WITHDRAWN:
Human Blood Type Influences the Host-Seeking Behavior and Fecundity of the Asian Malaria Vector *Anopheles Stephensi*.

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The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

The nutritional requirements of mosquitoes include both sugar (generally derived from the nectar of flowers) and blood (humans or animals). Mosquitoes express different degrees of preferences towards hosts depending on behavioral, ecological, and physiological factors. These preferences have implications for mosquito-borne disease risk. The present study is directed to reveal the effect of the human blood groups on the fecundity and fertility of the malaria vector *Anopheles stephensi*. In laboratory tests, mosquitoes were fed on ABO blood groups via artificial membrane feeders, and the level of attraction against different blood groups was tested by the electroantennogram and wind tunnel bioassay under control conditions. Overall, the human blood type had a significant impact on the fecundity and fertility of female *An. stephensi.* The highest numbers of eggs are laid, in the case of blood group B, (mean (±SD)) 203.19 (9.45) followed by the AB, 105.75 (4.51), and O, 98.8 (3.98). In the case of blood group B, females attain the highest fertility of about 89.98 (5.43%). This study provides novel insight into the ABO host choice of the mosquitoes that are still partially unknown and suggests more personal protection, which is a useful tool for the prevention of malaria.

1. Introduction

Mosquitoes pose a significant threat to human health due to the close association with the human habitat of many species, the propensity to bite people for blood, and their role in outbreaks of mosquito-borne diseases [1–3]. *Anopheles stephensi* Liston (Diptera: Culicidae) is among the critical vectors of malaria parasites in Pakistan, Iran, Afghanistan, and India [4]. Mosquitoes of the genus *Anopheles* are important vectors of the human malaria parasites *Plasmodium falciparum* and *P. vivax* [5]. As most of the vector mosquitoes are anautogenous, females require a blood meal for the oogenesis [6], and understanding blood-feeding preferences is critical for assessing mosquito-borne disease risk. The volume and quality of blood meal can play an important role in egg production and subsequently influence potential population dynamics and vector competence [7]. *Aedes aegypti* Linnaeus, a globally important vector of dengue viruses, when fed on animal blood, laid fewer eggs than after feeding on the blood of a human [8]. When *Culex quinquefasciatus*, a globally important mosquito of pest and public health importance, fed on bovine blood, it hurt fertility and fecundity compared to when provided chicken blood [9].

Similarly, the source of blood provided to *Anopheles* mosquitoes can impact fecundity with human, cow, and chicken blood, having differing effects over the egg production rate of *An. gambiae*, [10]. Beyond the source of blood, whether it is from animals or humans, the constituents of blood may play an important role with some studies indicating the importance of isoleucine, and amino acid, in the blood, influencing the rate of egg production in some species of mosquitoes [11–13].

An understanding of the attraction of many vector mosquitoes to human hosts could assist in determining the role of individual mosquito species in outbreaks of mosquito-borne diseases, and also

provide critical information to inform mosquito control and surveillance programs [14]. The preferences of some mosquitoes to feed on humans is well documented, especially *Ae. aegypti*, [15]. While many *Anopheles* mosquitoes display a similar anthropophilic feeding behavior, and this is a critical factor in determining the importance of their role in outbreaks of malaria, there is also often a demonstrated difference in host-feeding preferences of *Anopheles* mosquitoes. For example, *An. gambiae* has been shown to have strong anthrophillic feeding preferences while *An. stephensi* has zoophilic preferences [16]. Understanding how these mosquitoes, considered primarily zoophilic, may opportunistically, or at times preferentially, responding to humans in host-seeking behavior is critical.

Female mosquitoes use different cues to locate hosts; physical, visual, and most important are the chemical cues [17]. Organic molecules released from the host body are considered the most important with regard to mosquito attraction. Sweat excreted from the human body has various components that influence attraction, and even small variations in released organic chemicals influence host attraction by mosquitoes [18]. Further, variations in attraction to individual humans have been a source of great interest. Hematophagous female mosquitoes show different levels of attraction towards different human beings [19]. It is assumed that the host-seeking response of a mosquito to humans is directly linked with the body odor [19,20]. For example, *Aedes* and *Anopheles* mosquitoes are attracted more towards pregnant women, possibly because of their increased secretion of estrogen in urine [21,22]. The perception that some individuals are more likely to be bitten by a mosquito than others is frequently debated, and differences in human odor profiles and the response of mosquitoes have been a focus of research [23,24]. While an individuals' odor profile may be complex and vary due to a range of factors, blood type is often proposed as a determinant of propensity to be bitten by mosquitoes [25].

In this study, the effect of human ABO blood groups on the female mosquitos' oogenesis and fertility was investigated under laboratory conditions using methods of electroantennography and olfactometry bioassays. The investigation aimed to determine that a specific blood group represents the following qualities (a) more attractive than all other blood groups (phagostimulant); (b) fully engorged when feeding; (c) initiated the vitellogenin; (d) must have a positive effect on oogenesis; and (e) positive effects on health and fitness of offspring. There is a paucity of published data on the effect of human blood types on the fertility and fecundity of mosquitoes. We tested the hypothesis that human blood type groups significantly influence specific components of the life cycle of *An.stephensi* under laboratory conditions.

2. Results

2.1. Fecundity and fertility

Female *An. stephensi* that were fed on blood group B laid an average of highest numbers of eggs 203.18 (8.81), whereas those fed on blood groups O and AB laid an average of (mean (± SD)) 105.74 (7.04) and 98.8 (7.67) eggs per female (Fig. 1). The blood group B acted as the best nutrition for the female

mosquitoes laid the highest mean numbers of eggs per engorged female of 150 (11). Mosquitoes fed on blood group A laid the lowest mean numbers of eggs 65 (3); this is in line with the results from scanning electron microscope with the lowest development in the oogenesis was recorded. The data suggest that fecundity, fertility, and oogenesis are directly linked with the blood groups and the availability of the blood types.

2.2. Digestibility

A difference was observed in the rate of digestion of different blood groups after 12 h with visible differences in specimens of engorged mosquitoes according to the human blood group types provided. Approximately 6 h post-feeding, those fed on blood group A partially changed into black while those fed on B and O completely changed into black. However, the abdomen of females fed on the blood groups, AB, still maintained a reddish color. After 12 h of the female having O blood groups in their stomachs showed two-third of the abdomen distended. After 24 h, the O blood group was half-digested in the abdomen while the AB batch has changed the color of the abdomen red to black, and the females engorged on blood group B have abdomen slightly more than three quarter still filled with blood. After 48 h, the O blood group color completely disappeared from the abdomens of all the females. In contrast, the blood groups A and B disappeared after about 60 h of post fed. The AB blood group was the last one, which was disappeared after 72 h from all blood groups.

The percent rate of digestion of different ABO blood groups after feeding by female *An. stephensi* under control condition was shown in (Fig. 2). The blood group O has the highest rate of digestion, and it is significantly different from the other blood groups ($p < 0.0001$). The rate of digestion of 50% blood at a time in hours (h) by visual observation of the different blood groups varied from the 26 h for blood group O to 53% for blood group AB.

In the case of chemical tests, a positive reaction was observed for the precipitin and benzidine tests up to 100 h in the case of the AB blood group after the engorgement. Defaecated matter collected from the cages of each blood group also gave positive results.

