited		Number of eggs hatched		Hatching rate of eggs	
	likelihood ratio χ_2	*P*	likelihood ratio χ_2	*P*	likelihood ratio χ_2	*P*
Mating type (MAT)	**155.5**	**<0.0001**	**193.7**	**<0.0001**	**1859.2**	**<0.0001**
Female species (FSP)	**25.8**	**<0.0001**	**31.8**	**<0.0001**	**33.8**	**<0.0001**
Female wing length (FWL)	0.9	0.347	2.0	0.152	**10.2**	**0.001**
MAT x FSP	**19.2**	**<0.0001**	**26.0**	**<0.0001**	**14.4**	**<0.0001**
MAT x FWL	2.2	0.331	1.7	0.419	**24.3**	**<.0001**
FSP x FWL	0.3	0.582	0.1	0.704	**6.8**	**0.009**

Table 3. Percentage (%) of larval mortality (1st to 4th instar) of *Aedes albopictus* raised with different vegetation with result of two-way ANOVA analysis.

Temperature	Factor	Mortality (N=100 in each treatment)		
		df	F	*P*-value
22°C	Vegetation	3	2.19	0.08
	larval instar	2	0.73	0.47
	Vegetation x larval instar	6	0.12	0.99
28°C	Vegetation	3	2.69	**0.04**
	larval instar	2	0.43	0.64
	Vegetation x larval instar	6	0.32	0.92

*vegetation: bamboo, cherry and beech.

** larval instar: L1, L2, L3 and L4.

Table 4. Developmental time from the first instar to adult emergence (day), female wing size (mm), number of eggs laid per female in one gonotrophic cycle of *Aedes albopictus* raised with different vegetation with pairwise test results (Tukey Kramer honestly significant different test).

		Adult development time (day)				Female wing size (mm)		Number of eggs	
Temperature	Vegetation type	n_1	(Mean ± SD)	HSD test*	n_2	(Mean ± SD)	HSD test*	(Mean ± SD)	HSD test*
			ANOVA: F= 242.4, *P*<0.0001			F= 62.84, *P*<0.0001		F= 2.91, P= 0.06	
22°C	Bamboo	100	28.5 ± 2.35	A	20	3.10 ± 0.08	A	21.3 ± 11.7	A
	Cherry tree	100	40.2 ± 3.98	B	20	2.94 ± 0.06	B	13.6 ± 7.40	B
	Beech tree	100	34.9 ± 4.53	C	20	3.07 ± 0.07	C	20.2 ± 12.9	C
			F=43.52, *P*<0.0001			F= 25.11, *P*<0.0001		F= 1.12, P= 0.33	
28°C	Bamboo	100	18.0 ± 1.85	a	20	2.50 ± 0.07	a	26.1 ± 14.2	a
	Cherry tree	100	20.0 ± 4.35	b	20	2.38 ±0.07	b	19.3 ± 13.0	b
	Beech tree	100	23.5 ± 5.59	c	20	2.49 ± 0.04	c	23.2 ± 15.6	c

n_1: number of larvae observed.

n_2: number of adult females observed for wing size and number of eggs.

*HSD test: Tukey Kramer honestly significant difference test (different uppercase and lowercase letters denote significant difference (*P*<0.05).

Table 5. Wing length (mm), number of laid eggs, retained eggs, total number of eggs (laid + retained eggs), number of follicles and ratio of follicles in *Ae. albopictus* and *Ae. flavopictus* under different temperature and diet treatment.

Species	Temperature	Diet	Wing length (mm)		No. of laid eggs	No. of retained eggs	Total no. of eggs		No. of follicles	% of follicles developed to eggs
	(°C)		(Mean ± SD)	n_1	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)	n_2	(Mean ± SD)	
Ae. albopictus	25	High	2.91 ± 0.15	49	60.3 ± 42.4	19.0 ± 26.1	78.1 ± 10.8	30	131.1 ± 18.3	59.6
	25	Low	2.50 ± 0.07	50	17.5 ± 10.8	20.1 ± 21.7	37. 6 ± 19.1	30	78.8 ± 17.0	47.8
	28	High	2.86 ± 0.10	50	70.2 ± 32.1	12.6 ± 20.8	82.8 ± 23.9	30	102.1 ± 24.6	81.1
	28	Low	2.31 ± 0.10	50	20.8 ± 19.7	19.6 ± 24.2	40.4 ± 18.2	30	71.4 ± 21.5	56.6
Ae. flavopictus	25	High	3.40 ± 0.10	50	42.9 ± 25.4	17.1 ± 19.8	60.0 ± 23.8	30	104. 4 ± 20.1	57.4
	25	Low	2.89 ± 0.10	50	11.1 ± 8.1	24.2 ± 21.4	35.4 ± 19.8	30	71.5 ± 12.2	49.5
	28	High	3 ± 0.09	49	35.3 ± 25.1	18.4 ± 21.8	53.1 ± 25.2	30	95.4 ± 17.7	55.6
	28	Low	2.64 ± 0.11	47	5.75 ± 7.74	25.5 ± 21.3	31.1 ± 20.2	30	66.0 ± 20.2	47

n_1: Number of females observed for counting the number of eggs.
n_2: Number of females observed for counting the number of follicles.

Table 6. GLM for the number of laid eggs and Survival analysis (Cox`s proportional hazard) for longevity of *Ae. albopictus* and *Ae. flavopictus*.

Species	Factor	Number of laid eggs		Longevity	
		$\chi 2$	*P*-value	$\chi 2$	*P*-value
Ae. albopictus	Body size	95.8	**<0.001**	0.43	0.51
	Temperature * diet	3.76	**<0.05**	0.53	0.46
	Body size*temperature*diet	69.6	**<0.001**	0.58	0.44
Ae. flavopictus	Body size	83.0	**<0.001**	2.26	0.12
	Temperature * diet	1.60	0.2	0.18	0.66
	Body size*temperature*diet	20.8	**<0.001**	0.13	0.71

Table 7. Effect of temperature and relative humidity on oviposition variation, number of eggs, retained eggs and percentage of egg hatching of *Ae. albopictus* and *Ae. flavopictus*.

			Reproductive parameter				
	Temp (°C)	Relative humidity		Oviposition time	No. of eggs	Oviposition variation in females	% of hatching
Species			n	Mean ± SD	Mean ± SD	(%)	Mean ± SD
Ae. albopictus	25°C	52%	50	5.14 ± 0.63$_a$	15.6 ± 12.0$_a$	(46/50) 92%	89.8± 27.6$_a$
	28°C	47%	50	4.3 ± 0.58$_b$	19.4 ± 10.2$_a$	(48/50) 96%	81.1 ± 31.3$_b$
Ae. flavopictus	25°C	52%	50	5.28 ± 0.72$_A$	11.7 ± 7.81$_A$	(45/50) 90%	63.6 ± 37.7$_A$
	28°C	47%	50	4.94 ± 0.65$_B$	6.12 ± 8.31$_B$	(27/50) 54 %	29.4 ± 32.2$_B$

n= number of females. Different letters indicate values with significant differences (*t*-test, $P<0.05$). Regardless of the same letters indicate values without significant differences.

