Thermal Stress and Thermoregulation During Feeding in Mosquitoes

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1. Introduction

Many arthropods have acquired the ability to use the blood of endothermic vertebrates as their main or even unique food. Among insects, haematophagy has evolved independently in different groups [1], which have converged to this way of life under strong selective pressures that modelled many morphological, physiological and behavioural traits.

Blood is a rich source of nutrients and, except for the possible presence of parasites, otherwise sterile. However, being haematophagous is a risky task, as the food circulates inside vessels hidden beneath the skin of mobile hosts, able to defend themselves from biting or even predate on blood-sucking species. Thus, in order to minimize the contact with the host, blood-sucking insects need to pierce the host-skin without being noticed and gather blood in relatively high amounts and as quick as possible. Large blood-meals produce a strong osmotic misbalance at its ingestion and toxic metabolites as by products of its digestion. In addition, the rapid ingestion of a fluid which temperature can exceed that of the insects by 20°C or more and account for many times the insect's own body weight also 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, causing deleterious effects [2]. Numerous studies report the impact of temperature on different behavioural [3] and physiological processes such as development [4-6], metabolism [7, 8], blood-feeding and reproduction [9] of mosquitoes and insects in general.

Thermal stress may not only affect the insect itself but also its symbiotic flora [10-12] and the parasites that it transmits with an important impact on vector infectivity [13-15]. 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 is higher than that

of the surrounding environment, facilitating the horizontal transmission of parasites between vectors [16-17].

Provided their ectothermic nature, as well as their ability to colonize all kind of habitats, insects must cope with highly variable temperatures. Therefore, many insect species have developed particular physiological and behavioural mechanisms and strategies to avoid the risk to be submitted to thermal stress [18, 19]. To avoid the effect of environmental heat, insects can seek for fresher environments or adjust their water loss to increase evaporation. In the case of haematophagous insects such as mosquitoes, they must in addition confront the exposition to thermal stress at each feeding event.

The problem of heat transfer between hosts and blood-sucking insects during blood feeding remained largely overlooked until recently, when unexpected physiological mechanisms against thermal stress were unravelled in mosquitoes. We present in this chapter a brief account of these findings and the perspectives that they open in both, fundamental and applied research.

2. Thermal stress and protective strategies in *Anopheles*

The first evidences of thermal stress during feeding in haematophagous insects were obtained only recently [20]. The variation of the temperature of the body during the feeding process was measured in different species of blood-sucking insects, including two mosquitoes, *Aedes aegypti* and *Anopheles gambiae* using thermocouples. As soon as feeding begins, a steady increase of the body temperature occurs, reaching peak values of up to +10°C a few minutes later. After feeding, the temperature decreases gradually to come back similar to the environmental one. Depending on the values of environmental temperature, which is the initial temperature of the insect, and that of the blood, the amplitude and dynamics of heating and cooling vary.

Physiological responses of insects to heat include molecular changes, as is a rapid increase in the level of heat shock proteins (Hsps), which have a role as molecular chaperones that preserve the function of enzymes and other critical proteins [20]. More than a dozen Hsps are synthesized after exposure to high temperature, being the Hsp70 the most widely recognised as associated to thermal and other stresses. As in many other organisms, mosquito Hsp70s have been shown to increase during environmental stress [21, 22].

Benoit and co-workers [20] showed that, correlated with feeding and the associated elevation of the body temperature, a synthesis of heat-shock proteins occurs in *Aedes aegypti* in the few hours following a blood meal, in particular of Hsp70. In this species, the Hsp70 synthesis peaks 1 hour after feeding, reaching maximal expression in the mosquito midgut, where the relative amount of Hsp70 increases about 7 times after feeding. Similar increases in Hsp70 were showed immediately after blood feeding in *Culex pipiens* and in *Anopheles gambiae*, as well as in the bed bug *Cimex lectularius*. Nevertheless this increase, measured as the relative increase of mRNA by Northern blot, is not identical in the three mosquito species. Whereas in *Aedes aegypti* and

Culex pipiens the relative level increases between nine and ten times, in *Anopheles gambiae* only three times. This last result is particularly interesting, since it is probable that the last named species would be less submitted to thermal stress, as we will discuss in the following sections.

