

RESEARCH ARTICLE

Experimental analysis of the blood-sucking mechanism of female mosquitoes

Bo Heum Kim^{1,2}, Hae Koo Kim² and Sang Joon Lee^{1,2,*}

¹School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, Pohang Gyeongbuk 790-784, Republic of Korea and ²Center for Biofluid and Biomimic Research, Pohang University of Science and Technology, Pohang Gyeongbuk 790-784, Republic of Korea

*Author for correspondence (sjlee@postech.ac.kr)

Accepted 6 December 2010

SUMMARY

Pioneering studies have been conducted to reveal the functional characteristics of the two-pump system of the female mosquito. Mosquitoes are equipped with two pumping organs located in the head: the cibarial (CP) and the pharyngeal (PP) pumps. To analyze the functional relationship of these pumps during the blood-sucking process, micro-particle image velocimetry (PIV) and synchrotron X-ray micro-imaging were employed. The two pumps were found to be well coordinated with a phase shift (α) and time shift (β) but to have distinct functions in the liquid-sucking process. The first pump (CP) starts to expand first, and then the second pump (PP) expands in advance with a time shift (β) before the first pump (CP) begins to contract, playing a key role in improving pumping performance. The systaltic motion of the two pumps works systematically in a well-coordinated manner. In addition, the pumping performance of blood-sucking female mosquitoes is demonstrated to be superior to that of nectar-eating male mosquitoes. Intake flow rate is maximized by reducing the relaxation time of the CP and increasing the pumping frequency.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/214/07/1163/DC1>

Key words: female mosquito, two pumping organs, micro-PIV, X-ray micro-imaging technique.

INTRODUCTION

Only female mosquitoes bite humans to suck blood to obtain protein for their eggs. They have been studied mainly in connection with the transmission of tropical diseases, such as malaria and yellow fever (Dekker et al., 2005; Riehle et al., 2003). To suck blood from a host through a long and slender proboscis, female mosquitoes utilize pumping organs located in their head. The heart of a mosquito controls the circulatory system of the body (Glenn et al., 2010). However, their blood-sucking mechanism has not been studied yet. In addition, the main functions of pumping organs are different from those of the heart that controls the circulatory system of the body (Glenn et al., 2010).

The tiny pump system of female mosquitoes has the ability to suck more than three times the body mass of blood within a short period (Clements, 1992). Rapid intake of a sizeable amount of blood by blood-sucking insects is commonly induced by successive contractions and relaxations of their extrinsic visceral muscles, which function as dilators (Nayar and Sauerman, 1975). Pumping muscles in the head of fluid-feeding insects constitute a single pump (for example in the Reduviidae) or two pumps (e.g. Cicadidae and Culicidae) (Champman, 1998).

Mosquitoes are armed with a two-pump system and their systematic operation allows a large pressure gradient to develop. The two pumps, the cibarial dilator pump (CP) and pharyngeal dilator pump (PP) are located inside their head (Schiemenz, 1957). Blood is extracted from a host and transported to the main body of the insect through a food canal in the proboscis using the pressure gradient generated by the two pumps.

Researchers have attempted to explain the presence of the two pumps, especially in relation to the blood-sucking mechanisms

of female mosquitoes. The most persuasive argument to date is that they provide a rapid and highly efficient pumping mechanism that may reduce mortality significantly during the blood-sucking period (Gillett, 1969). Daniel and Kingsolver found that there is an optimal hematocrit at which the rate of protein intake, or the rate of blood cell intake, can be maximized for blood-feeding female mosquitoes (Daniel and Kingsolver, 1983). The intake rate of blood is important because it is directly related to egg production (Kingsolver and Daniel, 1995); however, there have been few investigations on the blood-sucking mechanism and functional features of the two-pump system of the female mosquito. There have been only a few theoretical studies on blood-feeding insects using morphological information and a numerical approach (Reddy and Kesavan, 1988). However, such approaches are limited in determining the *in vivo* processes (Kesavan and Reddy, 1985).

Recently, the transport of liquid in the food canal was investigated experimentally using a micro-particle image velocimetry (PIV) technique (Lee et al., 2009). Velocity information from the food canal can be used to study indirectly the main features of the blood-sucking phenomena of a female mosquito. In our study, the dynamic behavior of the two pumps in the head of the female mosquito was visualized using a synchrotron X-ray micro-imaging technique to show how the two pumps work during the blood sucking process. It is a powerful tool for observing the internal structure of opaque bio samples (Westneat et al., 2003; Westneat et al., 2008). The volume variation of the two pumping organs was investigated in relation to the streamwise velocity signal of the sucking flow inside the food canal of a mosquito.