Table 8. Multivariate ANOVA analysis for the number of eggs, hatching and survival analysis (Cox`s proportional hazard) for longevity of *Ae. albopictus* and *Ae. flavopictus*.

		Number of eggs		Ovipositon time		Hatching		Longevity	
Species	Factor	F	*P*-value	F	*P*-value	F	*P*-value	*χ2*	P-value
Ae. albopictus	Temperature x humidity	2.97	0.08	47.3	0.0001	2.14	0.14	2.94	0.86
Ae. flavopictus		11.9	0.01	6.03	0.01	23.7	0.0001	0.11	0.73

*Temperature: 25°C and 28°C; humidity: 52% and 47%. Temperature x humidity: interaction.

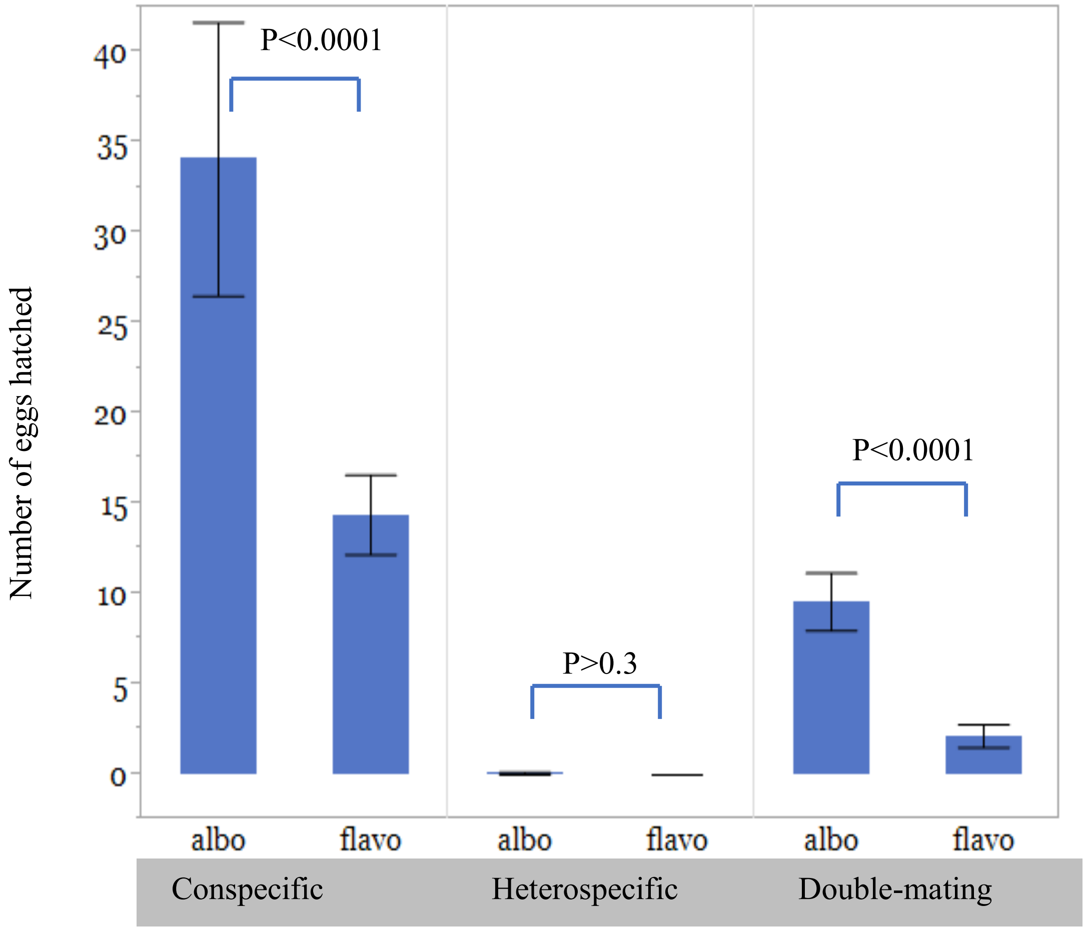


Figure 1. Number of eggs hatched of *Aedes albopictus* (albo) and *Aedes flavopictus* (flavo) in the three mating experiments with results of species-pairwise comparisons (Mann-Whitney Test).

