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Oxidative stress and apoptosis in Asian honey bees (*A. cerana*) exposed to multiple pesticides in intensive agricultural landscape

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Abstract – Pesticide usage associated with intensive agriculture is implicated as a major factor for pollinator decline. Apart from developmental and physiological impairments, pesticide exposure has been shown to cause major cognitive anomalies in honey bees. However, there are gaps in our understanding about the physiological and molecular mechanism that causes the impairments. The present study evaluates the tissue damages at the molecular level with focus on apoptosis in the wild population of honey bees exposed to pesticide cocktails corroborated by observations in laboratory experiments. The study, for the first time, reports increase in both mitochondria and endoplasmic reticulum-mediated apoptosis in the honeybee body tissues underpinned by significant oxidative stress leading to programmed cell death. There was heightened reactive oxygen species level and caspase3 activity in the tissue homogenates from bees exposed to pesticides. Western blot experiments showed a significant increase in the expressions of the apoptotic markers like p53, cytochrome C, and GADD153/CHOP (DNA damage 153 or C/EBP homologous protein) in the pesticide-exposed bee populations in both field and laboratory conditions. Expressions of these apoptotic markers and oxidative stress were more pronounced in the heads as compared to the abdomens of the pesticide-exposed bees. These findings clearly indicate greater tissue damage in the honeybee head tissue. The cognitive implication of this finding has been discussed.

Apis cerana / pesticides / reactive oxygen species / mitochondrial stress / apoptotic markers

1. INTRODUCTION

A large proportion of global crop production relies on insect pollinators (Gallai et al. 2009; Reilly et al. 2020). Human welfare depends on the crucial ecosystem services provided by the pollinator community as they directly influence agricultural yield (Klein et al. 2007; Aizen et al. 2009; Cohen et al. 2020). However, the

alarming decline in pollinator diversity and abundance and its adverse impact on agricultural sustainability is a major global concern (Kremen and Ricketts 2000; Aizen et al. 2009; Zattara and Aizen 2021). Among various key factors reported to be associated with declining pollinator populations worldwide, pesticide use is implicated as a major driver apart from habitat degradation, pathogens, etc. (Potts et al. 2016; Zhang et al. 2017; Baron et al. 2017; Migdal et al. 2018; Jactel et al. 2020).

Honey bees are responsible for pollinating approximately 66% of the 1500 species of crops worldwide and they epitomize the pollination

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crises that came to the fore over the last one and a half decades (Biesmeijer et al. 2006; Kremen et al. 2002; Guzman-Novoa 2016; Martin et al. 2021). There is a mounting evidence base regarding the negative impacts of synthetic chemical insecticides on honeybee health (Farooqui 2013; Chensheng et al. 2014; Magal et al. 2019; Chmiel et al. 2020). The major focus of the majority of previous studies has been on understanding the mortalities across the different stages of the bee life cycle (Doublet et al. 2015; Chakrabarti et al. 2018) investigating effects on brood development and impacts of sub-lethal pesticide exposures (Aupinel et al. 2007; Chauzat et al. 2009; Grillone et al. 2017). Various studies on sub-lethal toxicity of pesticides in honey bees have highlighted effects on learning performance, olfaction, visual ability, homing behavior, and neurophysiology (Palmer et al. 2013; Fischer et al. 2014; Goñalons and Farina 2018; Chakrabarti et al. 2019; Pudasaini 2020). However, a comprehensive understanding of the molecular underpinnings of the different physiological aberrations due to pesticide toxicity down to the cellular level is still emerging (Carnerio and El-Deiry 2020, Farder-Gomes et al. 2021a, b).