MATERIALS AND METHODS

Mosquito rearing and sample preparation

Mosquitoes (*Aedes togoi* Theobald 1907) were reared and maintained in an air-conditioned room at 27°C, 80% relative humidity, with a 16h:8h light:dark photoperiod. Larvae were hatched in distilled water and fed a slurry of ground fish food and baker's yeast. After pupation, mosquitoes were transferred to a cage made of a fine-mesh net. Upon emergence, they were fed using a 10% sucrose-soaked cotton rod placed on the bottom of the cage. Mosquitoes more than 4 days post-emergence were selected for the experiments.

The mouthparts (proboscis) of a mosquito consist of six piercing slender stylets that are appressed in a fascicle and this is surrounded by the labium. Among the six piercing stylets, the labrum, which serves as the food canal is the largest and stiffest (Clements, 1992). The labium has a feather-like opaque cuticle that prevents the observation of blood flow through the food canal (Fig. 1A). To visualize blood flow inside using the PIV technique, the opaque cuticle of the proboscis was removed microsurgically with sharp forceps. Through this treatment, the food canal becomes transparent as shown in Fig. 1B.

During the synchrotron X-ray imaging experiments, living mosquitoes were immobilized by sticking their legs with instant glue to a polyimide film, which transmits over 99.9% of X-ray energy. Mosquitoes were handled very carefully in order to minimize the stress caused by sample preparation and fixation on the sample holder.

Micro-PIV

To determine the temporal variation of the streamwise velocity component of the blood flow in the food canal, we employed a micro-PIV velocity field measurement technique (Adrian, 1991; Meinhart et al., 1999). The micro-PIV system consists of a vertical microscope (Eclipse 80i, Nikon, Nishiohi, Tokyo, Japan), a continuous Nd:YAG laser ($\lambda=532$ nm, SLOC, Shanghai, China) as a light source, a high-speed CMOS camera (Photoron ultima APX,

Fujimi, Tokyo, Japan) and a personal computer for data processing and control (Lee et al., 2009) (supplementary material Fig. S1). In the preparation of human blood samples, we followed the Declaration of Helsinki and the volunteer gave his consent prior to participation. A whole blood sample from a healthy male volunteer (age 28) was taken and stored in anti-coagulated Vacutainer® tubes (BD, Franklin Lakes, NJ, USA). The blood sample was mixed with fluorescent tracer particles (1.0×10^9 ml⁻¹) at room temperature (24°C) and then placed near the tip of a mosquito's proboscis. The fluorescent particles were made of polystyrene (Molecular Probes, Eugene, OR, USA) and were ~1.0 μm in diameter. Such particles absorb green light at a peak wavelength of $\lambda=534$ nm and emit at a peak wavelength of $\lambda=554$ nm. Fluorescence images of tracer particles were captured through a high-pass filter ($\lambda=550$ nm) attached to the microscope.

Synchrotron X-ray micro-imaging

Synchrotron X-ray microscopy was used to observe the internal structure of the insects. The experiments were carried out at the third generation synchrotron radiation source of the Pohang Accelerator Laboratory (PLS; Pohang, Republic of Korea). The X-ray beam at the 1B2 beamline was monochromatized using a W/B4C double-multilayer monochromator with peak energy at 10 keV. A scintillator composed of a CdWO₄ crystal was placed 70 mm behind the test sample to transform the X-ray images into visible light. The X-ray images were recorded using a CCD camera (MegaPlusII ES2001, Redlake, Tallahassee, FL, USA) at a frame rate of 30 frames s⁻¹ (Fig. 2). The field of view (FOV) for the X-ray imaging experiments with a ×4 objective lens was 2219 × 2219 μm. It took ~30 s from the setting of a mosquito on the sample stage to the start of X-ray imaging. A starved mosquito usually starts to suck as soon as the food reaches the proboscis. In order to record the blood-sucking process from the start, a diluted iodine solution was injected from outside the hutch using a silicon tube connected to a syringe pump. The sample holder was placed on a motorized stage to be able to remotely control the position of the test sample. To minimize the exposure time of the insect to the X-ray beam in order to maximize its survival time, a mechanical shutter was used so that the test mosquito was only exposed to the X-rays when recordings were being made. Mosquitoes remained alive for up to 5 min during the consecutive X-ray imaging experiments. Mosquitoes usually finished the blood-sucking process within 30 s to 1 min.

