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Time-of-day specific changes in metabolic detoxification and insecticide resistance in the malaria mosquito *Anopheles gambiae*

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ABSTRACT

Mosquitoes exhibit ~24 h rhythms in physiology and behavior, regulated by the cooperative action of an endogenous circadian clock and the environmental light:dark cycle. Here, we characterize diel (observed under light:dark conditions) time-of-day changes in metabolic detoxification and resistance to insecticide challenge in *Anopheles gambiae* mosquitoes. A better understanding of mosquito chronobiology will yield insights into developing novel control strategies for this important disease vector. We have previously identified >2000 rhythmically expressed *An. gambiae* genes. These include metabolic detoxification enzymes peaking at various times throughout the day. Especially interesting was the identification of rhythmic genes encoding enzymes capable of pyrethroid and/or DDT metabolism (CYP6M2, CYP6P3, CYP6Z1, and GSTE2). We hypothesized that these temporal changes in gene expression would confer time-of-day specific changes in metabolic detoxification and responses to insecticide challenge. *An. gambiae* mosquitoes (adult female Pimperena and Mali-NIH strains) were tested by gene expression analysis for diel rhythms in key genes associated with insecticidal resistance. Biochemical assays for total GST, esterase, and oxidase enzymatic activities were undertaken on time-specific mosquito head and body protein lysates. To determine for rhythmic susceptibility to insecticides by survivorship, mosquitoes were exposed to DDT or deltamethrin across the diel cycle. We report the occurrence of temporal changes in GST activity in samples extracted from the body and head with a single peak at late-night to dawn, but no rhythms were detected in oxidase or esterase activity. The Pimperena strain was found to be resistant to insecticidal challenge, and subsequent genomic analysis revealed the presence of the resistance-conferring *kdr* mutation. We observed diel rhythmicity in key insecticide detoxification genes in the Mali-NIH strain, with peak phases as previously reported in the Pimperena strain. The insecticide sensitive Mali-NIH strain mosquitoes exhibited a diel rhythm in survivorship to DDT exposure and a bimodal variation to deltamethrin challenge. Our results demonstrate rhythms in detoxification and pesticide susceptibility in *An. gambiae* mosquitoes; this knowledge could be incorporated into mosquito control and experimental design strategies, and contributes to our basic understanding of mosquito biology.

1. Introduction

There were around 219 million clinical cases of malaria in 2010; additionally, the parasite was responsible for over 600,000 deaths that year (World Health Organization, 2012). In sub-Saharan Africa, the *Anopheles gambiae* mosquito is the primary vector of the most deadly malarial parasite, *Plasmodium falciparum*. Vector control is achieved primarily through insecticide treated bed nets (ITNs) and indoor residual spraying (IRS). However, these methods primarily rely on pyrethroid insecticides, DDT (dichlorodiphenyltrichloroethane), organophosphates, and carbamates to which the *An. gambiae* vector is becoming increasingly resistant (The malERA Consultative Group on Vector Control, 2011). Thus, insecticide resistance is threatening the efficacy of these important control strategies. In addition, malaria parasite resistance to antimalarials is a serious concern in the effort to control malaria (The malERA Consultative Group on Drugs, 2011).

Pyrethroid insecticides remain popular for their fast action and low human toxicity. They remain the only option available for treating bed nets, and it is integral that the efficacy of this class of insecticides is maintained for as long as possible (Hemingway et al., 2004). Historically, IRS with DDT has been used effectively to reduce the incidence of malaria in endemic areas (Mabaso

Abbreviations: DDT, dichlorodiphenyltrichloroethane; GST, glutathione *S*-transferase; HFCS, high fructose corn syrup; IRS, indoor residual spraying; LD, 12 h light, 12 h dark cycle; ZT, Zeitgeber time.