Investigating the pesticide inflicted cell deaths in different body parts would constitute a major component of such an understanding. Reactive oxygen species or ROS has often been associated as a marker of oxidative stress in several organisms, including honeybees (Paynter et al. 2017; Paris et al. 2017; Richardson et al. 2018), and has been reported to trigger apoptotic signals (Kannan et al. 2000; Gupta et al. 2007; Khatun et al. 2018). Increased ROS level is also indicative of apoptotic cell death in insect tissues (Hasnain et al. 1999; Huang et al. 2013; Ma et al. 2021). Apoptosis has been shown to be mediated by mitochondria and/or endoplasmic reticulum (ER) (Clavier et al. 2016; Palu et al. 2020; Jong et al. 2017; Katsube et al. 2019). Previous studies have reported that high cellular oxidative stress results in ER-mediated apoptosis along with the mitochondrial death pathway (Simon et al. 2000; Malhotra and Kaufman 2007). It

was therefore hypothesized that determination of the status of the ER stress markers would help us analyze the lethality of the pesticide exposure-induced stress. A high level of pesticide exposure would induce high oxidative stress and result in ER stress-mediated apoptosis along with the activation of the mitochondrial stress pathway (Supplementary Table III). Since stress-mediated apoptotic cell deaths are dynamic and intricate processes, it is crucial to determine and localize the molecular markers of apoptosis by comparing their expressions among the various parts of the body of the honeybees exposed to pesticides in the intensive agricultural landscape. Comparative analysis of the expressions of these markers among the various experimental groups of the honey bees in the laboratory condition is equally essential to corroborate the findings from the in situ samples collected from the agricultural landscape.

It is widely documented that pesticides are capable of generating cellular ROS (Olgun et al. 2020). Cellular ROS in turn can switch on the ER stress hallmarks like GRP78 and CHOP/GADD153 and also cause mitochondria-dependent apoptotic pathway (Rasheva et al. 2009; Huang et al. 2013; Gu et al. 2016). The mitochondrial pathway involves the Bcl-2 family such as Bcl-2 and Bax and the high ratio of Bax to Bcl-2 can induce a loss of mitochondria membrane potential, leading to the release of cytochrome c into the cytosol (Martinou and Youle 2011; Aouacheria et al. 2017). Information regarding whether this cellular damage caused by pesticide exposure involves multimodular passages causing cell death is still emerging (Carnerio and El-Deiry 2020, Farder-Gomes et al. 2021a, b) and has not been studied in Asian native honey bees. Moreover, to curb the toxic cellular pathologies, the two probable molecular conduits need to be elucidated. In this study, we asked the following:

1. If there are increased oxidative stress and cell death in the honey bees in the pesticide-intensive agricultural landscapes

2. Which part of the honey bee body has maximum cell death in samples collected from pesticide-intensive agricultural landscapes

To answer these, we assessed total ROS and caspase-3 protein activity along with the main apoptotic markers in honey bees since they play crucial roles during the process of cell death.

Among mitochondrial apoptotic markers, p53, cytochrome C, Bcl-2 (anti-apoptotic marker), and Bcl-2-associated X-protein (Bax) were analyzed for their expression in honey bees exposed to differential levels of pesticide stress. For endoplasmic reticulum-mediated apoptosis, we analyzed GADD 153/CHOP and glucose-regulated protein GRP78. These protein markers were studied in the whole bee body as well as in the head and abdomen tissues in the field samples exposed facultatively to pesticides in the intensive agricultural landscape. Same markers were also assessed in the whole-body tissue of the honeybees exposed to field-realistic pesticide doses in the laboratory to corroborate the field results.

2. MATERIALS AND METHODS

2.1. Collection and establishment of baseline colonies

Ten wild *Apis cerana* colonies were collected from Kuldiha Wild Life Sanctuary, Odisha (21°20'31"21° to 21°29'08"N latitude and 86°25'23" to 86°44'50"E longitudes) where there was no possibility of ambient pesticide load. After collection, individual colonies were acclimatized in the sanctuary in ten five-frame nucleus hives for 4 months (September–December 2016), before the start of the experiments. A queen guard was placed at the entrance of each hive to prevent the queen from escaping and thus the colony from absconding.

