ANTIOXIDATIVE SYSTEMS DEFENSE AGAINST OXIDATIVE STRESS INDUCED BY BLOOD MEAL IN *AEDES AEGYPTI*

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Abstract. The release of iron from hemoglobin via the digestion of a blood meal in female mosquitoes can potentially induce oxidative damage and even death. These mosquitoes need an effective antioxidant to prevent this. We carried out this study to determine the antioxidant activities of ferritin, glutathione peroxidase (GPx), glutathione S-transferase (GST) and catalase, and glutathione (GSH). These enzymes had their greatest activity among 4 day old virgin female mosquitoes. Using a single blood feed model, groups of female mosquitoes were tested at 4, 7 and 20 days post-emergence. They were allowed to feed on a hamster for 1 hour. The engorged mosquitoes were collected at 48 and 72 hours after their blood meal. There were no changes in GSH, GPx, GST or catalase levels, but ferritin levels increased markedly (about 2-3 fold) by 48 hours post blood-feed in all mosquito age groups. On repeated blood-feed experiments, mosquitoes aged 4 days were blood fed, once every 3 days and were collected 48 hours after their most recent blood meal. A significant decrease in GSH and GPx activity and a further increase in ferritin, were detected. Ferritin levels were 0.19 ± 0.03 and 0.14 ± 0.02 ng/ g protein in the repeat and single blood-feed groups, respectively. These results suggest ferritin is an inducible, sensitive defense system protecting against oxidative stress caused by iron derived from blood meals in *Aedes aegypti* mosquitoes.

Keywords: *Aedes aegypti*, blood feeding, catalase, ferritin

INTRODUCTION

Female mosquitoes need blood meals to provide the high amount of iron needed for egg development (Nichol *et al*, 2002). Mosquitoes take about 3 l of blood per meal (Shahabuddin *et al*, 1995), equal to about 18.5 g of iron (Pascoa *et al*, 2005). Excess iron induces free radical production via the Fenton-reaction and consequently causes oxidative damage (Halliwell and Gutteridge, 1990). To manage the deleterious effects of oxidation, antioxidant defense mechanisms are needed by mosquitoes.

Mosquitoes protect themselves against oxidative stress induced by blood meal with the peritrophic metrix (PM)

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(Bhatnagar *et al*, 2003). The PM is composed of chitin and a protein-containing layer lining the midgut of most insects; it has a role in protecting the insect from invasive pathogens (Tellam *et al*, 1999). The PM is dramatically thickened after a blood meal (Freyvogel and Jaquet, 1965).

In addition to the PM, enzymatic and non-enzymatic antioxidant mechanisms may also be involved in protection against oxidative stress induced by a blood meal. Several studies have demonstrated antioxidant enzymes and ferritin play a crucial role in the survival of mosquitoes (Zhao *et al*, 2001; Dunkov *et al*, 2002; Geiser *et al*, 2006; Kasai *et al*, 2009).

Catalase is an antioxidant enzyme that decomposes hydrogen peroxide into water and oxygen, contributing a reactive hydroxyl radical (Aebi, 1984) and has been reported to protect *Anopheles gambiae* oocytes and early embryos from oxidative damage (DeJong *et al*, 2007). Mosquitoes without catalase had higher mortality after a blood meal (Magalhaes *et al*, 2008).

Glutathione S-transferase (GST) is involved in detoxification of both endogenous and exogenous compounds and has been implicated in insecticide metabolism (Hayes *et al*, 1995; Ranson and Hemingway, 2005). The GST gene was found to be up-regulated after induction using a xenobiotic in *Aedes aegypti* mosquitoes (Strode *et al*, 2008). The GST gene in *Aedes aegypti* mosquitoes is able to bind hematin, which is thought to be a protective mechanism after a blood meal (Lumjuan *et al*, 2007).

Responses to oxidative stress induced by a blood meal were investigated in the main vector for dengue fever, *Aedes aegypti*. The effects of the antioxidant enzymes glutathione peroxidase (GPx), glutathione S-transferase (GST) and catalase (CAT) were determined. Changes in glutathione (GSH), a low molecular weight antioxidant and co-enzyme for GPx and GST, and the effect of ferritin were determined.

