

Chapter 13

Heat Shock Proteins and Blood-Feeding in Arthropods



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Abstract The blood of endothermic vertebrates constitutes the main, or even the only food for many arthropod species. Even though blood is a food rich in nutrients and in most cases sterile, its consumption is associated to many stressing factors. Energetic, thermal, osmotic and oxidative stresses are among the consequences for arthropods of the rapid ingestion of large amounts of warm blood. To cope with these stressors, these animals have developed different physiological and molecular mechanisms allowing the reduction of the stress or the reparation of the infringed damage. Among the first, specific mechanisms of thermoregulation and rapid excretion have been identified. The rapid synthesis of HSP following each feeding event make parts of the mechanisms of molecular reparation. The increase in the HSP70 levels varies across species from about 3 to around 17 times the basal level. This variability in the molecular response is plausibly associated to the occurrence or not of complementary mechanisms for reducing the effect of the stressor, as for instance, thermoregulation. The reduction of HSP70 or HSP70/HSC70 expression does not affect the blood meal size, but impairs blood digestion by the insect.

Keywords Disease vectors · Haematophagy · HSP · Kissing-bugs · Mosquitoes · Thermal stress · Thermoregulation

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Abbreviations

AM	Anterior midgut
dsRNA	Double-stranded
RNA	HCP70
HSCP	Heat-shock cognate protein 70
HSP	Heat shock proteins
HSP70	Heat shock protein 70
IMD	Immune deficiency
mRNA	Messenger RNA
PM	Posterior midgut
RNAi	RNA interference

13.1 Introduction

The blood of vertebrate animals represents a highly nutritive element where proteins account for more than 90% of total dry weight and, except for the eventual presence of parasites, otherwise sterile. This made many arthropods to adopt it as a main (e.g., mosquitoes) or even only food along their whole life (e.g., kissing-bugs). Nevertheless, blood is not freely available in nature, but it circulates inside vessels hidden under the skin of active animals, capable of defending themselves, and normally larger than the blood-sucking organism. To feed on vertebrate blood is a risky task, which requires being able of solving several major problems related with obtaining food from hosts, which can play, at the same time, the role of predators. As a consequence, strong selective pressures have modelled specific morphological, physiological and behavioural adaptations in animals having a haematophagous way of life.

13.1.1 Blood-Feeding as a Stressful Event

Most blood-sucking arthropods take big amounts of blood, in relatively short times, in order to minimize the risks associated to frequent host search and to reduce the duration of contact with hosts. This rapid ingestion of a mass of fluid, which can account for many times their own body weight also implies a significant stress for the animal. This stress has multiple forms and physiological targets. First, the pumping of a large amounts of blood to the intestine in a short time generates an important mechanical stress associated a sudden increase in metabolic rate (Leis et al. 2016). Second, the intake of blood from endothermic vertebrates implies the transfer of a big amount of heat and, as a consequence, thermal stress (Benoit et al. 2011; Lahondère and Lazzari 2012, 2013; Lahondère et al. 2017). Third, the water

and ion balance due to the contrast between the excess water input with the blood intake and the dehydration during the off-host periods (Maddrell 1991; Benoit et al. 2010). Fourth, big amounts of blood imply high amounts of toxic or potentially noxious elements entering into the body (Sterkel et al. 2017). Finally, as haemoglobin is a major blood protein, representing about 60% of the proteins in the blood, its digestion produces big amounts of heme, which is a pro-oxidant molecule (Oliveira et al. 2011).

In addition, vertebrate host blood is the route used by several species of parasites to infect hematophagous arthropods, which development will trigger a variety of cellular stresses causing an immune response in these invertebrates.

All these stressors induce specific adaptive responses reducing their impact and also act as selective pressures for the development and support of particular physiological strategies for overcoming them. Among these responses, the synthesis of HSP triggered by feeding plays a major role in protecting the cellular integrity and reducing the physiological impact of stress associated to the ingestion of a blood meal.

