Tropical Biomedicine 32(1): 65–75 (2015)

Feeding stage, species, body part and sex-specific activity of Glutathione S-transferase in mosquito

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Received 25 January 2014; received in revised from 27 May 2014; accepted 29 May 2014

Abstract. In the present study, the feeding stage, body parts, development and sex specific activity of Glutathione S-transferases (GSTs) were observed in different mosquito species (*Aedes aegypti*, *Culex quinquefasciatus*, *Anopheles stephensi*, *An. culicifacies*, *An. annularis*, *An. subpictus*, *An. vagus*). GST activity was assayed spectrophotometrically at 23°C, using a UV Max microplate Reader, to measure the rate of conjugation of GSH to CDNB. A significant species-specific difference in the activity of GST was noticed, highest being in unfed *Ae. aegypti* (41.2 nmol/min/mg) followed by unfed *Cx. quinquefasciatus* (7.9 nmol/min/mg) and the least in unfed *An. stephensi* (5.8 nmol/min/mg). In all the species the GST activity was found to be significantly higher in fully fed and gravid stages compared with the unfed, while the enzyme activity was reduced after egg laying either to the level of unfed animals or well below its level in all the experimental species. The GST activity was found to be higher in the abdominal region of all the experimental species in comparison with the other body parts (head and thorax). The GST activity of *An. stephensi* increased gradually through the larval stages and reached the maximum level in the pupae and remained at that level in the newly emerged adults. However, its activity declined markedly (10 fold) with ageing from 5 to 40 days. A significant sex-related difference in the specific activity of GST was found in *An. stephensi* where approximately 3.5 fold lower activity was observed in males compared with its females, whereas no significant variation was noticed in *Ae. aegypti* and *Cx. quinquefasciatus*. The study corroborates the fact that GSTs are differentially regulated by multiple mechanisms in response to xenobiotics modulation in situation-specific manner such as species, sex, feeding and developmental stage. The knowledge of situation-specific modulation of GST will provide a better understanding of GST based insecticide resistance mechanism which is essential for the design of sensitive monitoring methods and for an effective insecticide resistance management. The findings are significant in terms of the methods used to control mosquito vector population.

INTRODUCTION

The Glutathione-S-transferases (GSTs) are a diverse family of enzymes involved in a wide range of biological processes, many of which involve the conjugation of the tripeptide glutathione to an electrophilic substrate (Ranson & Hemingway, 2005). These enzymes form an integral part of the phase-II detoxification system (Kostaropoulos *et al.*, 1996). They play a central role in detoxification of both endogenous and xenobiotic compounds and are also involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress (Enayati *et al.*, 2005). The enzyme does have a role in maintaining the redox status of the mosquito cell, particularly in relation to vectorial capacity and resistance (Ranson & Hemingway, 2005). It is also believed that these enzymes are a family of detoxification enzymes that play essential roles in the survival of insects exposed to endogenous and erogenous xenobiotics (Kostaropoulos *et al.*, 1996). Moreover, many studies show a decrease in

reduced glutathione (GSH) concentrations during ageing (Hazelton & Lang 1980). It is also hypothesized that the presence of GST-based insecticide resistance affects the survival of mosquito pathogens and their vectorial capacity (McCarroll & Hemingway, 2002; Ranson & Hemingway, 2005). The elevated GST activity in the organism is a resistant phenotype and may account for longevity, which is directly related to vectorial capacity and competence (Tripathy *et al*., 2011). The overall importance of glutathione-S-transferases in detoxification suggested the need for a study of these enzymes during mosquito development from egg through larvae, pupa to adult and also its role in a mosquito during its blood feeding conditions and ovarian maturation, to study the situation-specific, developmental stage specific and species-specific role of this multi-tasking enzyme. The knowledge of situation-specific modulation of GST will provide a better understanding of GST based insecticide resistance mechanism which is essential for the design of sensitive monitoring methods and for effective insecticide resistance management.

