Mosquitoes Cool Down during Blood Feeding to Avoid Overheating

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Summary

Temperature is one of the most important factors affecting the life of insects [1]. For instance, high temperatures can have deleterious effects on insects’ physiology. Therefore, many of them have developed various strategies to avoid the risk of thermal stress [2]. They can seek a fresher environment or adjust their water loss, but hematophagous insects, such as mosquitoes, must confront the issue of thermal stress at each feeding event on a warm-blooded host [3]. To better understand to what extent mosquitoes are exposed to thermal stress while feeding, we conducted a real-time infrared thermographic analysis of mosquitoes’ body temperature during feeding on both warm blood and sugar solution. First, our results highlighted differences in temperature between the body parts of the mosquito (i.e., heterothermy) during blood intake, but not during sugar meals. We also found that anopheline mosquitoes can decrease their body temperature during blood feeding thanks to evaporative cooling of fluid droplets, which are excreted and maintained at the end of the abdomen. This mechanism protects the insect itself, probably as well as the sheltered microorganisms, both symbionts and parasites, from thermal stress. These findings constitute the first evidence of thermoregulation among hematophagous insects and explain the paradox of fresh blood excretion during feeding.

Results

In female mosquitoes, the blood meal constitutes a highly nutritive element that provides the nutrients required for egg production. However blood feeding is a risky task, given that the host may turn into a potential predator [4]. Therefore, these insects must ingest relatively large amounts of blood in a short period of time in order to minimize hosts’ antiparasitic behavior [5]. The rapid ingestion of a fluid that can exceed temperatures of 40°C implies a rapid transfer of heat into the insect’s body. Thus, the inner temperature of the insect could exceed the physiological limits of certain functions [6]. Numerous studies report on the impact of temperature on different behavioral [7] and physiological processes such as development [8–10], metabolism [11, 12], blood feeding, and reproduction [13]. Furthermore, it has been recently demonstrated that a blood meal triggers off the synthesis of heat-shock proteins in mosquitoes, providing evidence of thermal stress while feeding and the existence of a molecular protective mechanism [3]. Thermal stress may not only affect the insect itself but also its symbiotic flora [14, 15] and the parasites that it transmits with an important impact on mosquito infectivity [16–18]. Finally, heat constitutes a main cue to find a food source (i.e., a warm-blooded vertebrate). Consequently, a recently fed insect could be exposed to cannibalism if its body temperature was higher than that of the surrounding environment, facilitating the horizontal transmission of parasites between insects [19, 20].

Mosquitoes such as Anopheles stephensi Liston (Diptera: Culicidae) and Aedes aegypti Linnaeus (Diptera: Culicidae), are vectors of pathogens responsible for the death of millions of people every year. Thus, there is an urgent need to explore every avenue for developing unique control strategies against mosquito-borne diseases, and much work has been conducted on different aspects of the physiology of mosquitoes. Here, we delved deeper into the understanding of the mosquitoes’ physiology, examining to what extent these insects are exposed to heat stress while feeding and how they can cope with it.

Heterothermy during Feeding

Thermal imaging analysis has first revealed that during feeding, the different regions of the mosquito’s body exhibited different temperatures. In Anopheles stephensi, the head temperature ($T_h$) remained close to that of the ingested blood ($T_b$), whereas the thorax temperature ($T_t$) and more particularly the abdomen temperature ($T_a$) stayed closer to that of the ambient temperature ($T_a$) (Figure 1A). The thermal profile of a mosquito during feeding (Figure 2A), whatever the temperature of the host ($T_{host}$) presented, can be summarized by: $T_h > T_t > T_a$ (Figures 1D and 1E). We recorded an average difference of 3.3°C between $T_h$ and $T_a$ when the $T_{host}$ was 34°C (Figure 1D) and 2.2°C when $T_{host}$ was 28°C (Figure 1E).