MATERIAL AND METHODS

Mosquito rearing

Aedes aegypti laboratory bred mosquitoes were used for all the experiments. The colony was maintained at the Insecticide Unit of the Department of Entomology, Faculty of Tropical Medicine, Mahidol University. Eggs were hatched synchronously by submerging egg papers in deoxygenated water. Larvae were reared at low density in a plastic tray (33 cm x 25 cm x 11 cm) containing 3 liters of aged tap water with a density of 180-210 larvae per container, fed sufficiently with pulverized fish food to produce similarly sized adults. Within 24 hours after pupation, they were sexed based on secondary sexual characteristics size. Males and females were reared seperately in cages (30 cm x 20 cm x 20 cm). The adult mosquitoes were the maintained on a sucrose diet using a cotton ball soaked in 10% sucrose and placed inside a small glass bottle. All specimens were maintained under semi-controlled environmental conditions (25°-27°C, 60-80% relative humidity) in a 12 hours light-12 hours dark photoperiod.

Blood feeding

The day the mosquitoes were fed with a blood meal the sucrose soaked cotton was removed from the cage 2-3 hours before the blood meal. The mosquitoes fed on a restrained hamster put in the cage (6 cm x 15 cm x 5.50 cm), at a ratio of 100 females per hamster for a feeding time of 1 hour. The engorged mosquitoes were then collected. In the control group (no blood meal) the mosquitoes were kept on a sucrose diet, and examined at age 1, 4,7

and 20 days. Both the control and blood fed mosquitoes, were collected and immediately frozen at -20ºC until tested for catalase (CAT), glutathione (GSH), glutathione S-transferase (GST) and glutathione peroxidase (GPx) levels. Total protein concentration and ferritin levels were also determined. The study was approved by the Faculty of Tropical Medicine-Animal Care and Use Committee (FTM-ACUC), Mahidol University, Thailand.

Single blood feed. Female mosquitoes were blood fed on a restrained hamster when they were 4, 7, and 20 days old. After the 1 hour feed, the engorged mosquitoes were collected and transferred to cylindrical pint cardboard cups covered at one end with a fine non-wettable nylon mesh. During the rest of the experiment the mosquitoes were fed with 10% sucrose soaked cotton placed on the cover of the cup. At 6, 24, 48, and 72 hours post-blood feed, mosquitoes were randomly picked, immediately frozen and stored at -20ºC for further analysis.

Repeat blood feeding. To minimize the effect of mosquito age only female mosquitoes the same age (±1 day) were used. Mosquitoes were blood fed every 4th day. Thus, at blood feeding time the mosquitoes were 4, 7, and 10, days old; after each blood meal only fully engorged mosquitoes were transferred to another mosquito rearing cage and maintained on a 10% sucrose diet until the next blood feed. Forty-eight hours after the last blood feed (age 12 days old), the mosquitoes were collected, immediately frozen and stored at -20ºC for further analysis.

Preparation of crude homogenates

Pools of five mosquitoes were prepared and homogenized with a pellet mixer in a microtube containing 500 l of 50 mM potassium buffer at pH 7.4, then clarified by centrifugation for 15 minutes at 16,300*g*. The clear supernatant was transferred to a new microtube and kept at -20ºC until analysis (within 1 week).

Determination of glutathione and antioxidant enzyme activity

Glutathione. Total glutathione was determined based on the DTNB-glutathione reductase recycling method as described by Anderson (1985). Briefly, an aliquot of 30 l mosquito supernatant was diluted with 470 l of 50 mM potassium buffer at pH 7.4. The reaction mixture contained 2 ml of sodium phosphate buffer, 50 l of sample or standard glutathione, 200 l of 12 U/ml glutathione reductase and 200 l of 4 mM NADPH. All the reagents were mixed and incubated at 30ºC for 5 minutes before adding 200 l of 4M 5,5′-dithiobis-2-nitrobenzoic acid (DTNB; Sigma, St Louis, MO). The rate of *p*-nitrophenol formed from DTNB was monitored at 412 nm for 3 minutes with a UV-visible spectrometer (GBC Cintra 10e, Melbourne, Australia). The glutathione concentration was calculated with a standard curve using a standard glutathione reduced form (Sigma, St Louis, MO) and reported in mol/mg protein.

Glutathione peroxidase. GPx activity was measured using the method of Paglia and Valentine (1967) based on the oxidation of nicotinomide adenine dinucleotide phosphate (NADPH) at 340 nm in the presence of *tert*-buthyl hydroperoxide (*t*-BuOOH) as a substrate. Briefly, 1 ml of the total reaction mixture was prepared by mixing 100 l of 1 M Tris-HCl containing 5 mM EDTA buffer at pH 8.0, 20 l of 0.1 M of GSH, 100 l of 10 U/ml GSH reductase, 100 l of 2 mM NADPH, 10 l of sample and 670 l of deionized water. The mixture was then incubated at 37ºC for 2 minutes, then the reaction was started by adding 10 l of 7 mM *t*-BuOOH. The rate of reduction of

NADPH was monitored by absorbance at 340 nm for 2 minutes with a UV-visible spectrometer. GPx activity was calculated using the molar extinction coefficient of NADPH 6.22 $mM^{-1}cm^{-1}$ and reported as mol NADPH/min/mg protein.