2.3. Effect on oogenesis

By the use of a scanning electron microscope (SEM), two clusters of ovarioles were identified in the ovaries of the female fed over blood group B, 36 h post engorgement. While the development of ovaries commenced after 48 h in the case of blood groups A and AB. The result indicated that after the single engorgement of blood group O, the development of ovaries had not started. But here in this study, the females were selected after 36 h and were presented to SEM to test the ovary development stages. The ovarioles were situated in the middle of the spongy fat bodies like structure (Fig. 3). After 36 h, the ovaries development in female mosquitoes fed on blood group B looks mature. While the females fed over blood groups A, AB and O are still under immature conditions 36 h post-feeding (Fig. 3).

The total number of ovarioles that were in all females mosquitoes fed on different blood groups ranged from 100 to 600, strictly linked upon the blood groups [26]. The highest numbers of ovarioles were observed in females fed on blood group B; all the three females have, on average of 550 ovarioles. The lowest numbers of ovarioles were counted in the female fed on blood group O about 150 ovarioles (Fig. 3).

2.4. Electroantennography

The electroantennography (EAG) experiments were conducted to test the response of different olfactory receptors on the antennae. First of all, the electroantennogram responses of *An. stephensi* were recorded against the known olfactory stimuli, including lactic acid, 1-octen-3-01, and isovaleric acid. After recording the results of control stimuli, the treatment stimuli were applied, and the response was recorded. The 5–7 days old unfed female *An. stephensi* mosquitoes gave highly blood group linked EAG response. Air current from the blood group B indicated a significantly stronger response over the other blood groups. The amplitude response for the other blood group O is lower than the B. While the A blood group is the least attractive one blood group because the female showed the lowest level of response amplitude (Fig. 4A). The electroantennogram response is directly related to the time of air current in seconds, as the time of air current increases, the olfactory response of antennae was also increased. All the blood groups led to a significant response against the control of air currents (Fig. 4B).

2.5. Wind Tunnel Bioassays

In wind tunnel bioassays, *An. stephensi* exhibited a clear preference for human blood type B, as evident by the significantly higher number of mosquitoes attracted to that human blood type (Fig. 5). The second and third most attractive blood groups were the O and AB, while there was no significant ($P = 0.1454$) difference between the number of mosquitoes attracted to blood group A compared with control.

Similarly, *An. stephensi*, when exposed to olfactory cues derived from volunteers of differing blood group types, displayed a strong preference for blood group B compared to other blood groups (Fig. 5). The second most important stimulus was the O blood group, while the A and AB were the least attractive blood groups. The blood group B was highly significant for all five replications, resulted in the following mean percentages of female mosquitoes flying into the five-arm wind tunnel treatment and the control.

2.6. Mosquito Fitness

The female fed on blood groups B and O showed the highest level of mosquito fitness, as evident by the lowest mortality rates for eggs, larvae, pupae, and adults (Fig. 6). When compared with the females fed on blood, groups A and AB have a significantly lower preference, and they have high larval mortality, egg infertility, and adult death rate.

2.7. Feeding rate and egg production

About 91% of the female fed on blood group B were fully engorged compared with the 70%, 54%, and 45% in the case of blood groups O, AB, and A (Fig. 7). On the other hand, the percent numbers of eggs were also higher in the case of females fed on blood group B (Fig. 8A). The rate of egg production and engorgement with blood group B increased more than 12% relative to blood group O and 55% in the case of A blood type (Fig. 8B), suggesting that the blood group B can be highly beneficial and attractive to female mosquitoes.

3. Discussion

This is the first time the host-seeking and reproductive response of *An. stephensi* to human blood group types has been undertaken. The findings of these laboratory studies suggest a strong preference in host-seeking of individuals with type B blood group with concomitant increased fecundity of mosquitoes feeding on that blood. Our results reflect those of other researchers who have found that the source of blood-fed on by mosquitoes of pest and public health importance influences fecundity and other aspects of their life cycle.

The amount and the quality of blood meal is a vital thing to female mosquitoes for their gonotrophic cycles and rate of egg production [27]. However, the variation in the egg production rate and the number of females moving towards their first gonotrophic cycles is strictly linked with the amino acids and the protein contents in each blood meal [28]. Djamila et al. (2016) evaluated the effects of two blood meals; the chicken and cattle blood, on the egg development rate of *An. maculipennis* and found fecundity were significantly lower with the chicken blood as compared with the cattle blood, while fertility is not associated with blood meals [29]. In contrast, *Ae. aegypti* has significantly higher fecundity and fertility when fed on human blood as compared to the pig and sheep blood [30]. The hatching rate of the eggs was also associated with the type and the size of blood meals [30]. The fertility, fecundity, hatching rate, developmental time of the larvae of *Cx. theileri* is strongly associated with the source of blood meals. The fecundity of females fed on chicken blood was significantly higher than the females fed on human and cow blood [31].

We have analyzed the rate of digestion of different blood types by *An. stephensi* female mosquitoes by several methods; observational studies, precipitin test, and benzidine test [32–34]. The visual and the tests indicated that the blood group O is the blood that could easily be digested by females in all three replications. This may be because of the chemical structure of the O blood group; it is simpler than the other blood groups in case of antigen absence.

All the anautogenous female mosquitoes required blood meals to start and complete their oogenesis or egg development mechanism [35]. Most of the previous studies concluded that the proteins contained within the blood meal have a vital role in the start of oogenesis [35]. However, not all the blood meals are equal in energy for all the anautogenous species of mosquitoes, and switching sources of blood meals from preferred hosts may adversely impact reproductive capacity in some mosquito species. The female

Ae. aegypti fed on bovine blood starts early oogenesis than fed on human blood [8], while the fecundity of the *Cx. pipiens* fed on chicken blood is significantly higher than the females fed on animal blood [13]. The female *Anopheles* mosquitoes fed on a rich liquid diet (amino acids, vitamin, carbohydrates, proteins, cholesterol, and phagostimulant) shows the oogenesis at a comparable rate to the females fed on blood [36]. Several methods are used to study the egg development mechanism or oogenesis in female mosquitoes. But most important and widely used of them are a light microscope, scanning electron microscope and fluorescent confocal microscopy [37–39]. In this study, we demonstrated that SEM was an effective method to study the ultrastructure of ovaries of female mosquitoes fed on different blood groups.

Experiments on females fed on ABO blood groups and control reveal the different levels of changes in the structure of reproductive organs that lead to the series of changes in cell structure. Blood type B elucidated the significantly higher changes in the cell structure and ovaries development. One of the protuberant changes in the cell structure of females fed on blood group B a large number of un-oriented microvilli in the area of oocytes (Fig. 3). The same type of microvilli was also observed in the case of females fed on blood groups O and AB but few in numbers and small in size. Results indicated that these microvilli are not uniform in length, and these are not regularly present in all females fed on different blood groups. This type of results is in line with the Brandt [40] microvilli are not uniform in length [39]. There are two clusters of ovarioles identified under an SEM in the ovaries of females mosquitoes fed on blood group B, and slightly smaller clusters are detected in the case of blood groups O and AB. Females ingested the blood type A showed the contrary results; there are no or very few microvilli are identified all the females of all the replications. Sponge-like fat bodies surround these clusters of ovarioles. The results are also in line with the scanning electron microscopy results obtained by Clements [35]. The total numbers of ovarioles were ranged from 120 to 650 in the case of blood group B and the 60 to 400 in the case of O and AB blood groups. There are only 20–30 ovarioles were identified in the case of females fed on blood group A. The results are also in agreement with Roth and Porter [41], who described the oogenesis in *Ae. aegypti*.