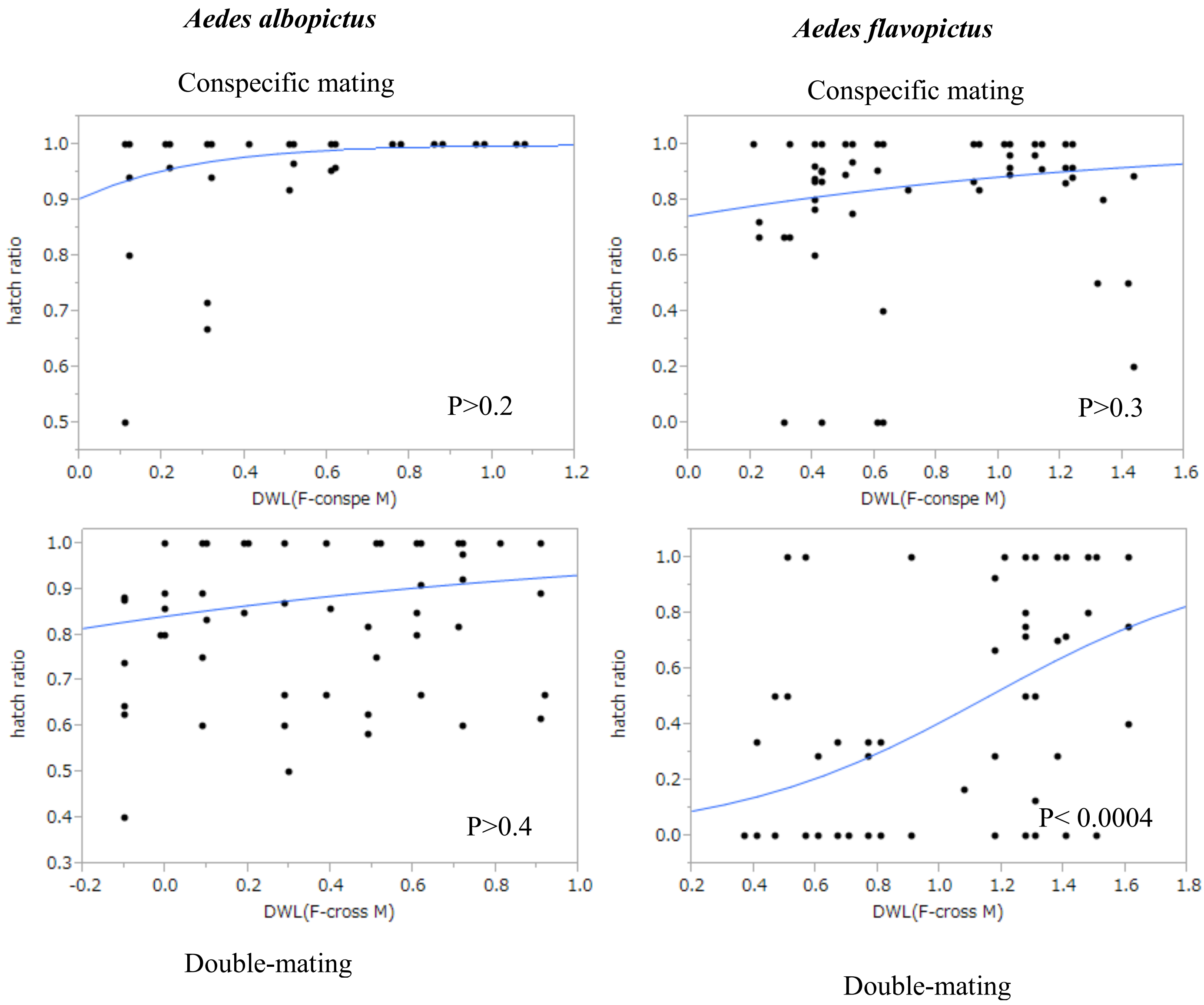


Figure 2. The relationship between egg hatch ratio and difference of female wing length in *Aedes albopictus* (albo) and *Aedes flavopictus* (flavo) in conspecific and double-mating experiments.

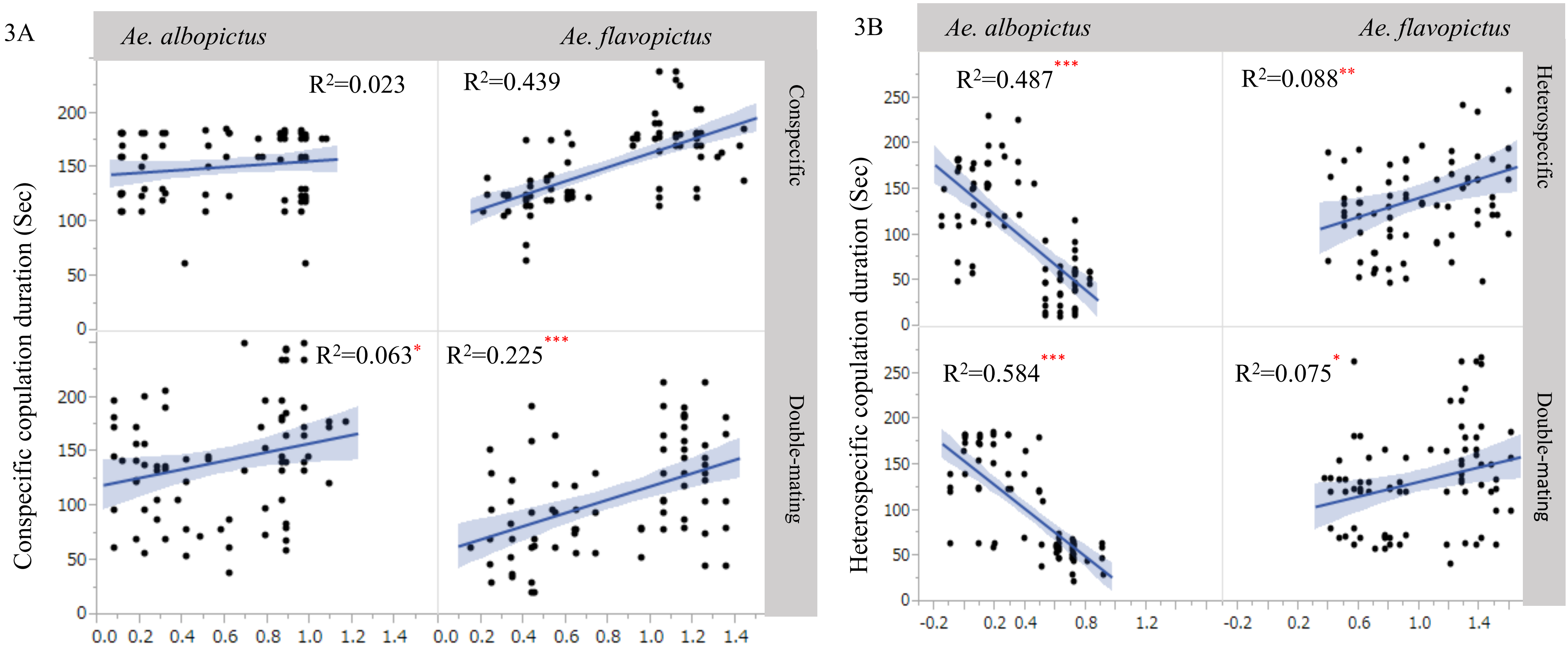
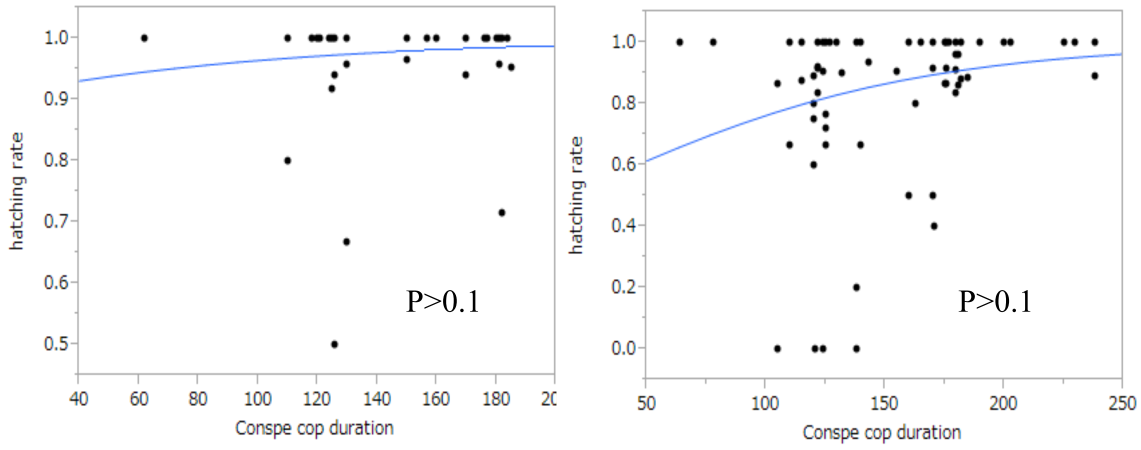
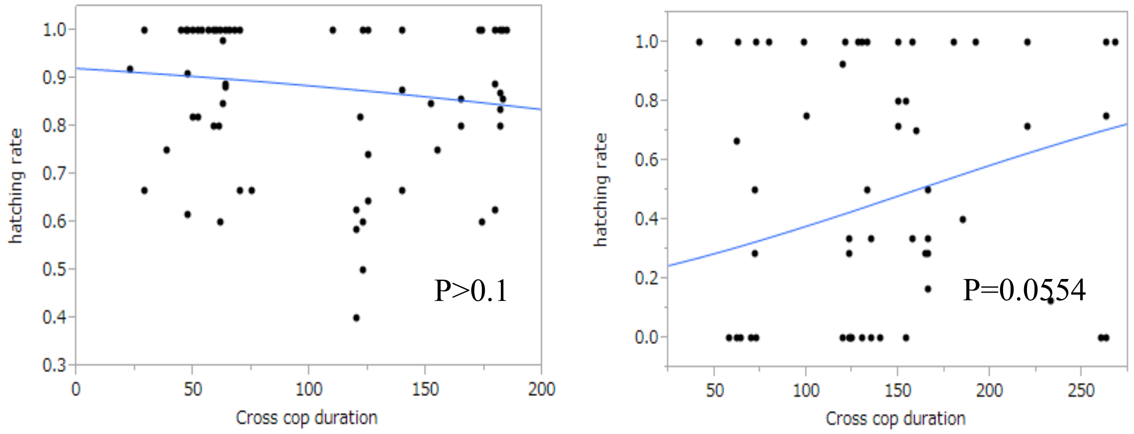