3. Heterothermy during feeding in *Anopheles*

To better understand to what extent mosquitoes are exposed to thermal stress during feeding, we recently conducted a real-time infrared thermographic analysis of the evolution of the body temperature of *Anopheles stephensi* during feeding on live hosts at different skin temperatures and using an artificial feeder [23].

Thermal imaging analysis has first revealed that during feeding, the different regions of the mosquito's body exhibited different temperatures. When *Anopheles stephensi* fed on mice or human volunteers, their head temperature remained close to that of the ingested blood while the abdomen temperature stayed closer to that of the ambient temperature (Figure 1). The thermal profile along a mosquito's body during feeding, notwithstanding the exact temperature of the host skin, can be summarized as in this: $T_{head}^{\circ} > T_{thorax}^{\circ} > T_{abdomen}^{\circ}$. The fact of maintaining different temperatures in different regions of the body by an animal is named "regional heterothermy" and it is common in vertebrates living in cold aquatic or terrestrial environments. When the body temperature changes with time, this condition is called "temporal heterothermy". A combination of both types of heterothermy is frequently found in insects that perform pre-heating of flight muscles before taking off. By means of simultaneous isometric contractions of antagonist muscles, insects like bumble-bees and moths heat their thorax up to reach the optimal temperature for muscular work [18].

In the case of *Anopheles stephensi*, an average difference of 3.3° C between T_{head}° and $T_{abdomen}^{\circ}$ was measured when the T_{host}° was 34° C and 2.2° C when T_{host} was 28° C. At the end of feeding, when mouthparts are retracted from the skin, the mosquito temperature returns rapidly to environmental temperature (ectothermy).

Infrared thermography revealed a quite different pattern of body temperature in *Aedes aegypti*. In this species, the abdominal temperature during feeding remains close to that of the host, rather than to that of the environment as in *Anopheles stephensi* [23]. On the other hand, when the two species fed on sugar solution, despite the muscular activity of the ingestion pump, no heterothermy occurs: the temperature of the whole body remained that of the environment. As a consequence males, which don't feed on blood, exhibit a typically ectothermic thermal profile even when resting on a warm host, demonstrating that heating is only due to blood ingestion and not to the proximity of the host [23].

4. Prediuresis and drop-keeping

During blood feeding, most haematophagous species excrete drops of fluid, a process referred in mosquitoes as "prediuresis". The physiological function of prediuresis has been related to

Figure 1. Thermographic image of an *Anopheles stephensi* female at the beginning of feeding on an anesthetized mouse ($T_{\text{host}}^{\circ} = 28^{\circ} \text{C}$, $T_{\text{environment}}^{\circ} = 22^{\circ} \text{C}$). The temperature of the head is very close to the mouse one and a temperature gradient along the mosquito body can be observed (i.e., heterothermy).

erythrocytes concentration and elimination of water excess. The eliminated fluid is in most insects composed of urine, but in some blood-sucking species, such as mosquitoes and sandflies, it also contains fresh ingested blood that gives to the drop a bright red appearance. In mosquitoes, which feed not only on vertebrate blood, but also on nectar, prediuresis occurs during blood-feeding but it is rare or absent when they take a sugar meal.

In *Anopheles stephensi*, notwithstanding the nature of the host, blood-feeding almost always proceeds in a similar way: drops of fluid start being excreted during the first or second minute after the insect begins to feed. Frequently, a drop remains attached to the end of the abdomen for several minutes, increasing its size during feeding. Eventually the drop falls, and a new one is emitted and retained at the abdomen's end. The number of drops produced until complete gorging may vary.