MATERIALS AND METHODS

Laboratory rearing and collection of mosquito sample

Anophelese stephensi, *Culex quinquefasciatu and Aedes aegypti* colony were reared in the laboratory, Department of Entomology, Regional Medical Research Center (RMRC), Bhubaneswar, at a temperature of 28 ± 2°C and relative humidity 80 ± 10%. For the study of developmental stages of mosquito and Glutathione S-transferase activity, the eggs of the species were collected and, after hatching the 1st instar larvae were transferred to enamel trays containing tap water with yeast granules (as a source of food) for further development. Each experiment included samples from different age groups (egg, 1st instar, 2nd instar, 3rd instar, 4th instar, pupa and adults till 40 days old). After the emergence of adults they were divided into three sets. One set of the samples was kept unfed for 48 hours, the other set of the samples was given glucose feeding and the 3rd set was allowed to feed on rabbit blood. Out of these blood-fed samples, some were allowed to grow till the gravid stage. Then some of these gravids were reared after egg laying. Biochemical assay was performed in each set of the samples comprising of 5 pools each having 5-6 individuals. All biochemical processing of samples were carried out at 0-4°C.

Collection of mosquitoes from field (no insecticide was applied in the collection area)

The *Anopheles* and Culicine mosquito species were collected from Khurda district of Orissa. The identification of species (*Cx. quinquefasciatus*, *Ae. aegypti*, *An. stephensi*, *An. opheles culicifacies*, *Anopheles annularis*, *Anopheles subpictus*, *Annopheles vagus*) were carried out using the keys of Christophers, (1933) and they were separated on the basis of abdominal conditions (unfed, blood-fed, gravid). Some of the gravid and blood-fed were allowed to lay eggs and, after egg laying, the adults were taken for biochemical studies. The remaining samples (cold-inactivated mosquitoes) were dissected to obtain head, thorax and abdomen. s.

Chemicals

Reduced glutathione (GSH), 1-chloro-2, 4-dinitrobenzene (CDNB) and dimethyl sulfoxide were procured from M P Bio-medicals Pvt Ltd, Mumbai, India. All other chemicals used were of analytical grade.

Tissue preparation and biochemical assay of GST

The tissue homogenate was prepared according to the protocol of Hazelton & Lang (1983) and as modified by Tripathy *et al*., (2011). In brief, GST activity was assayed spectrophotometrically at 23°C, using a UV Max Microplate Reader, to measure the rate of conjugation of GSH to CDNB. Final substrate concentrations were 1 mM (CDNB) and 5mM (GSH) in 0.1 M sodium phosphate buffer, pH 6.0. Reagents were prepared prior to use (1 hour). Three replicates of 1.2-ml reaction mixtures (1mM CDNB and 5mM GSH in 0.1M sodium phosphate buffer, pH 6.0) were

placed in glass vials. Insect homogenate (60 µl) was added to the reaction mixture. Three blanks were prepared for each experiment with 60 µl distilled water and 1.2 ml of reaction mixture. Aliquots (210 µl) from each of the above reactions were placed in micro titer plate and the reaction rates were measured at 340 nm for 5 min. The GST activity per individual was calculated in µmol CDNB conjugated/min/well using the extinction coefficient ($e=9.6\ mM^{-1}cm^{-1}$). Specific activity was calculated by estimating the protein content. Protein was estimated according to the method of the Bradford (1976).

Statistics

Data were expressed as mean ± standard deviation of 5 to 10 pools per group. Six to ten individual samples combined per pool. A two-way analysis of variance followed by Duncan's new multiple range test was used to test the differences between four feeding stages (Unfed, fullfed, gravid and after egg laying), species (*Ae. aegypti*, *Cx. quinquefasciatus*, *An. stephensi*, *An. culicifacies*, *An. annularis*, *An. subpictus*, *An. vagus*) and region (head & thorax and abdomen) distribution of GST. Sex related differences of the enzyme were calculated by unpaired t-test. A difference was considered significant at $P<0.05$.