Most of the autogenous mosquitoes detect the volatile semiochemicals by the use of olfactory neurons present on antennae. Earlier studies indicated the EAG response of *An. gambiae* towards the volatile compounds of human sweat and the carboxylic acid from the cheese [42,43]. About eight volatile compounds were identified from the chicken feces that provoked the electroantennogram response from the *Cx. quinquefasciatus*, [44]. These volatiles also derived the behavior of female *Cx. quinquefasciatus* under control conditions. The female *Ae. albopictus* showed the inverse dose-dependent EAG response against certain fatty acids and alcohols derived from the human skin emanations [45].

In the case of the present study, substantial electroantennogram responses were displayed by the antennae of halted mosquitoes against known stimulants L-lactic acid, 1-octen-3-01, and isovaleric acid. After confirming the sensitivity and response of antennae and the EAG towards the human-specific known stimulants, treatment stimuli ABO blood groups were applied and demonstrated a positive

response of *An. stephensi* to the blood group B. The results from the electroantennogram response clearly stated that the blood groups have significant effects on the behavior of female *An. stephensi*; the blood group A is the least attractive. The unique negative behavior was also observed when a blend of odors of human blood was released; it indicates that the females might be able to differentiate the odor of different blood groups and also alter the response accordingly. However, it will be crucial to understand the relatedness of blood type and mosquito attractiveness given there may be additional considerations, such as skin biota or other skin-associated chemicals, beyond blood group type that may influence the host-seeking behavior of mosquitoes.

Artificial blood-feeders with collagen membranes are an effective method to evaluate attraction responses of blood and the chemical compounds released by the human skin and the sweat. The chemical volatiles permeated from the collagen membrane attracted the female mosquitoes and indicated the landing behavior in choice assays. In most of the previous studies, the collagen membrane was used in artificial membrane feeders to study the hematophagous behavior of female mosquitoes [46]. The blood emitted chemical volatiles that attracted the hungry female mosquitoes. Female *Cx. quinquefasciatus* and *Ae. aegypti* showed different levels of attraction towards the avian and bovine [46]. Blood composition and concentration are the key drivers of this type of behavior in female mosquitoes, mainly in a wide choice. Along with the blood second, the most important driver is sweat volatiles; these volatiles affects the behavior and landing response of *An. gambiae* and *Ae. albopictus* [43,45]. Both the electroantennogram and wind tunnel bioassays were conducted based on the strength of the anemotactic response of *An. stephensi*. In the case of the present study, both tests conducted in the olfactometer indicated that blood groups have a substantial impact on the behavior and fertility and fecundity of the female *An. stephensi* mosquitoes. In both, cases blood group B acted as a mosquito magnet so that the human-specific blood groups may act as an important part for the identification of autogenous female mosquitoes such as *An. stephensi.*

In conclusion, the present study supports the hypothesis that blood groups have effects on the fertility, fecundity, and behavior of female *An. stephensi*, just like *An. albimanus* [46]. *An. stephens*i is a well-known vector involved in the transmission of malarial parasites (*P. falciparum* and *P. vivax*) and given blood from different vertebrates affects the behavior, fertility, and fecundity of mosquitoes, our results suggest this may be due to differences in the chemical composition and concentration of blood of vertebrate [29,46]. Significant differences were observed in both the host-seeking behavior in response to differing ABO blood group types together with concomitant differences in fecundity following blood-feeding by *An. stephensi* in a laboratory setting. The results of this study represent an important breakthrough in the field of parasitology and malaria control. It provides clear insights about the behavior of female mosquitoes that not only may have implications for determining in those in the community most at risk of exposure to malaria parasites, and consequently possibly prioritized for anti-malarial medication. It opens potential opportunities for the development and adaptation of novel mosquito control and surveillance strategies that exploit the host-seeking behaviours demonstrated here.

4. Methods

4.1. Rearing

All mosquitoes used in these experiments were derived from a laboratory colony of *An. stephensi* is initially established in University College Agriculture, University of Sargodha. Uninfected mosquitoes were maintained in the laboratory; in gauze covered boxes (30 cm width × 30 cm height × 50 cm depth) under control condition 27 ± 2 ºC temperature and 75–80% relative humidity. Auto ON/OFF switches with the timer were used to break the scotophase (dark) period in the control conditions of the laboratory with the light: dark cycle set to 12:12 h [47]. A 10% fructose solution supplemented with 0.05% para-minobenzoic acid (PABA) was provided to mosquitoes. The adult mosquitoes were reared on blood provided via an *in situ* electronically derived artificial membrane feeder, set at 37 ± 1 ºC, and offered twice a week given their need for another blood meal approximately 5–6 h after the first [48]. Multiple blood feeding is vital for *Anopheline* species as it has been demonstrated as influencing reproductive behavior [49]. Oviposition cups were provided two days following the second blood meal. The larvae were reared under laboratory conditions as described above and provided a certified Laboratory Rodent Diet (LRD) Lab Diet 5001 [50].

4.2. Fecundity and fertility

To determine differences in fecundity (number of eggs) and fertility (percentage of fertile eggs) in *An. stephensi* mosquitoes, cages of mosquitoes were provided ABO blood groups and control (distilled water) via artificial membrane feeders (as described above for mosquito rearing). The blood was obtained from the blood bank of DHQ (one batch of each blood group was used throughout the study), Chakwal, Punjab Pakistan. A total of three replicate cages were used for each blood type and control. Feeding success was determined to calculate the percentage of fed mosquitoes, and also the numbers of fully engorged female mosquitoes were recorded.

To determine fecundity, females from each blood group removed, killed, and dissected under a microscope, and the numbers of eggs per female were counted 60 h post-blood-feeding. Additionally, to determine oviposition and larval development, 40 fully fed mosquitoes were caged in one of three replicate glass cages with gauze (25 cm width × 25 cm height × 25 cm depth) and provided wet filter papers were placed for egg-laying. The total number of eggs was counted after every 12 h from 48 h until 96 h post-blood-feeding under a light microscope. The total numbers of eggs / 40females / box for each human blood group type for three replicates were calculated.

For fertility estimation, an additional 40 gravid *An. stephensi* from each treatment and replication, including the control group, the females were gently transferred to the cages having triangular Whatman filter paper No.1 by using the mouth aspirator. The egg laid in each experimental and control group was reared in plastic cups filled with distilled water. The numbers of hatched larvae were recorded for a fertility test. While the eggs that could not be hatched into larvae up to day seven were considered as infertile. The number of fertile and infertile eggs was recorded from all experimental and control cages.

The collected eggs from each experimental box were placed into the plastic trays (24 cm width × 12 cm height × 7 cm depth) with water, and the development of mosquitoes was observed until adult mosquitoes had emerged from all pupae for each blood group. The water in these plastic trays was maintained at a constant level throughout immature mosquito rearing. The larvae were fed a certified Laboratory Rodent Diet (LRD) Lab Diet 5001[50]. The rearing was done according to the standard mass rearing of *Anopheles* techniques [51]. Pupae were counted and removed from the tray and placed in cages according to each human blood group type feeding, to allow emergence, and the percent of male and female mosquitoes was recorded. Adult mosquitoes were maintained on a 10% fructose solution supplemented with 0.05% para-minobenzoic acid (PABA) but were not provided with a blood meal. The mortality of adult mosquitoes was recorded daily until total mortality reached 100%.