2.2. Site selection and distribution of acclimatized colonies

Two study areas were chosen in the Eastern Indian state of Odisha. One of the sites was a low cropping intensity area (LIC) or control and another was a high cropping intensity area (HIC). Farming in LIC (adjacent to the Kuldiha Wild Life Sanctuary) predominantly constituted subsistent home gardens with no or little pesticide use and has diverse natural forage options (Chakrabarti et al. 2015b, 2019). The HIC (adjacent to Jaleshwar town; 26°38'49.6"N latitude and 85°48'2.92"E longitude) is an area where commercial farming is practiced with heavy pesticide usage. The main chemical groups of the pesticides used and their target organism, as well as mode of action, are shown in Table I. There is little semi-natural vegetation in this area. Major crops in the area included rice paddy and vegetables. The details of pesticides used in the studied region (specifically in the HIC areas) are listed in Table I. Five sampling sites were selected in each of the LIC and HIC locations and one acclimatized colony of *Apis cerana* was placed in each of the ten sites. The distribution of colonies was random, to negate any bias. Colonies were at least 2.5 km apart from each other to minimize overlapping in the foraging range of *Apis cerana* (Punchihewa et al. 1985; Reddy et al. 2015; Yang et al. 2020). After placement, all colonies were allowed to sustain for 12 months. Additionally, six more colonies from LIC were procured for laboratory pesticide exposure studies.

2.3. Colony establishment in laboratory and pesticide treatments

Six colonies (collected from LIC) were acclimatized and established in the laboratory flight cages (having dimensions 2.5 m * 2.5 m * 3.7 m for length * width * height) based on previous studies (Chakrabarti et al. 2019). Individual *A. cerana* colonies brought from LIC zones were maintained in separate cages where they were acclimatized for 2 days. Both control (LC) and pesticide-exposed (LT) colonies were fed with

3 M sugar syrup presented outside the hive in 40 2-ml Eppendorf tubes inserted in artificial flowers kept inside the respective flight cage. Bees were also offered dry multi-floral pollen procured from M/S Ambrosia Natural Products (India) Pvt. Ltd. The Eppendorf tubes for the treatment colony (LT) had field mimicking concentrations of pesticides and were exposed for the entire duration of the experiment. The bees undergoing treatment (LT) were provided only with pesticide-laden resources for their obligatory intake (Chakrabarti et al. 2015a, b). The experimental duration was 2 months in the flight cages (Chakrabarti et al. 2015a).

Since the principal focus of the study was field populations, treatments were selected using concentrations known to be used by farmers in the field. The pesticides used by farmers, which were also found in soil and honey bee whole-body pesticide residue analyses, were procured from local pesticide outlets.

2.3.1. Pesticide dose calculation

Three pesticides in combinations were used (Chakrabarti et al. 2015a, b): an organophosphorus (OP) pesticide – dimethoate 30% emulsifiable concentrate (EC) (Tafgor, Rallis India Limited, Akola, Maharashtra), a synthetic pyrethroid (SP) – cypermethrin 10% EC (Ustaad, United Phosphorus Limited, Vapi, Gujarat), and an organochlorine pesticide (ES) – endosulfan 35% EC (Thiodan, Bayer, Ankleshwar, Gujarat) in our study (Table I). The field use pattern of all three pesticides is 15% OP + 5% SP + 17.5% ES in HIC sites in Odisha. For the LIC site in Odisha, the pattern is 5% SP + 17.5% ES. Although the only difference between HIC and LIC appears to be the use of OP in terms of pesticide type used (which came out from previous survey Chakrabarty et al. (2015a) and Pers. Comm.) overall pesticide exposure of the bees in HIC was much higher than in LIC as mentioned

Table I. List of pesticides used in the field of study with their mode of action and application regime (mL/10L)

SI	Brand name and manufacturer	Type of pesticides and chemical nature of active ingredient	Emulsifiable concentrate (EC)	Target organism	Medium	Application regime in the field (mL/10L)		Mode of action
						LIC	HIC	
1	Tafgor, Rallis India Limited, Akola, Maharashtra	Organophosphorus (OP) – Dimethoate	30%	Insects	Water	-	18	Neurotoxic, by damaging acetylcholinesterase. (Karalliedde and Senanayake 1988; Sharma et al. 2020)
2	Ustaad, United Phosphorus Limited, Vapi, Gujarat	Synthetic Pyrethroid (SP) – Cypermethrin	10%	Insects	Water	3	10	Neurotoxic, by interacting with voltage-gated sodium channels in neurons. (Ref. Soderlund et al. 1989, 2012)
3	Thiodan, Bayer, Ankleshwar, Gujarat	Organochlorine (ES) – Endosulfan	35%	Insects	Water	12	15	Neurotoxic, by inhibition of carboxyl ester hydrolases, particularly acetylcholinesterase (AChE) (Ref. Costa et al. 2008; Richardson et al. 2018)