Figure 3. Regression analysis of two parameters by experiment type and female species. Regression analysis between difference of female wing length and conspecific male wing length and conspecific copulation duration (Fig. 3A); Regression analysis between difference of female wing length and different species male wing length and heterospecific copulation duration (Fig. 3B); *: $P<0.05$, **: $P<0.01$, ***: $P<0.0001$.

Aedes albopictus ***Aedes flavopictus***



Conspecific mating Conspecific mating



Double-mating Double-mating

Figure 4. GLM between conspecific copulation duration and hatching rate of eggs; between double-mating copulation duration and hatching rate of eggs of *Aedes albopictus* and *Aedes flavopictus*.

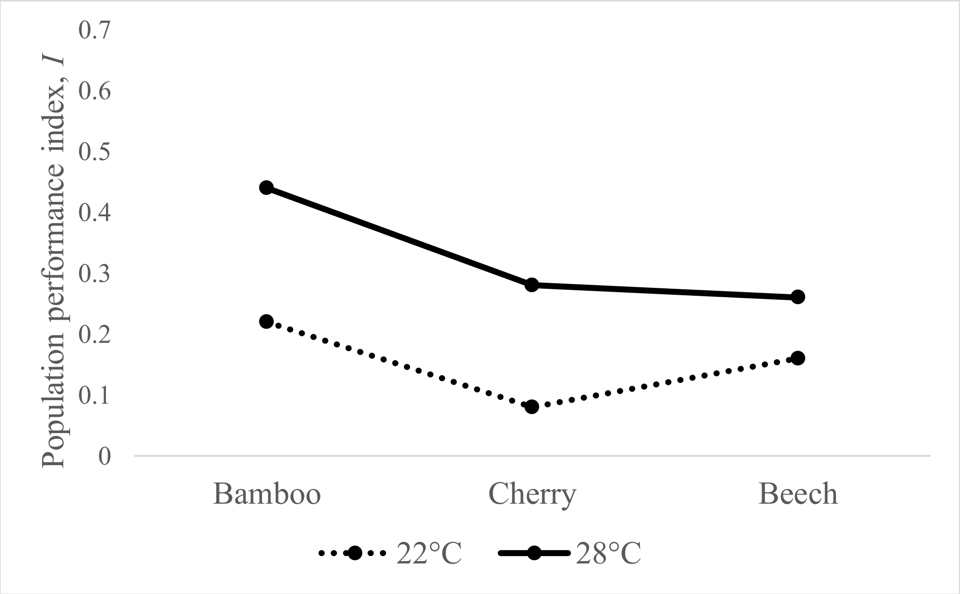


Figure 5. Estimated population performance index (*I*) values of *Ae. albopictus* were reared under three vegetation treatments with two temperature at 22°C and 28°C.

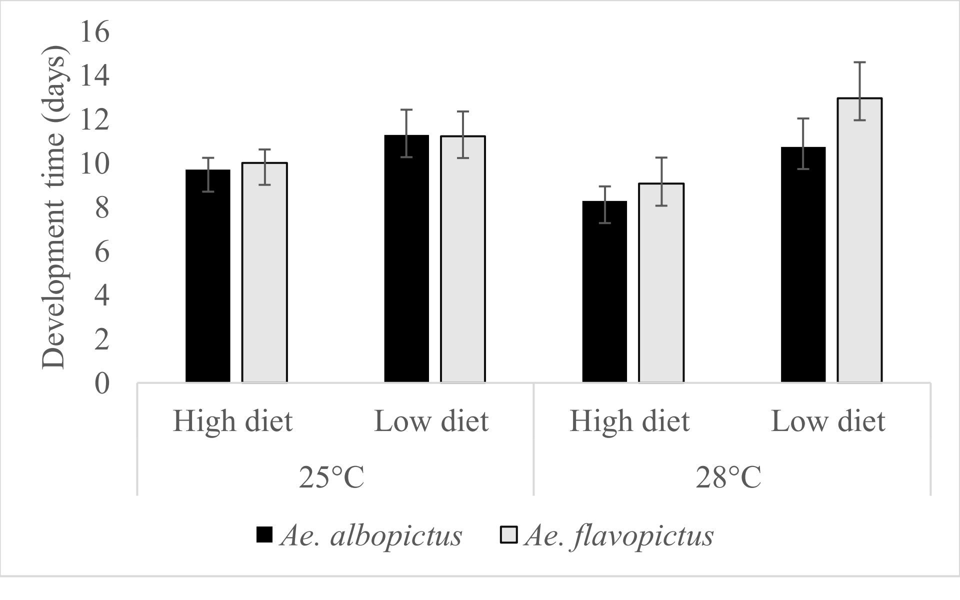
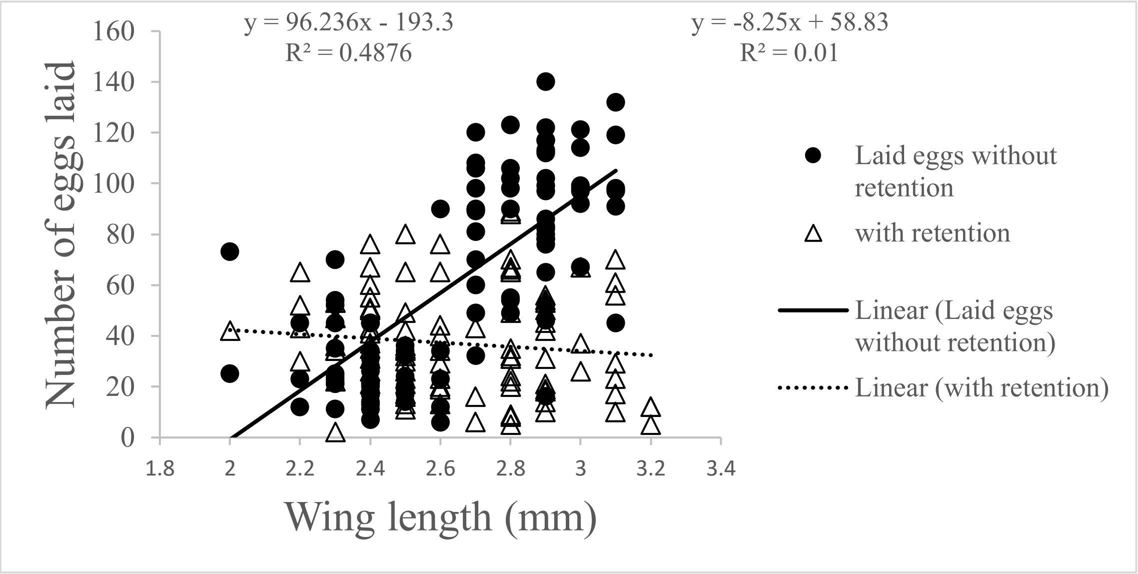


Figure 6. Mean development time (days), by larval rearing temperature (25°C and 28°C) and level of diet (high, low) of *Ae. albopictus* and *Ae. flavopictus*.