We also noted some variations in the $T_h$, which corresponds to the probing periods when insects tasted the meal (red curves, Figures 1D and 1E). The $T_h$ seems to be more affected than $T_t$ and $T_a$ because the head is closer to the host. At the end of feeding, when mouthparts got out, the mosquito $T_t$ (i.e., body temperature) remained close to $T_a$ (ectothermy). We obtained similar results during Aedes blood feedings with the exception that the $T_a$ remained close to the $T_{host}$ as a result of the typical position adopted by this mosquito species (i.e., closer to the substrate or the host) (Figure 3; Table 1). However, when the two species fed on a sugar solution, no heterothermy occurred: the temperature of the whole body remained close to $T_a$, despite the muscular activity of the ingestion pump (Figure 1C).

Moreover, males, which don’t feed on blood, exhibit a typically ectothermic thermal profile (Figure 3) even at rest on a warm host, demonstrating that heating is only due to blood ingestion and not to the proximity of the host.

Drop-Keepers Perform Evaporative Cooling

During blood feeding, most hematophagous species excrete drops of fluid [21–25]. In mosquitoes, this process is called prediuresis and aims at concentrating the erythrocytes (nutrients) contained in the blood meal and at restoring the water balance [26, 11]. This fluid is mostly composed of urine, but in some cases (e.g., mosquitoes, sandflies), it also contains freshly ingested blood that gives a bright red appearance to the drop. In mosquitoes, which feed not only on vertebrate...
blood but also on nectar, prediuresis can occur during blood feeding but is very limited or absent when these insects take a sugar meal [11]. In *Anopheles stephensi*, whatever the host type is, blood feeding almost always proceeded in a similar way: drops of fluid were excreted through the insect anus between 1 and 2 min after feeding started, sometimes sooner (Table 1). In many cases, a drop remained attached to the end of the abdomen for several minutes. Eventually the drop fell, and a new one was emitted. The number of drops produced until complete gorging varied. Thermography revealed that when mosquitoes performed prediuresis and kept the drop, a transient fall of an average of 2°C occurred (Figures 1D and 1E) and heterothermy was even more pronounced (Figures 1B and 2A). The same results were obtained while feeding on both human beings and mice with different $T_{host}$ and on an artificial feeder. Furthermore, the rates of both prediuresis (Fisher’s test, p value = 0.1149) and drop keeping (Fisher’s test, p value = 0.2106) didn’t differ significantly between both types of experimental blood feeding in *An. stephensi*. The $T_{host}$ of drop keepers (blue line and dots, Figure 2B) is significantly lower (test for equality of slopes, F = 4.0215, p value = 0.0484) than those of non-drop-keeping mosquitoes (red line and dots, Figure 2B). These results demonstrate the existence of a physical cooling process in *Anopheles stephensi*. Conversely, we never observed drop keeping in *Aedes aegypti* among the tested mosquitoes that produced “preurine” while feeding (Table 1) even if the frequency of prediuresis was not significantly different between the two species of mosquitoes that were fed on a living host (Fisher’s test, p value = 0.3008).

Figure 1. *Anopheles stephensi* Feeding on an Anesthetized Mouse
(A–C) Thermographic images of *Anopheles stephensi* females during feeding on an anesthetized mouse ($T_{host}$ = 32°C, $T_a$ = 22°C) or sugar solution. (A) The mosquito does not perform evaporative cooling. The temperatures of head and thorax are very close to the mouse one and the temperature gradient along the mosquito body is limited. (B) The mosquito performs evaporative cooling. The retention of the fluid drop attached to the abdomen end leads to a fall of the abdomen temperature causing a clear temperature gradient along the mosquito body. N.B., the color of the droplet does not reflect the real temperature, because of the difference in the emissivity between the cuticle of the mosquito and the drop surface. (C) An *Anopheles stephensi* female during feeding on a sugar solution. Neither a difference between the insect temperature and that of the environment nor prediuresis can be observed.
(D and E) Variations in the body temperatures of *Anopheles stephensi* during feeding on an anesthetized mouse. (D) The arrows indicate excretion of a droplet (1), loss of the drop (2), emission and quick loss of a small droplet (3), and end of feeding (4); $T_{host}$ = 34°C; $T_a$ = 24°C. (E) The arrows indicate excretion (1), drop volume increase (2), and loss of the fluid droplet (3), and end of feeding (4); $T_{host}$ = 28°C; $T_a$ = 22°C.
Mosquito’s Body Cooling during Blood Feeding

