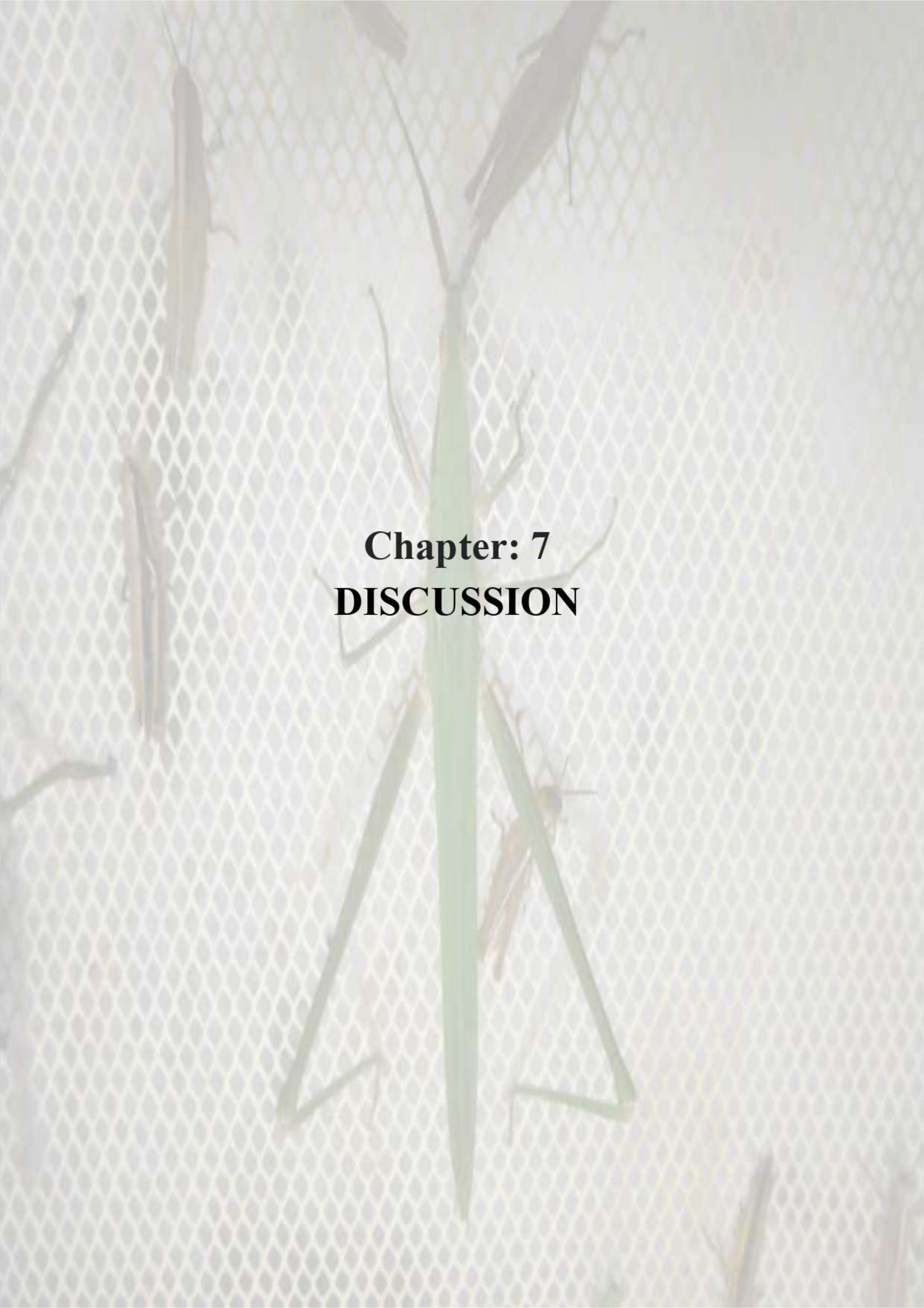

Chapter: 7
DISCUSSION

7. DISCUSSION

The screening study was conducted to assess the toxic efficacy of commonly used pesticides in in-vitro tissues of common agri-field animals. The actual study was conducted to assess the toxic efficacy of azadirachtin (AZT) in orthopteran grasshopper. The results obtained in the study under in-vivo and in-vitro toxicity are discussed here.

7.1. Oxidative stress (OS) induced in-vitro toxicity in common agri-field animals

The liver is the largest organ in the vertebrate body and it is the major site of xenobiotic metabolism and excretion. Hepatic injury is a common pathological outcome which exists in many liver diseases. Cows (*Bos taurus*), goats (*Capra aegagrus hircus*) and poultry birds (*Gallus gallus domesticus*) have been recognized as the most effective common agri-field animal for promoting health and economy, worldwide. These animals are predominantly common in agri-field and consume those products. In the current study, the catalase (CAT) activity in goat liver decreased after in-vitro incubation with pesticides. In addition, this inhibition was prominent in case of chlorpyrifos (Fig. 1.a) and AZT (Fig. 1.c). The activity of superoxide-dismutase (SOD) in goat liver distinctly decreased after chlorpyrifos intoxication (Fig. 1.f). But, the statistical calculation did not present significant changes. However, in case of gut tissues in the present experimental insect, both CAT and SOD activities increased after in-vivo and in-vitro AZT exposure which is found to be highly significant (Fig. 1.d and e; Fig. 1.i and j). Moreover, the extent of increase of enzymatic activities with comparison to control is higher in female insect (lane 1-6) than that of male insect (lane 7-12). This may suggest that these pesticides were less effective in the mammals and birds to generate radical toxicity, so the adaptive responses were less prominent. But in case of insect the toxicity and the adaptive responses both were significant. Oxidative stress (OS) has been shown to play a crucial role in the cellular toxicity generated by a large number of pesticides (Stevenson et al. 1995). Free radicals (FRs) especially reactive oxygen species (ROS) are essential for different metabolic functions including the cell signalling and other physiological processes (Li et al. 2016). It has been already reported that the role of AZT as a potential inducer of mitochondrial superoxide anion radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (Huc 2007). This has been clearly noticed in our present study.