earlier. LIC farmers grew crops at a subsistence level only in some seasons of the year, used only very little quantity of pesticides per se, and had more natural vegetation in the landscape providing alternative food sources for bees. On the other hand, HIC zone farmers practiced yearlong commercial cultivation and consumed more pesticide by frequency as well as types and volume. The HIC landscape had very little natural vegetation and offered little alternative food sources for the bees. At all HIC sites in Odisha, direct spraying occurs at least twice weekly. As a result, foraging bees in the field come into direct contact with these pesticides (Chakrabarti et al. 2015a). Accordingly, a comparable treatment was calculated for feeder tubes each of 2 ml containing 3 M sugar syrup (Supplementary Table I) (Chakrabarti et al. 2015a, b). Each LT hive was exposed to 21.6 mg OP/colony/day, 4 mg SP/colony/day, and 21 mg ES/colony/day.

2.4. Protein extraction and estimation

For randomly sampling bees from the field (LIC and HIC) and laboratory colonies (LC and LT), a sub-sample of 10 adult foraging individuals was taken from a larger sample of foragers collected from the colony entrances. This sub-sample of 10 individuals is hence a random sample drawn from all colonies in field and laboratories, respectively, that were used for tissue lysate preparation. This whole process was repeated thrice for use in the analyses. Samples were immediately dissected on ice for heads and abdomens. Proteins from the tissues were extracted following the previous protocol (Mitra et al. 2013; Chakrabarti et al. 2019). The measurements of sample length and weights averaging from all experimental batches of *Apis cerana* are depicted in Supplementary Table IV.

2.4.1. Western blotting

Western blot for protein expression studies from tissues of bees was performed using protocols adapted from previous studies (Chakrabarti et al. 2019; Bansal et al. 2017; Banerjee et al. 2020). Details of the primary antibodies p53, Cyt

C, Bax, Bcl-2, GRP78, GADD153, and GAPDH are listed in Supplementary Table II. GAPDH was used as an internal loading control.

2.4.2. Cell lysate preparation for ROS and Caspase-3 assays

At the end of the experimental duration, the protein was extracted from honey bee whole bodies (LIC, HIC, LC, and LT) as well as respective heads and abdomens from each experimental group (LIC and HIC) following previous protocol (Mitra et al. 2018).

2.4.3. Assessment of reactive oxygen species (ROS)

ROS was quantified from field and laboratory experimental groups of bee populations using a fluorometric 2',7'-dichlorodihydrofluorescein diacetate (DCFDA)-based assay (Abcam, Cambridge, MA) following a previous protocol (Datta et al. 2017).

2.4.4. Caspase-3 activity assay

Caspase-3 activity in bee tissues was determined using ApoAlert caspase-3 fluorescent assay kit (Clontech Q25 Laboratories, Mountain View, CA) following the manufacturer's protocol (Chatterjee et al. 2011).

2.4.5. Statistical analysis

Data were analyzed using R V.3.5.3 and GraphPad Prism version 8.1.0. Shapiro–Wilk test was performed to test for normality. For comparing two variables, two-tailed *t*-tests were performed and for comparing multiple variables, one-way ANOVA was done with Tukey's Post hoc test. Mean values are presented as \pm standard errors of means.

Additional details can be found in the Supplementary methods.

3. RESULTS

3.1. Expression of mitochondria-mediated apoptotic markers in honey bee groups

Mitochondria-mediated apoptotic marker showed significantly higher upregulation in the HIC field population (p53: 3.08 ± 0.11 ; Cyt C: 6.03 ± 0.19 , and Bax: 9.24 ± 1.1) compared to the LIC (Figure 1a, b). Similarly, the pesticide-treated laboratory populations (LT) also exhibited significantly higher expression of these marker proteins (p53: 2.69 ± 0.1 , Cyt C: 2.22 ± 0.15 and Bax: 2.49 ± 0.05) compared to the LC samples (Figure 1a, c). Interestingly, the expression of anti-apoptotic protein Bcl-2 showed significant upregulation in HIC group (1.65 ± 0.11 folds) compared to LIC groups (Figure 1A, B) (Berkers et al. 2013; Abdullah and Ravanan 2018; Ahmed 2019; Cabirol and Haase 2019). Similarly, LT group showed significant upregulation in Bcl-2 expression (1.81 ± 0.09 folds) compared to LC samples (Figure 1a, c).