Discussion

In insects, evaporative cooling is accomplished by producing and retaining a drop of fluid (nectar, honey-dew, water, or “prerurine,” depending on species), which causes a decrease of the $T_{an}$ by conduction and evaporation. It is a widespread mechanism in insects that can access water easily (e.g., succulent plant feeder) [27]. Evaporative cooling constitutes a beneficial and effective response to high temperature associated risks and has been observed in different groups of insects [2, 27]. This decrease of temperature helps them avoid the deleterious physiological consequences of thermal stress. Some insects such as honeybees [28] and bumblebees [29] produce heat with their thoracic muscles while flying (endothermy) and regurgitate a droplet of nectar through their mouthparts to cool down their head, thus keeping the brain safe from overheating. Moths emit fluid, which is retained on the proboscis [30], whereas others, like aphids [31], excrete honey-dew through their anus; these drops consequently refresh their head and their abdomen, respectively. The loss of temperature varies between 2°C and 8°C depending on species [27], and during our experiments we found that, in mosquitoes, the $T_{ab}$ of drop keepers falls of about 2°C during drop retention. For mosquitoes and all hematophagous insects that need to manage an excess of water during feeding and keep a well-adjusted water balance [11], evaporative cooling represents an efficient protective mechanism against overheating. It is well known that the rate of production and the size of the droplets excreted in mosquitoes during prediuresis differ not only between species but also within the same species [11].

Our results also facilitate the understanding of two puzzling aspects of prediuresis in mosquitoes. The first one is the elimination, during feeding, of some of the just ingested blood containing erythrocytes. It is widely accepted that strong selective pressures made blood-sucking insects minimize their contact time with a host in order to reduce the risk of being predated [32]. Thus, throwing away some of the food they ingest can appear, at first glance, as a maladaptive strategy. From a thermoregulative point of view, however, this “waste” makes sense, because it allows a quick increase in the volume (and evaporative surface) of the droplet and perhaps the surface properties of the drop, influencing its retention. Evaporative cooling could thus explain the excretion of fresh blood during feeding in mosquitoes. The second puzzling aspect of prediuresis is that not all species of mosquito undergo it. Actually, species that perform prediuresis need more time to reach repletion during a blood meal than species that do not produce preurine [26, 33]. Thus, the production of preurine could be seen, again, as a maladaptive strategy. However, our results suggest that this strategy could represent a trade-off between feeding quickly and avoiding overheating in species that are particularly sensitive to thermal stress. Drop keeping as cooling mechanism is also consistent with the particular position adopted by Anopheles species, which keep their abdomen away from the host surface. This results in the exposition of the drop to the ambient air, facilitating cooling and also avoiding its loss by contact with the host skin. In addition, prediuresis has been associated with the loss of weight that would be necessary to allow mosquitoes to fly away from the host to minimize predation risks [21]. Moreover, this cooling mechanism may also have consequences on pathogens present in the blood meal.

Figure 2. Mosquito Temperature during Feeding on Blood

(A) Thermographic sequence showing the production of a drop during feeding and the subsequent cooling of the abdomen in a mosquito (Anopheles stephensi). The insect fed on a human host ($T_{host} = 36°C$, $T_a = 23°C$). Images were taken every 5 s. Before the emission of the droplet (1), the abdomen could be clearly distinguished. Then, the female excreted a drop (2) that grew gradually (3, 4, 5) and a marked heterothermy was observed (6, 7). Finally, the drop fell and another one was instantly produced (8, 9).