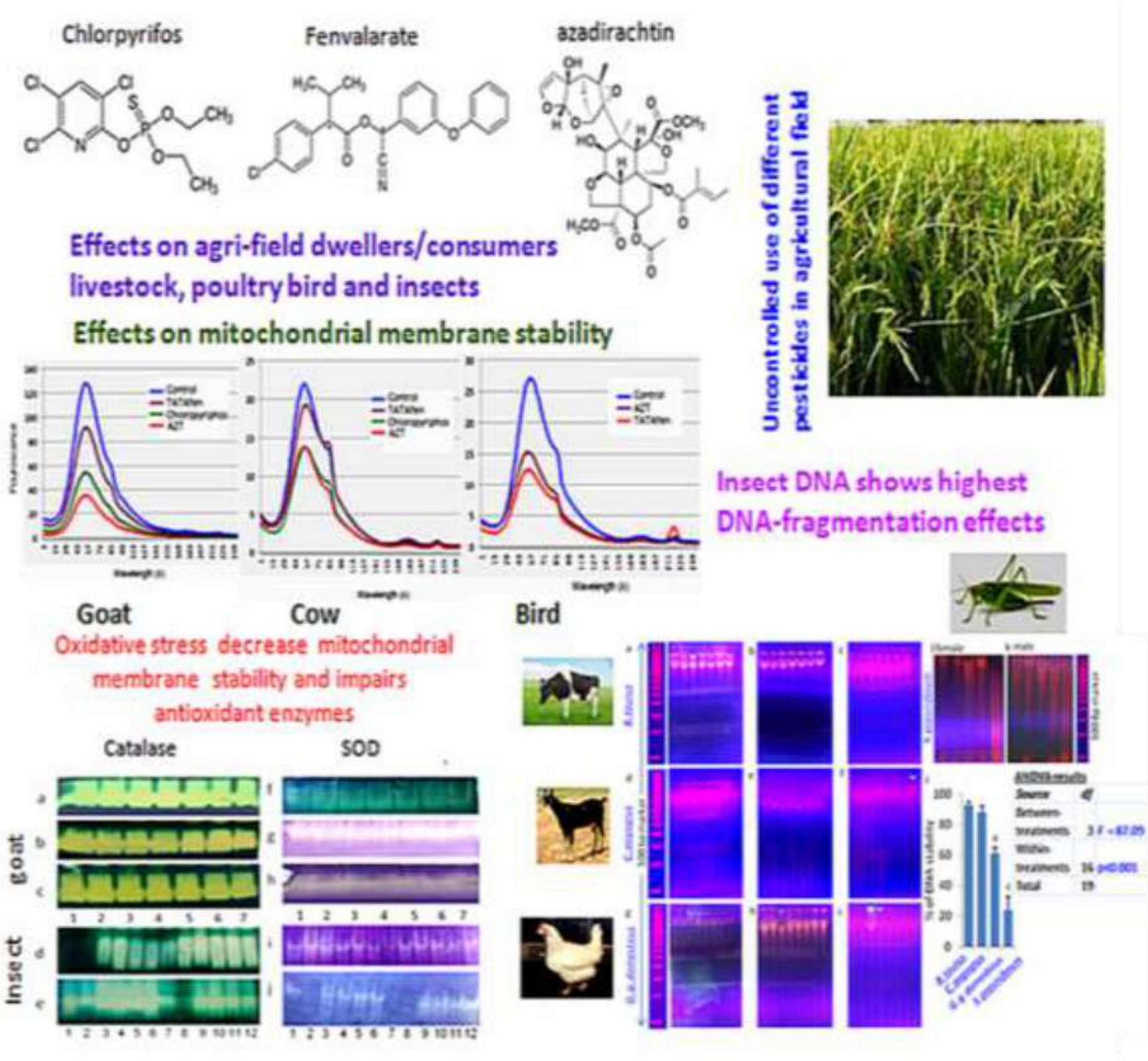

Plate 27. Oxidative stress induced toxicity and DNA stability in some agri-field based livestock/insect by widely used pesticides.

The impairment in antioxidant defence mechanism is evident by the cellular damage caused by pesticides (Lobo et al. 2010). This has been reflected in the mitochondrial membrane degeneration studies (Fig. 3). This degeneration is responsible to develop a FR-cascade possibly at the cytosolic level. Plasma membrane and organelle membrane stability is a determinant of cytotoxicity. Earlier it has been suggested that altered membrane fluidity can be responsible for the decreased activity of pesticide in mouse (Maiti et al. 1995) and in experimental chicken model (Maiti et al. 1996). So, pesticide may impair the physiological and biochemical processes across the species and that may be influenced by the cellular physiology. The CAT activity was impaired in the experimental organisms after exposure to different concentrations of pesticides i.e. chlorpyrifos, fenvalerate, nimbecidine. It might be due to the binding of the pesticide residue to CAT or by inhibiting this enzyme synthesis (Tripathi and Verma 2000). An appreciable amount of decrease in SOD activity was noticed in the liver of cattle (Fig. 1.f and 1.g). But a significant dose dependant increase of SOD activity was noticed after both in-vivo and in-vitro AZT exposure to the insect of both sexes. This suggests that antioxidant enzymes are more responsive in environmental stress in the lower organisms and it may be selected as a biomarker in variety of OS. The similar decrease in CAT activity was observed in brain, liver and kidney of *Channa punctatus* exposed to triazophos (Naveed and Janaiah 2011). The CAT activity reduced in the brain, gill, liver and skeletal muscles of α-methrin treated *Channa punctatus* also noticed (Tripathi and Singh 2013). The degree of effects of chlorpyrifos, fenvalerate, and nimbecidine on CAT and other protein profile was variable in different common agri-field animal-tissues because it may depend on the location and metabolic involvement of the tissues (Doss et al. 2008). Tissue specific antioxidative responses were known to take place during stress exposure.

In the present study, we have established that pesticides influence antioxidative enzymes in liver of higher organisms and gut of the insects and therefore, liver injury associated with this insecticide may be due to oxidative tissue damage. There is little evidence of change in antioxidant system after intoxication with pesticides to agri-field dweller organism. An increase of SOD and CAT activities in the liver of dichlorvos-treated carps observed (Hai et al. 1997) and also found elevated levels of malondialdehyde (MDA) in the hepatic tissue of dichlorvos-treated fish. Fish is the organism of aquatic ecosystem and exposure via contamination in water might have instance effect. The mechanism of intoxication might suggest the pattern of toxicity in our present experimental organism. The organophosphates (OPs) and carbamates, besides its inhibitory effect on acetyl-cholinesterase (AChE), initiate the accumulation of FRs leading to lipid peroxidation (LPO) on such organism (Yang et al. 1996). Mitochondrial stability and function has not been observed in those previous experiments. In the current investigation, we evaluated mitochondrial membrane potential in cattle and bird-hen and considered noticed that pesticides have a strong destabilizing activity. Due to very low yield of mitochondria the signal was undetectable in the insect. Nevertheless, direct evidence of antioxidative responses (CAT/SOD) and deoxyribonucleic acid (DNA) instability was noticed in insect after AZT exposure.

Antioxidant protection is accomplished by many enzymatic and non-enzymatic factors which maintain the physiological level of reactive oxygen metabolites (Ondreicka et al. 1998). The antioxidant defence system includes SOD, CAT and GPx enzymes. I measured the activity of two antioxidative enzymes; SOD and CAT. Superoxide-dismutase plays an important role in the first line of the antioxidant defence system by catalysing the dismutation of $O_2^{\bullet -}$ to form H_2O_2 and molecular oxygen (O_2). Further this

H_2O_2 undergoes FR-cascade reacting with transition metals or LPO products are reactive aldehydes, such as MDA (Ho et al. 1998).

In the screening study, a significant DNA-laddering was found in the experimental insect induced by AZT (Fig. 2.j and 2.k). A moderate DNA-laddering was also noticed in the bird-hen (Fig. 2.g, 2.h and 2.i) for three pesticides. But in the higher vertebrate, DNA was found to be more stable (Fig. 2.a to 2.f). The pesticide treatment induces DNA-fragmentation which is one of the criteria of necrotic or apoptotic cell death. This finding would be related to the magnitude of the exposure to pesticides and was directly involved in DNA-fragmentation. So, the major changes observed in DNA-fragmentation of liver may suggest a direct effect of the pesticides. Fungicide-induced DNA damages were caused by OS, and might be responsible for the higher occurrence of apoptotic cell death (Schwarzbacherová et al. 2017). Similar to our present study, electrophoretic analysis confirmed the potential role of epoxiconazole to induce DNA-fragmented ladder that re-garded as the marker of apoptosis (Šiviková et al. 2018).

The screening study was conducted to investigate the pesticides in alleviating OS in liver injury and DNA-fragmentation. Liver is the site of biotransformation by which a toxic compound is transformed to a less harmful form to reduce toxicity (Hodgson 2004). However, during these processing hepatic cells may itself be damaged to some extent and develop chronic hepatotoxicity. Pesticides induce OS, which leads to the generation of FRs, changes in antioxidants levels and LPO (Ender and Onder 2006) thus causing dam-age to proteins, lipids and DNA (Kale et al. 1999). Report reveals that dimethoate-induced DNA damage in the haemocytes of the insect *Chorthippus biguttulus* (Karpeta-Kaczmarek et al. 2016). Some critical analysis has been shown earlier in *Drosophila* sp., but not in the present experimental grasshopper species. Dichlorvos is an OP-pesticide was reported to induce DNA damage and also affect pre and post replication repair mechanism in *Drosophila* sp. (Mishra et al. 2014). The normal error rectification processes were thus distorted and a certain degree of mutagenicity was generated at the level of bio-transformation by the application of the pesticides.

The DNA-fragmentation observed in the screening study has very wider significance. It is the normal consequences of OS that was demonstrated through inactivation of antioxidant enzymes (CAT/SOD) in metabolic organ. This is also consistent with previous studies where DNA-fragmentation was induced by pesticides in rat lymphocytes (Sharma et al. 2010) and in rat brain by cypermethrin (Hussien et al. 2013).