Aedes albopictus

A.



Aedes flavopictus

B.

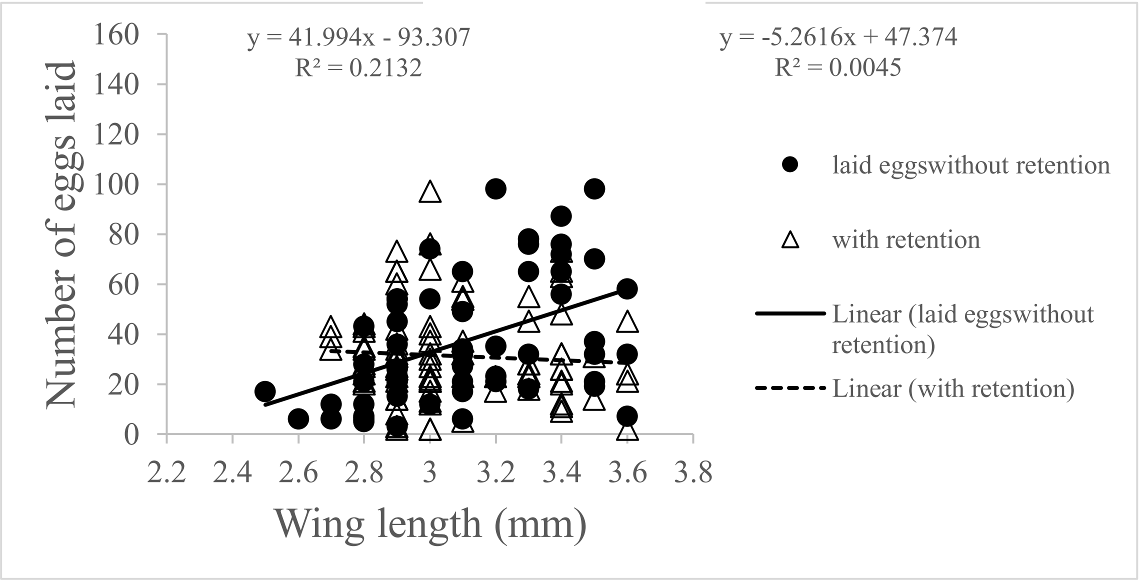


Figure 7. Body size (wing length) and number of eggs laid (retention and without retention) in (A) *Ae. albopictus* and (B) *Ae. flavopictus*. Dark circle indicates female laid eggs without retention and open triangle indicates female with retained eggs.

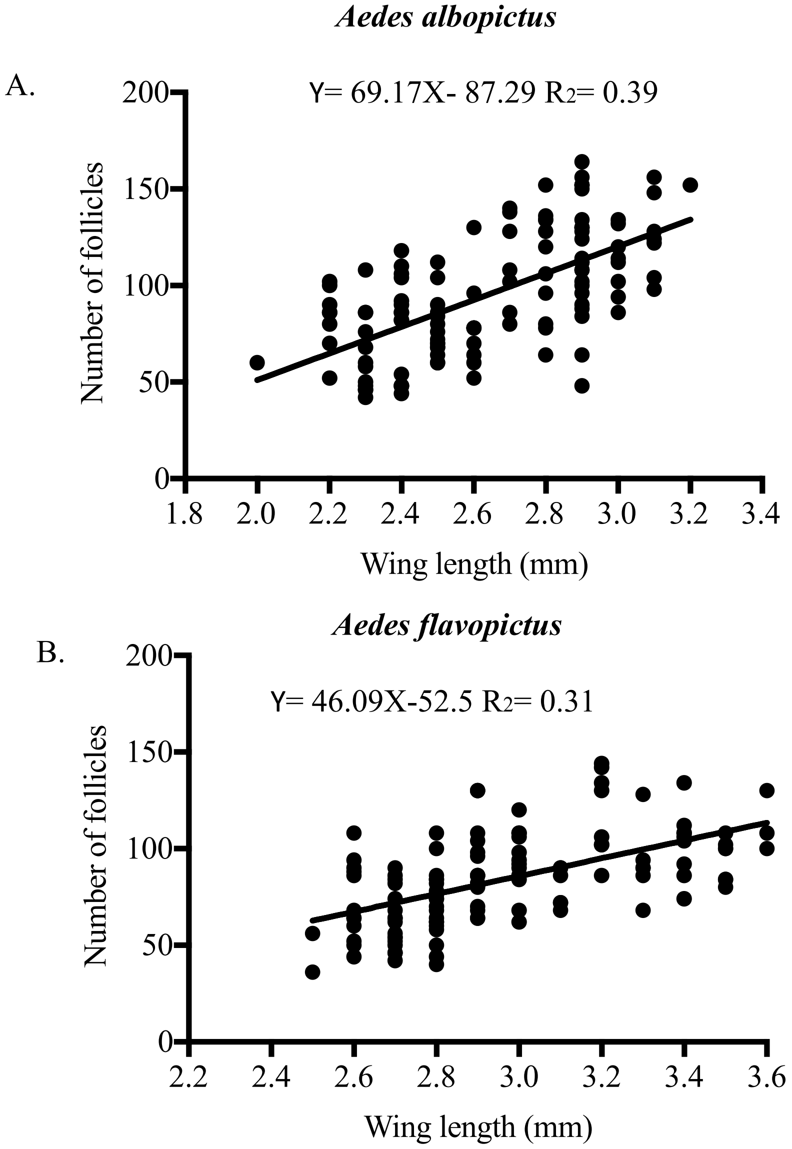


Figure 8. Body size (wing length) and number of follicles in (A) *Ae. albopictus* and (B) *Ae. flavopictus*.