RESULTS

The specific activity of GST in different stages (unfed, fully fed, gravid, after egg laying) of different mosquito species (*Ae. aegypti*, *Cx. quinquefasciatus*, *An. stephensi*, *An. culicifacies*, *An. annularis*, *An. subpictus*, *An. vagus)* were studied. A significant species-specific difference in the activity of GST was observed with the highest activity in unfed *Ae. aegypti* (41.2 nmol/min/mg) followed by unfed *Cx. quinquefasciatus* (7.9 nmol/min/mg) and the least in unfed *An. stephensi* (5.8 nmol/min/mg). In all the species the GST activity was found to be high in fully fed and gravid stage compared with the unfed while the enzyme activity fell after the egg laying either to the level of unfed animals or well below its level in all the experimental species. However, the important and interesting finding is that in all the species, the specific activity of GST increased in fully fed and gravid stage compared with the unfed and after egg laying stage (Figure 1). The species-specific difference in different stages may be due to environmental or genetic factors.

To determine whether the decrease of GST activity in the adult were localized (head+thorax and abdomen) or general (whole body), different body regions of *Cx. quinquefasciatus*, *Ae. aegypti*, *An. stephensi*, *An. culicifacies*, *An. annularis*, *An. subpictus*, *An. vagus* were analyzed. GST activities were found in both parts of the body region of the adult, the activity was very high in the abdominal region and around 4.5 fold when compared with the head thorax region. In all species similar results were observed. In *Ae. aegypti* the specific activity of GST was found to be highest in the whole body of unfed, blood-fed, gravid and after egg laying (41.2nmol/min/mg, 64.2nmol/min/mg, 62.3nmol/min/mg, 39nm/min/mg respectively) and its regional distribution showed only 1.2-1.4nmol/min/mg in the head and thorax region but in the abdominal region the enzyme distribution was significantly high (data ranged from 38nmol/min/mg – 61.9nmol/min/mg). Thus, it indicated that, the GST activity was localized in the abdomen (Figure 2).

The glutathione S-transferase activity during the life-span of the mosquito, *An. stephensi* was studied. The GST activity of *An. stephensi* increased gradually through the larval stages and reached maximum in the pupae (120.36 nm/min/mg) and slightly decreased in newly emerged adults (111.85 nm/min/mg). However, its activity declined markedly (10 fold) and reached a plateau by the fifth day of the adult stage (10.004nm/min/mg) and a gradual decline occurred with ageing from 5 to 40 days (Figure 3).

The GST activities in laboratory bred female and male *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* were compared. A significant sex related difference in the specific activity of GST was found in *An. stephensi*, approximately

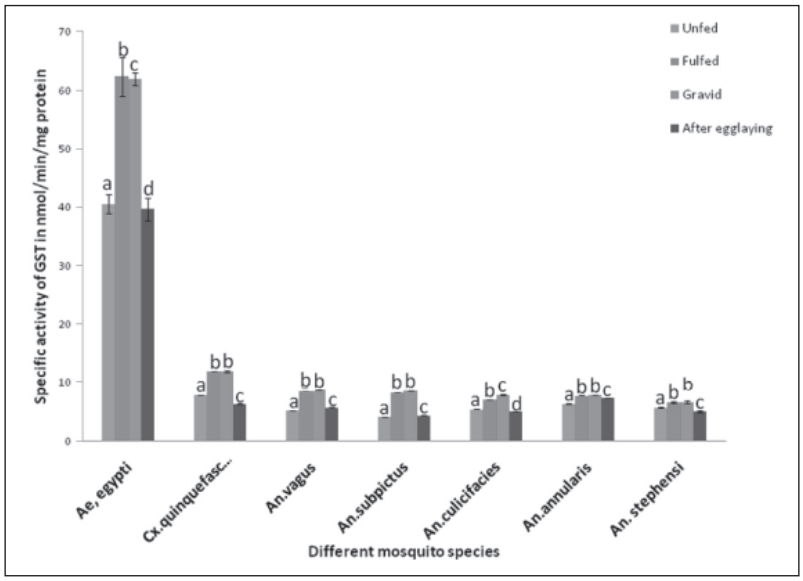

Figure 1. Specific activity of GST in different feeding stages of different mosquito species
(Data having superscripts of different letters are significantly different from each other (P<0.05))