Not during the single generation period, even maternal pesticide (fenvalerate) exposure during pregnancy-period may impair growth and brain development in mouse off-spring (Liu et al. 2018). Hampering in the brain behaviour is shown to trigger Parkinson-like symptom through initiation of autophagy and p38 MAPK/mTOR signalling pathway (Zhu et al. 2020). Moreover, interferences by fenvalerate in the expression of pro-apoptotic Bcl-2 family proteins increase the carcinogenicity potential of this pesticide (Cui et al. 2018). The differences in the DNA stability of different organisms may be due to differences in DNA sequences between lineages which noticed in our current study. These facts influence the stability, mutability and error-correction efficiency of the DNA. In lower organisms the DNA was found to be less stable in response to xenobiotic/pesticide exposure (Fig. 2j and k). The insects originated between the Cambrian period (Euarthropoda, 535 MY) and the Devonian period (385 MY). During this period and further during the long tenure of speciation, insect has gone through less diversification (Plate 23). So the adaptive modification and DNA stabilization is less likely to happen here. In contrast, Aves originated 135 MY ago in the first part of Cretaceous period and went through stages of more diversification (Plate 23). Comparatively, less DNA-fragmentation is noticed in *G. g. domesticus*, the bird-hen (Fig. 2.g, h and i). Report reveals that in prokaryotes like bacteria, environmental stress and other 'mutants' has influenced on the selection pressure. Nevertheless, those have lower DNA repair efficiency, hence higher mutation rate (Denamur and Matic 2006). In cattle very less DNA-fragmentation is noticed (Fig. 2.a to 2.f). An extensive diversification and adaptive modification is noticed in mammalian, originated in Cenozoic period, 65 MY and these mammals were diverged in rodents, primates, cattle and others in a very short (Cenozoic) time period (Plate 23). Adaptation in higher eukaryote may be governed more by natural selection-pressure than mutation associated forced error on DNA sequences/stability. Even species can differ in rate of copy error detection and repair. Number of DNA replications per generation can also vary with population structure and mating system (Bromham 2009). Beside the toxicity and adaptation studies and their comparative analysis in different living system, there may have some wider messages in our study. This work may resemble a reference model of environmental stress and its ecological/evolutionary impact dictated by the xenobiotic interfered DNA stability.

As for example, Daphnia haemoglobin (Hb) has been established as the widely recognized respiratory pigment. As a reliable biomarker it can predict the water contamination with Bisphenol A (BPA), Benzo[a]pyrene (B[a]P), chlorpyrifos (Ha and Choi 2009). Azadirachtin has been shown to suppress the growth and development of *Bactrocera dorsalis* by releasing cathepsin (Zhao et al. 2019) and induce apoptosis in *Bombyx mori* by releasing extra Ca^{2+} (Zhang et al. 2017).

Lower organisms are more socially behaving and actively participate in the formation of a composite niche/ecosystem. So, any kind of changes or deterioration in ecosystem may affect their fecundity, reproductive ability, life span and mortality. The changes in their DNA structure/stability may modulate their phenotypic behaviour and their interaction with neighbouring organisms.

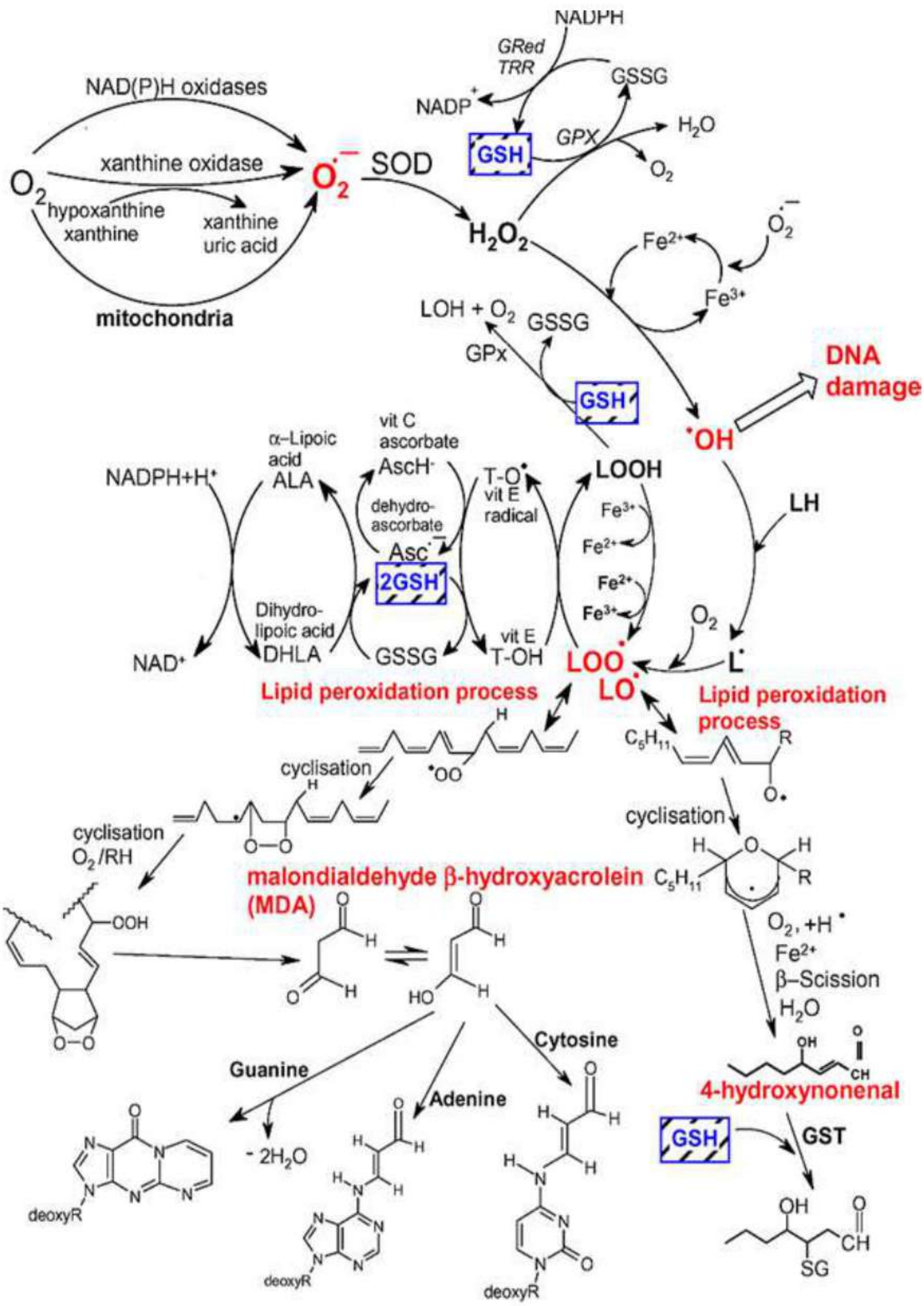

Plate 28. Free radicals (FRs) generation and reactive oxygen species (ROS) formations in biological system. (Valko et al. 2007)