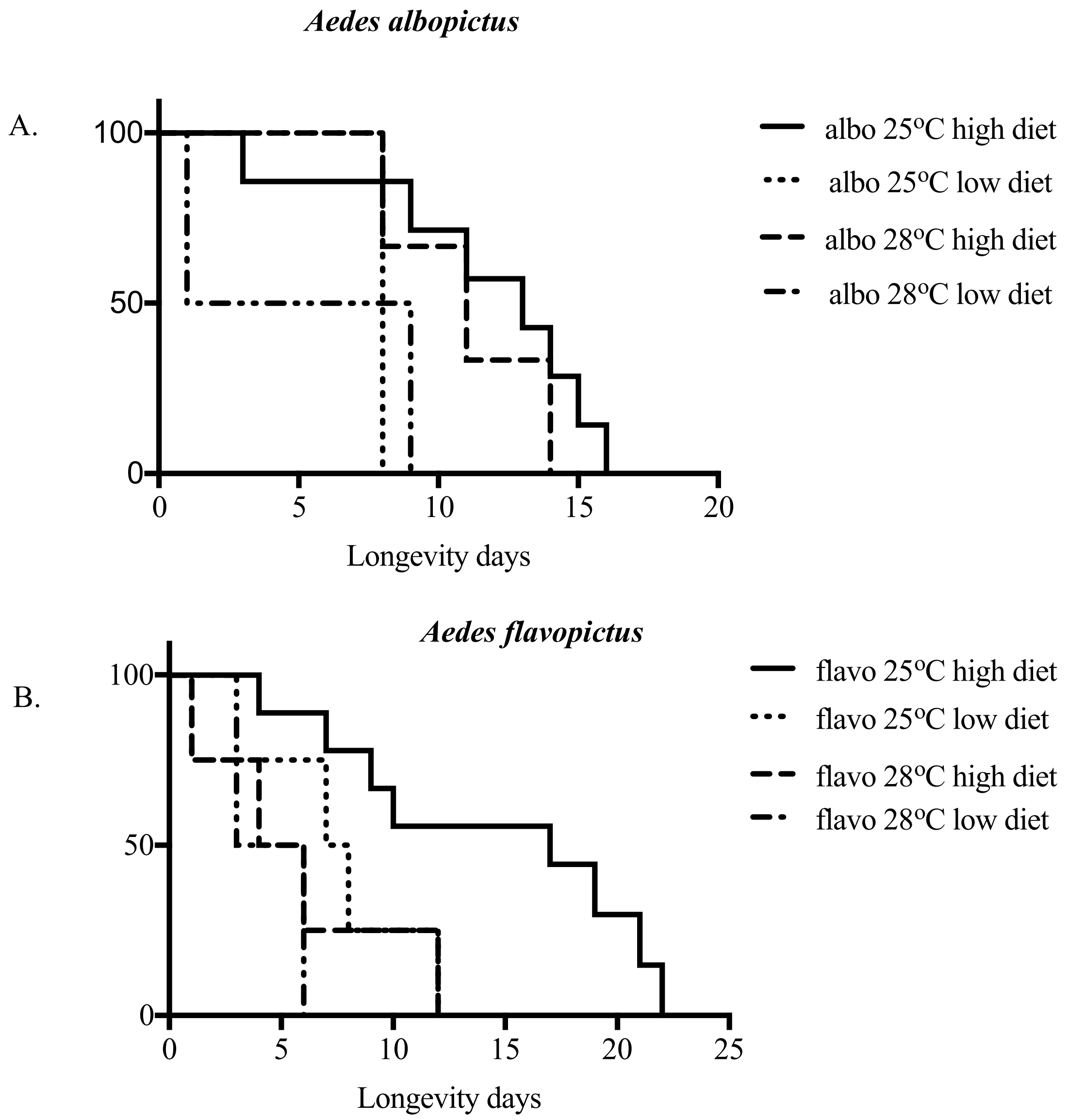


Figure 9. Kaplan-Meier plots of the longevity (days) of (A) *Ae. albopictus* and (B) *Ae. flavopictus* at two temperatures (25°C and 28°C) with two different diets (low and high).

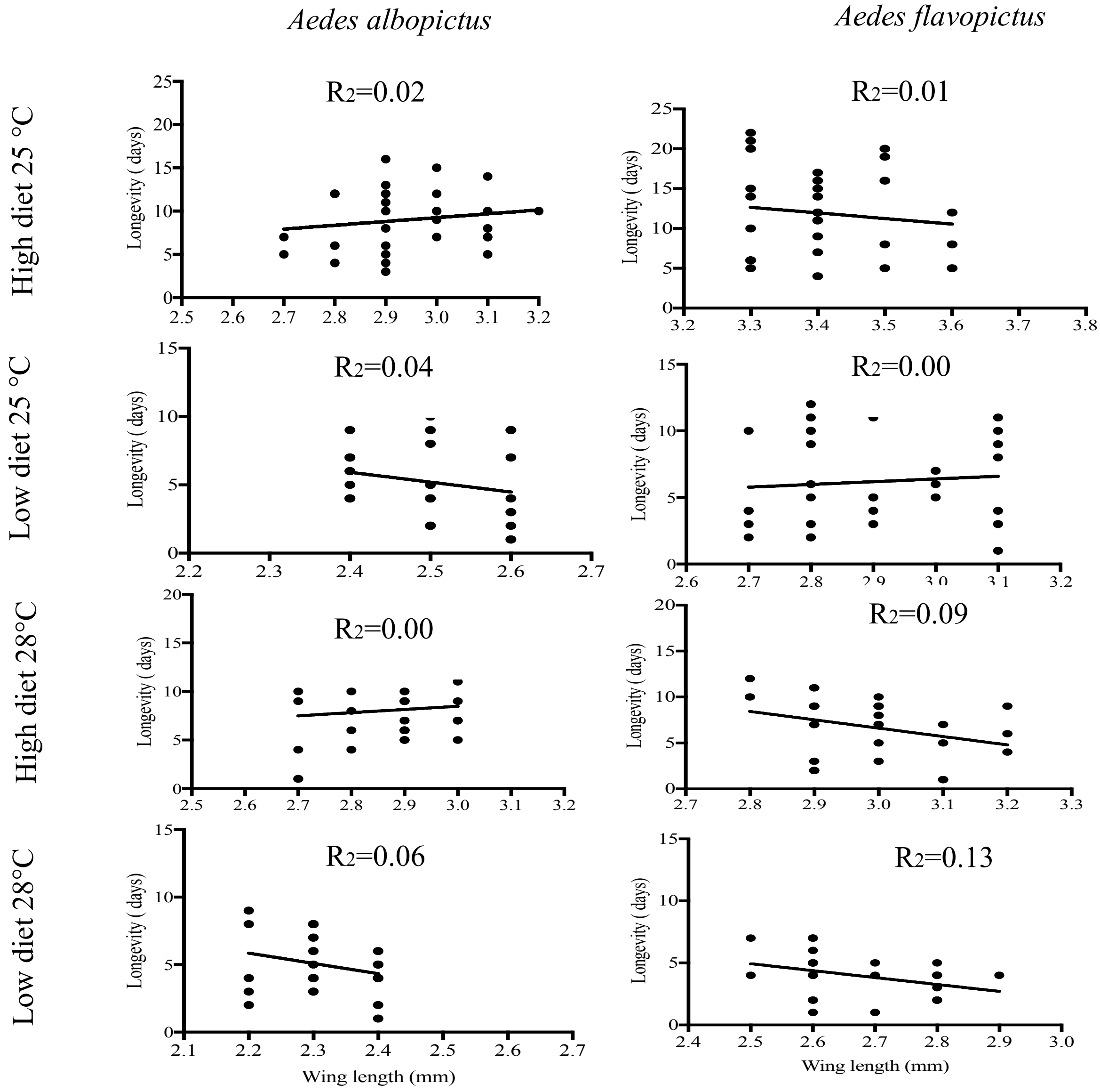


Figure 10. Relationship between longevity (days) and body size (wing length) of *Ae. albopictus* and *Ae. flavopictus* by larval rearing temperature and diet treatment. The equation of the regression of longevity on wing length is given with the R2 value.