3.2. Expression of mitochondria-mediated apoptotic markers in the head and abdomen of the honey bees under differential stress condition

When comparing the head and abdominal regions of the honey bees sampled from field sites, p53, Cyt- C, Bcl2, and Bax showed significant upregulation in HIC heads (2.91 ± 0.29 fold; 3.42 ± 0.2 fold; 4.26 ± 0.22 fold; 1.88 ± 0.01 fold, respectively) compared to LIC heads. These markers showed small yet significant upregulation in the expressions in the HIC abdomen (p53: 1.86 ± 0.12 folds; Cyt-C: 3.68 ± 0.46 folds; Bax: 1.31 ± 0.07 folds) compared to LIC abdomen excepting Bcl2 which did not show any significant difference in expression between HIC and LIC abdomens (Figure 2). The expression of these markers also showed significant upregulation in HIC heads (p53: 2.02 ± 0.01 fold, Cyt C: 2.62 ± 0.38 fold, Bcl2: 2.54 ± 0.08 fold, and Bax: 1.51 ± 0.06 fold) when compared to HIC abdominal samples (Figure 2).

3.3. High pesticide exposure in field and laboratory also induces ER-mediated apoptotic markers

The protein expressions of ER-mediated apoptotic markers – GRP78 and GADD153/CHOP – in the whole-body tissue lysates of HIC honey bees showed significant upregulation (GRP78: 2.19 ± 0.14 ; GADD153: 4.76 ± 0.24) as compared to LIC (Figure 3a, b). LT samples showed significant upregulation of GRP78 (4.39 ± 0.11 folds) and GADD153/CHOP (3.19 ± 0.03 folds) as compared to LC, corroborating the field results (Figure 3a, c).

3.4. Head tissues express higher ER-mediated apoptotic marker expressions compared to abdomen

HIC heads showed upregulated expressions of GRP78 (12.95 ± 2.9 folds) and GADD153 (9.01 ± 2.4 folds) compared to LIC heads (Figure 4). Next, the HIC abdomen showed significant upregulation of GRP78 (6.8 ± 1.85 folds) and GADD153 (3.76 ± 0.3) as compared to LIC abdomens (Figure 4). Similarly, heads of HIC samples showed significant upregulation in the expressions of GRP78 (1.98 ± 0.16 folds) and GADD153 (2.71 ± 0.65 folds) compared to HIC abdomens. However, LIC bee heads did not show any significant upregulation of GRP78 or GADD153 when compared with LIC abdominal samples (Figure 4).

3.5. High pesticide exposure increases the ROS levels in tissues of the honey bees

Fluorometric quantification of ROS in the whole-body tissue lysate of honey bees showed significant upregulation of ROS activity in HIC (3.63 ± 0.96 folds) as compared to LIC (Figure 5a), implying unmitigated oxidative stress in bee tissues from intensive agricultural zones. Moreover, an assay of ROS activity in the heads and in the abdomens of the bees