Glutathione-S-transferase. GST activity was assayed by 1-chloro-2,4-dinitrobenzene (CDNB) as described by Habig *et al* (1974). A diluted sample was prepared by mixing 30 l of supernatant with 50 l of 50 mM potassium buffer at pH 7.4. The total volume of reaction mixture was 2.8 ml and consisted of 20 l of the diluted sample, 40 l of 0.1 M GSH concentration and 50 mM potassium buffer at pH 7.4. The mixture was incubated at 25°C for 2 minutes and then 60 l of 70 mM CDNB was added to start the reaction. The enzyme activity was quantified by monitoring the disappearance of CDNB at 340 nm for 3 minutes with a UV-visible spectrometer. The activity of GST was calculated using a molar extinction coefficient of CDNB 9.60 $mM^{-1}cm^{-1}$ and reported as mol/min/mg protein.

Catalase. Catalase activity was determined based on monitoring the rate of hydrogen peroxide decomposition, as described by Aebi (1984). Four hundred ninety microliter of 1 M Tris-HCl containing 5 mM EDTA pH 8.0 was mixed with 500 l of 10 mM H_2O_2 in a quartz cuvette and incubated at 37°C for 5 minutes before adding 10 l of the sample. The decrease in absorbance at 240 nm was measured continuously for 2 minutes with a UV-visible spectrometer. Catalase activity was calculated using a molar extinction coefficient of 43.3 mM^{-1} cm^{-1} and reported in mol H_2O_2/min/mg protein.

Ferritin and protein. Ferritin was determined using an immunoenzymatic colorimetric method with a ferritin kit (Diametra, Foligno, Italy) following the manufacturer's instructions. The protein concentration was determined using a modified method of Lowry (Markwell *et al*, 1978). Bovine albumin was used as a standard protein solution.

Statistical analysis

Statistical analysis was performed using SPSS version 11.5 (SPSS, Chicago, IL). The mean values were analyzed using one-way analysis of variance (one-way ANOVA), and a Tukey multiple comparison was used as a post test. A *p*-value <0.05 was considered statistically significant. Data were expressed as means ± standard deviations (SD).

RESULTS

Total GSH and antioxidant enzyme activities of GPx, GST and catalase in adult virgin female *Aedes aegypti* mosquitoes at ages 1, 4, 7 and 20 days old are shown in Table 1. Under laboratory conditions, sugar fed mosquitoes expressed antioxidant enzymes from the first day with maximum activity at four days after emerging. Aging showed no significant changes to antioxidant enzyme activity or GSH. A single blood meal did not alter the activities of the enzymes or GSH at any age group.

Despite the sugar feeds, ferritin levels tended to increase in older mosquitoes. Ferritin levels were 0.04 ± 0.03, 0.07 ± 0.02 and 0.17 ± 0.05 ng/ g protein at 4, 7 and 20 days, respectively. Significant increases in ferritin were found at 48 and 72 hours (0.14 ± 0.02 and 0.12 ± 0.02 ng/ g protein, respectively) after a single blood meal in 7 day old mosquitoes. A 3 fold increase in ferritin levels was observed at age 20 days after a single blood meal (Fig 1). However, ferritin levels then decreased 72 hours after the blood meal. This result repeated among 20 day old mosquitoes confirmed these results.

Table 1
Glutathione and antioxidant enzyme activities in sugar-fed adult female *Aedes aegyti* mosquitoes.

Antioxidant	Age (days)			
	1	4	7	20
GSH (mol/mg protein)	2.0 ± 0.4	2.9 ± 0.7	2.6 ± 1.5	2.6 ± 0.8
GPx (mol/min/mg protein)	11.6 ± 0.7	16.3 ± 1.8	16.3 ± 2.5	15.4 ± 1.9
GST (mol/min/mg protein)	100.4 ± 15.7	148.1 ± 30.9	112.3 ± 45.7	120.8 ± 47.4
CAT (mol/min/mg protein)	9.2 ± 1.3	8.0 ± 0.4	6.9 ± 1.0	7.4 ± 1.6

Data are presented as mean ± SD of the 3 pools of 5 mosquitoes.