7.2. Biodiversity of acridids species

Biodiversity involve construction of urbanization in the agri-field areas, which collectively exerts an intense influence on the entire natural habitat of grasshoppers. The encroachment of man into the habitual areas of grasshoppers leads to a considerable reduction of both the number of grasshopper species and the total number of grasshoppers in the adapted field. The other effect of the aforesaid activities on the grasshopper species is that these species were forced to concentrate and develop the potentiality for mass multiplication in favourable year and move to neighbouring area of cultivation and emerge as pests. The occurrence of large number of *Spathosternum* sp. and *Oxya* sp. supports this contention (Tandon and Khera 1978). And also reported such change in the habitat of grasshoppers and observed the changes in the behaviour of certain orthopteran insects of different stations, in dense stands of grass (Uvarov 1961). These species behave as phytophytes, while in light stands these are geophytes (Bei-Bienko 1958). Tandon and Khera (1978) also reported this kind of behaviour in case of *Trilophidia annulata*, *Aulacobothrus luteipes*, *Gesonula punctifrons*, which dealt in this study was also affected by changes in environment leading to its emergence as a serious pest in the neighbouring agri-field. Further it is worth mentioning here that some of the species namely *Oxya velox*, *Locusta migratoria*, *Eucoptacra saturata*, *Eyprepocnemis alacris alacris* etc. which were available in the past from the agri-fields in and around in this area were little found in the screening study. It may be decided from this study that the vegetation in combination with the other physical factors considered in this study collectively influence the population diversity of grasshoppers in this area. The temperature showed direct influence on the population structure of grasshoppers but failed to show any significant correlation (Hazra 1984) because temperature alone is of little influence in governing the abundance and distribution pattern of grasshoppers. The relative humidity content of the surface soil was positively correlated with the grasshopper population. During monsoon, higher content of relative humidity enhanced the growth rate of vegetation (Hazra 1984). The species like *Spathosternum prasiniferum prasiniferum* and *Oxya fuscovittata* were present in agri-field throughout the year and such a year round occurrence with different nymphal stages suggest the continuous breeding indicative of the existence of multi-voltine cycle in these species (Uvarov 1977). Similarly, *Acetabularia crenulata*, *Phlaeoba infumata*, *Aiolopus thalassinus tamulus*, *Oxya hyla hyla* were bi-voltine and *Aulacobothrus luteipes luteipes*, *G. punctifrons*, *Acrida exaltata* were uni-voltine. Therefore, *S. pr. prasiniferum* was selected as model organism for evaluation of toxicity of AZT because of this insect easy culture in laboratory conditions, short generation time and ease of genetic manipulation.

7.3. General observation

The observations made in the present study on the behaviour of the insects after exposure to AZT include irregular movements, impairment of locomotion, convulsions and weak response for external stimuli, knockdown and finally paralysis. Since behaviour is one of the important functional outputs of central nervous system (CNS), the observed behavioural changes attributed to disturbances in central nervous coordination. The behavioural changes can also be attributed to the alterations in the AChE activity (Fig. 12.e and 13.d) and in the nervous system of insects observed in the present study (Plate 30) following exposure to AZT. It has been demonstrated earlier that pyrethroid exerts inhibitory action in AChE. The observed hyper-activity in the animals may be due to high amount of monoamines accumulated in nervous and muscle tissue and due to the excitatory action of AZT on the nerve activity observed in the present study.

7.3.1. Toxic effects on insect's behaviour and physiology

Major insects are pollinator in plants besides existence pests of plants. Most orthopteran insects are generally very high reproductive potentials with short life cycles and produced high biomass. An abnormal behaviour like restlessness, sudden quick, jerky movements are observed in the insects at low concentration of AZT whereas increased movements accompanied with swarming, loss of equilibrium and abnormal oviposition are observed in higher concentration of AZT exposed insects. In the present study, corrected mortality (%) of AZT have been determined to select experimental concentrations for assessment of its toxic impacts at molecular levels of *S. pr. prasiniferum*. I watched insect mortality increases with the doses of AZT treatment in both sexes and it is noticed that male is more sensitive to the AZT and female is more tolerant to AZT (Plate 31). The mortality increases almost linearly and after the 5 ppm dose, mortality rate becomes higher (Fig. 17.b and Fig. 5.a). From the Fig. 17.c it can be advocated that the selected acridid-species and other similar acridid-species have high nymphal life span (≥80 days), but in Fig. 17.d it is clear that adult female has appreciably higher life span than its male and this is true for all the primarily studied organism from that habitat. This finding is in correspondence with our biochemical and molecular study; that is female has a greater adaptability in terms of OS and antioxidant activities. The behavioural changes noticed in insects treated with AZT were more pronounced under different doses. The insects recovered from behavioural changes after low doses. Non-recovery to behavioural normality following higher doses may be due to the irreversible changes occurred in the functions of the nervous system (Plate 25). The recovery under low doses is presumably due to detoxification mechanisms existing in these animals (Diaz et al. 2000).

The increase of AZT in the insect's body impaired their metabolism and resistance against the toxic effects of AZT for that reason insects become susceptible to degenerative tissue damages. Food ingestion (contaminated with AZT) and preliminary digestion were significantly ($P<0.001$) increased at higher doses of the pesticide. In general male found to be more sensitive to toxic exposure in relation to brain tissues.

7.3.2. Toxic effects on insect's nutritional-indices

The antifeedant or reduced food intake response observed in the cockroaches exposed to azadirachtin or cypermethrin has been reported. *Spodopfera littoralis* treated with AZT showed greater antifeedant response. Research on the biological activity and chemistry of antifeedants is heavily emphasized because of their biodegradable nature and relative safety for useful organisms in the environment. Unlike insecticides that kill absolute both pests and predators, the antifeedants obtained from plants are relatively safe for natural enemies which survive on pests rather than on plants. Feeding and reproduction in insects are very closely related to nutritional factors. The consumption of food is necessary for all the aspects of insect's performance, i.e., growth and development, reproduction, defence, movement and survival (Slansky and Rodriquez 1987).

There was significance change in feed consumption of all the treated groups compared to control group. The literature on the effect of AZT efficacy in this insect is scanty. From the present study, it was found that AZT at the applied concentration was highly effective against these insects. As an index to control the crop insect, nutritional-indices have been analysed in adult stages of *S. pr. prasiniferum* after dose dependant AZT toxicity (Fig. 5.b). Food ingestion, digestion and consumption index (CI) which has necessarily increased in higher doses is statistically significant ($P<0.001$) for stimulating the enzymes activities during feeding whereas that the approximate digestibility (AD) is not significantly increased (Fig. 5.b) may be because of the fact that physiological event of insects to consume AZT containing relatively less quantity of food is distributed to body (Sundararaj et al. 1995). Whereas efficiency of conversion of digested-food (ECD) and efficiency of conversion of ingested-food (ECI) are reduced after treatment for availability of flavonoid act as toxins to number of insects (Harborne 1982) suggested that the utilization of food for conservation of energy for various life activities have been challenged by the AZT. The increase in CI and AD was stimulating for enzymes activities during feeding. Comparison of CI and AD among several field insects suggests that when the CI is higher in this insect the AD is very much lower with comparison to other similar field insects. This suggests they damage plants more than they are actually capable to digest it. Nevertheless, this hypo-metabolic state increases the mortality of the insect as a result of AZT toxicity occur. Azadirachtin interferes with growth via digestive impairment by inhibiting the secretion of proteinases from gut cells (Koul et al. 1996) and similar result observes on *Manduca sexta* L. (tobacco hornworm) (Timmins and Reynold 1992). Decrease level of ECD (Hummel 1989) suggests that AZT have toxic effects on *S. pr. prasiniferum* like other conventional pesticides. Earlier studies indicate that low level of feeding may be due to the fact that the AZT block the bioavailability of nutrients (Broadway and Duffey 1986) whereas in the present study the higher concentration of AZT significantly increases the consumption level so AZT may be a phago-stimulant/deterrent which played an important role in the consumption, digestion and alteration the feeding rate of this species. Azadirachtin also reduced weight gain and nutritional-indices in an insect. This is possibly due to the reduction in digestive enzymes found in the larval mid-gut (Nouri-Ganbalani et al. 2016).

7.3.3. Toxic effects on insect's gut

The in-vivo and in-vitro both sexes gut protein contents has been significantly differing in comparison to control (Fig. 6.a and 7.a) by interfere with the production of certain types of proteins. An increased level of LPO was found in in-vivo female and male gut (Fig. 6.b and 7.b). Azadirachtin administration resulted in a significant increase in MDA production (El-Demerdash 2007) arises from the reaction of FRs with lipids and this is considered to be an important feature of the cellular injury by FR-attack (Hoek and Pastorino 2002). In the present study, an increased level of non-protein-soluble-thiols (NPSH) was found in in-vivo and in-vitro both sexes gut cells which suggest possible adaptive strategies against AZT toxicity (Fig. 6.c and 7.c). This biomolecule can serve as a sink for FRs and reactive species against OS induces toxicity by its protective and adaptive roles (Otto and Moon 1995). Alkaline-phosphatase (ALP) is a primary hydrolytic enzymes of insect's gut (Sakharov et al. 1989) that hydrolyses phosphate-monoesters under alkaline conditions and its activity is decreased by AZT like neem products (Senthil-Nathan 2006). Our results indicate that dose dependant AZT reduced ALP activity in in-vivo both sexes gut cells (Fig. 6.d) that changing the physiological balance and physiological events of *S. pr. prasiniferum* with ion transport to the gut cell. Most aromatic plant products have inhibitory activity of AChE (Shaaya and Rafaeli 2007). Acetyl-cholinesterase activity is significantly decreased in female by non-specific binding with AZT other than active site (Mordue et al. 2010) therefore acetylcholine (ACh) cannot be degraded and insects will die with toxic symptoms (Tang 2000) whereas male gut represent higher activity (Fig. 6.e and 7.d).