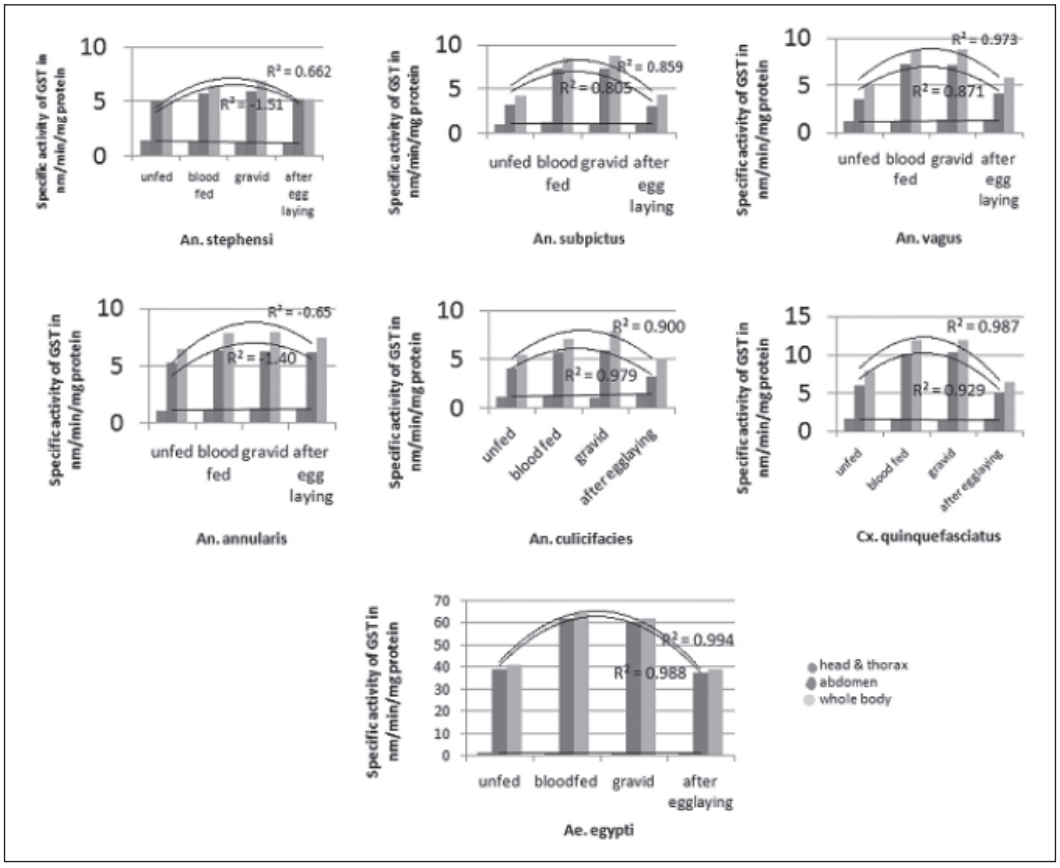

Figure 2. GST activity in different body parts of mosquito

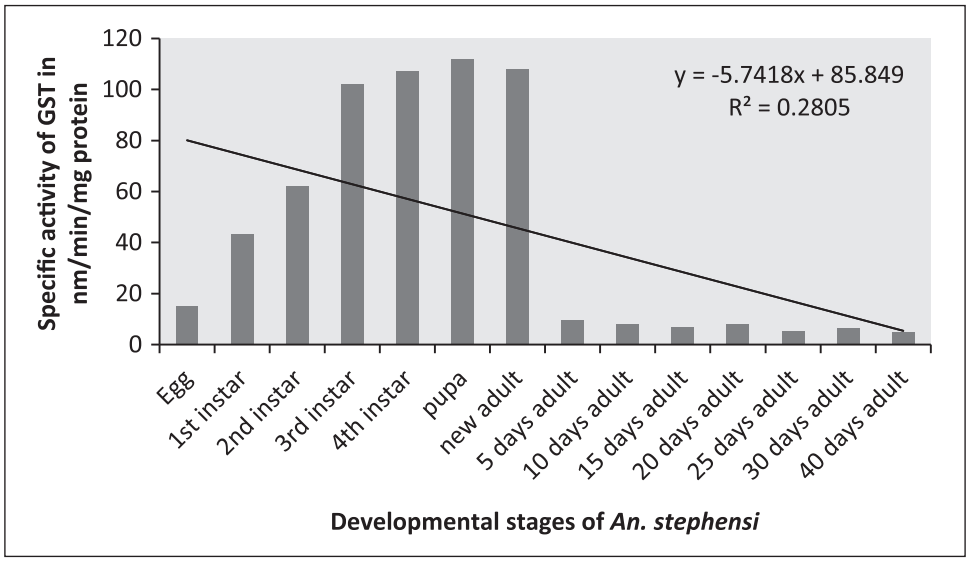

Figure 3. Glutathione S-transferase activity during the life-span of the mosquito, *An. Stephensi* (egg to 40 days Adult)

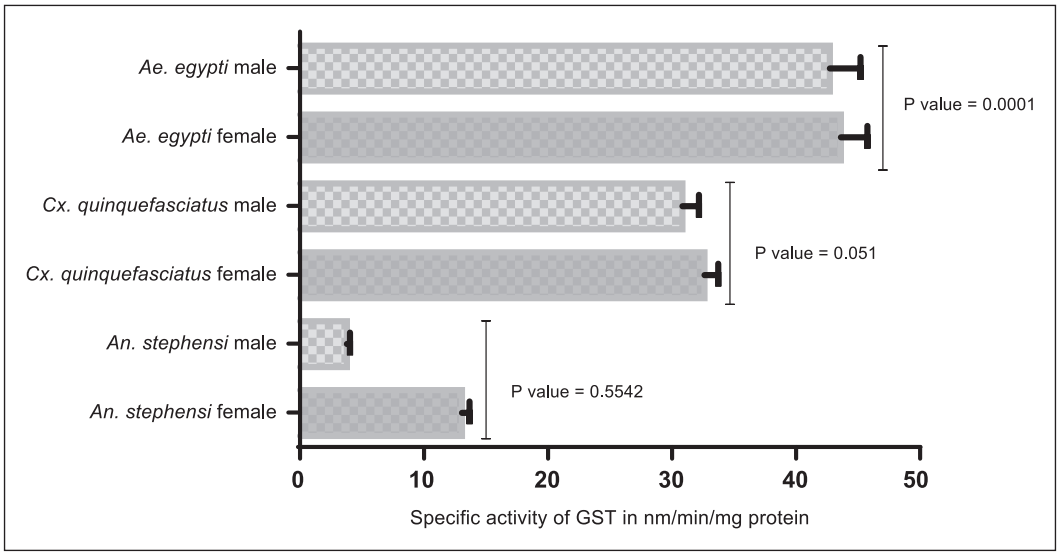

Figure 4. Specific activity of GST in Males and Females of *An. stephensi*, *Cx. quinquifaciatus* and *Aedes egypti*

3.5 fold lower activities was observed in males compared with its females whereas no significant variation was noticed in *Ae. aegypti* and *Cx. quinquefasciatus* (Figure 4). The lower specific activity of GST may account for lesser life span in male species as male mosquitoes are shorter lived than the females. In the laboratory the three experimental species were reared till their death to study their longevity. It was found that the maximum longevity of *An. stephensi* males was 8 days while the females lived up to 36 days (the mean longevity of males and females was 5.6±0.8 and 27.5±0.76 respectively. However, the male and female species of *Ae. aegypti* and *Cx. quinquefasciatus* did not show significant differences in their longevity.