The boundaries of the two-pump system could not always be detected clearly because of the complicated anatomy of the pumping organs that are covered with dilator muscles. In order to visualize the fluid flow inside the pumping organs, a diluted iopamidol solution (600 mg ml⁻¹) was used as a contrast medium. The iopamidol solution has a similar viscosity range to that of the test blood sample, indicating similar shear thinning effects of a non-Newtonian fluid (supplementary material Fig. S2). Therefore, the iopamidol solution represents, reasonably well, the biophysical characteristics of blood under the experimental condition of the present study.

Data processing of X-ray micro-imaging

During the blood-sucking process, the deformation index and light intensity in X-ray images of the two pumping organs were carefully examined. The deformation index is defined as the ratio of the major axis to the minor axis of each pumping chamber. Although the temporal variation of the deformation index sufficiently matches the variation of the light intensity, the latter was found to provide clearer signal variation (supplementary material Fig. S3). The light

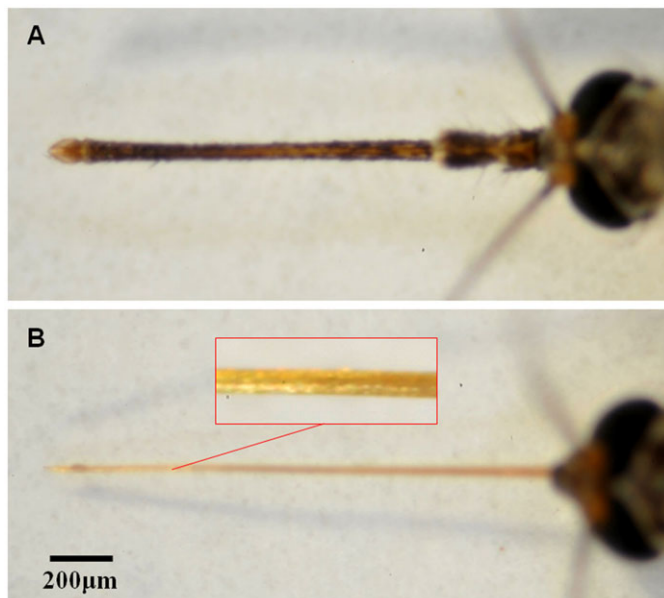


Fig. 1. Removal of the opaque cuticle of a female mosquito's proboscis for micro-PIV experiments. (A) A proboscis covered with the feather-like cuticle. (B) The opaque labium layer (outer most part) was stripped off to visualize blood flow inside the food canal.

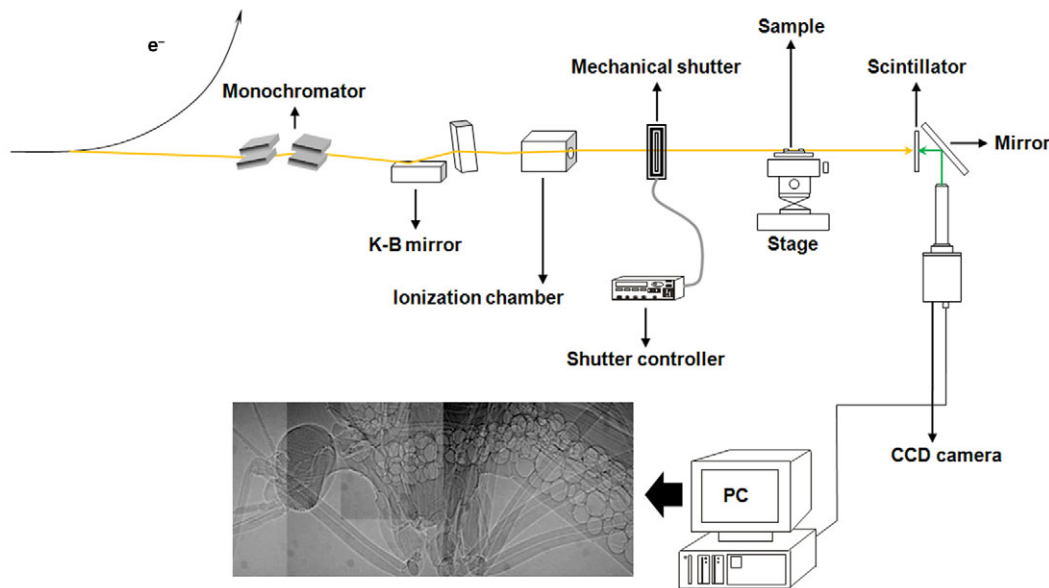


Fig. 2. Schematic diagram of the synchrotron X-ray micro-imaging technique. An X-ray beam penetrating a test sample is converted into visible light after passing through the scintillator. The temporal variation in the internal structures and dynamic motion of pumping organs were visualized under *in vivo* conditions.

