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# Deprivation of both sucrose and water reduces the mosquito heart contraction rate while increasing the expression of nitric oxide synthase

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ABSTRACT

Adult female mosquitoes rely on carbohydrate-rich plant nectars as their main source of energy. In the present study we tested whether the deprivation of a carbohydrate dietary source or the deprivation of both carbohydrate and water affects mosquito heart physiology. Intravital video imaging of *Anopheles gambiae* showed that, relative to sucrose fed mosquitoes, the deprivation of both sucrose and water for 24 h, but not the deprivation of sucrose alone, reduces the heart contraction rate. Measurement of the protein, carbohydrate and lipid content of mosquitoes in the three treatment groups did not explain this cardiac phenotype. However, while the deprivation of sucrose reduced mosquito weight and abdominal width, the deprivation of both sucrose and water reduced mosquito weight even further without augmenting the change in abdominal width, indirectly suggesting that starvation and dehydration reduces hemolymph pressure. Analysis of the mRNA levels of crustacean cardioactive peptide (CCAP), FMRFamide, corazonin, neuropeptide F and short neuropeptide F then suggested that these neuropeptides do not regulate the cardiac phenotype observed. However, relative to sucrose fed and sucrose deprived mosquitoes, the mRNA level of nitric oxide synthase (NOS) was significantly elevated in mosquitoes that had been deprived of both sucrose and water. Given that nitric oxide suppresses the heart rate of vertebrates and invertebrates, these data suggest a role for this free radical in modulating mosquito heart physiology.
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## 1. Introduction

The insect circulatory system functions in the transport of nutrients, waste and hormones (Chapman et al., 2013; Klowden, 2013). This system also circulates the cells and humoral factors that initiate and maintain the immune responses required for host defense and wound healing (King and Hillyer, 2012). The insect circulatory system is composed of a fluid called hemolymph, an open body cavity called the hemocoel, and a series of pumps, of which the dorsal vessel is the most important (Chapman et al., 2013; Jones, 1977; Klowden, 2013). The dorsal vessel is a muscular tube that extends along the dorsal midline of the insect and is divided into a thoracic aorta and an abdominal heart (Ejaz and Lange, 2008; Glenn et al., 2010; League et al., 2015; Leodido et al., 2013; Wasserthal, 2007). In general terms, hemolymph enters the heart of adult insects through valves called ostia that are located in the anterior portion of each abdominal segment and at the thoraco-abdominal junction. Once in the lumen of the dorsal vessel, wave-like contractions of cardiac muscle translocate the hemolymph from one end of the body to the other (Glenn et al., 2010; League et al., 2015; Lee and Socha, 2009). To ensure the thorough dissemination of hemolymph, in many insect orders the adult heart periodically alternates between contracting in anterograde (toward the head) and retrograde (toward the posterior of the abdomen) directions (Gerould, 1933; League et al., 2015), leading to the discharge of hemolymph into the hemocoel through excurrent openings located in both the head and the terminal abdominal segment (Glenn et al., 2010). Upon discharge into the hemocoel, hemolymph flows back toward the heart or enters the appendages with the assistance of accessory pulsatile organs (Andereck et al., 2010; Boppana and Hillyer, 2014; Hustert et al., 2014; Pass, 2000).

The insect heart is myogenic (Jones, 1977; Slama and Lukas, 2011), but several neuropeptides and neurotransmitters modulate heart rhythmicity and directionality (Cuthbert and Evans, 1989; Dulcis and Levine, 2005; Ejaz and Lange, 2008; Estevez-Lao et al., 2013; Hillyer et al., 2014; Johnson et al., 1997). The circumstances that lead to changes in the endogenous production of cardiomyotropic or cardioinhibitory factors remain largely unknown. However, several environmental and physiological conditions are

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known to affect heart physiology. For example, increased ambient temperature induces an increase in the insect heart rate (Edwards and Nutting, 1950; Lagerspetz and Perttunen, 1962; Richards, 1963). Similarly, experimental heating of the thorax, but not the abdomen, increases the heart rate of *Manduca sexta*, which suggests that the circulatory system also has a thermoregulatory function (Heinrich, 1971).

Although the correlation between temperature and heart rate has been demonstrated in several insect orders, most of what we know about the effect of diet on heart physiology comes from studies done on the fruit fly, *Drosophila melanogaster*. In this fly species, balanced low calorie diets result in lower myocardial lipid levels and increased cardiac performance (Bazzell et al., 2013; Birse et al., 2010; Lim et al., 2011), whereas diets that are high in sugar induce cardiomyopathy (Na et al., 2013). In *Periplaneta americana* nymphs, food deprivation does not induce noticeable changes in heart physiology, but food deprivation in aquatic *Anopheles quadrimaculatus* larvae results in a decrease in the heart rate (Jones, 1956, 1977). In adult mosquitoes the effect of nutrition on heart physiology remains unknown, but nutritional status is known to affect survival, flight performance, fecundity, and vector competence (Gary and Foster, 2001; Kaufmann et al., 2013; Naksathit and Scott, 1998; Vaidyanathan et al., 2008; Zhao et al., 2012).