By the third blood meal, oxidative stress was evidenced by a significant reduction in GSH and GPx activities (Fig 2a, b). The ferritin level further increased after repeat blood meals. It was significantly higher than after a single blood meal (0.19 ± 0.03, 0.14 ± 0.02, ng/mg protein, respectively) (Fig 2c). However, there were no significant differences in GST and catalase activites.

DISCUSSION

Antioxidant defense mechanisms are crucial for development and survival of mosquitoes. We found non-blood fed virgin female *Aedes aegypti* mosquitoes produced antioxidant enzymes, including GPx, GST, catalase GSH as "early as the 1 day post-emergence and maximum anti oxidant levels were steady until at least 20 days after emergence. Antioxidant enzymes have been detected in various stages of mosquito development. A study of *Culex quinquefasciatus* mosquitoes found the GST gene was expressed during three developmental stages: larvae, pupae and adult (Kasai *et al*, 2009). Peroxidase protein was also found present in all developmental stages of *Aedes aegypti* mosquitoes (Zhao *et al*, 2001).

Adult female mosquitoes require a blood meal for egg production (Geiser *et al*, 2003). However, the ingested blood meal results in a release of heme and iron, which are able to catalyze free radical reactions and consequently cause oxidative damage. Hence, mosquitoes need effective mechanisms to protect against iron-induced oxidative stress after a blood meal.

Digestion of a blood meal is complete within 48 hours. Akov (1965) and Briegel and Lea (1975) have found 24 hours after a blood meal erythrocytes can still be observed in the food bolus of *Aedes aegypti* mosquitoes. We also observed increasing protein levels to a maximum from 6 to 24 hours after a blood meal, then these returned to baseline by 48 hours after a blood meal.

We presumed the amount of oxidative stress depended on the frequency of oxidant exposure, which is evidenced by a decrease in GSH and GPx after repeated blood meals, but not after a single blood meal. A decrease in GSH is a marker of high oxidative stress or an indication of disrupted redox balance (Townsend *et al*, 2003). With a large amount of oxidative stress, some proteins may become damaged and lose their activity. Zhao *et al*

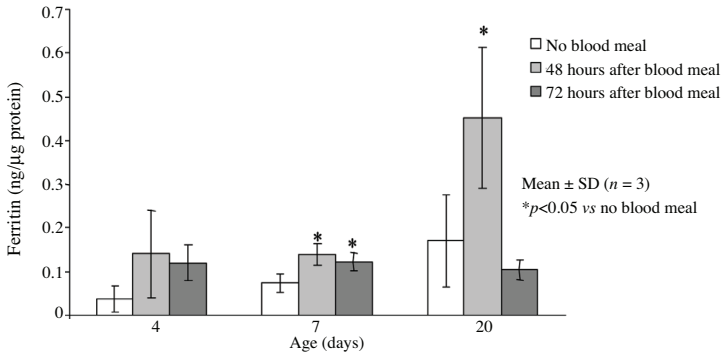

Fig 1–Ferritin levels in virgin adult female *Aedes aegyti* mosquitoes at 48 and 72 hours after a single blood meal.