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et al., 2004). Insecticides rapidly incapacitate insects under normal circumstances. Resistance has developed, however, due to the use of DDT and pyrethroids, especially when used to control agricultural pests in West Africa (Hemingway et al., 2004). DDT and pyrethroids both target the insect voltage-gated sodium channel (Chinn and Narahashi, 1986; Davies et al., 2007). In *An. gambiae*, resistance can develop as a result of a single leucine-phenylalanine point mutation, termed *knock down resistance* (*kdr*). This mutation in the voltage-gated sodium channel gene, *para* (AGAP004707), confers resistance to all pyrethroids and DDT through target site insensitivity (Martinez-Torres et al., 1998).

Another form of resistance is metabolic resistance, in which the mosquito more efficiently breaks down or inactivates insecticides. In mosquitoes, this includes elevated levels of cytochrome P450 mono-oxygenases (CYP), glutathione *S*-transferases, and carboxylesterases (Hemingway et al., 2004). In *An. gambiae*, the glutathione *S*-transferase, GSTE2, was able to metabolize DDT and an increase in expression of this enzyme corresponded with DDT-resistant strains (Ranson et al., 2001b). Additionally, various cytochrome P450s in *An. gambiae* have been shown to be involved in resistance and are capable of metabolizing pyrethroids and DDT. Several were found with elevated expression levels in wild-caught or recently colonized pyrethroid-resistant populations of *An. gambiae* (Djouaka et al., 2008; Müller et al., 2008, 2007). CYP6P3 has been shown to metabolize pyrethroids (Müller et al., 2008) and CYP6M2 has been shown to metabolize type I and type II pyrethroids (the class of pyrethroids to which deltamethrin belongs), as well as DDT (Mitchell et al., 2012; Stevenson et al., 2011). Similarly, CYP6Z1 has shown binding specificity to and metabolism of DDT (Chiu et al., 2008), and *CYP6Z1* expression is also found elevated in pyrethroid-resistant mosquitoes (Chiu et al., 2008; Nikou et al., 2003; David et al., 2005).

Mosquito physiology and behavior are under 24 h rhythmic control; these behaviors include feeding on sugar and blood-meal hosts, dusk mating, oviposition, nocturnal flight activity, antennal protein rhythms, and host odorant sensitivity (Clements, 1999; Jones and Gubbins, 1978; Keating et al., 2013; Rund et al., 2011, 2012, 2013a). Our previous studies in *An. gambiae* have revealed ~24 h rhythmicity at the level of RNA expression for numerous biological processes under diel [environmental light:dark (LD)] and circadian [constant dark] conditions. Many of these genes are regulated by the coordinated action of the endogenous circadian clock and the environmental LD cycle (Rund et al., 2011, 2013b), a phenomenon that has been reported in other organisms (Ceriani et al., 2002; Lin et al., 2002; Albers et al., 1991; Chen et al., 2009; Michael et al., 2008; Hastings, 1960; Wijnen et al., 2006).

We have previously reported rhythmic RNA expression in both *An. gambiae* head and body samples of enzymes integral to insecticide breakdown (Rund et al., 2013b, 2011). These include genes encoding enzymes known to metabolize insecticides: CYP6Z1 (AGAP008219), CYP6P3 (AGAP002865), CYP6M2 (AGAP008212), and GSTE2 (AGAP009194) (Rund et al., 2013b, 2011). See Fig. 1 for a complete summary of detoxification genes of the CYP, GST, and carboxylesterase classes we have previously identified as rhythmic, and their peak phases. Furthermore, several of these rhythms persist under constant dark conditions, suggesting that they are driven by the endogenous circadian clock (Rund et al., 2013b, 2011).