intensity averaged in a square center section ($16 \times 16 \mu\text{m}^2$) of each pump was used to indicate the rhythmical volume variation of the two pumps (supplementary material Fig. S4). To analyze in detail the functional characteristics of the two pumps, the intensity values in the center section for every cycle of each test sample were conditional-averaged by assigning the temporal location of the maximum intensity value in each period as the reference time ($t=0$). The intensity values used for analyzing the systaltic motion of the pumps are the reciprocal of light intensity, so the increase in light intensity values corresponds to the expansion of the pump volume. As the volume of each pump increases, the X-ray image of the sucked iodine solution in the center region becomes darker, i.e. the light intensity decreases linearly. Time series data were also obtained from each sample to determine the pulsation characteristics such as pulsatile frequency and pumping duration (supplementary material Movie 1). The intensity values of CP and PP were normalized by their maximum values (I_0). The intensity variation of both pumps was recorded for at least 20 cycles.

RESULTS

Three elementary phases of blood-sucking process

Fig. 3 shows the internal structure of the food canal and head of a female mosquito sucking iodine solution. At the beginning of the food-sucking cycle, the CP and PP are empty (Fig. 3A). As the CP begins to expand, liquid is sucked into the CP chamber through the food canal, while the PP remains relaxed (Fig. 3B). As a result, the light intensity in the center region of the CP increases gradually as the iodine solution is accumulated. Subsequently, it rapidly decreases as the solution is drained towards the PP through the canal connecting the two pumps (Fig. 3C). Finally, following fast contraction and expansion, the PP completes one feeding cycle. This systaltic muscular motion of the two pumping organs creates a strong hydrostatic negative pressure (Borrell, 2006), sufficient to suck liquid-phase food from the outside into the digestive organ through a long food canal (length $\sim 2000 \mu\text{m}$, diameter $\sim 30 \mu\text{m}$). The pumping process of mosquitoes can be divided into three phases. To analyze these phases in detail, the streamwise velocity signal of the flow in the food canal was synchronized with the corresponding variations in the volume of the two pumping organs in X-ray images (Fig. 4).

Phasic variation of the CP

Phase 1 corresponds to the period during which the CP takes in fluid from the outside through the food canal (Fig. 4). In this pumping phase, flow velocity in the food canal exhibits a temporary stoppage from the initial low-level velocity. This is attributed to the shrinkage of the end of the elastic food tube that occurs because of the strong negative pressure induced by expansion of the CP. As the shrunken passage near the CP is opened, the flow velocity is increased dramatically to 1.6 cm s^{-1} . The opening of the shrunken elastic tube by dilator muscles was observed in a previous anatomical study (Schiemenz, 1957). During phase 1, the expansion period of the CP is considerably longer than that of the PP. The expansion time of the CP of female mosquitoes is 2.6 times longer than that of the complementary PP. The CP/PP expansion ratio for male mosquitoes is ~ 4.2 . It is also worth noting that the contraction times of both pumps are almost the same. The CP/PP contraction ratio is 1.0 and 1.1 for male and female mosquitoes, respectively.

Systematic operation of two pumping organs

In phase 2, as the CP reaches a crucial threshold volume, the PP start to draw liquid food from the CP when the cibarial-pharyngeal valve (C-P valve), located between the two pump organs, is opened (Schiemenz, 1957; Waldbauer, 1962). This valve is actively controlled by dilator muscles, depending on the operational condition of the two pump organs. It facilitates the flow between the two pumps. In this pumping phase, the maximum expansion of the two pump organs has a definite phase shift (α). This implies that the temporal volume variations of the two pump organs are rhythmically coordinated and synchronized in each feeding cycle. The duration of the CP contraction is almost the same as that of PP expansion. However, the PP starts to expand in advance of the CP, with a time-shift (β ; Table 1). When the C-P valve is opened after the CP starts to contract, the volume of liquid sucked up by the PP is somewhat smaller than the volume of the CP. If the time shift is zero ($\beta=0$), CP contraction and PP expansion occur simultaneously. In addition, the reverse flow discharged from the CP chamber toward the food canal causes negative inertia against the main feeding flow. In this case, the PP expends more energy than in the earlier expansion of the PP, with a pertinent time shift of β . The earlier expansion of