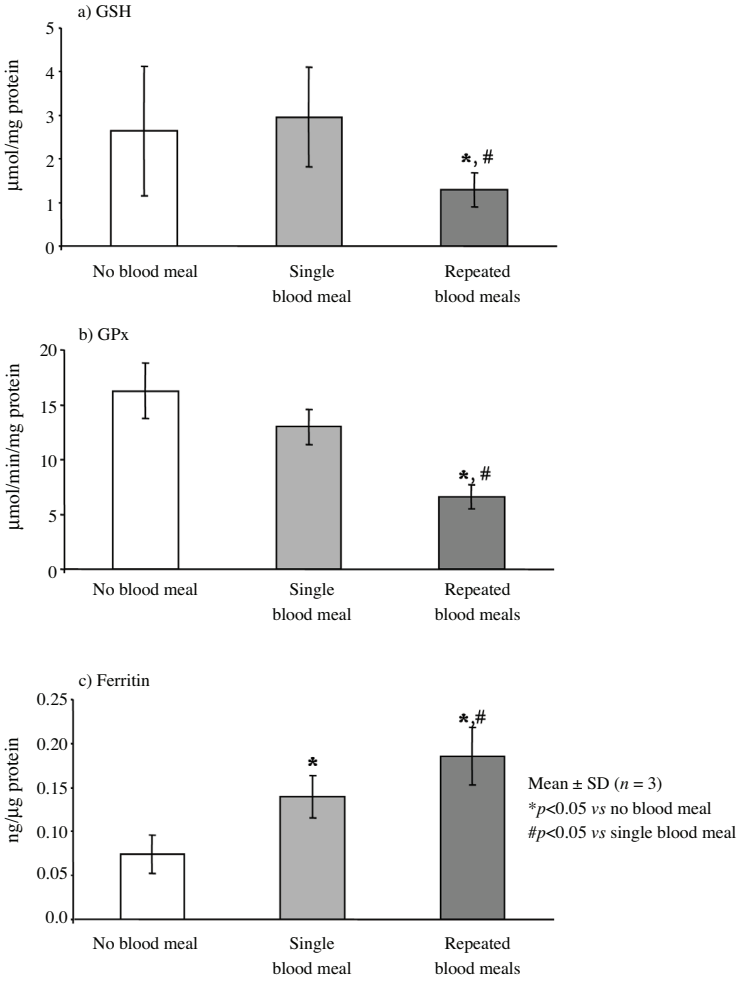

Fig 2–Glutathione, glutathione peroxidase and ferritin levels in virgin adult female *Aedes aegyti* mosquitoes 48 hours after a single or third repeated blood meal.

(2001) demonstrated the peroxidase gene is transcribed only in mosquito larvae and pupae, but not in adults or in early embryos. GPx activity is lost as a result of oxidative stress from repeated blood meals.

Ferritin is an iron sequester protein. In mosquitoes, ferritin functions not only as an iron storage protein, as it does in vertebrates, but also acts as an iron transporter (Pharm and Winzerling 2010). Ferritin in *Aedes aegypti* mosquitoes, is composed of several subunits of heavy chains (HC) and light chains (LH) (Geiser *et al*, 2003). Heavy chain subunits exhibit ferroxidase catalytic activity and light chains subunit serve as structural proteins. Ferritin is transcribed in *Aedes aegypti* mosquitoes during all developmental stages: eggs, larvae, pupae and adult mosquitoes, including sugar-fed mosquitoes (Dunkov *et al*, 2002). We found ferritin levels increased with increasing age among sugar-fed mosquitoes. It also increased dramatically with blood meals, especially among 20 day old mosquitoes. This phenomenon needs further investigation. It

unlikely to be related to high oxidative stress, since no changes in GSH or GPx were seen.

Ferritin is a sensitive defense mechanism against oxidative stress induced by blood meals. Higher ferritin levels were seen in female mosquitoes with repeated blood meals. Sander *et al* (2003) studied gene expression after blood meals in adult female *Aedes aegypti* mosquitoes by microarray analysis. Their study did not found up-regulation of genes encoding for GST and GPx; however, they found the ferritin heavy chain homologue (HCH) gene was up-regulated by 3 hours and ferritin levels were significantly increased 24 hours after a blood meal. In our study ferritin was detected by ELISA 48 and 72 hours after blood meal.

The role of ferritin to protect against oxidative stress induced by iron has been studied in *Aedes aegypti* larval cells (CCL-125) treated with iron (Geiser *et al*, 2006). They found ferritin heavy chain (HCH) synthesis was induced resulting in an increasing of secreted ferritin in a linear relationship to intracellular iron concentration. Inhibition of secreted ferritin by CI-976 affected cell viability, iron delivery to the ovaries and the number of eggs produced (Geiser *et al*, 2009). Dunkov *et al* (2002) reported a marked increase in HCH gene transcription in mosquito larvae cell growth in iron-enriched medium or with blood meals among adult female mosquitoes.

We conclude in adult female *Aedes aegypti* mosquitoes ferritin is an inducible, sensitive defense mechanism protecting against oxidative stress induced by a blood meal. This knowledge may lead to development of target antibodies or insecticides for mosquito population management.

ACKOWLEDGEMENTS

L.S. is grateful for receipt of The 60th Year Supreme Reign of His Majesty King Bhumibol Adulyadej financial grant from The Faculty of Graduate Studies, Mahidol University, for the academic years 2008 to 2010. We also thank Mrs Jittrarat Srithong and Mrs Aisa Togvang, staff of the Department of Medical Entomology, Faculty of Tropical Medicine for mosquitoes rearing.

REFERENCES

Aebi H. Catalase in vitro. *Methods Enzymol* 1984; 105: 121-6.