Rhythmic RNA expression levels are likely to correspond to rhythmic protein abundance. For example, in mosquito antennae we have detected rhythmic protein levels of CYP6P3 corresponding with the occurrence of a *CYP6P3* rhythmic RNA expression profile. In total head appendages (maxillary palps, antennae, and proboscis), CYP6P3 and GSTD7 (AGAP004163) were found to be rhythmic (Rund et al., 2013a). However, GSTE3 (AGAP009197), which has also been implicated in insecticidal detoxification (Ding et al., 2005), was not found to have rhythmic protein levels in total head appendages despite rhythmic gene expression in analysis of whole mosquito heads (Rund et al., 2013a).

Rhythmic susceptibility to insecticides, or "chronotoxicity," has been investigated in other insects (Eesa and Cutkomp, 1995; Halberg et al., 1974; Yang et al., 2010; Bainbridge et al., 1982; Hooven et al., 2009; Batth, 1972; Hamby et al., 2013; Pszczolkowski et al., 2004; Shipp and Otton, 1976). In *Drosophila melanogaster*, rhythmicity in susceptibility to pesticides was shown, with the highest dose–response derived LC_{50} to propoxur and fipronil at ZT 4 (morning) (Hooven et al., 2009). No temporal variation in LC_{50} response to deltamethrin was observed in *D. melanogaster* (Hooven et al., 2009). No time-of-day specific changes in resistance to the pyrethroid fenpropathrin in the agricultural pest, *D. suzukii*,

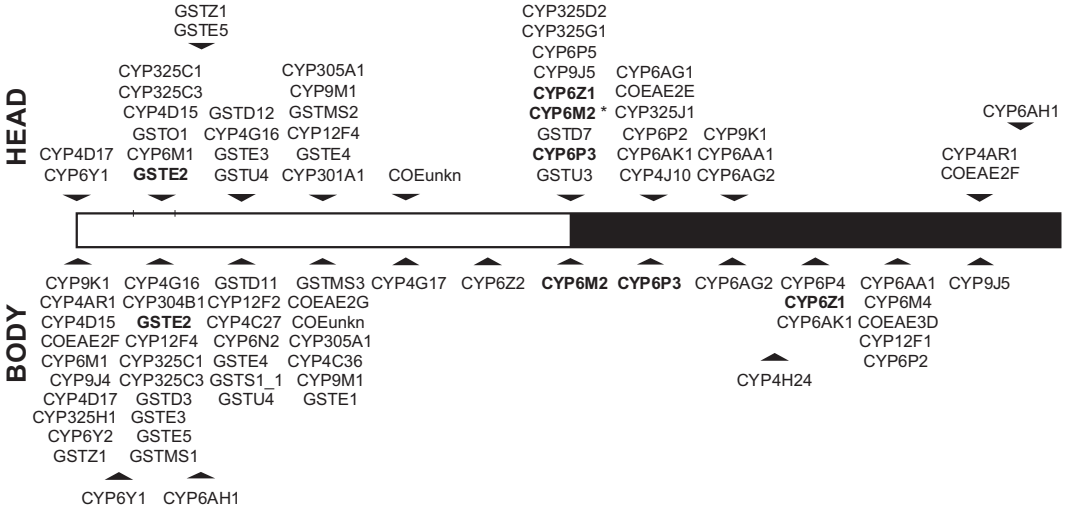

Fig. 1. Peak expression times of rhythmically expressed genes with putative roles in metabolic detoxification. Peak times of rhythmic gene expression from *An. gambiae* (Pimperena strain) in heads (upper) and bodies (lower) as determined from gene expression analysis in Rund et al. under LD conditions (Rund et al., 2013b, 2011). Only cytochrome P450 mono-oxygenases, carboxylesterases, and glutathione *S*-transferases are shown. Gene names in bold denote genes encoding enzymes with characterized insecticide detoxification function (Ranson et al., 2001b; Djouaka et al., 2008; Müller et al., 2008; Stevenson et al., 2011; Chiu et al., 2008). Genes selected based on JTK_CYCLE (Hughes et al., 2010) algorithm $q < 0.05$. *Expression determined using a pulsatile expression pattern algorithm (Rund et al., 2013b).