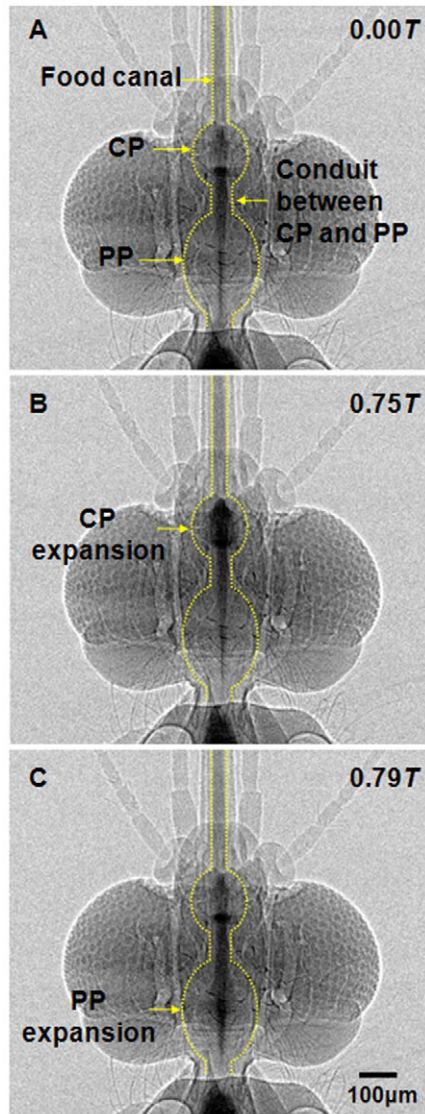


Fig. 3. Systaltic motion of the two pumping organs of a female mosquito during sucking of diluted iodine solution. Dotted yellow lines indicate the boundaries of food canal, two pumping organs at maximum expansion and the conduit between the CP and PP. (A) Both pumps are contracted initially. (B,C) Iodine solution passing through the food canal. The temporal changes of the CP and the PP are clearly demonstrated by area variation in the dark regions. T , period of the cyclic motion. $0.007T$ denotes the start of one cycle and $1.007T$ is the end of the cycle.

the PP with β saves energy by adding inertial force to the main flow. It also accelerates the expansion and contraction process of the CP, increasing the feeding frequency. Consequently, mosquitoes can suck large amounts of liquid efficiently in a short period.

Reverse flow in mosquitoes

A slight reverse flow (-0.15 cm s^{-1}) was observed in the food canal during phase 2. However, the amount of the reverse flow was very small, $\sim 1\%$ of the total amount of liquid sucked up by CP in phase 1. In phase 3, the PP pushes liquid towards the body of the insect and the CP discharges the remaining fluid in both directions. The velocity of reverse flow inside the food canal in phase 3 is slightly smaller (-0.27 cm s^{-1}) than that in phase 2. This reverse flow from

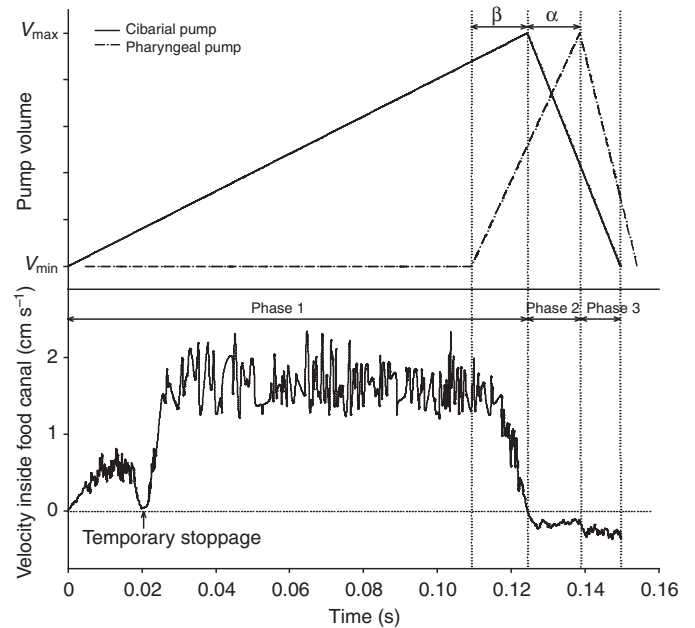


Fig. 4. Typical flow velocity inside the food canal of a female mosquito and the corresponding volume variation of the two pumping organs. One sucking period is divided into three phases.

the CP to the food canal is generated because the negative suction pressure caused by the dilator muscles of the PP is not sufficient in phase 3. However, the amount of reverse flow in phase 3 is also very small, that is, $\sim 3\%$ of the total amount of liquid sucked up by the CP in phase 1.