DISCUSSION

Blood feeding or hematophagy is a behavior exhibited by female mosquitoes which is required for reproduction and transmission of pathogens (Dana *et al*., 2005). The cycle of blood feeding, egg development and oviposition is generally called gonotrophic cycle, a term coined by Beklemishev (1940). For most mosquitoes living in optimal field or laboratory conditions, this cycle requires about 72 hours and involves a complex series of biological events including peritrophic matrix formation, blood digestion, oocyte development, vitellogenesis and excretion (Dana *et al*., 2005). Acquisistion of a blood meal stimulates midgut proteolytic activity such that approximately 80% of the protein content is digested within 24 hours (Fisk, 1950; Briegel, 1975; Billingsley & Hecker, 1991; Lemos *et al*., 1996; Jahan *et al*., 1999). Multiple aminopeptidases have been isolated from hematophagous insects and it has been suggested that they may play different roles in digestion (Hori *et al*., 1983; Cheeseman & Gooding, 1985; Ferreira & Terra, 1986; Billingsley, 1990), thus the GST activity may be associated with the physiological process of the mosquito as they (GSTs) are involved in metabolic cycle for the transport of certain amino acids across membrane of the malpighian tubules. Hence the study was carried out to ascertain the biochemical response of GSTs to the physiological process of blood feeding, blood digestion and egg development in *An. stephensi* (urban malaria vector in India).

Mosquitoes such as Anophelines must ingest a blood meal to obtain the nutrients required for oogenesis. The blood meal itself brings metabolic changes and induces a state of oxidative stress (Felix *et al*., 2010). The blood is digested by the midgut and nutrients are transported to the fat body where vitellogenin and other major proteins of the egg yolk are synthesized (Attardo *et al*., 2005). Blood-fed females respond to oxidative stress by increasing systemic expression of ROS detoxification enzymes (Molina-Cruz *et al*., 2008). In *An. gambieae*, Dejong *et al*. (2007) observed that H_2O_2 levels in hemolymph increased dramatically after a blood meal, due to increased metabolic activity during the process of blood digestion and oogenesis. Thus, it corresponds to the present finding i.e. increased GST activity during the full fed stage and oogenesis. Hence, we postulate that higher GST activity during gonotrophic cycle is strongly associated with the enhanced levels of H_2O_2 as GST scavenges H_2O_2. According to Dejong *et al*. (2007), ROS detoxification by catalase increases the reproductive output by protecting the ovary and the early embryo from oxidative damage but the transient and local accumulation of ROS appears to be necessary for normal mosquito physiology. Kumar *et al*. (2003) analyzed ROS levels in hemolymph of the refractory strain of *An. gambie* and suggested that clearance of H_2O_2 plays a rate limiting step in free radical detoxification. Their study also confirmed that H_2O_2 level increases in response to blood feeding. According to Lumjuan *et al*. (2007), out of the three mosquito specific GSTs, GSTX2-2 showed an affinity for hematin and this indicates a possible role of these enzymes in protecting mosquitoes against heme toxicity during blood feeding.

The finding is significant in context of vector, as the vector gets infected after taking an infected blood meal and at that time it remains under double stress due to blood-fed condition and presence of parasite in the blood meal. However, the present observation has shown that during the blood-fed condition the specific activity of GST is high which might be required to neutralize the ROS generated during the stress condition. The intake of blood meal by the mosquito brings metabolic changes and induces a state of oxidative stress and further it is increased by the presence of *Plasmodium* parasites in the blood meal (Felix *et al*., 2010). Elevated level of reactive oxygen species is a major factor contributing to the parasite melanotic encapsulation (Kumar *et al*., 2003, Christophides *et al*., 2004). Previous studies have shown that higher levels of reactive oxygen species (ROS) in mosquito hemolymph limit *Plasmodium* development (Dejong *et al*., 2007; Molina-Cruz *et al*., 2008).