Sex dimorphic role of some of the adaptive response is evident in the current study such as increase of CAT activity in female but decrease in male insect. During the present investigation it is observed that the AZT promotes changes in the antioxidant defences in gut. Catalase is the first line biomarker against cellular oxygen toxicity (Pandey et al. 2003). Present investigation represents concentration dependent increases CAT activity in female but decreased in male. Superoxide-dismutase activity after AZT treatment is significantly higher than that of controls (Fig. 8). Report suggests that, pesticides intoxicate-OS leading to generation of FRs that change in antioxidants levels and LPO (Ender and Onder 2006). The degeneration of mid-gut tissue was accompanied by the transcriptional regulation of mitogen-activated protein kinase (MAPK) and calcium apoptotic signalling (Shu et al. 2018). Heavy metal induced MAPK P38 kinase activation is found to be linked.

Inhibition of insect pest amylase by plant derived inhibitors (Sivakumar et al. 2006) has been established. It was observed that increased amylase activity in female gut at higher dose of AZT is most probably due to consumption and utilization of large quantities of food whereas AZT inhibition effect is in male gut (Fig. 9) suggest that AZT is insecticidal because they form stable complexes with digestive enzymes which could inhibit the formation of enzyme-substrate complex. Thus leading to blocking of digestive enzymes result in poor nutrition, growth retardation and leading to death of the insect (Bezzar-Bendjazia et al. 2017). Neem extract has an ability to inhibit the cellulase activity (Sami and Shakoori 2007) and inhibition of cellulases by some of its phenolic compounds is evident (Ximenes et al. 2010). Decreased activity of cellulase in female gut (Fig. 9) hydrolysing enzyme activity suggests that plant derives molecules mimic the substrate molecule (Sami and Shakoori 2011). Imbalance in enzyme substrate complex might have inhibited the enzyme activity in the treated insects and enzyme production is related to the amount of food CI (Chapman 1985).

Pesticides indirectly generate various radicals that damage proteins, lipids and DNA by oxidation (Kale et al. 1999). Present work indicates that, OS-induces DNA-laddering/lowers DNA stability (Fig. 10) and less mRNA/protein production. Macromolecular disruption by AZT-induced MDA elevation has shown in some insect species (Sharma et al. 2010).

Oxidative stress occurs when the balance between the productions of ROS replace the antioxidant defence capability of aerobic cell (Rice-Evans et al. 1996). Thus generated ROS may interact with cellular proteins, lipids and DNA which results in alter cell functions. Accumulation of ROS has been implicated in almost all the stages of the process of carcinogenesis (Klaunig 1998). Azadirachtin is a potential inducer of ROS like mitochondrial superoxide anion (O_2•-) and hydrogen peroxide (H_2O_2) (Huc et al. 2007) which can diffuse from the site of generation to other targets within the cells or even propagate the injury to other intact cells. These ROS produce deleterious effects by initiating LPO directly or by acting as second messengers for the primary FRs that initiate LPO (Das 2002). Oxidative stress plays an important role in the cellular toxicity of various pesticides (Stevenson et al. 1995). Measurement of LPO products, like MDA and oxidative redox parameters like SOD and CAT has increased after exposure to pesticides (Prakasam et al. 2001) which is found to be elevated in our present study. It suggests that the ability of intoxicated gut with dose dependant AZT to protect against oxidative damage in female is more than in male. It happens by the stimulation of the body defending antioxidant mechanisms to overcome the induction of OS.

7.3.4. Toxic effects on insect's brain and haemolymph

The increase of the insect mortality was noticed in the current study with the increase of AZT. The increase of AZT in the insect's body impaired their metabolism and resistance against the toxic effects of AZT. Moreover, the antioxidant defence system and the neuroendocrine regulations remain significantly incapable to counteract the toxic effect of AZT. And for that reason insects become susceptible to degenerative tissue damages. Food ingestion and preliminary digestion were significantly ($P<0.001$) increased at higher doses of the pesticide. Due to higher intake of green leaves contaminated with AZT insects accumulate higher amount of AZT. This initiated hyper-metabolic state in association with higher intake of toxic substance. Notwithstanding, this state failed higher cellular metabolism due to a significant increase of toxicity generated from OS. One report reveals that impairment in AD with higher concentrations of neem extracts might induce toxic consequences in the grasshopper organs (Sundararaj et al. 1995).

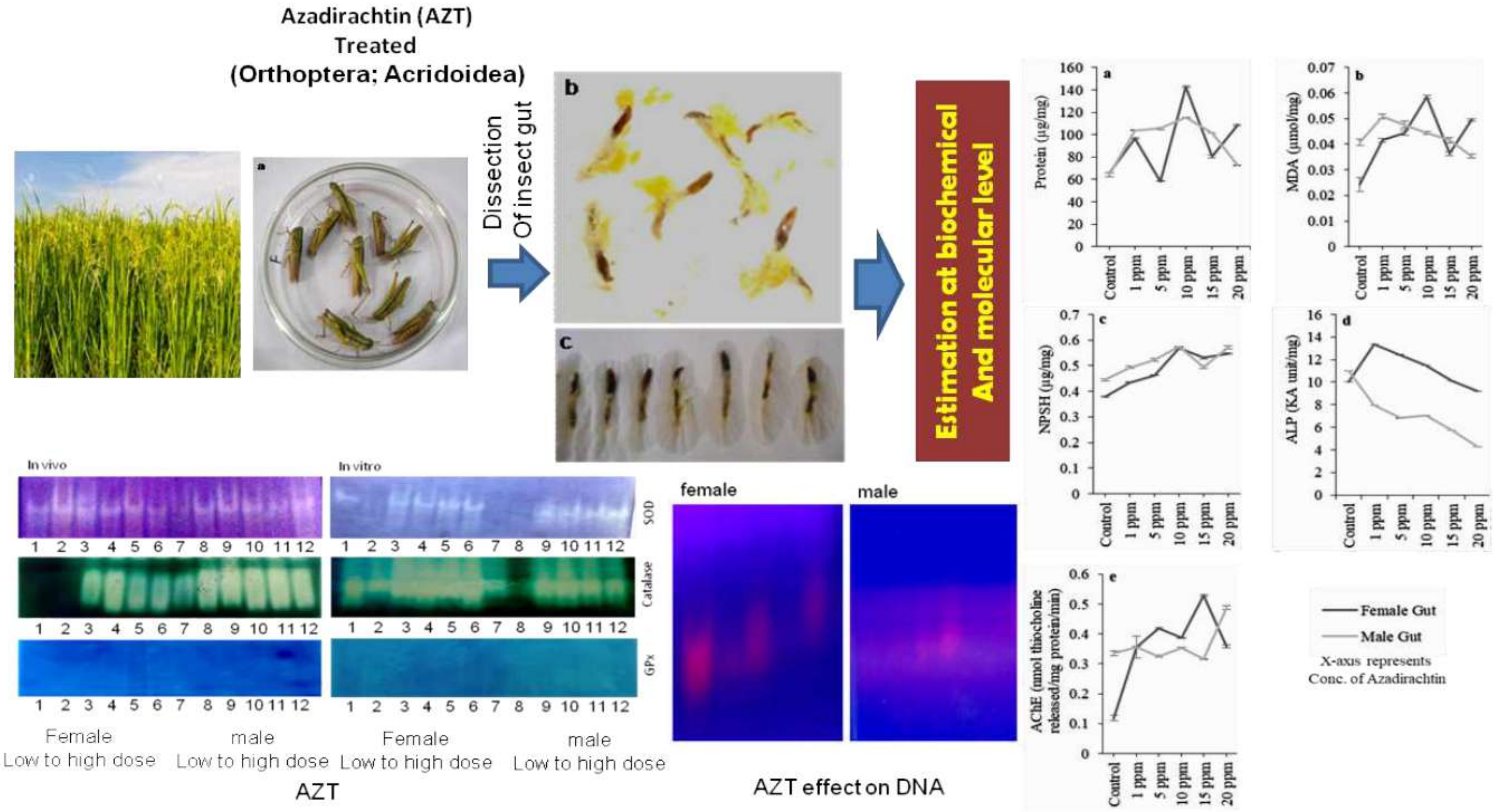

Plate 29. Sex dimorphic adaptive responses against azadirachtin toxicity in gut tissues of *Spathosternum prasiniferum prasiniferum* (Orthoptera: Acridoidea).