13.1.2 Thermal Stress, Thermoregulation and Molecular Reparative Measures

The first evidence of the occurrence of thermal stress during feeding in haematophagous insects were obtained relatively recently (Benoit et al. 2011). The variation of the temperature of the body during the procurement of a blood meal was measured in different species of blood-sucking insects, including two mosquitoes, *Aedes aegypti* and *Anopheles gambiae* using thermocouples. A steady increase of the body temperature, reaching peak values of up to 10 °C above the initial temperature of the insect body, was verified a few minutes after the beginning of blood ingestion. After gorging, the body temperature decreases gradually to come back similar to that of the environmental. 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 may vary due to thermodynamic reasons, as the heat conductivity of the body wall or the presence or absence of wind.

In some species, however, specific mechanisms for dissipating the excess of heat exist. For instance, in the malaria vector *Anopheles stephensi*, a thermoregulatory mechanism allows dissipating a part of the heat entering into the mosquito body with the ingested blood, reducing the potential thermal stress to which internal organs may be exposed. During feeding, this mosquito emits droplets of a fluid composed of urine and blood, which are retained at the end of the abdomen. The evaporation of the fluid dissipates the excess of heat, reducing the temperature of the whole body; cooling in particular the abdomen of the mosquito (Lahondère and Lazzari 2012, 2013).

Other physiological responses of insects to overheating during feeding include molecular changes, as a rapid increase in the level of HSP (Benoit et al. 2011). As in many other organisms, mosquito Hsp70s have been shown to increase during environmental stress (Gross et al. 2009; Benoit et al. 2010). Benoit and co-workers (2011) 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 h after feeding, reaching its 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 different species. Whereas in *Aedes aegypti* and *Culex pipiens* the relative level increases between nine and ten times, in *Anopheles gambiae* only three times. The vector of leishmaniosis *Lutzomia longipalpis* increases by a factor of 17 the level of HSP70 after the haematophagous act (Aguiar-Martins 2015), whereas in the kissing bug *Rhodnius prolixus* increases about 3.5-fold in the insect midgut (Paim et al. 2016). This important difference in the synthesis of HSP across species, could be related to the adaptation to different environments of different species, but also to the strategy to cope with thermal stress, i.e., thermoregulation or molecular reparative measures.

13.1.3 HSP70 (HSP70/HSC70) and Blood Feeding

One of the common physiological responses of various organisms to stress situations is the rapid increase in the expression of Heat Shock proteins (HSP) transcripts. Among the various types of Heat Shock proteins, HSP70 has been the most well characterized and studied in several insect species. As mentioned above, the hematophagous insects show an increase of 3-17fold in HSP70 expression after the blood meal.

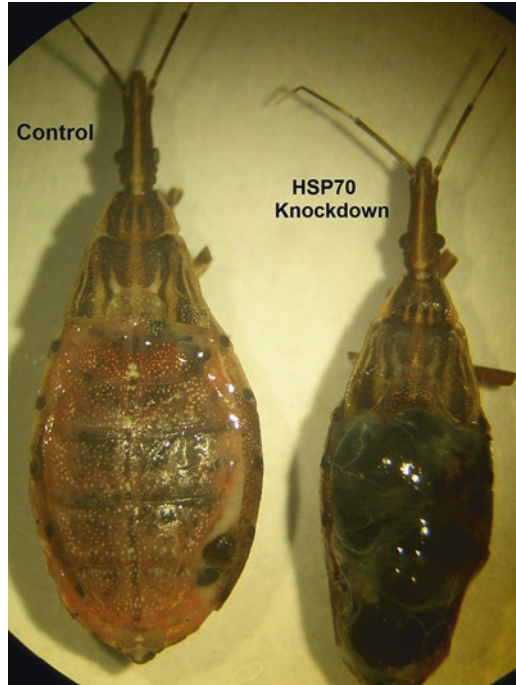
In *R. prolixus* knocked down for HSP70/HSC70 maintained in starvation after HSP70 knockdown prematurely died in a short interval of 8 days (within 32–40 days after HSP70 dsRNA injection), whereas control insects gradually died over an interval of 44 days, from 42nd to the 86th day after injection (Paim et al. 2016). The authors suggested that these insects may be dying due to their inability to counteract the stress caused by prolonged starvation. Kollien and Billingsley (2002) had reported the up-regulation of HSP70 expression in *Triatoma infestans* under long-lasting starvation. Triatomine bugs ingest water only during the blood feeding so the hydric stress induced by long periods of starvation is very critical for these insects (Rolandi et al. 2014). HSP70 RNAi knockdown in *Aedes aegypti* significantly reduced the dehydration tolerance in these haematophagous insects (Benoit et al. 2010).