Real-time thermography revealed that when *Anopheles stephensi* performs prediuresis and keeps a drop attached to its anus, a transient fall of 2°C or more of the abdominal temperature occurs and the characteristic heterothermy along its body becomes even more pronounced (Figure 2). The same phenomenon was observed in females of this species feeding in mice, human volunteers or using an artificial feeder [23]. Besides, when ingest-

ing blood at the same temperature, the abdominal temperature of drop-keepers is significantly lower than that of mosquitoes that just perform prediuresis but that do not keep drops. These results demonstrate the existence of a physical cooling process in *Anopheles stephensi*. Conversely, drop-keeping was never observed to occur in *Aedes aegypti* among the individuals producing pre-urine while feeding, even if the frequency of prediuresis is the same in both mosquito species [23].

Figure 2. Thermographic image of the same *Anopheles stephensi* female as Figure 1, but during prediuresis. 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. The colour 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.

5. Thermoregulation in *Anopheles*

Many insects, in particular those having easy access to water, produce and retain drops of fluid, such as nectar, honey-dew, water or urine, depending on species, which evaporates in contact with the air, causing heat loss by evaporative cooling and the consequent decrease of

the temperature of the insect body. Evaporative cooling constitutes an adaptive and effective response to risks associated to high temperature and has been observed in different groups of insects [24, 25].

This decrease of temperature helps them to avoid the deleterious physiological consequences of thermal stress. Some insects such as honeybees and bumblebees 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 [26, 27]. Moths emit fluid, which is retained on the proboscis to refresh their head whereas others, like aphids, excrete honey-dew through their anus that consequently refresh their abdomen. The recorded loss of temperature is between 2 and 8° C depending on species [28].

Figure 3. Evolution of the body temperatures of *Anopheles stephensi* during feeding on an anesthetized mouse. The arrow indicates the excretion of a droplet. ($T_{\text{host}} = 35.5^{\circ}\text{C}$, $T_{\text{environment}} = 28^{\circ}\text{C}$)

In *Anopheles* mosquitoes, the abdominal temperature of drop-keepers decreases of about 2° C during drop retention. For mosquitoes and in general for all haematophagous insects that need to manage an excess of water into their body during feeding and keep a well-adjusted water balance, evaporative cooling represents an efficient protective mechanism against overheating.

To what extent prediuresis and drop-keeping occurs is variable and it is 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, as also differs the amount of erythrocytes from the ingested blood [29].

Figure 4. Sequence of thermographic images showing the production of a drop during feeding and the subsequent cooling of the abdomen in an *Anopheles stephensi* female. The insect fed on a human host ($T_{\text{host}}^{\circ} = 36^{\circ} \text{C}$, $T_{\text{environment}}^{\circ} = 23^{\circ} \text{C}$). Images were taken every 5 seconds.

6. A novel significance of prediuresis

Even though the occurrence of prediuresis and the elimination of fresh blood have been largely reported, it has been always considered just a way of concentrating erythrocytes and reducing the insect weight for take-off [30]. Nevertheless, two puzzling aspects of prediuresis in mosquitoes remained unsolved. The first one is the elimination during feeding of some of the just ingested blood containing erythrocytes [29]. 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 [1]. Thus, throwing away some of the food they ingest appears, at first glance, as a maladaptive strategy. From a point of view of thermoregulation, however, this “waste” makes sense, since 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. Thus, the excretion of fresh blood during feeding in mosquitoes can be explained in terms of an adaptive response of evaporative cooling when exposed to thermal stress associated to feeding.

The second puzzling aspect of prediuresis is that not all mosquito species perform it. In fact, it has been shown that species that perform prediuresis need more time to reach repletion during a blood meal than species that do not produce pre-urine [31, 32]. Thus, the production of pre-urine could be seen, again, as a maladaptive strategy. However, an increase in feeding time could represent a trade-off between feeding quickly and avoiding overheating in species that are particularly sensitive to thermal stress. Others may be less sensitive or, as *Aedes* minimize the consequences of thermal stress by synthesizing more heat-shock proteins as, for example, *Anopheles* mosquitoes.