In general male is found to be more sensitive to toxic exposure in relation to brain tissues. This is reflected in terms of ALP activities and less gross peroxidation outcome.

Oxidative stress is reported that are initiated by pesticides and it plays a significant role in the cellular toxicity (Stevenson et al. 1995). Azadirachtin has been shown to be a potential inducer of ROS and it is strongly inducing mitochondrial $O_2{\bullet}^-$ and H_2O_2 (Huc 2007). In the current study increase in neural protein content (Fig. 12.a and Fig. 13.a) and haemolymph protein content (Fig. 14.a) from both sexes were found to be significantly higher after dose dependant AZT toxicity indicates that AZT may interfere with the protein synthesis or post translational phenomenon at certain life span of the insect (Senthil-Nathan et al. 2004).

Lipid peroxidation products increases from the reaction of FRs with lipids and is considered to be an important marker of the cellular OS (Hoek and Pastorino 2002). In the current study lipid peroxide products were measured in the form of thiobarbituric-acid-reactive-substances (TBARS). Malondialdehyde, is a distinct representative of the TBARS cellular oxidation product. These are generated from the peroxidised polyunsaturated fatty acids (PUFA) (Freeman and Crapo 1981). It is reported that pesticides can induced LPO by the enhancement of TBARS production (Catalgol et al. 2007). In our observations, increased levels of LPO were found in the insect brain of both sexes exposed to AZT (Fig. 12.b). The LPO was also increased in haemolymph of the insect of either sex (Fig. 14.b). This suggests that, AZT was distributed and accumulated in the brain tissues, at the same time it initiated systemic stress. This fact was justified by the results of increased rate of insect mortality at higher AZT doses. In the reported that ROS induced LPO directly or via some second messenger initiates cellular toxicity (Das 2002). In addition, brain is reported to have low antioxidant defence system (Ward et al. 1994), that's why ROS could induce a certain level of toxicity. The dose dependent increase of LPO was also noticed in other report (Farombi et al. 2008). Protective and adaptive roles of thiol substantial against OS-induced toxicity (Otto and Moon 1995). In the present study, a significant ($P<0.001$) increase of NPSH was noticed in in-vivo insect brain (Fig. 12.c), haemolymph (Fig. 14.c) from both sexes. A significant decrease of NPSH was observed in only in-vitro male brain. Thus, NPSH level in tissues are considered a critical determinant of the cellular protective measures against toxicity (Meister and Tate 1976). Our results show that AZT toxicity increased the ALP activity in in-vivo male brain (Fig. 12.d) and decreased in haemolymph of both sexes of the treated group (Fig. 14.d). The toxicity was more distinct in higher doses (Senthil-Nathan et al. 2004). Acetyl-cholinesterase is an important neurotransmitter in the developed living organism mainly distributed in the brain and CNS (Kim et al. 2006). It performs during neural communications and neuromuscular activities (Yin et al. 2008). Due to the results of this enzymatic cleft and cannot be recaptured by the postsynaptic terminal. So, the ACh is not degraded and recycled. As a result, disconnection the insects' manifests toxicity symptoms and dies in severe condition (Tang 2000). Several essential oils from aromatic plants have all been shown to be inhibitors of AChE (Shaaya and Rafaeli 2007). Azadirachtin binding with AChE is shown to be non-specific. It occupies various sites at the surface of AChE molecule instead of a specific binding to active site (Mordue et al. 2010). In this study AChE activity significantly decreased in in-vivo male brain (Fig. 12.e) and AChE inhibition of the in-vitro brain of both sexes after dose dependant AZT toxicity compared to that of control (Fig. 13.d) indicates damage to nerve cells. That eventually induces impairment of recycling AChE and disruption of normal nerve conduction. The AChE activity of haemolymph from both sexes was significantly become the port of the systemic circulation depressed (Fig. 14.e) which indicated that the toxicity by AZT (Lynn et al. 2012).

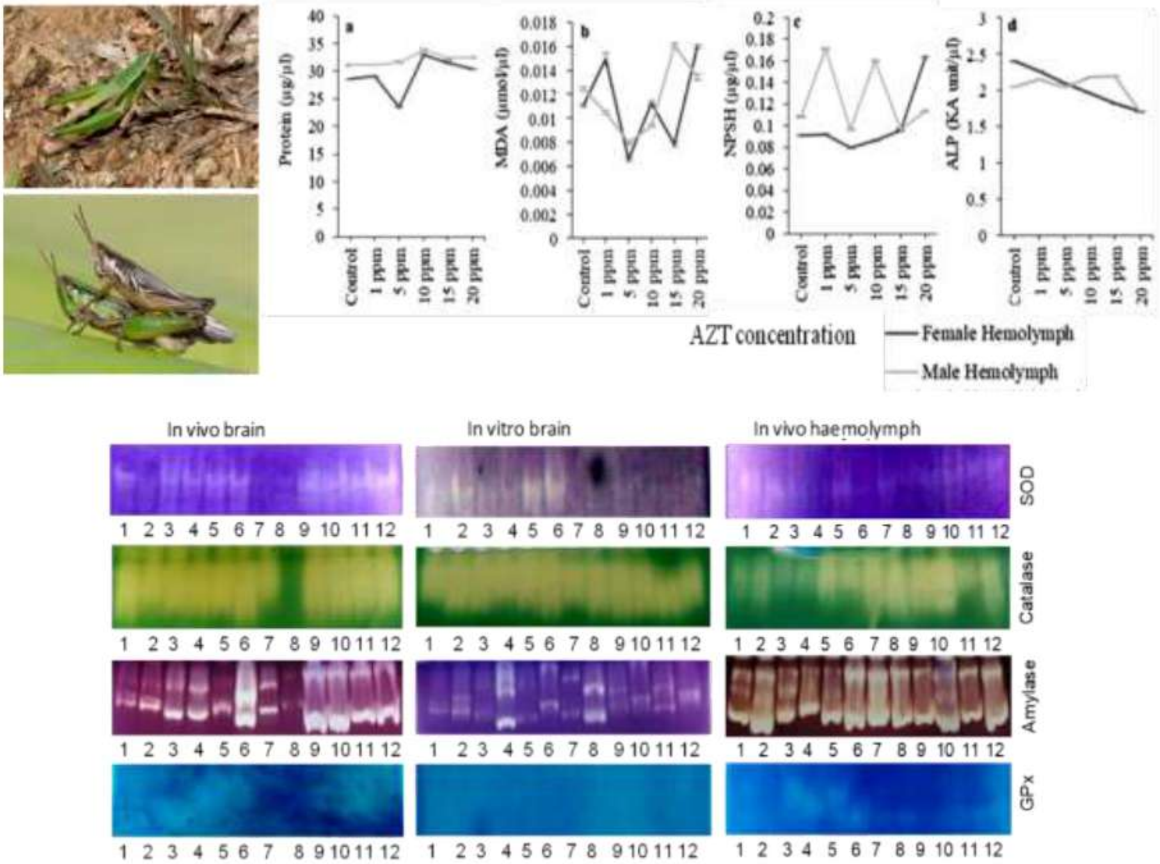

Plate 30. Neural oxidant-stress by AZT induces antioxidative enzymes evincing bi-omarker potential in paddy pest, *Spathosternum prasiniferum prasiniferum* (Orthoptera: Acridoidea).