Furthermore, it was observed that *R. prolixus* fed after HSP70 knockdown died even earlier, within 21–27 days post dsRNA injection. Blood feeding induced 100% mortality in engorged HSP70 knockdown insects, whereas all controls were able to normally moult to the adult phase in the same period. A mortality rate of 100% was also observed in engorged HSP70-knockdown adult females (which need to feed but do not perform ecdysis).

Physiologically normal *R. prolixus* nymphs, during their feeding, may ingest up to 9 times their initial host blood weight. This blood passes through the anterior intestine and reaches the first portion of the midgut: the anterior midgut (AM), where it is stored (Orchard 2006). In its engorged state, the insect has limited mobility and becomes more vulnerable. Thus, the insect need to quickly eliminate water and salts excess to reduce its body volume (Maddrell and Gardiner 1976). In this context, the AM acts to promote rapid diuresis of the water and ions present in the blood through the AM epithelium to the haemolymph and subsequently, from the haemolymph to the Malpighi tubules. Urine flows to the distal portion of the Malpighi tubules, where K^+ is reabsorbed, and continues to the rectum, where it is eliminated at regular intervals. The integration of the events associated with diet and diuresis is coordinated by a combination of hormones, with serotonin being one of the main factors of this coordination, being present in the nervous system, salivary glands and AM of *R. prolixus* (Brugge et al. 2008). Among other functions, serotonin stimulates the contraction of the digestive tract muscles, induces the cuticle plasticization, allows the insect to accommodate a larger volume of ingested blood and stimulates the transport of water and ions from the AM to the haemolymph (Orchard 2006). In addition to regulating the volume of water and ions present in the blood repellent, AM is still involved in carbohydrate digestion, haemolysis, and storage of glycogen and lipids (Billingsley 1988). Consequently, the blood nutrients are concentrated in the AM and pass in small portions to the posterior midgut (PM), where digestive proteases are secreted and also where nutrient uptake and storage occurs (Billingsley 1988). The digestion process in PM is probably mediated by the endocrine system, since Okasha (1964, 1970) demonstrated that decapitated insects had low levels of proteases in PM. In *R. prolixus* adults, the peak of protease activity occurs on the 4th day after feeding and then gradually decreases. In adult females, this peak of protease activity in PM refers to the vitellogenesis cycle in the ovaries, where several parameters such as protein transfer in the oocytes and trophocytes growth reach a peak on the 4th day after feeding. Although the regulation of these two events is independent, this apparent relationship between them is quite appropriate, since high protein activity in the PM leads to increased amino acid availability in the haemolymph for protein production during vitellogenesis (Persaud and Davey 1971).

In mosquitoes, it is known that there are pathways of digestion regulation in the midgut involving the interaction of insulin and TOR (“target of rapamycin”). Insulin-like peptides are released from the brain of mosquitoes after blood ingestion, interact with TOR and induce the expression of late trypsin-like enzymes in the midgut, which are the enzymes responsible for most of the digestion in the mosquitoes midgut (Gulia-Nuss et al. 2011). In the transcriptome of *R. prolixus* digestive

Fig. 13.1 Internal aspect of *R. prolixus* 5th instar nymphs dissected at the 14th day after blood feeding (the dorsal exoskeleton was removed in each bug), showing a new pink cuticle forming under the older cuticle in the control nymph (*left bug*) and the midgut still full of blood, occupying most of the abdominal cavity of the HSP70 knockdown insect (*right bug*), indicating that the ingested blood was not adequately processed and digested when HSP70 was suppressed in this insect



tract, TOR transcripts coding for a regulatory protein kinase of several cellular processes were found, such as cell growth, proliferation and survival (Ribeiro et al. 2014). The presence of TOR transcripts in *R. prolixus* gut suggests a possible role of the TOR signalling pathway in the regulation of protein digestion in the PM of this triatomine. However, the functioning of the mechanism of digestion signalling in *R. prolixus* is still scarce understood.