Comparison of pumping performance of male and female mosquitoes

Fig. 5 compares the cyclic volume variation of the two pumps of a representative female mosquito with that of a typical male mosquito (supplementary material Fig. S5). The experimental data were statistically averaged from 10 samples for each species and the results are summarized in Table 2. The liquid-sucking period of male mosquitoes is $\sim 202 \text{ ms}$ and that of female mosquitoes is 160 ms . The total amount of liquid sucked up by mosquitoes can be estimated by multiplying the volume of liquid drawn by the CP in one period (net flow rate; Q_{net}) by the number of pumpings ($f \times t_{\text{tot}}$, where f is the pumping frequency and t_{tot} is the total feeding time for liquid food). Q_{net} is equal to the amount of liquid drawn by the CP in phase 1. The pumping frequency of female mosquitoes is $4.1 \pm 1.3 \text{ Hz}$ (mean \pm s.d.) and that of male mosquitoes is $2.9 \pm 0.8 \text{ Hz}$.

Q_{net} can be obtained by multiplying the flow rate (Q) by the net suction time of the CP in phase 1 during one feeding period (t_{cp}). The total amount of liquid sucked for a given feeding time can be expressed as $Q \times t_{\text{cp}} \times f \times t_{\text{tot}}$. For a non-Newtonian fluid, the flow rate is related to pressure difference as follows:

$$Q = \frac{\pi R^3 \rho}{1/n + 3} \left(\frac{\Delta P R}{2mL} \right)^{1/n}, \quad (1)$$

where n is the index of the power-law modes ($=1$) and m is a model constant ($=\mu$) for Newtonian fluids (Richard and Kenneth, 1979) and L is length of the food canal, P is pressure, R is tube radius, and ρ is density of fluid. If the force induced by the pumping organs is the same for both female and male mosquitoes, the following relation can be obtained.

Table 1. Statistical data charting the phasic portion, phase shift and different characteristics of the two pumps in both male and female mosquitoes (*Aedes togoi*)

	Phase 1 (ms)	Phase 2 (ms)	Phase 3 (ms)	α (ms)	β (ms)	CP _{idle} (ms)	CP/PP	
							Expansion	Contraction
Male	266±42	53±4	63±3	53±4	10±3	64±16	4.2	1.0
Female	171±24	46±3	62±1	46±3	19±6	11±7	2.6	1.1

Values are means ± s.e.m.

α , phase shift; β , time shift.

CP, cibarial pump; PP, pharyngeal pump; CP_{idle}, idle period during which CP does not suck liquid.

Table 2. Statistically averaged diameter and length of food canal and pumping frequency of both male and female mosquitoes (*Aedes togoi*)

Animal no.	Females			Males		
	<i>D</i> (μm)	<i>L</i> (μm)	<i>f</i> (Hz)	<i>D</i> (μm)	<i>L</i> (μm)	<i>f</i> (Hz)
1	30.2	2415.2	3.9	21.8	2312.9	1.8
2	35.2	2235.8	3.2	25.1	2437.0	3.2
3	31.9	2240.8	6.2	16.8	2268.7	3.0
4	38.6	2472.2	6.2	25.2	2429.0	3.3
5	33.5	2432.0	3.7	18.5	2526.8	3.6
6	28.5	2009.3	4.0	15.1	2314.6	2.0
7	32.0	2279.3	3.0	20.1	2381.7	2.4
8	33.5	2400.1	3.0	18.4	2705.4	2.2
9	27.3	2497.4	4.6	23.5	2012.7	3.8
10	27.0	2183.7	2.7	23.3	2407.4	4.1
Mean ± s.d.	31.8±3.5	2316.6±145.8	4.1±1.3	20.8±3.4	2379.6±170.3	2.9±0.8

$$Q \propto \frac{D^{3-\frac{1}{n}}}{L^{\frac{1}{n}}}, \quad (2)$$

where *D* is the diameter of the food canal. The average diameter of the food canal of 10 female mosquitoes was 31.8±3.5 μm and that of 10 male mosquitoes was 20.8±1.1 μm. The tube length of female mosquitoes was 2316.6±46.1 μm and that of male mosquitoes was 2379.6±53.8 μm (Table 2). The *n*-value of bovine blood at 25°C has been reported as 0.89 (Kim et al., 2002). With an *n*-value of 0.89, the total volume of liquid food sucked by a female mosquito is ~2.5 times larger than that taken up by a male mosquito.