Significant alterations are noticed in the antioxidant systems after AZT treatment in the insects. Superoxide-dismutase activity of in-vivo and in-vitro brain from both sexes was significantly higher after AZT exposure (Fig. 15). This suggests that AZT has stimu-lated the body defending antioxidant mechanisms to overcome the OS-induced by AZT. Superoxide-dismutase activities of AZT treated male haemolymph were significantly higher whereas the significant low level of SOD was recorded in female haemolymph (Fig. 15). Superoxide-dismutase is responsible for the dismutation of superoxide radicals

($O_2•^-$) to H_2O_2. The CAT system primarily defences against the oxygen related toxicity and this enzyme is used as biomarkers of OS (Pandey et al. 2003). In the present investigation, the variable response in the CAT activity was noticed in opposite sexes and in in-vivo/in-vitro experimental conditions. It may be related to the variable production of ROS which might be influenced by the metabolic and cellular respiratory rate. These rates are dependent on gender and neuro-endocrinal regulations of physical functions. Report reveals that CAT activity was decreased in mammalian brain intoxicated with different pesticides (Mekail and Sharafaddin 2009). It is supported by the earlier report (Monteiro et al. 2006) which demonstrate that higher level of $O_2•^-$ might be produced by different pollutants (Ahmad et al. 2000).

The CAT activity showed a marked decrease in male insect treated with AZT as compared to that of control. The decreased activities of these two antioxidant enzymes (SOD and CAT) contributed increased more OS in male insect. This observation suggests decline in the ability of the enzyme to might have impaired cellular glutathione and thus decreasing the antioxidant capacity of the nervous tissue. This speculation was confirmed after estimating the levels of NPSH. Glutathione-peroxidase however, showed no change in activity after exposure to AZT as compared to control in the female (Fig. 15). Thus we may speculate that AZT exerts its toxicity by generating excessive OS via increased ROS and compromised antioxidant defence mechanism.

Azadirachtin form stable complexes with amylase which could inhibit the formation of substrate enzyme complex (Sami and Shakoori 2011). By this way it inactivates digestive enzymes and cause poor nutrition, growth retardation and death. Previously it was reported the inhibition of insect pest amylases by plant some derived inhibitors (Sivakumar et al. 2006) because plant extracts are insecticidal.

Extensive DNA-laddering was noticed in AZT intoxicated brain of both sexes (Fig. 16). This is due to the OS that was demonstrated through elevation in TBARS. Free radicals is known to damage the DNA stability. This is also consistent with previous studies where DNA-fragmentation was induced by pesticide in mammalian lymphocytes (Sharma et al. 2010) and in mammalian brain tissues (Hussien et al. 2013). Pesticides may cause damage to proteins, lipids and DNA by oxidation (Kale et al. 1999). Elevation of TBARS and oxidative redox parameters like SOD, CAT in female brain suggests that female can respond in better way to overcome the OS against such pesticide.

7.3.5. Toxic effects on insect's gonad and juvenile tissue

It is evident that AZT toxicity strongly interferes with the production of total cellular protein of gonads (Fig. 18.a and 19.a) and juvenile tissue (Fig. 21.a) and during this time, insects are very susceptible after exposure (Senthil-Nathan et al. 2004). Pesticides toxicity leading to OS by generation of FRs, changes in LPO and antioxidants levels (Ender and Onder 2006). Pesticides did not directly generate FRs but generates numerous radicals indirectly like $O_2•^-$ and hydroxyl radical (OH•) that damage cellular proteins, lipids and DNA by oxidation (Kale et al. 1999). Our result shows that, gonadal MDA level increases with the increased doses in in-vivo and in-vitro experiment by LPO arises from the reaction of FRs with lipids and this is regarded as an important sign of the cellular injury brought by free radical attack (Hoek and Pastorino 2002). Sometimes the AZT effect seems biphasic and higher in male (Fig. 18.b). Fetoui et al. (2009) reported that concentration dependent pesticides toxicity resulted in a significant increase in MDA production in justifying of our result may accumulate FRs or ROS by impairment of enzymatic antioxidant system. Increased ROS production may thus be associated with the AZT metabolism leading to the peroxidation of membrane lipids of the gonads finally causing to cell damage or hampered reproduction.

In case of NPSH the result is opposite (Fig. 18.c) that higher increase in female than male suggesting a better protective response to nullify MDA level keeping in ovary in healthier physiological state due to its utilization to challenge the predominant OS under influence of ROS generated from AZT. Previous investigators have also reported the significant decrease in NPSH level is concentration and time dependent (Sharma and Ansari 2013) by elevation of MDA.

In-vivo experiment in female shows stronger adaptive responses (Plate 31). The densitometry data of all enzymatic activities were analysed (Fig. 20 and 22). These data suggest that acute exposure even in isolated tissue is responsive in case of female but not in male. Higher adaptability in female may be governed by its specific pattern of hormonal metabolism which needs to be further explored (McCabe et al. 2017). The strong increase/activation of amylase suggests an induction of hyper-metabolic state for enhanced energy catabolism and energy utilization to maintain the metabolic integrity is higher in female than male, that may have resulted in higher male mortality.

In case of instar-II is found to be more sensitive with dose dependant AZT toxicity. The higher level of NPSH and MDA at this stage emphasizes the occurrence of toxicity and thiol-mediated adaptive responses against it. The decreased NPSH level of in-vitro instar-IV indicates that protection against the ROS is more required. Interestingly, higher AChE in instar-IV promote depletion of active neurotransmitter ACh (Fig. 21.d) might impair property neural transmission and adaptive organ wise communication. Instar-IV represents decreased AChE activity indicating ACh is not broken and accumulates within synapses, causing overall decline in neural and muscular control (Dutta and Arends 2003) and initiates accumulation of FRs leading to LPO (Yang et al. 1996).

Antioxidant protection consists of many enzymatic and non-enzymatic factors which maintains the physiological level of reactive oxygen (Ondreicka et al. 1998). Superoxide-dismutase plays a major role in the first line antioxidant defence system by catalysing the dismutation of O_2•- to form H_2O_2 and molecular O_2 (Ho et al. 1998). Tsan (1993) found that SOD activity increased during hypoxia. Catalase is a ubiquitous enzyme present in cells of all aerobic organism, which converts two molecules of the strong oxidant, H_2O_2 to molecular O_2 and two molecules of H_2O (Laszlo et al. 1991). Kono and Fridovich (1982) found that O_2•- inhibited CAT activity and the presence of H_2O_2 inhibited the action of dismutase. The CAT activity increased in female gonads is not essentially related to reduction of MDA levels. In other words, the MDA level is elevated in in-vivo female gonads when CAT activity reached maximum in female gonads. The increased MDA level indicates an enhancement of LPO by prooxidant substances react with unsaturated fatty acids of biological membrane due to AZT intoxication. These results suggest the possibility of peroxidation process of AZT intoxication at higher dose. In this study we observe decreased CAT activity in in-vitro female and male as well as in-vitro instar-IV of AZT intoxication. This indicate a reduced ability to protect against H_2O_2, whereas increased SOD activity indicates that protection against O_2•$^-$ is more prominent.

Catalase is a common antioxidant enzyme that catalyses the decomposition of H_2O_2 to molecular O_2 and H_2O. Increased in-vivo and in-vitro CAT activity in female suggests more adaptive response by depletion or removal of FRs than male after dose dependent AZT toxicity (Fig. 20). Both SOD and CAT activities of in-vitro testis (Fig. 20) and instar-II (Fig. 22) decreased significantly and that evidently shows that AZT impairs the male reproductive function in grasshopper and instar specific juvenile tissue is probably related to the enhancement of LPO. The CAT activity was reduced by pesticides induced enhancement of superoxide radicals (Yu 1994) or might be due to binding of AZT to CAT or by inhibiting CAT synthesis (Tripathi and Verma 2004). In the present study, low dose of AZT cannot adversely effect on reproductive functions on both gonads.

However, a significant decline CAT in male alter the membrane configuration can either by direct effect of this xenobiotic on the cell membrane or due to the increased LPO (Al-sharif et al. 1990) and the degree of toxicity on CAT and protein profile is altered on different tissues by depending on the location and metabolic significance of the tissues (Doss et al. 2008).