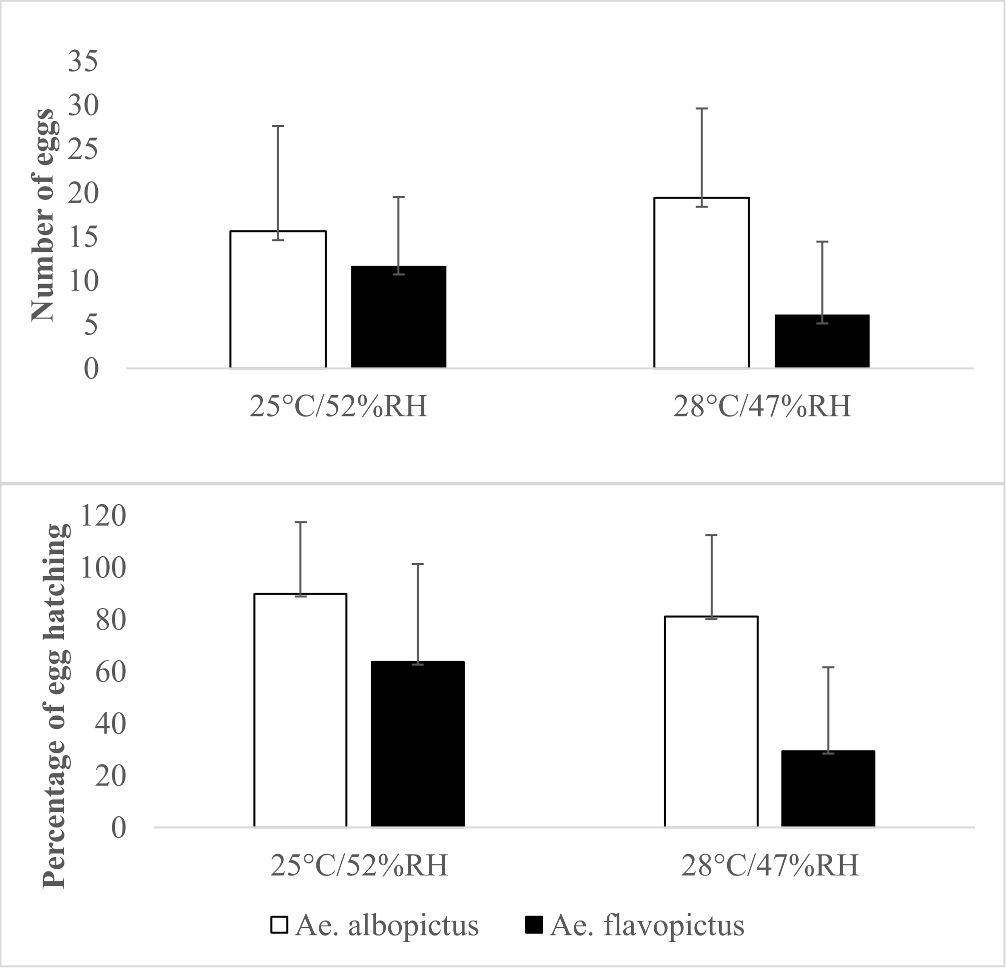


Figure 11. Number of eggs and egg hatching response of *Ae. albopictus* and *Ae. flavopictus* at two temperatures (25°C and 28°C) and humidity (52% and 47%RH).

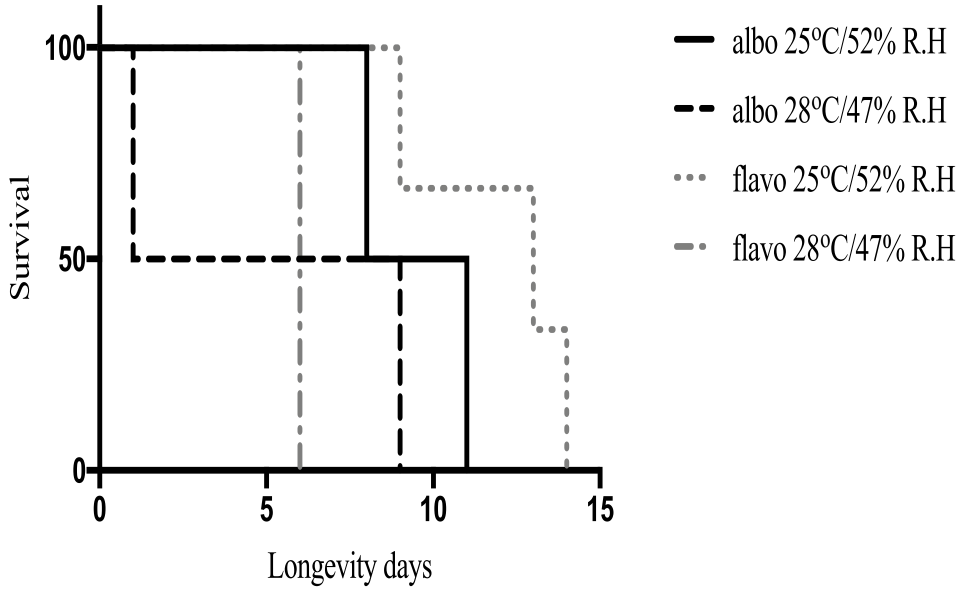


Figure 12. Kaplan-Meier survival plots of *Ae. albopictus* (albo) and *Ae. flavopictus* (flavo) females at two temperatures (25°C and 28°C) and humidity (52% and 47%RH).