4.3. Digestibility tests

To test the effect of human ABO blood groups (on the rate of digestion in mosquitoes), the precipitin and benzidine tests were used. The experiment was conducted in controlled laboratory conditions where the temperature, humidity, and day and night periods were maintained as described above. Mosquitoes that had not been fed previously on either a sugar solution or blood were used in experiments. Mosquitoes were provided one of four different human blood group types, as previously described. After feeding the female were kept in the same boxes without any further food and water, and boxes were placed in an incubator where the temperature and the relative humidity was at a constant level (28 ± 2 ºC & 80 ± 5%). The engorged female adult mosquitoes were killed at 8 h intervals, rubbed over the filter paper [52], and the filter papers were placed inside the refrigerator until the test could be conducted. Approximately 48 female mosquitoes were used in each boxed marked for each blood group.

To perform the precipitin test, the physiological saline, along with the filter paper smears, were extracted in a small capillary tube. The specific antiserum was also extracted in the same capillary tube at the end; the change in color, clumping, and cloudiness of the solution indicates the presence of human blood in the tissue smears [53]. To apply the benzidine test, the collected material was heated in a steam oven for 10–12 min at 108–110 ºC. The test was used to check the traces of iron porphyrins in the abdomen of mosquitoes.

4.4. Effect of blood groups on oogenesis

To test the blood specific effects on the development of the ovaries of *An. stephensi*, the ovaries of fully fed female mosquitoes were collected separately from the box of each blood group 36 hours post engorgement. For scanning electron microscopy (SEM), the whole female mosquitoes were selected from each box of every blood group separately. The preparation of specimens for the scanning electron microscope the whole process is divided into two fixations viz, the primary and the secondary fixation. For the primary fixing process, the 2.5% glutaraldehyde in 0.1 M cacodylate buffer was used for the period of 2 h, followed by the three consecutive washing with the same buffer for the 30 min. While for the secondary fixation process, 1% osmium tetroxide was used for the 2 h. Then the samples were rinsed for the final time with the 0.1 M cacodylate buffer three times for 30 min.

The ovaries were dehydrated by using the graded series of acetone (50%, 70%, 80%, 90%, and 100%). The dehydrated ovaries were then transferred to the critical point drying apparatus. The recommended quantity of acetone solution was also poured into the drying chamber to avoid over-drying. Liquid nitrogen was also added into the drying critical point drying chamber. The CO_2 and acetone were allowed to be mixed freely; the same process was repeated eight times to confirm the drying of the specimen. The dried mosquito specimens were mounted over the stubs, and the specimens tubes were coated with a thin layer of silver. Gold-spotted SCD005 was used, and then the samples were photographed with SEM.

4.5. Electroantennography (EAG)

To measure the response of mosquitoes to each human blood group type, EAG recordings from one or two antennae of *An. stephensi* female mosquito was made. Unfed female mosquitoes were anesthetized by the use of CO_2 and were permanently fixed with the reference electrode by the use of spectra 360 electrode gel. It was made sure that the mosquito was completely immobile except the antennae. The tips of the antennae were pressed into the small drop of electrode gel on the recording electrode. Both of the electrodes are silver wires coated with silver chloride with a diameter of 0.2 mm. All of the experimental preparations were done in continuous airflow (600 mL/min, 1.5 m/s) by the Teflon tube of 0.7–0.8 cm diameter, containing about 100mL/min dry air and the 600 mL/min moist air passed through the charcoal filter. At this stage, little modification was done in the structure of the electroantennogram, and an artificial blood feeder of mosquitoes with the membrane was attached to the system.

The blood feeder was packed in a glass jar through which continuous airflow was passed, and this air flow ends as stimuli near the mounted mosquito. The diameter of the glass tube was 0.5 mm, and the flow of air was controlled by the use of ON/OFF switch; three bursts of 0.5 sec of air from the blood jar were provided as a stimulus to host-seeking mosquitoes. All the blood groups were tested systematically together with the control group. The amplifier amplified the generated signals while the recordings were decoded by the well-known software (EAG 2000, Syntech, Hilversum, and the Netherlands). All of the test blood groups were also dissolved separately in tetryl-butyl-methyl ether (MTBE), and about 30 ul of this test solution was applied on to a piece of filter paper (1.5-2 cm). About 20 min was given to the TMBE solution to evaporate from the filter paper leaving behind the blood; then, this piece of filter paper was placed into the Pasteur pipette. In the case of control treatment, distilled water was used, and the same treatment was applied with the distilled water for the test compounds. The stimulus controller C5-01/b, Syntech, was used to inject the odor cues originating from the treated filter paper in the Pasteur pipette into the humidified and filtered air stream directed towards the antennae of immobilized mosquitoes. Olfactory stimuli were tested randomly against different mosquito specimens with a total of five specimens exposed to each of the human blood type groups and control.

To minimize the chances of error and to test the electrophysiological activity of the stuck female mosquito, lactic acid, 1-octen-3-01, and isovaleric acid were used as known stimulants [43,54]. After that, each blood group was replicated three times to record the activity of olfactory neurons of antennae. All the treatments of blood groups were tested randomly, and a regular interruption of control stimulus (0.1%

lactic acid) was done. The regular interruption of the control stimulus was used to control the activity of the antennae. All the stimulants were expressed as a mean percent response to the control treatment. The response of different female mosquitoes to human blood groups was indicated as a mean percent response. The results were analyzed by the use of Student's t-test.

4.6. Wind tunnel bioassays

Wind tunnel bioassays were used to determine the response of *An. stephensi* to four human blood groups (A, B, AB, and O) and control stimuli (distilled water). Wind tunnel bioassays have been used to evaluate the response of *Ae. aegypti, Cx. quinquefasciatus* and *Cx. nigripalpus* towards the blood volatiles [46]. A dual choice wind tunnel was converted into a "five-choice" tunnel with all five glass tubes having glass jars at their end with openings to accommodate an artificial blood feeder. A continuous flow of warm water ensured the blood remained in liquid and produced its specific smell.

A batch of approximately 100 female mosquitoes was released at the downwind end of the tunnel in the air stream coming from the five upwind end chambers. After 30 min, the numbers of mosquitoes in each of the five glass jars were counted. The mosquitoes were then sent back towards the downwind end of the wind tunnel, and the positions of odor cues were changed, including the control. Before the second time release, the fresh air was passed through the tunnel. Again the mosquitoes were released from the releasing box, and the response of the mosquitoes towards the new cues and the number of mosquitoes in each chamber at the upwind end was counted after 30 min; the same process was repeated, and for the third time with randomization.

To test the response of female mosquitoes towards human-emitted olfactory cues, an olfactometer was used in previous studies [43,45]. The olfactometer test was conducted in the control room; the temperature was 27 ± 2 ºC with 70–80% relative humidity. The optimum activity of the *An. stephensi* was observed late at night, so the experiment was conducted at 2–6 AM [55].

Steel balls rubbed in the hands of persons (ten volunteers per blood group) of having ABO blood groups along with the few drops of blood group-specific sweat were placed in the glass jars at the upwind end of the olfactometer. Approximately 100 female mosquitoes were released at the downwind end of the olfactometer from the releasing cage. After 30 min, the total numbers of mosquitoes in each box at the upwind end were counted, including the control. After that, the mosquitoes were returned to the releasing cage; after that, the positions of steel balls at the upwind end of the glass jar of olfactometer were changed randomly to decrease the biases from the data. To remove the smell of a sweat from the olfactory tube after cleaning, the fresh air was passed for about 10 min continuously. Mosquitoes were then again allowed to enter into the olfactometer, and after 30 min, the total number of mosquitoes was counted. The same process was repeated for the third time. The same experiment was repeated five times with different persons and mosquitoes to decrease the chances of error. A new batch of mosquitoes was selected for each experiment.