Dose dependant higher rate of DNA-degradation is noticed in all cases of in-vivo and in-vitro studies (Fig. 23.a). Especially, in nymph the DNA-degradation is found to be more in both stages after being treated with higher concentration of AZT (Fig. 23.b) which is the normal consequence of OS and the same is demonstrated through elevation in MDA and reduction in antioxidant enzymes. This is also consistent with previous studies where DNA-fragmentation is induced by pesticide in mammalian lymphocytes (Sharma et al. 2010) and brain (Hussien et al. 2013).

In the present investigation, although the activities of SOD and CAT are increased, possibly as an adaptive response to the generated FRs (Somashekaraiah et al. 1992) in female, the total antioxidant defence mechanism fails to protect the male gonads of this insect from molecular damage caused by AZT as evidenced by increased MDA. Lipid peroxidation has been used as an OS-marker and elevated levels of LPO, induced by environmental toxins, may differ from species to species. The present study shows a reduction of CAT activity in in-vitro instar-II related to elevation of MDA levels observe in agreement with Sayeed et al. (2003). According to Zhang et al. (2004) severe OS suppresses antioxidant defence enzyme activities due to oxidative damage and loss of compensatory mechanisms. In our study, ovary and instar-IV of grasshopper exhibiting a recovery response by CAT activity is observed which was adjustable and effective to protect from cellular injury. However, AZT effect on this species making them more sensitive to environmental changes and the parameters evaluated can be used to monitor pesticide toxicity in environment. The maximal suppression of antioxidant enzyme activity is obtained by dose dependant AZT toxicity on male than female gonads (Fig. 20) that may affect reproductive physiology of male and greatly delay the survival and fitness finally ends up in less net reproducibility of the species in toxicity. But protective strategies in female may be of interests to study the adaptive mechanisms which could have been implemented to augment the adaptive behaviour in either sex of this species or other species. Periodic changes of activities were noticed in almost all enzymatic action and in other biological parameters also in the current study. This is revealed from the zymographic analysis and the corresponding densitometry data. This type of drug action has been reported earlier (Martinez et al. 2013). Thus, AZT intoxication of grasshopper caused disturbances in oxidative-antioxidative balance in the male gonads and leads to high mortality level than female as well as instars wise tissue damage. The interpretation of these results will be more complete after further investigation of other antioxidant enzymes.

The production of FRs increases with stress, while some of the endogenous defence mechanisms decrease (Plate 32). This imbalance leads to progressive damage of cellular structures, presumably resulting in the sex dependant. The antioxidant defence system must thus minimize the levels of most harmful ROS on one side while still permit enough ROS to remain for their useful purposes (e.g., cell signalling and redox regulation). Cells usually tolerate such mild OS; this stress can even up-regulate cellular repair processes and other protective systems of a specific sex of this species.

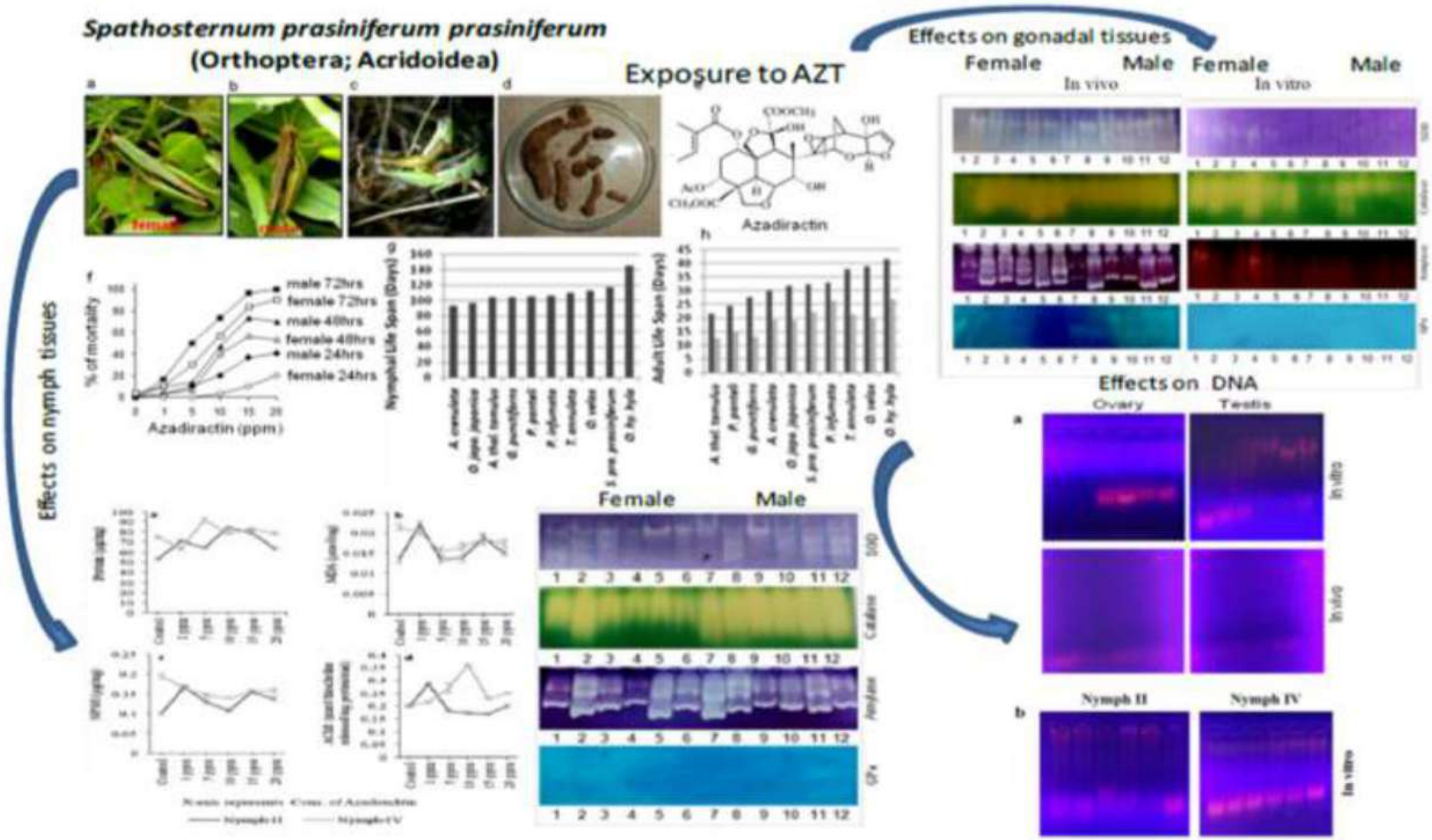

Plate 31. Bio-indicator potential of *Spathosternum prasiniferum prasiniferum* (Orthoptera: Acridoidea) in pesticide (azadirachtin)-induced radical toxicity in gonadal/nymphal tissues; correlation with eco-sustainability.

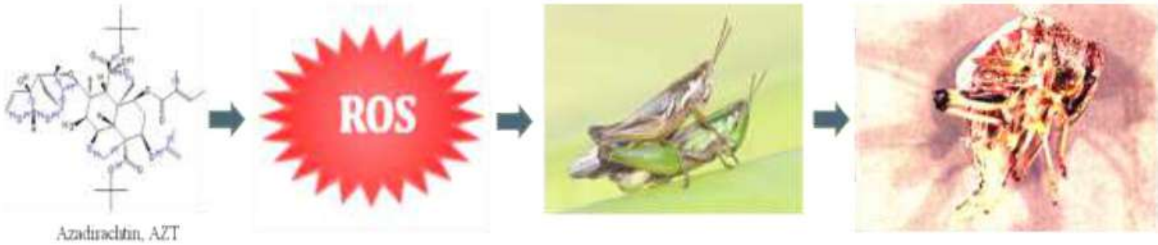

Plate 32. Diagram demonstrates the summary of the present study (Treatment with AZT might have produced ROS within the insect's body. These radicals could have induced excessive mortality of treated adults and nymphs. Altered morphology was marked with the appearance of difficulties in ecdysis).