Akov S. Inhibition of blood digestion and oocyte growth in *Aedes aegypti* by 5-fluorouracil. *Biol Bull* 1965; 129: 439-53.

Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 1985; 113: 548-55.

Bhatnagar RK, Arora N, Sachidanand S, Shahabuddin M, Keister D, Chauhan VS. Synthetic propeptide inhibits mosquito midgut chitinase and blocks sporogonic development of malaria parasite. *Biochem Biophys Res Commun* 2003; 304: 783-7.

Briegel H, Lea AO. Relationship between protein and proteolytic activity in the midgut of mosquitoes. *J Insect Physiol* 1975; 21: 1597-604.

DeJong RJ, Miller LM, Molina-Cruz A, Gupta L, Kumar S, Barillas-Mury C. Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito *Anopheles gambiae*. *Proc Natl Acad Sci USA* 2007; 104: 2121-6.

Dunkov BC, Georgieva T, Yoshiga T, Hall M, Law JH. *Aedes aegypti* ferritin heavy chain homologue: feeding of iron or blood influences message levels, lengths and subunit abundance. *J Insect Sci* 2002; 2: 7-17.

Freyvogel TA, Jaquet C. The prerequisites for the formation of a peritrophic membrane

in culicidae females. *Acta Trop* 1965; 22: 148-54.

Geiser DL, Chavez CA, Flores-Munguia R, Winzerling JJ, Pham DQ. *Aedes aegypti* ferritin. *Eur J Biochem* 2003; 270: 3667-74.

Geiser DL, Zhang D, Winzerling JJ. Secreted ferritin:mosquitos defense against iron overload? *Insect Biochem Mol Biol* 2006; 36: 177-87.

Geiser DL, Shen MC, Mayo JJ, Winzerling JJ. Iron loaded ferritin secretion and inhibition by CI-976 in *Aedes aegypti* larval cells. *Comp Biochem Physiol B* 2009; 152: 352-63.

Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; 249: 7130-9.

Halliwell B, Gutteridge JMC. The role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990; 186: 1-85.

Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* 1995; 30: 445-600.

Kasai S. KO, Okamura Y, Tomita T. Alternative splicing and developmental regulation of glutathione transferases in *Culex quinquefasciatus* Say. *Pestic Biochem Physiol* 2009; 94: 21-9.

Lumjuan N, Stevenson BJ, Prapanthadara LA, *et al*. The *Aedes aegypti* glutathione transferase family. *Insect Biochem Mol Biol* 2007; 37: 1026-35.

Magalhaes T, Brackney DE, Beier JC, Foy BD. Silencing an *Anopheles gambiae* catalase and sulfhydryl oxidase increases mosquito mortality after a blood meal. *Arch Insect Biochem Physiol* 2008; 68: 134-43.

Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978; 87: 206-10.

Nichol H, Law JH, Winzerling JJ. Iron metabolism in insects. *Ann Rev Entomol* 2002; 47: 535-59.

Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythocyte glutathione peroxidase. *J Lab Clin Med* 1967; 70: 158-69.

Pascoa V, Oliveira PL, Dansa-Petretski M, *et al*. *Aedes aegypti* peritrophic matrix and its interaction with heme during blood digestion. *Insect Biochem Mol Biol* 2002; 32: 517-23.

Pharm DQD, Winzerling JJ. Insect ferritin: Typical or atypical. *Biochim Biophy Acta* 2010; 1800: 824-33.

Ranson H, Hemingway J. Mosquito glutathione transferases. *Methods Enzymol* 2005; 401: 226-41.

Sanders H, Evans AM, Ross LS, Gill SS. Blood meal induces global changes in midgut gene expression in the disease vector, *Aedes aegypti*. *Insect Biochem Mol Biol* 2003; 33: 1105-22.

Shahabuddin M, Kaidoh T, Aikawa M, Kaslow DC. *Plasmodium gallinaceum*: mosquito peritrophic matrix and the parasite-vector compatibility. *Exp Parasitol* 1995; 81: 386-93.

Strode C, Wondji CS, David JP, Hawkes NJ, *et al*. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. *Insect Biochem Mol Biol* 2008; 38: 113-23.

Tellam RL, Wijffels G, Willadsen P. Peritrophic matrix proteins. *Insect Biochem Mol Bi*ol 1999; 29: 87-101.

Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed Pharmacother* 2003; 57: 145-55.

Zhao X, Smartt CT, Li J, Chistensen BM. *Aedes aegypti* peroxidase gene characterization and developmental expression. *Insect Biochem Mol Biol* 2001; 31: 481-90.