Several parameters related to the ingestion, processing and blood digestion were investigated to understand the mechanisms by which the blood ingestion caused the premature death of HSP70-suppressed insects. The weight loss in the first hours after blood intake (which corresponds to the diuresis peak) was not affected by HSP70 suppression, but the blood processing and digestion were significantly impaired. At the 14th day after blood feeding, while the anterior midgut of the control insects presented normal appearance and volume, and a thin pink cuticle relative to the new exoskeleton was already formed under the abdomen (indicating that these insects were entering in the ecdysis process), the anterior midgut of HSP70 knockdown insects was still full of blood, occupying virtually all the abdominal cavity and there was no sign of a new cuticle formation (Fig. 13.1).

The permanence of most of the blood proteins in the anterior midgut over the time after feeding indicated that the blood transport to the posterior midgut was greatly reduced in HSP70 knockdown insects. From the 10th day after feeding, there was a significant reduction in the amount of total proteins in the AM of control insects, which normally passed to PM to be digested. The HSP70 knockdown in

Aedes aegypti mosquitoes showed similar results and the total protein levels in the AM remained high for a long time after blood-feeding, indicating that blood processing and digestion was impaired in knockdown mosquitoes, with 25% reduction in egg production (Benoit et al. 2011).

The impairment of digestion in HSP70 knockdown *R. prolixus* was clearly demonstrated by reduction in the protease activity in the posterior midgut. Among the 3rd and the 14th day after feeding, a significant increase occurs in the endoprotease activity in the posterior midgut of control insects, while in HSP70 knockdown insects, the protease activity exhibited levels close to zero.

In addition to the digestion process, the post-feeding respiratory metabolism of HSP70 knockdown insects was also disturbed. In insects, the gases exchange between the atmosphere and metabolically active tissues is accompanied by air filling in the tracheal system, which consists of spiracles, trachea, and tracheoles. Spiracles are external openings that act as valves present in the abdomen and in the thorax of the insects, and tracheal trunks that are subdivided into smaller tracheas are found in the inner part (Contreras and Bradley 2010). Monitoring insects in a respirometer can generate basically three different breathing patterns. The discontinuous pattern is characterized by three well-defined phases: a phase in which the respiratory spiracles are completely open and the gases are free to diffuse between the insect tracheal space and the external atmosphere, a phase in which the spiracles are completely closed, and another phase in which the spiracles open and close quickly. This last respiratory pattern has been observed in several insects, including *R. prolixus*, in situations where the metabolic rate is low (when they are not moving or not fed), and seems to be important to avoid unnecessary water loss during respiration. In the cyclic pattern, peaks of CO₂ release occur with a certain regularity, however, between the peaks, the respiratory spiracles never close completely and the CO₂ release does not reach zero. This respiratory pattern can be interpreted as an oscillation between the phase in which the spiracles are fully opened and a phase of reduced CO₂ release. In the continuous respiratory pattern, CO₂ release is continuous and there is no period of total closure of the spiracles. Cyclic and continuous respiratory patterns are usually observed in situations of high energy costs (Contreras and Bradley 2009).

R. prolixus bugs monitored in a respirometer during feeding on an artificial feeder (blood heated at 37 °C) presented a continuous respiratory pattern, keeping the spiracles open or partially open during the whole feeding period, which is a high energetic cost for the insect metabolism. The respiratory pattern during *R. prolixus* nymphs blood ingestion is quite similar to the breathing pattern observed by Contreras and Bradley (2009), when this same triatomine was monitored in the respirometer during continuous exposure at a temperature of 35 °C.