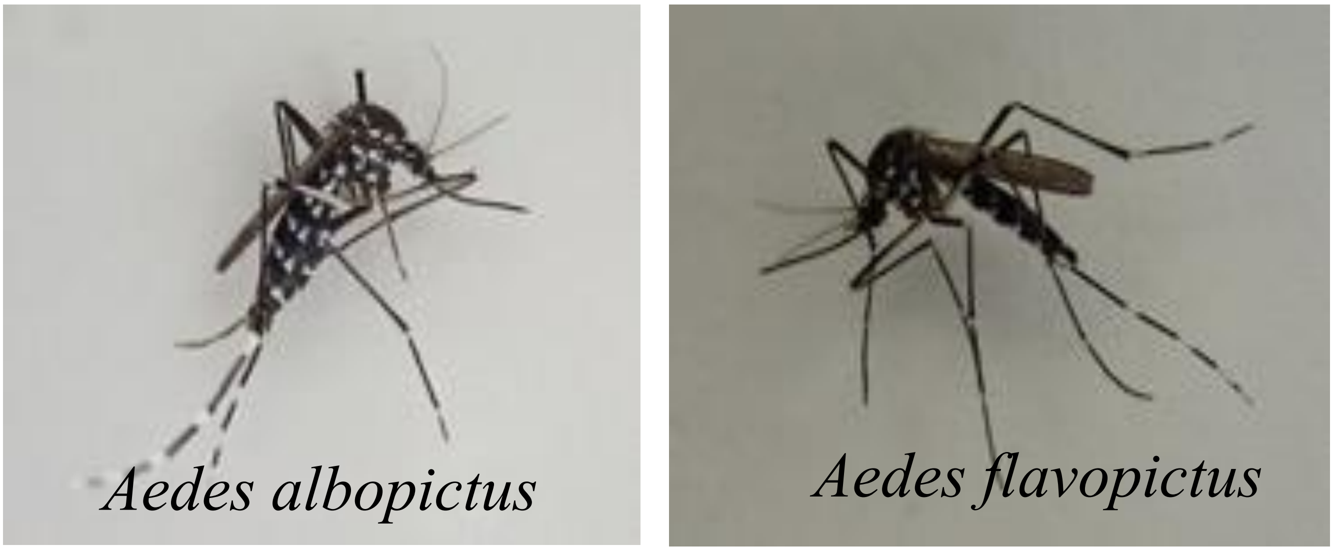


Image 1. Photograph of *Ae. albopictus* and *Ae. flavopictus*.

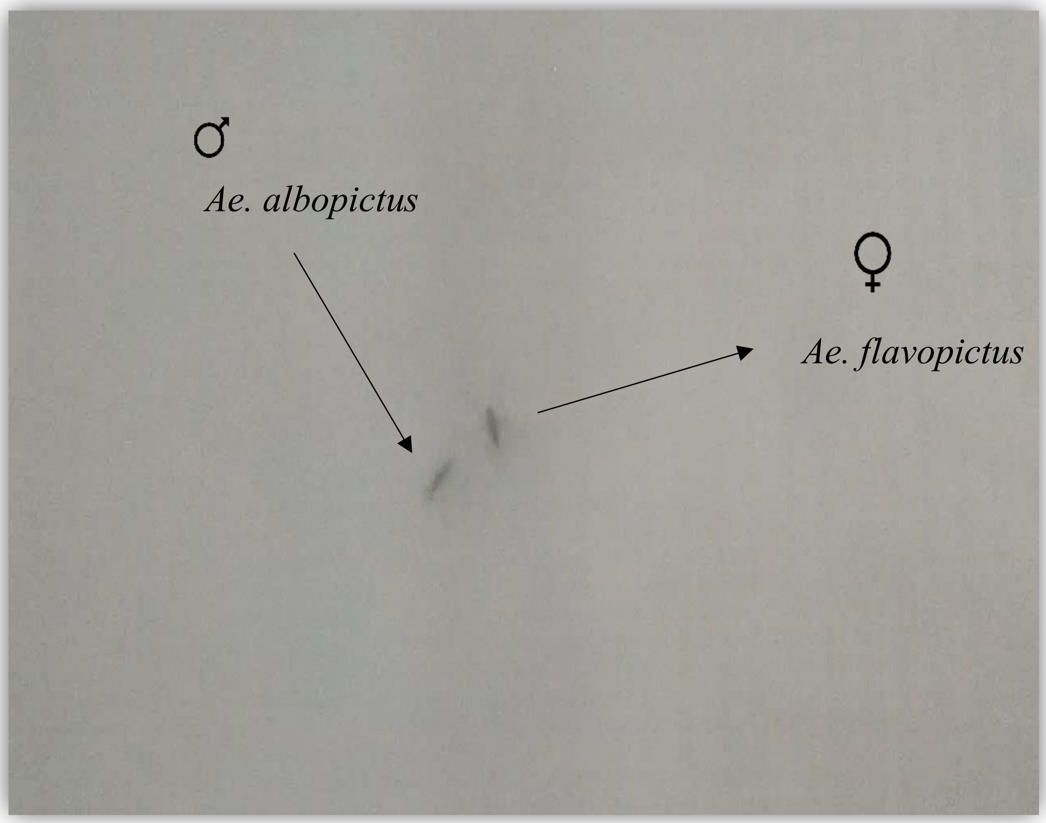
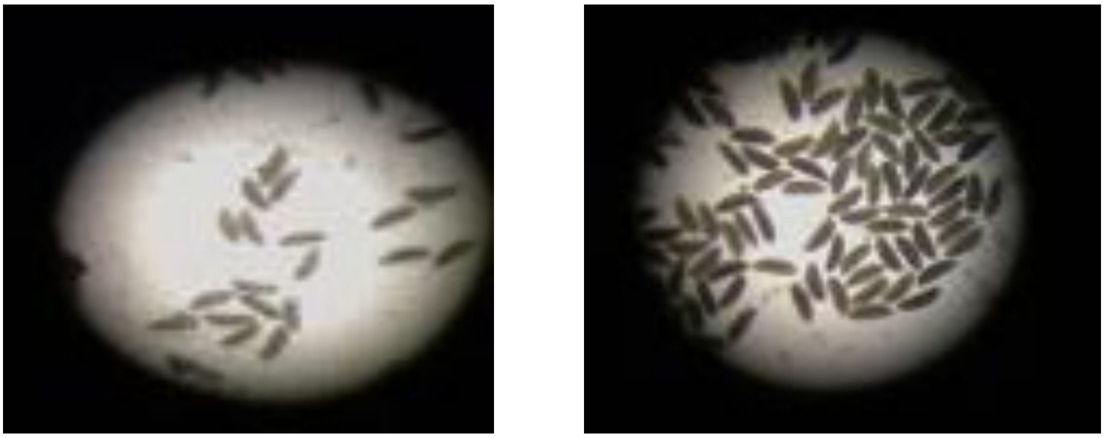


Image 2. Photograph of heterospecific mating between *Ae. albopictus* and *Ae. flavopictus*.

Image 3. Photograph of sterile eggs.



Aedes albopictus *Aedes flavopictus*

Image 4. Photograph of retained eggs in ovaries.

Biographical Sketch

Kanazawa, Japan, 2020

The author, **Sultana Afroza**, was born on March 1, 1983. She took her Primary education from Rajarchar Primary School, Secondary education from Makahati G.C. High School, and Higher Secondary education from Begum Badrunnessa Mohila college. She graduated from Dhaka University, Dhaka, Bangladesh, with a Bachelor of Science degree in Zoology and completed a Master of Science in Entomology, where she received Dean award, Rajia Memorial Gold Medal and Silver Jubilee award. After completing graduation, she joined in Bangladesh International School and College, as an Assistant teacher (Biology). After that, she had joined in the Department of Zoology, Jagannath University as a lecturer in 2013 and promoted as an Assistant Professor in 2017. She got final selection for MEXT fellowship, Japan to pursue Ph.D.

Afroza Sultana

THE END