In the present study we tested the effect of acute deprivation of sucrose and water on the heart of the malaria mosquito, *Anopheles gambiae*. By measuring heart physiology and nutritional status in the adult stage we show that sucrose deprivation for 24 h has little effect on heart physiology but that the combination of sucrose and water deprivation for the same period results in a reduction in the heart contraction rate. Neither sucrose nor water deprivation induces significant changes in the expression of myotropic neuropeptides but the combination of sucrose and water deprivation leads to elevated transcription of nitric oxide synthase.

## 2. Materials and methods

### 2.1. Mosquito rearing and maintenance

*A. gambiae sensu stricto* (G3 strain) were reared and maintained as described (Glenn et al., 2010). Briefly, eggs were placed in water and larvae were fed a slurry of baker's yeast and Koi fish food. Following pupation, mosquitoes were transferred to 2.3 L plastic containers with a fine mesh marquisette top, and upon emergence a cotton ball soaked in a 10% sucrose solution was placed directly on the marquisette lid such that mosquitoes could feed ad libitum. Mosquito rearing and maintenance was performed in an environmental chamber (Percival Scientific Inc., Perry, IA) at 27 °C and 75% relative humidity, under a 12 h:12 h light:dark cycle.

### 2.2. Experimental treatments

At three or four days post-eclosion, female mosquitoes that had emerged on the same day and had been housed in the same 2.3 L container were divided into three 0.47 L cardboard containers with a fine mesh marquisette top. The first container, the control group, was provided a cotton ball soaked in a 10% sucrose solution. The second container, the sucrose deprived group, was provided a cotton ball saturated in deionized water. The third container, the sucrose and water deprived group, was not provided any food or water. Organismal, physiological and molecular recordings were performed at 24 h following the beginning of treatment. For experiments measuring parameters other than mosquito survival, the animals included in the analyses were those that when assayed appeared healthy, as determined by their ability to fly. In these experiments, mosquitoes were never provided a blood meal.

### 2.3. Mosquito survival

At 24 h post-treatment, the number of live and dead mosquitoes in each container was counted and the percent survival was calculated. Four independent trials were conducted, each with an average of 45 mosquitoes per treatment. Data were analyzed by one-way ANOVA, which was followed by a Tukey's multiple comparisons test.

### 2.4. Heart physiology

Heart physiology was measured essentially as described (Estevez-Lao et al., 2013; Glenn et al., 2010; Hillyer et al., 2014). Each mosquito was positioned dorsal side up with wings spread laterally. One 0.15 mm diameter pin was inserted through a non-vascular region of each wing, and two pins were placed against (not through) the anterior pronotal lobe of the thorax. The heart of live and intact mosquitoes was then viewed using trans bright field illumination on a Nikon SMZ 1500 stereomicroscope (Nikon Corp., Tokyo, Japan) connected to an ORCA-Flash 2.8 high-speed monochrome CMOS camera (Hamamatsu Photonics, Hamamatsu City, Japan) and Nikon Advanced Research NIS-Elements software. For each mosquito, a 60 s video was recorded and each video was manually analyzed to obtain the following cardiac parameters: (1) total, anterograde and retrograde contraction rate, (2) percent of contractions propagating in the anterograde and retrograde directions, (3) percent time spent contracting in the anterograde and retrograde directions, and (4) frequency of heartbeat direction reversals. Between 49 and 51 mosquitoes were analyzed per treatment, with individuals originating from 6 independent cohorts. All videos were acquired during the daytime. For each trial, videos for all treatment groups were acquired sequentially and manually analyzed at a later time. Data were statistically analyzed using one-way ANOVA, and when significant intersample variation was detected ($P < 0.05$) this was followed by Tukey's multiple comparisons test.

### 2.5. Biochemical assays to determine nutritional composition

Quantification of total protein content was determined using the Bradford assay (Bradford, 1976). Four mosquitoes were homogenized in 200 μl of distilled water using a pestle and motor. The homogenate was centrifuged at 15,000 rcf for 1 min at 4 °C. A volume of 2 μl of supernatant was transferred to a 1.5 ml tube and 60 μl of Coomassie Blue G-250 were added (all reagents in this section were purchased from Thermo Fisher Scientific, Waltham, MA). The solution was mixed, and after 5 min of color development the absorbance was read at 595 nm using an Eppendorf Biophotometer Plus (Eppendorf AG, Hamburg, Germany). The absorbance values were compared to a standard curve of known concentrations of bovine serum albumin to calculate the total protein content per mosquito.

Quantification of total carbohydrate content was determined using the phenol–sulfuric acid method as described (Ahmed, 2013), with minor modifications. Four mosquitoes were homogenized in 200 μl of distilled water, the homogenate was centrifuged at 15,000 rcf for 1 min at 4 °C, 5 μl of supernatant were transferred to a 1.5 ml tube, which was followed by the addition of 200 μl of water and 5 μl of an 80% phenol solution in water. A volume of 500 μl of sulfuric acid was then added and 10 min later the absorbance was read at 490 nm. The absorbance was compared to a standard curve of known concentrations of glucose to calculate the total carbohydrate content per mosquito.

Quantification of total lipid content was determined using the sulfo-phospho-vanillin method (Cheng et al., 2011; Vanhandel, 1985). Four mosquitoes were homogenized in 200 μl of 2:1 chloro-

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