(B) Regression lines illustrating abdomen temperature for Anopheles stephensi drop-keeper mosquitoes, i.e., performing evaporative cooling (EC) (EC, blue circles, n = 15) or not (no EC, red circles, n = 41), as a function of blood-meal temperature. The dotted line represents points where $T_{ab} = T_{blood}$. Parallel lines analysis indicates that the abdomen temperature of mosquitoes that performed evaporative cooling is significantly lower than that of mosquitoes that did not (test for equality of slopes, $F = 4.0215$, $p = 0.0484$). Mosquitoes were fed on a human host.
When anopheline mosquitoes ingest a blood meal from an infected host, mature and functional Plasmodium gametocytes are present in the erythrocytes and undergo differentiation in the mosquito midgut. Numerous developmental processes of the parasites are influenced by temperature [34]. Indeed, high temperatures negatively affect early stages of the parasite life cycle. No exflagellation occurs above 30 °C, leaving parasites in an inactive state [17]. Later processes such as ookinete formation or migration of sporozoites toward the salivary glands are also influenced by temperature [18, 35, 36]. Furthermore, it has been well demonstrated that different species of Plasmodium are thermosensitive and that temperature has a direct impact on the incubation period of parasites in the mosquito [16]. The proliferation and dispersion of flaviviruses in Aedes mosquitoes is also under the influence of temperature, but contrary to Plasmodium, this latter constitutes one of the most important factor positively influencing the extrinsic incubation period (EIP). It has been shown that high temperatures are important for flaviviruses, influencing the rate of viral multiplication and consequently on the vector competence [37, 38].

Moreover, Plasmodium parasitoids have to cope with the formation of the peritrophic matrix following each blood meal, which restrains their penetration through the gut wall [39, 40]. During the process of differentiation, Plasmodium ookinetes have to cross the peritrophic matrix and the midgut epithelium, before they turn into oocysts [41]. The time needed for the formation of the peritrophic matrix positively correlates with the vectorial capacity of mosquitoes, taking a longer time in Anopheles species than in species of Aedes or Culex [32]. Thus, for Plasmodium parasites, insect’s heterothermy could represent an important advantage, because when they are ingested, they are exposed to a rapid fall in temperature, which could immediately trigger off exflagellation. Parasites could therefore penetrate the gut wall before the peritrophic matrix is fully formed.

From an evolutionary point of view, it makes sense given that Plasmodium parasites certainly take an advantage to be associated with species that can undergo evaporative cooling, protecting them from lethal temperatures. Furthermore, flaviviruses associated with non-drop-keeper species benefit from a necessary warmer environment. Besides, evaporative cooling could also protect the symbiotic microorganisms, which can play an important role in hematophagous insects, from heat stress. Asiaa bacteria have been found in high density in the gut of Anopheles stephensi females, as well as in ovaries [14], and numerous microorganism species have also been isolated in Aedes aegypti [15].

Finally, we can wonder about one further implication of our interpretation of the functionalities of prediuresis. It concerns the way environmental temperature may affect the survival of less thermotolerant mosquito species. If we consider that the species performing evaporative cooling could be more sensitive to heat, any change in the environmental temperature due to local or global warming would have a higher impact on them than on species that do not perform it. Other species, such as Culex spp., feed quickly and do not perform prediuresis while feeding [42]. We expect that such species have been selected to reduce the contact time with their host and consequently to be more thermotolerant to temperature increase. Indeed, it has recently been shown that heat-shock proteins are synthesized as a consequence of feeding in Aedes aegypti [3].

In conclusion, our results show for the first time that hematophagous insects such as anopheline mosquitoes are capable of thermoregulation by evaporative cooling during blood intake. It results in a marked heterothermy during feeding. Prediuresis has deeper physiological consequences than only diuresis. In addition to excretion, it implies blood concentration and thermoregulation. This mechanism protects the insect itself, probably as well as the associated microorganisms (both symbionts and parasites) from thermal stress.

Table 1. Comparative Rates of Prediuresis and Drop Keeping during Feeding in Anopheles stephensi and Aedes aegypti

<table>
<thead>
<tr>
<th>Mosquito Species</th>
<th>Anopheles stephensi</th>
<th>Aedes aegypti</th>
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</thead>
<tbody>
<tr>
<td>Type of experimental blood feeding</td>
<td></td>
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<tr>
<td>Living host</td>
<td>Artificial feeder</td>
<td></td>
</tr>
<tr>
<td>Prediuresis</td>
<td>77% (n = 43)</td>
<td>87% (n = 75)</td>
</tr>
<tr>
<td>Drop keeping</td>
<td>35% (n = 15)</td>
<td>24% (n = 18)</td>
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<tr>
<td>Feeding body position</td>
<td></td>
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<tr>
<td>Away from the host surface</td>
<td>Near the host surface</td>
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Rates of both prediuresis (Fisher’s test, p value = 0.1149) and drop keeping (Fisher’s test, p value = 0.2106) didn’t differ significantly between the two types of experimental blood feeding in An. stephensi. The prediuresis rate was not significantly different between the two species of mosquitoes that fed on a living host (Fisher’s test, p value = 0.3008).