These results demonstrate that the pumping performance of female mosquitoes is better than that of male mosquitoes. Considering the duration of each phase, as summarized in Table 1, phase 1 is the major phase governing the pumping performance of mosquitoes. In the dominant phase 1, the PP is in the relaxed idle state, while the CP is in the expansion state. The CP has a short relaxation time as well. On average, phase 1 of male mosquitoes (266 ms) was 1.6 times longer than that of female mosquitoes (171 ms). For male mosquitoes, the idle state in phase 1 occupied 24% of the feeding period, whereas that of female mosquitoes was merely 6%.

DISCUSSION

In this study, we employed advanced flow visualization techniques to analyze the functional features of the two pumping organs in the head of female mosquitoes. By synchronizing the velocity signal of the flow inside the food canal, the volume variation of the two pumping organs was classified into three phases. To our best knowledge, this is the first study to observe *in vivo* the processes involved in female mosquitoes sucking viscous liquid during feeding.

There have been a few earlier studies on the blood-sucking phenomena of mosquitoes, but these were based on anatomic observation (Patton and Cragg, 1913; Schiemenz, 1957). Pappas analyzed the electromyography signals of food-sucking mosquitoes

and reported that the CP initiates the pumping sequence earlier than the PP (Pappas, 1988). This report agrees well with the present results, even though there is no detail description on the blood-sucking mechanism. The present study made it possible to explain

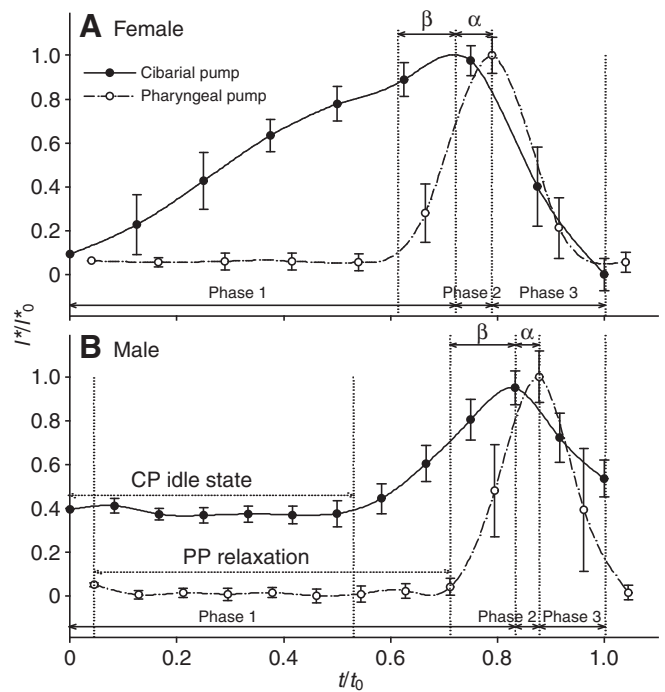


Fig. 5. A typical cyclic volume variation of the two pumps, CP and PP, of (A) a female mosquito and (B) a male mosquito. The CP has a longer expansion time compared with the PP in both male and female mosquitoes. Female mosquitoes are characterized by a short idle state. This is advantageous for taking up large amounts of fluid into the pumping chamber. Values are means ± s.e.m. Intensity (*I**/*I*₀) is normalized intensity; *t*/*t*₀ is dimensionless time.