4.7. Statistical analysis

The mean numbers of eggs of *An. stephensi* were evaluated with the help of a linear model (ANOVA) and Tukey's test on Minitab® software (12.2, version, Minitab). Before performing the ANOVA, with the help of angular transformation (arcsine $\sqrt{x}$), egg viability was also transformed [56] for fertility.

4.8. Ethics statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Declarations

Authors' contribution

Shahmshad Ahmed Khan, Muhammad Anjum Aqueel, Saboor Ahmad, Taimoor Hussain, and Sadia Malik planned the experiments. MAA and SAK conducted the experiments, and SAK wrote the manuscript. Nur Faeza Abu Kassim, Cameron Ewart Webb, MAA, and SA participated in review and editing. NFAK submitted the manuscript.

Conflict of interest

There is no conflict of interest between the authors

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Figures

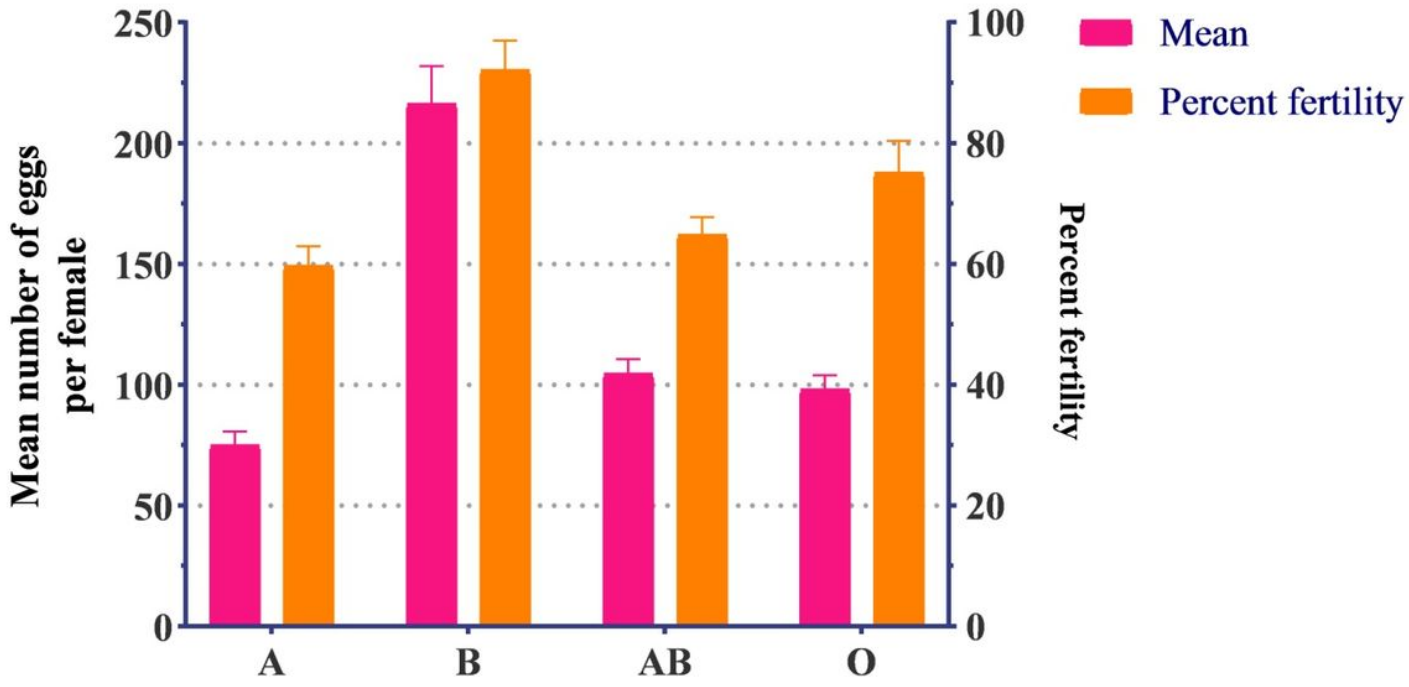

Figure 1

The mean numbers of eggs produced by each female reared on ABO blood groups, and the percent fertility of the next generation on the right Y-axis. Females fed on B blood group have the highest numbers of eggs and the highest ability to produce fertile next generation and the higher number of female mosquitoes while females fed on A group produced lowest numbers of eggs and the lowest ability to produce fertile next generation.