Experimental Procedures

Mosquito Rearing
The experimental insects were uninfected females of Anopheles stephensi Liston (Diptera: Culicidae) SDA 500 strain produced and provided by the CEPRA – Institut Pasteur (Center of Production and Infection of Anophelines, Paris). Aedes aegypti (Bora strain) mosquitoes were provided by the Institut
Mosquito’s Body Cooling during Blood Feeding

de Recherche pour le Développement (IRD) Center of Montpellier (France). Females of the two species were maintained in our laboratory in 30 × 30 × 30 cm cages at 24 °C ± 1 °C, under conditions of 70 ± 5% relative humidity and a 12-12 h light-dark cycle. A 10% wt/vol sucrose solution was provided for females ad libitum and then removed from the cage 24 hr before experiments. Females aged between 3 and 7 days postemergence were used for the experiments. Only females that had never been previously blood fed and 24 hr sucrose starved were used for all tests.

Experimental Blood Feeding

Experiments were conducted at room temperature (24 °C ± 1 °C), 60 ± 5% relative humidity. Mosquitoes were allowed to engorge on two different hosts, anesthetized SKH1-E hairless mice (Charles River Laboratories International) and human hands at different temperatures ranging from 28 °C to 37 °C. This enabled us to compare prediuresis production between the two types of hosts and measure the impact of the host’s body temperature (T_host) on the female mosquito’s one (T_m). All tested females were individually placed within the experimental blood-feeding cage in order to measure with a higher precision their body temperature while feeding. When a host was presented, each female was allowed to feed to repletion on the host, and only fully engorged mosquitoes were kept for prediuresis and body temperature analysis. All experiments were performed in accordance with institutional and national guidelines and regulations on animal care.

We also conducted experimental blood meals with Anopheles stephensi females using an artificial feeder. Heparinized sheep blood was placed in a heated reservoir and covered by a Parafilm ™ "M" membrane. The temperature of the blood was controlled by using a temperature sensor placed into the blood reservoir. The blood-meal temperatures ranged from 33 °C to 37 °C. Females were placed one by one in the cage to feed on the membrane, and this allowed us to compare the rate production of prediuresis between females fed on a live host and those fed on an artificial feeder.

We performed Fisher’s test (with an α risk of 5%) to compare the prediuresis and the drop-keeping rates between species and types of experimental blood feeding. These statistics were done with R (R Development Core Team, http://www.R-project.org).

Thermographic Recordings and Thermal Data Analysis

Temperature measurements were made by using a thermographic camera (PYROVIEW 380L compact, DIAIR infrared GmbH, Germany; spectral band: 8–14 μm, uncooled detector 2D, 384 × 288 pixels) equipped with a macro lens (pixel size 80 μm; A = 60 mm; 30 × 23). Thermal data were acquired and recorded with the RTV Analyzer RT software (IMPAC Systems GmbH, Germany). The emissivity was fixed at 0.98 as determined by Stabaneither and Schmaranzer [43]. Thermographic images were captured every 5 s along the whole feeding process of mosquitoes. The temperatures of the center of the head, the thorax, and the abdomen of each mosquito were measured frame by frame from the recordings with the RTV analyzer (see above).

Parallel Line Analysis was used to determine whether linear regression slopes and intercepts were different. We compared the T_m of our two mosquito groups, drop keepers and non-drop keepers with an α risk of 5%. These graphs and statistics were done with SigmaPlot V.12.

Supplemental Information

Supplemental Information includes one movie and can be found with this article online at doi:10.1016/j.cub.2011.11.029.

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