From 72 h after blood feeding, HSP70 knockdown insects presented an altered energetic metabolism. Normally, *R. prolixus* nymphs maintained their typical cyclic breathing pattern after blood feeding. In contrast, insects that were silenced for HSP70 can maintain their cyclic respiratory pattern, but with a much lower frequency of CO₂ release peaks or altered their profile to a continuous respiratory pattern with no clear peaks of CO₂ release. The rate of CO₂ production by the control

insects increased significantly 72 h after feeding, probably due to the beginning of the blood processing and digestion, processes that require an increase in the metabolic rate, while in HSP70 knockdown insects, the amount of CO₂ produced was significantly reduced, indicating a need for energy savings in these insects, which is probably a consequence of the various physiological disorders that appear to occur in these insects. In hematophagous flies of the genus *Glossina*, Taylor (1977) observed that there was a correlation between the amount of blood ingested during feeding and oxygen consumption, and suggests that the high metabolism of these insects after feeding probably reflects the energy demand for the digestion process in these flies.

In *R. prolixus*, lysozyme expression in the midgut tends to increase more than 500-fold after blood feeding in normal conditions, while in HSP70 knockdown insects, lysozyme transcripts appeared reduced to levels close to zero. Lysozyme is an important enzyme that catalyses the hydrolysis of glycoside bonds of the peptidoglycan layer present in the cell wall of some bacteria, causing the lysis of these bacterial cells (Kollien et al. 2003). In addition to provide protection against these microorganisms present in the environment, lysozymes also act in the digestion of symbiotic bacteria, which develop at high densities in the anterior midgut after blood (Ribeiro et al. 2014). Although lysozyme appeared more expressed in anterior than in posterior midgut, it is in the posterior midgut that most symbionts are digested, mainly because lysozyme acts better in slightly acidic pH (Balczun et al. 2008). It has been demonstrated that lysozyme expression can be activated in the medium intestine of *R. prolixus* through the injection of bacteria or through *T. cruzi* feeding (Ursic-Bedoya et al. 2008).

The temporal analysis of the lysozyme expression at different periods before and after feeding indicated that the blood supply induces an increase in lysozyme expression in the midgut of *R. prolixus* 5th instar nymphs, with a peak approximately 5 days after feeding, while starved insects presented a basal expression of this enzyme. Kollien et al. (2003) reported that the peak of higher expression of intestinal lysozyme in *Triatoma infestans* is on the 15th day after feeding. This high expression of lysozyme on the 15th day after feeding may be related to the process of ecdysis, which coincides with this time. In this situation, lysozyme could act as a protection for the insect, which during the exoskeleton exchange may be more susceptible to infection by environmental bacteria.

After the blood ingestion, an immune response is stimulated in *R. prolixus* midgut. The expression of genes related to relish, IMD, Defensine and Lysozyme were up-regulated after blood feeding (Paim et al. 2016). In addition to ecdysteroid control, the blood meal components are important to *R. prolixus* immune response induction (Azambuja et al. 1997). On the other hand, in HSP70 knockdown bugs, the immune response was impaired, once immune genes related to lysozyme, immune deficiency (IMD) and Relish were not up-regulated following the blood meal, as if the insect had not responded to the arrival of blood in the intestinal tract.

In blood-sucking arthropods midgut, blood meal induces bacterial proliferation. The activation of immune responses in insects depends mainly on two intracellular immune cascades: the Toll and the IMD pathways (Vieira et al. 2014). Relish and

IMD are molecules involved in the IMD pathway. Relish is a transcription factor that induces antimicrobial peptide expression, especially against gram-negative bacteria. Lysozymes participate in the hydrolysis of the bacterial cell wall peptidoglycans, preventing the colonization of the digestive tract by pathogens, in addition to also have a digestive function in triatomine bugs. The up-regulation of these antimicrobial genes may be important to control the bacterial population that may develop in high densities on the midgut after a blood meal.

13.2 Conclusions

Haematophagy has independently evolved many times among arthropods. Yet, feeding on blood requires specific adaptations to cope with the many risks and stressors associated to feeding on the blood of endothermic vertebrates. Heat Shock Proteins play a crucial role in protecting the physiological integrity of blood-sucking animals at each feeding event. The level of different HSP significantly increases during the hours following the intake a blood meal, constituting a measure of molecular reparation. Their manipulative reduction also impacts several physiological processes, such as blood-digestion, moult, metabolism and even survival.

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