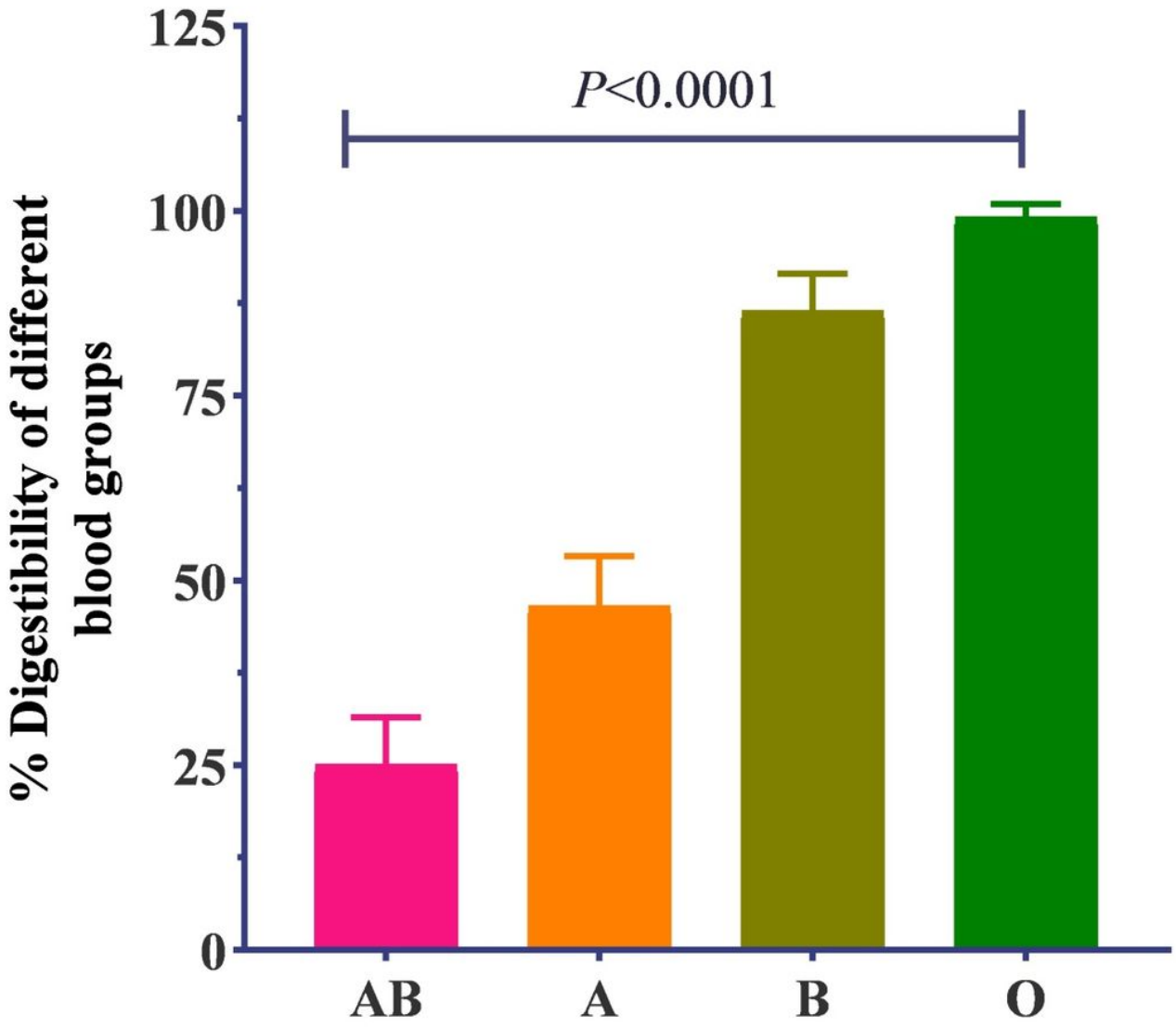

Figure 2

The percent rate of digestion of different blood groups after feeding by female Anopheles stephensi under control conditions. The blood group O has the highest rate of digestion, and it is significantly different from the other blood groups (P<0.0001) A, B, and O, while blood group AB has the lowest rate of digestion.

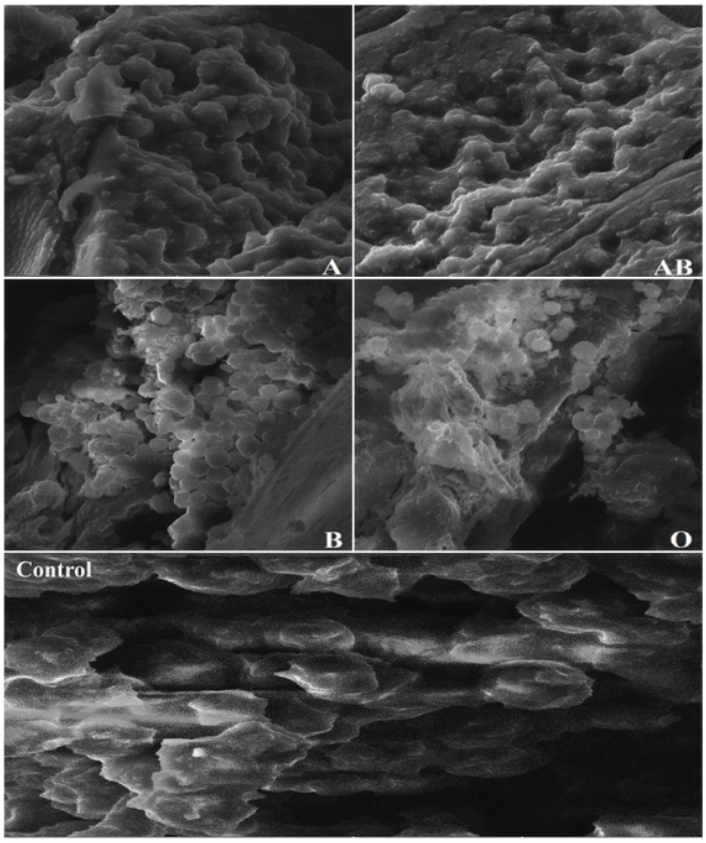

Figure 3

Scanning electron microscope results of the effect of different blood groups (A, B, AB, and O) on the oogenesis (SEM Magnification 2.5kx, 20µm).

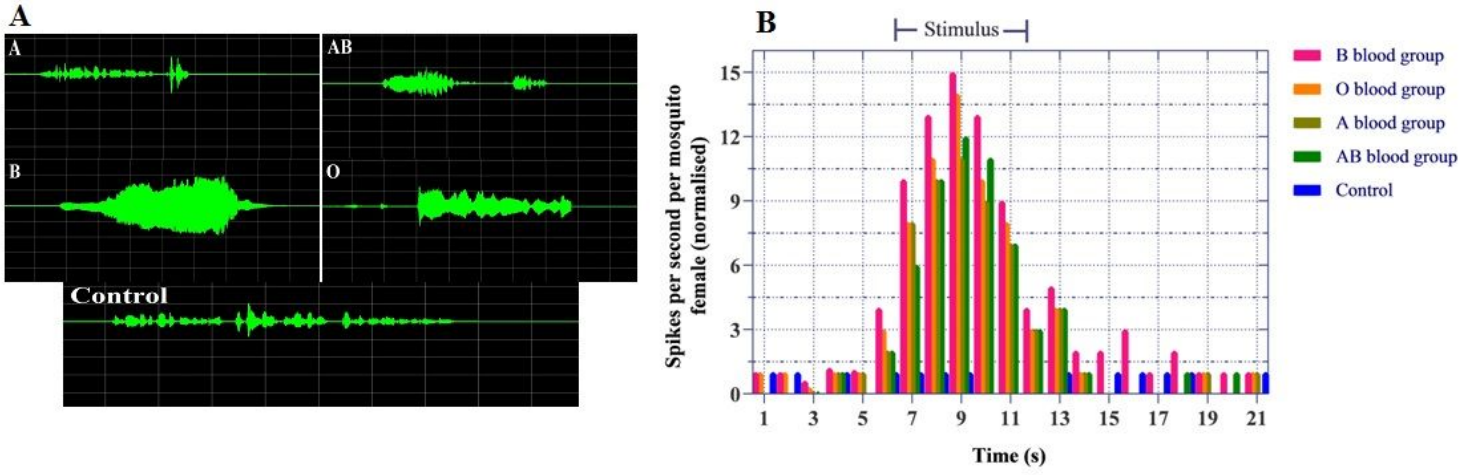

Figure 4

Electroantennogram response of Anopheles stephensi. (A) The electroantennogram response of female Anopheles stephensi antennae to all ABO blood groups along with these scented air puffs was also used to test the response of hanged females. (B) The electroantennogram response to a fumed air puff passed through the ABO blood groups. The graph is showing the observed changes in numbers of spikes of antennae to the different blood group stimuli (applied during the gray box). The value shown is the number of spikes per second, per female mosquito (Anopheles stephensi). A value of 1 (dashed line) indicates no change in spike rate. The control stimuli applied to the antenna: Puffs of unscented air in black color demonstrate the lack of response of the antenna.