Further it is supported by the evidence that ROS is generated in defense mechanism against pathogen infections in insects (Ha *et al.*, 2005a, b) including mosquitoes (Dimopoulos *et al.*, 2002; Kumar *et al.*, 2003).

The occurrence of high concentration of GSTs in the abdominal region is because of the fact that the mosquito abdomen contains the midgut, fat-body and malpighian tubules, which are analogous to mammalian organs that have high transferase levels and are essential for absorption of nutrients and the excretion of waste products (Hazelton & Lang 1983). Thus, the high enzyme concentration in this region is perhaps related to their functional roles.

In *An. stephensi*, the GST activity progressively increased from the level in egg through the larval stages to reach the maximum in pupa and remained at that level in the newly emerged adult after which its activity fell drastically. These differences in the specific activity might be due to different isozymes present in the tissues at different developmental stages of *An. stephensi*, and their preference for binding to CDNB or any inhibitor. The observed specific activity for *An. stephensi* larvae is similar to the findings in the drosophila *H. armigera* and *H. zea* (Rajurkar *et al.*, 2003). The drosophila eggs exhibit highest specific activity of GSTs while their adults show lowest specific activity. The maximum activity of the enzyme was seen in the 4th instar larvae. Similarly, in *H. armigera* maximum activity was found in two day old 5th instar larvae and in *H. zea* it was also highest in 5th instar larvae (Rajurkar *et al.*, 2003). This indicates that the quantity of enzyme increased as the larvae grew older. Some studies have also reported a higher specific activity in the pupae, such as in *Lucilia cuprina*, *Ae. aegypti*, *Tenebrio molitor* (Kostaropoulos *et al.*, 1996), which is similar to our findings. This may be explained in many ways: pupae are less mobile and consequently are more vulnerable to unfavorable environmental conditions, including the presence of toxic substances (Kostaropoulos *et al.*, 1996); secondly, the elevated biosynthesis and construction of adult tissues in the pupal stage (Doctor & Fristrom, 1985). Thus high specific activity could mean higher ability of detoxification and as a consequence protection of important and crucial biosynthetic pathways from inhibition by toxic substances (endogenous and exogenous). These evidences indicate a less effective GST detoxification system in the adult stage. However, lowest specific activity in the larval stage was found in *H. armigera* (a polyphagus pest of agricultural importance) (Rajurkar *et al.*, 2003). Papadopoulos *et al.* (2004) studied the pattern of GST in the course of the development of *Apis mellifera maccdonica* where the highest activity towards the substrate CDNB was found in adult stage and the lowest in egg. The major consequence of the lower GST activity is that the capacity for GSH linked detoxification of xenobiotics is diminished during senescence. There is a decrease in the overall potential for conjugation of foreign compounds, which then are metabolized and excreted as mercapturic acids. For these reasons old organisms would be at a higher risk to toxicants than the young ones. Thus a phase-II reaction of detoxification process is altered during senescence. However, a decrease in microsomal cytochome P450 dependent reactions in phase-I has been shown to occur with ageing (Hazelton & Lang 1983). The developmental changes in glutathione S-transferase i.e. high GST activities observed during larval growth and metamorphosis are due to the reason that this period is characterized by high biosynthetic activities (Hazelton & Lang 1983). Thus a higher capacity for detoxification is needed at this stage. A similar view of glucuronidation conjugation reactions in developmental stages has been proposed for *Ae. aegypti* (Hazelton & Lang 1983). Such evidence could be related in part to the lack of DDT tolerance with age in adult mosquitoes (Lines & Nassor, 1991). Blood ingestion had been reported to increase the tolerance to DDT and PYRs (Halliday & Feyerisen, 1987) and GSTs could be involved since several GSTs genes are up-regulated after blood ingestion in mosquitoes (Marinotti *et al.*, 2005).