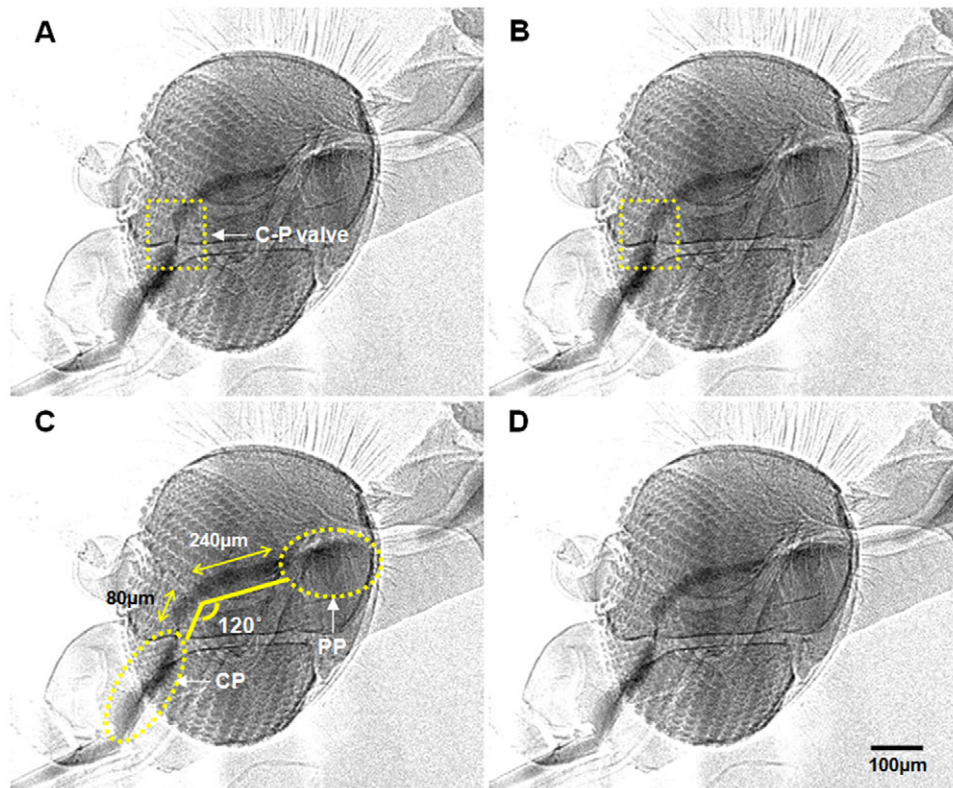


Fig. 6. Lateral view of the head of a mosquito sucking a dilute iodine solution. (A,B) The fluid path is opened as the dilator muscle of the PP is stimulated. A cibarial-pharyngeal valve (C-P valve) exists at the posterior of the CP just before the PP. (C) The food conduit connecting the CP and the proboscis has a turning angle of about 120 deg with the conduit connecting the PP and the slender neck of the mosquito. (D) When the PP contracts, the iodine solution is more clearly observed in the conduit connecting the PP and the neck.

the detailed phasic variation of each pump of the two pumping organs and the functional characteristics of each pump, based on direct visualization of the pumping organs of blood-sucking female mosquitoes.

In the normal blood-sucking process of a mosquito, the unevenness and stoppage of flow due to the non-Newtonian aspect of blood, are disadvantageous because the blood viscosity increases as the flow speed decreases or under reversed flow conditions. The two pumps of a mosquito seem to contribute to maintaining the forward flow. As a result, unevenness and stoppage in the flow are minimized in the three phases of the systolic motion of the two pumps. In phase 1, the PP sucks the liquid with forward inertia due to the earlier expansion with a time shift (β). The expansion of the PP contributes to reduce reverse flow, compared with the degree of reverse flow in phases 2 and 3. At the end of phase 3, the CP starts to expand while the PP contracts, accumulating blood in the CP and then transporting it to the PP.

Morphologically, a two-pump system is necessary. The CP is located at a curved in the path, where minor energy loss occurs. This curved flow path is inevitable for the connection of the proboscis and the digestive organs of the main body. If the flow path from the food canal to the main body was straight, a flying mosquito would encounter many difficulties in hunting and piercing the skin of a host to suck blood (Fig. 6). If mosquitoes were equipped with only one pump, it would be difficult to draw food from outside and to regulate the flow effectively.

In summary, the functional characteristics of the two-pump system of mosquitoes were analyzed in detail during the sucking process by estimating the volume variation of the two pumping organs and the velocity change of blood flow in the food canal. Mosquitoes suck viscous liquid very efficiently using the systematic operation of the two pumping organs (CP and PP) and a smart valve located between CP and PP. The systolic motion of the two pumps has a phase shift, α and one food-feeding period is divided into

three phases. It is noteworthy that the second pump (PP) starts to expand in advance, with a time-shift, β . This earlier expansion of the PP reduces the pumping period and saves energy used for regulating the flow through the two pump organs.

Based on statistical analysis, the pumping performance of blood-sucking female mosquitoes is superior to that of nectar-eating male mosquitoes. A major difference results from the sustained muscular motion of the CP of female mosquitoes during phase 1. In conclusion, the two pumps of female mosquitoes work systematically in a well-coordinated manner.

ACKNOWLEDGEMENTS

This work was supported by the Creative Research Initiatives (Diagnosis of Biofluid Flow Phenomena and Biomimic Research) of the Ministry of Education, Science and Technology/National Research Foundation of Korea. X-ray imaging experiments were performed at 1B2 and 7B2 beamlines of the Pohang Accelerator Laboratory (PAL), Pohang, Korea.

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