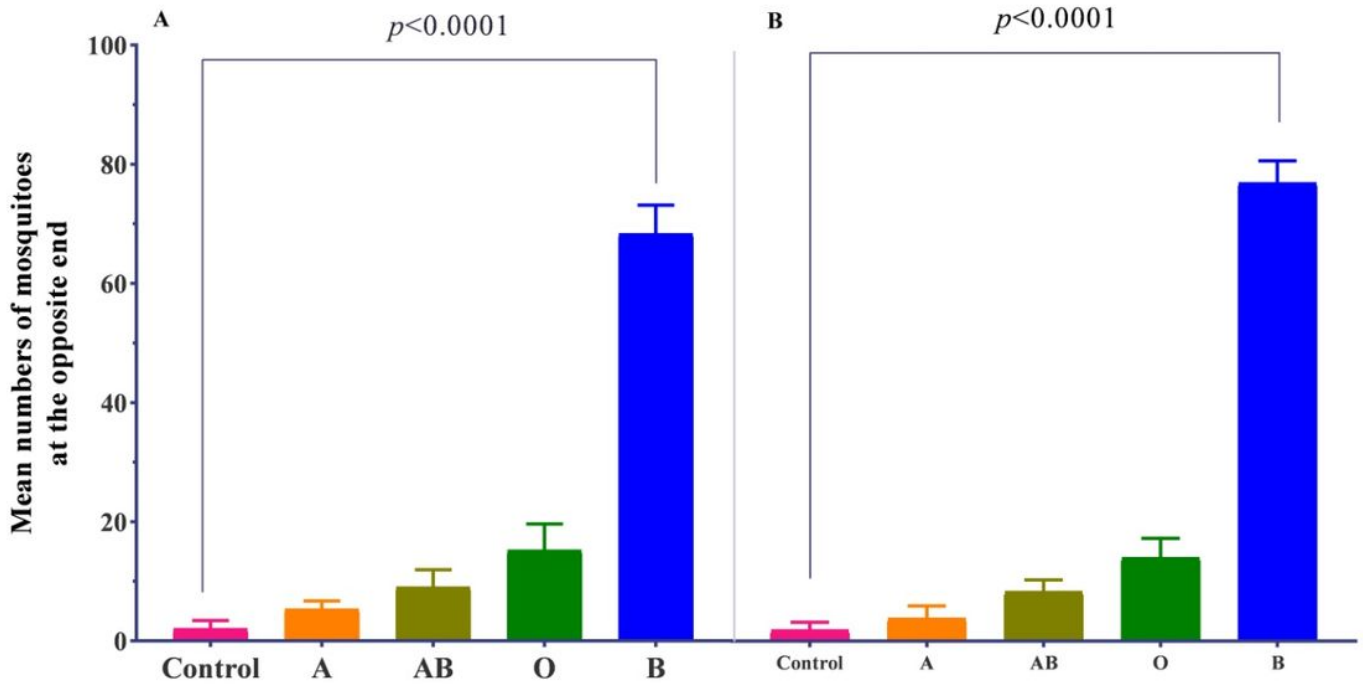

Figure 5

The response of female mosquitoes in wind tunnel bioassay. The plot is showing the response of female mosquitoes against different ABO blood groups. The Y tube was specially modified, and it has five arms at the opposite ends with other modifications to pass through the scented air from all arms. (A) There is no significant difference observed between the unscented air and the blood group A (P=0.0172), and there is also no significant difference was also present between the blood groups A and AB (P<0.0053).The arm having the blood group B have significantly higher numbers of mosquitoes at the opposite end (P<0.0001). (B) The response of female mosquitoes against filth collected from the hands and the armpit of the person having ABO blood groups. The Y-tube was specially modified, and it has five arms at the opposite ends with other modifications to pass through the scented air from all arms. There is no significant difference observed between the unscented air and the blood group A (P=0.1454). The number of female mosquitoes at the opposite end was significantly higher than other blood groups (P<0.0001).

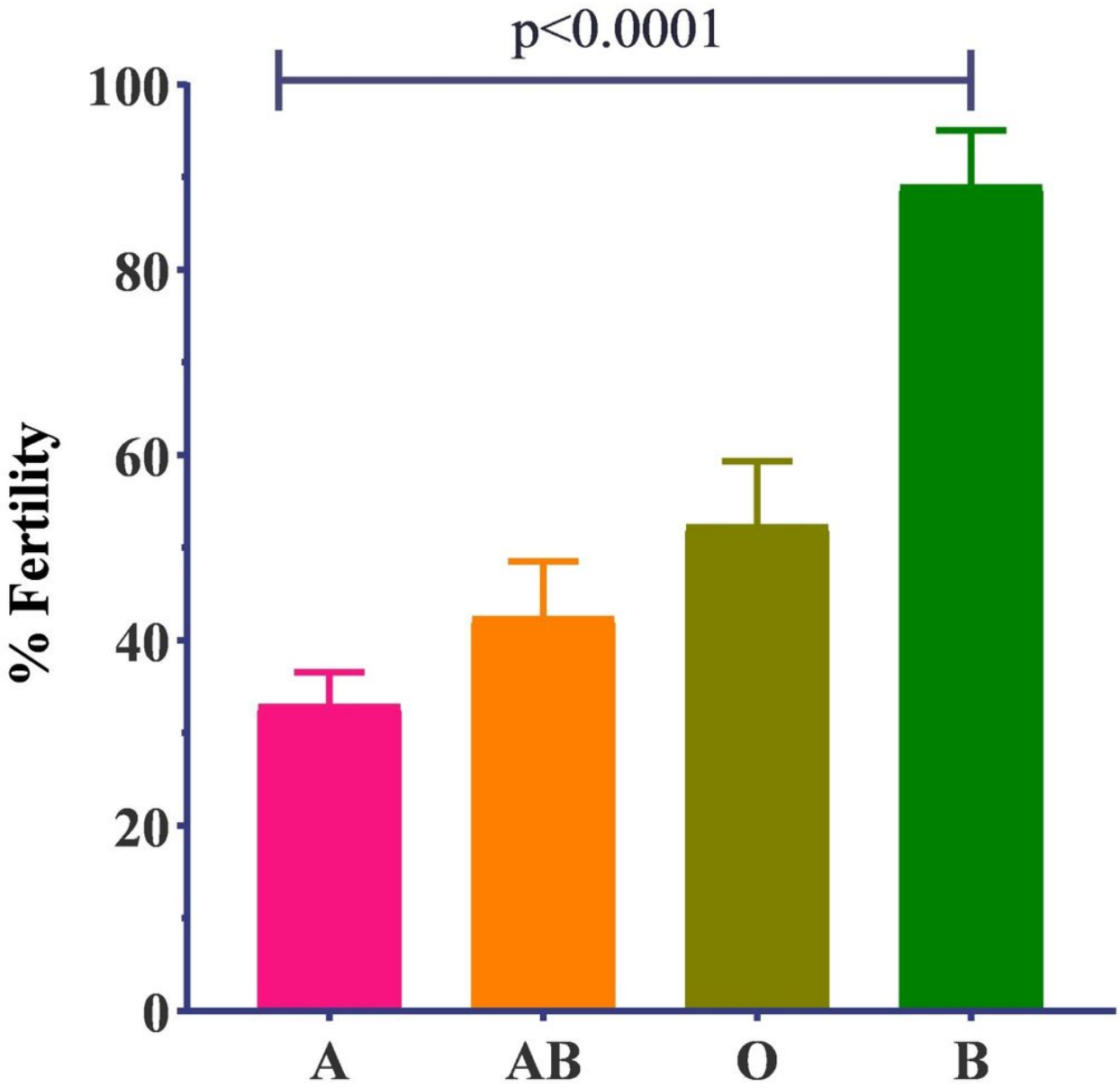

Figure 6

The percent fertility in all the rearing boxes and the in all three replications. The larvae were collected from the females reared on different blood groups and were reared under the same conditions as their parents and were allowed to feed on the same blood groups. To test the fertility level and the percentage fertility in all blood groups, including the A, B, AB, and O. All the blood groups are significantly different from each other, and also the blood group “B” has the highest percentage fertility in the next generation.