The low specific activity of GST in the male species of Anopheline may account for their shorter lifespan. It can be supported by the fact that proteins involved in the defense against ROS (Reactive Oxygen Species) have major effects on longevity in the mosquitoes (Tripathy *et al*., 2011). Further studies have demonstrated that some GSTs are up-regulated with aging (Zou *et al*., 2000), and their over-expression could cause a life span extension in flies (http://www.uams.edu/biochem.Hbenes.asp).

In Tanzania, for instance the survival of marked males of *An. gambeae* was only slightly less than that of the females and the two oldest mosquitoes recaptured after 27 and 30 days and both were males (Wernsdorfer & Mc Gregor, 1988). Similarly, in Pakistan, the survival of males of *An. stephensi* was identical with that of the females and the maximum longevity of male *An. stephensi* was 12 days (Wernsdorfer & Mc Gregor, 1988). Thus, the present study suggests that the physiological effects may be less in females than the males of *An. stephensi.*

The study by Spillings *et al*. (2008) suggested that insecticide detoxification mechanisms involved in insecticide exposure leads to enhanced expression of the resistant phenotype (as GST). Thus, the present findings seems significant in the context of the methods used to control the populations of the vector species if the mass killing effect of insecticide application proves increasingly inadequate against the *Ae. aegypti*, *Cx. quinquefasciatus* adult blood-fed and gravid females, larvae and pupae which are already carrying the insecticide resistant phenotype (carrying a high specific activity of GST). An enhanced ability of the insecticide resistant insects to tolerate oxidative stress has also been implied by the protective role of glutathione S-transferases (Vontas *et al*., 2001). Mittapalli *et al*. (2007) studied the GST expression in the Hessian fly and found that the product of the Delta GST genes aid in detoxifying exogenous allelochemicals from the host plant (wheat), while that of a Sigma GST could provide protection against toxic oxygen species generated endogenously during development. Our previous study showed that GST played a significant role in insecticide resistance of the vector and an enhanced ability of the insecticide resistant mosquitoes to tolerate oxidative stress was implied by the protective role of Glutathione S- transferase (Tripathy *et al*., 2011). Todate, the anti-plasmodial role of GST in the mosquito vector is not known. The recent study by Oliveira *et al*. (2012) revealed that Nitric Oxide Synthetase (NOS) induction is not sufficient to achieve an effective antiplasmodial response. Thus it draws our attention to the protective role of Glutathione S- transferase which may have antiplasmodial role in the mosquito vector. Thus, further studies are needed to explore the mosquito immune defense mechanism, genetic pathway of vector physiology, vectorial capacity and competence. Recently, in epsilon and delta GST gene promoters from Anophelines, putative binding sites and regulatory/ response elements involved in the induction of GST expression in response to oxidative stress have been found supporting the antioxidant physiological role of GSTs (Ding *et al*., 2003; Udomsinprasert *et al*., 2005). This suggests that GSTs are differentially regulated by multiple mechanisms in response to xenobiotics modulation and/or in a tissue- or developmental-specific manner. The knowledge of regulatory elements involved in the induction of GST's, will provide a better understanding of the molecular basis in the GST-based insecticide-resistance mechanism essential for the design of sensitive monitoring methods and then for an effective insecticide resistance management.

Acknowledgement. Financial assistance from Indian Council of Medical Research (New Delhi) to the Regional Medical Research Centre (Bhubaneswar) is gratefully acknowledged. We thank the Director, Regional Medical Research Centre (Bhubaneswar), for providing necessary facilities. We also thank the staff members of the division of Medical Entomology, RMRC (Bhubaneswar) for their cooperation.

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