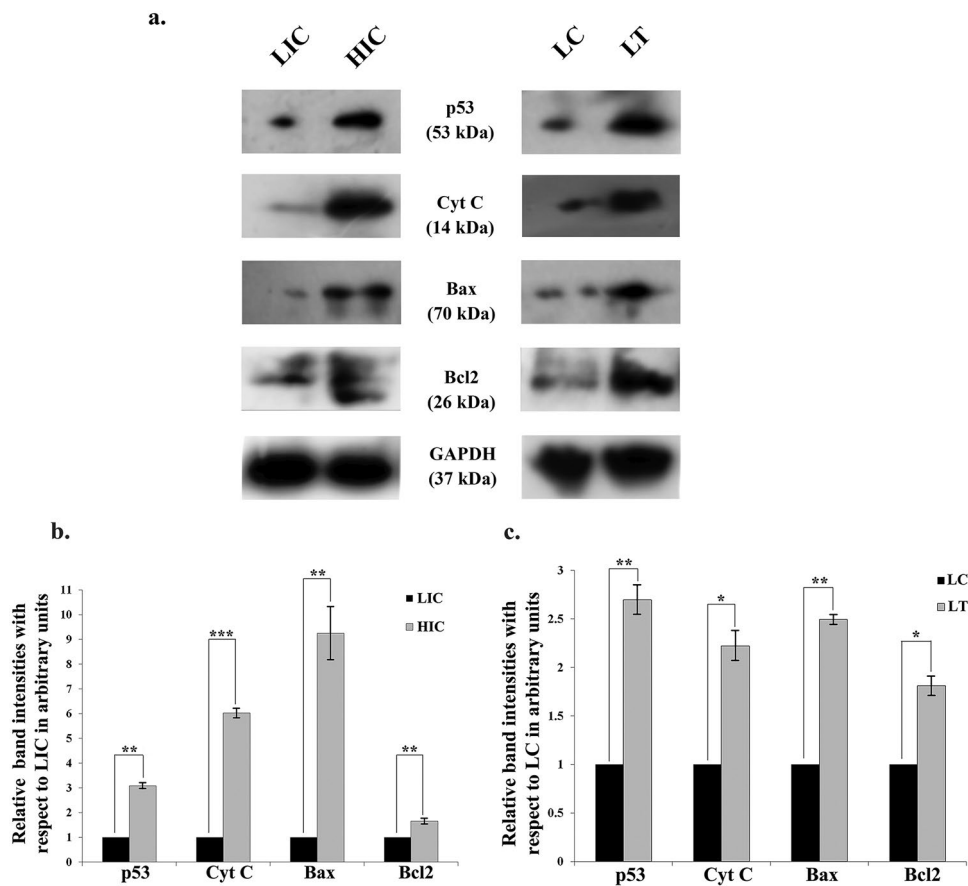


Figure 1. Expression of mitochondria-mediated apoptotic markers in the whole body of honey bees. **a** Representative blots showing the protein expressions, normalized by GAPDH expression in different experimental groups. **b** Graph showing the relative fold change in the expressions of the proteins in the bees collected from field as compared to LIC group. **c** Graph showing the relative fold change in the protein expressions in the bees treated in laboratory condition as compared to LC group. LIC, low intensity cropping site; HIC, high intensity cropping site; LC, laboratory control; LT, laboratory treated. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and $n = 3$).

collected from the LIC and HIC sites showed significantly higher ROS activity in HIC heads (5.6 ± 0.7 folds) as compared to LIC heads (Figure 5b). Also, HIC abdomens exhibited significantly higher ROS activity (1.54 ± 0.18 folds) as compared to LIC abdomens. Honey bee heads from HIC samples showed a significantly higher ROS activity compared to their respective abdominal parts (28.4 ± 0.9 folds). Interestingly, LIC heads showed a significant increase in ROS activity (8.03 ± 0.97 folds) as compared to LIC abdomens (Figure 5b). These

results corroborated with the site-dependent alteration in the ROS activity in the whole body of the bees (Figure 5a).

Similarly, the pesticide-treated bees under laboratory condition (LT) showed significant upregulation of ROS activity (5.3 ± 0.08 folds) as compared to untreated control group (Figure 5c). The differential rise in the ROS levels in the body parts, i.e., ROS activity was higher in the heads as compared to the abdomen in bees under laboratory conditions, revalidating the results obtained from field samples (data not shown).