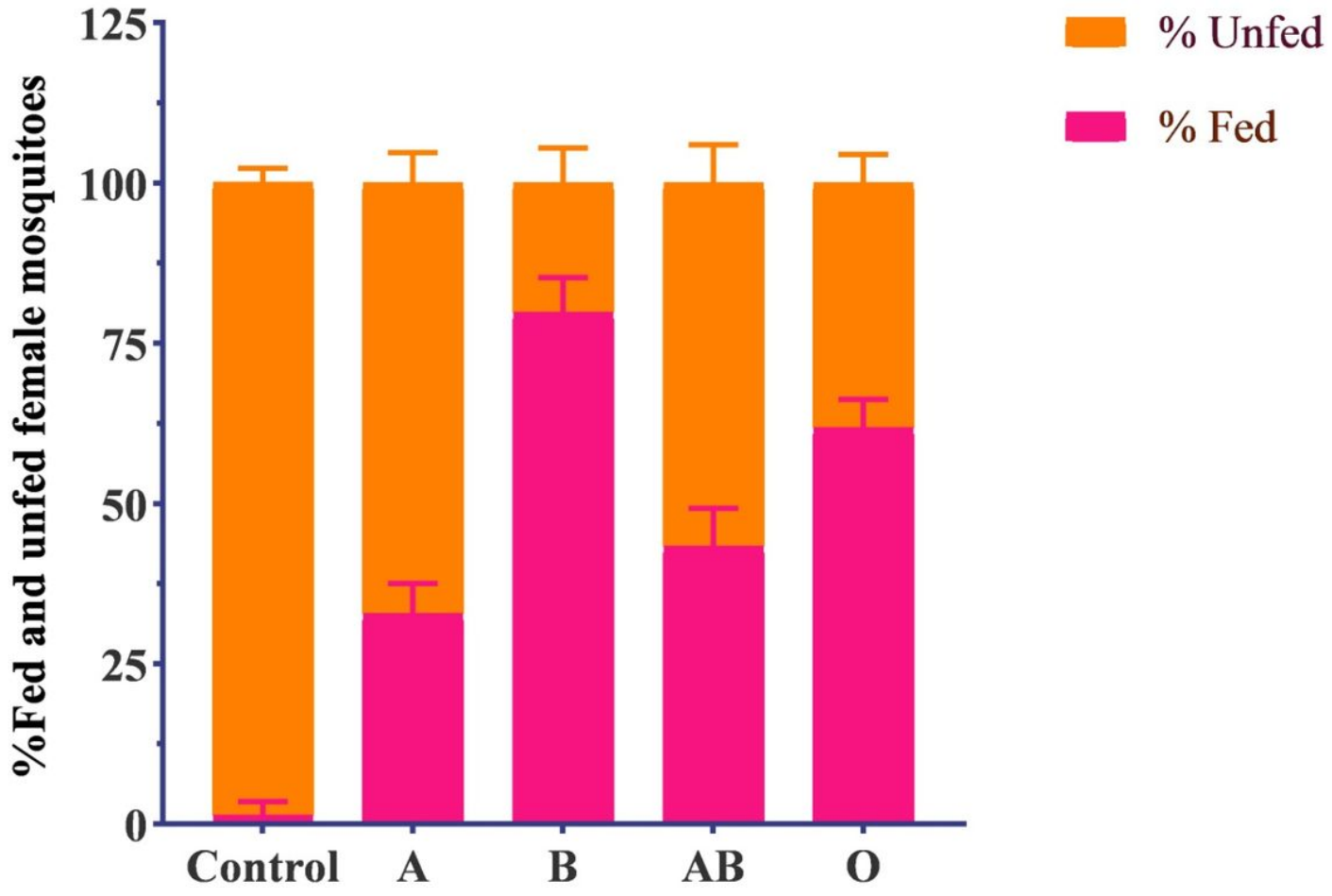

Figure 7

The percent fed and unfed female mosquitoes. The female Anopheles stephensi mosquitoes were reared under different control conditions on different blood groups. The female mosquitoes preferred to feed on blood group B, even significantly higher than the blood group O. The lowest numbers of females were preferred to feed on blood group even lower than the AB.

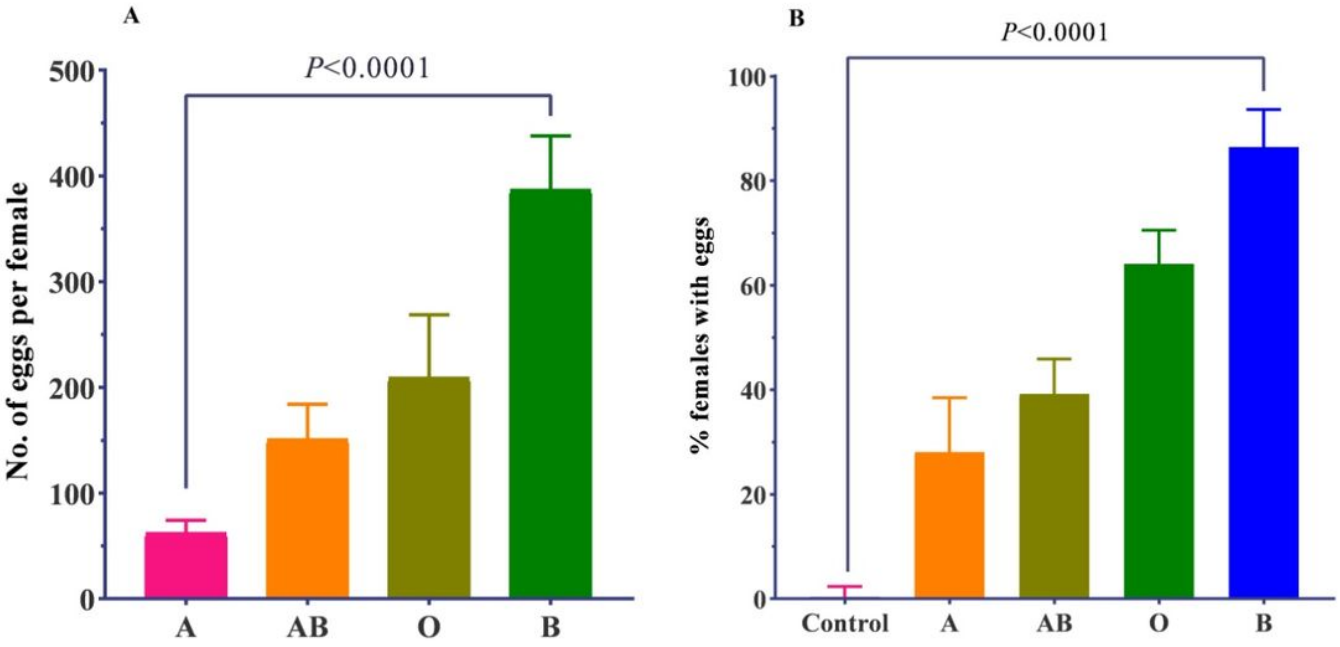

Figure 8

Association between the different blood groups and the fecundity of Anopheles stephensi. (A) The females fed on different blood have different numbers of eggs laid by female Anopheles stephensi. The females fed on blood groups A and AB do not have significantly different numbers of eggs (P=1350). While the highest numbers of eggs were laid by the female fed on blood group B, and it is significantly higher than all other blood groups, including AB, O, and A. (B) About 100 female Anopheles stephensi mosquitoes were reared on different treatments, including ABO blood groups and one control 10% sugar solution. Female with eggs from each treatment were identified under a microscope and counted. So the % female with eggs indicates the numbers of females with eggs from each blood group. Results indicated that the numbers of females with eggs were significantly higher than all other blood groups in case of blood group B. About 80% of the females fed on blood group B have the eggs while the lowest numbers of females with eggs were identified in the case of females fed on blood group A.