Drop-keeping as evaporative cooling mechanism is in accordance with the particular position adopted by *Anopheles* species, which keep their abdomen away from the host surface. This causes the drop to be more exposed to the ambient air facilitating evaporation and cooling, and also avoiding the drop to be lost by contact with the host skin.

7. Thermoregulation and pathogens transmission

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, a process that is influenced by temperature. Indeed, high temperatures negatively affect early stages of the parasite life cycle and no exflagellation occurs above 30° C, holding parasites in an inactive state [14]. Later processes such as ookinete formation or migration of sporozoites towards the salivary glands are also influenced by temperature [15, 33, 34]. Furthermore, it has been well demonstrated that different species of *Plasmodium* are thermo-sensitive and that temperature has a direct impact on the incubation period of parasites in the mosquito [13]. On the other hand, 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, acting on the rate of viral multiplication and consequently on the vector competence [35, 36, 37].

Moreover, *Plasmodium* parasites have to cope with the formation of the peritrophic matrix that follows each blood meal, which restrain their penetration through the gut wall [38, 39]. During the process of differentiation, *Plasmodium* ookinetes have to cross the peritrophic matrix and the midgut epithelium, before they turn into oocysts [40]. 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* [1, 41]. Thus, for *Plasmodium* parasites, insect's heterothermy could represent an important advantage, since when they enter into the mosquito's body, they are exposed to a rapid fall in temperature, which could immediately trigger exflagellation. Parasites could therefore penetrate the gut wall before the peritrophic matrix is fully formed.

From an evolutionary point of view, it makes sense that *Plasmodium* parasites take an advantage to be associated with species that undergo evaporative cooling, protecting them from lethal temperatures. On the other hand, flaviviruses associated with non drop-keeper species would benefit from a necessary warmer environment.

Evaporative cooling could also protect from heat stress the symbiotic microorganisms associated to mosquitoes and that can play an important role in haematophagous insects [10]. *Asaia* bacteria have been found in high density in the gut of *Anopheles stephensi* females as well as in ovaries [11]. Recently many genera have been identified in the midgut of natural populations of *Anopheles gambiae* [42]. In particular, the abundance of *Enterobacteriaceae* in the mosquito midgut has been found to correlate significantly with the *Plasmodium* infection status [42].

8. Thermoregulation and thermotolerance in mosquitoes

Finally, it is possible to speculate on two further implications of our interpretation of the functionality of prediuresis as thermoregulatory mechanisms. The first one concerns how environmental temperature may affect the survival of less thermotolerant mosquitoes. If we consider that the species that perform 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, as for example *Culex spp.* that feed quickly and do not perform prediuresis while feeding [43]. It can be predicted that such species have been selected to reduce the contact time with their host and consequently to be more thermotolerant to temperature increases. Indeed, *Aedes aegypti* and its ability to produce Hsps represent an example of this [20].

The second implication of our finding is related to the control of mosquito populations. Prediuresis has deeper physiological consequences than just diuresis. In addition to excretion, it implies blood concentration and thermoregulation. The exploitation of the knowledge about excretion physiology to control disease vector insects by interfering with the function of Malpighian tubules has been already proposed for other haematophagous insects [44], and the same can be expected for mosquitoes. In this case, blocking or delaying the production of urine would have a double impact on disease transmission by affecting microorganisms transmitted by prediuresis [45, 46] and/or affecting the survival of mosquitoes exposed to overheating.

9. Conclusion

Anopheles mosquitoes are capable to perform thermoregulation by evaporative cooling during blood intake. This mechanism protects the insect itself, as well as the associated microorganisms (both symbionts and parasites) from thermal stress. Thus, prediuresis which plays such different roles in the mosquito physiology, appears one more time as an interesting possible target for the control of disease vectors.

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