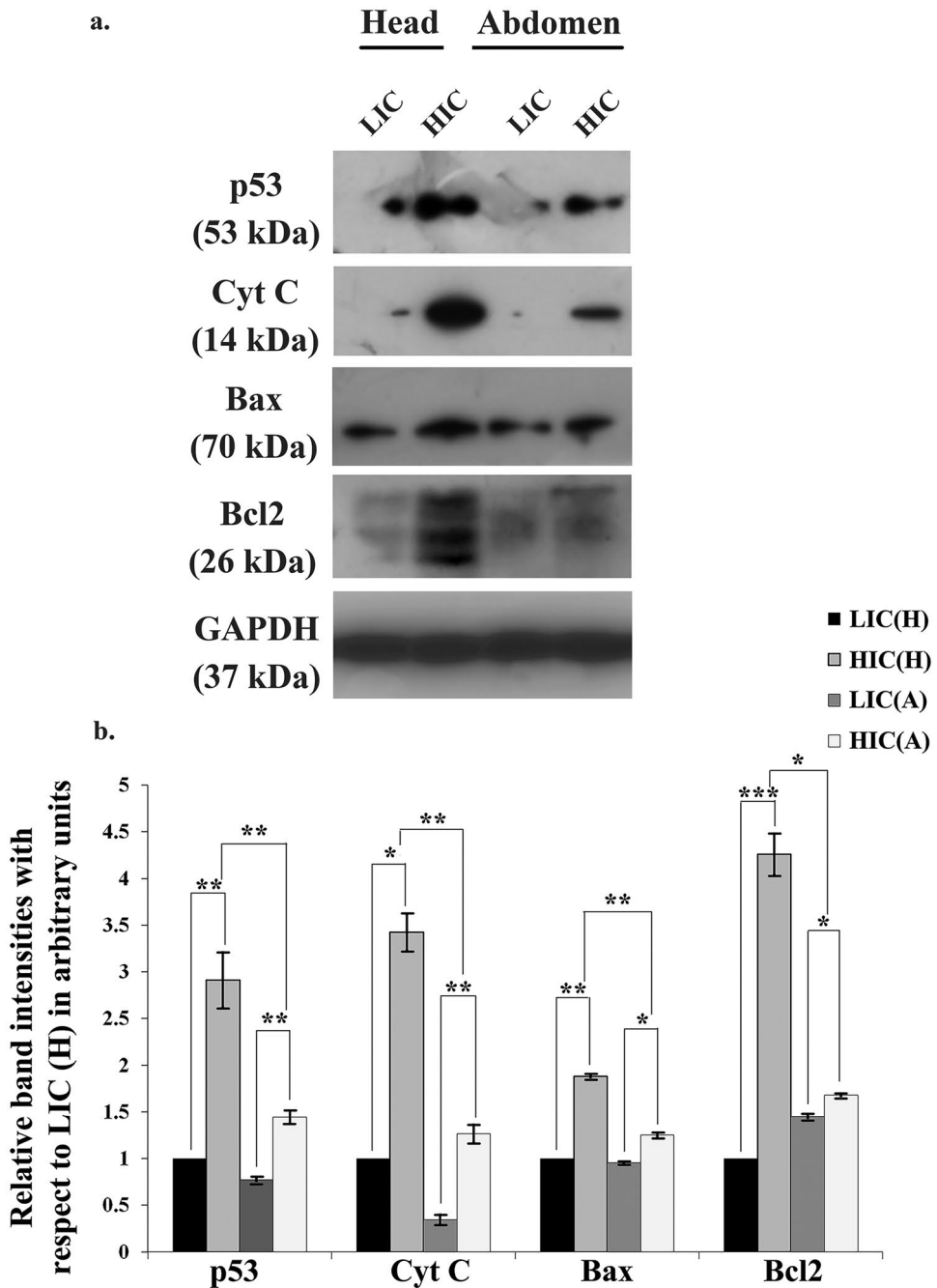


Figure 2. Differential expressions of mitochondria-mediated apoptotic markers in head and abdomen of the honey bees. **a** Representative blots showing the expressions of the proteins, normalized by GAPDH expression in the heads and abdomens of different groups of bees collected from field. **b** Graph showing the relative fold change in the expressions of the proteins in the heads and abdomens of honey bees collected from field as compared to LIC(H) group. LIC, low intensity cropping site; HIC, high intensity cropping site; LIC(H), heads of bees from LIC; HIC(H), heads of bees collected from HIC; LIC(A), abdomens of bees collected from LIC; HIC(A), abdomens of bees collected from HIC. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and $n = 3$).

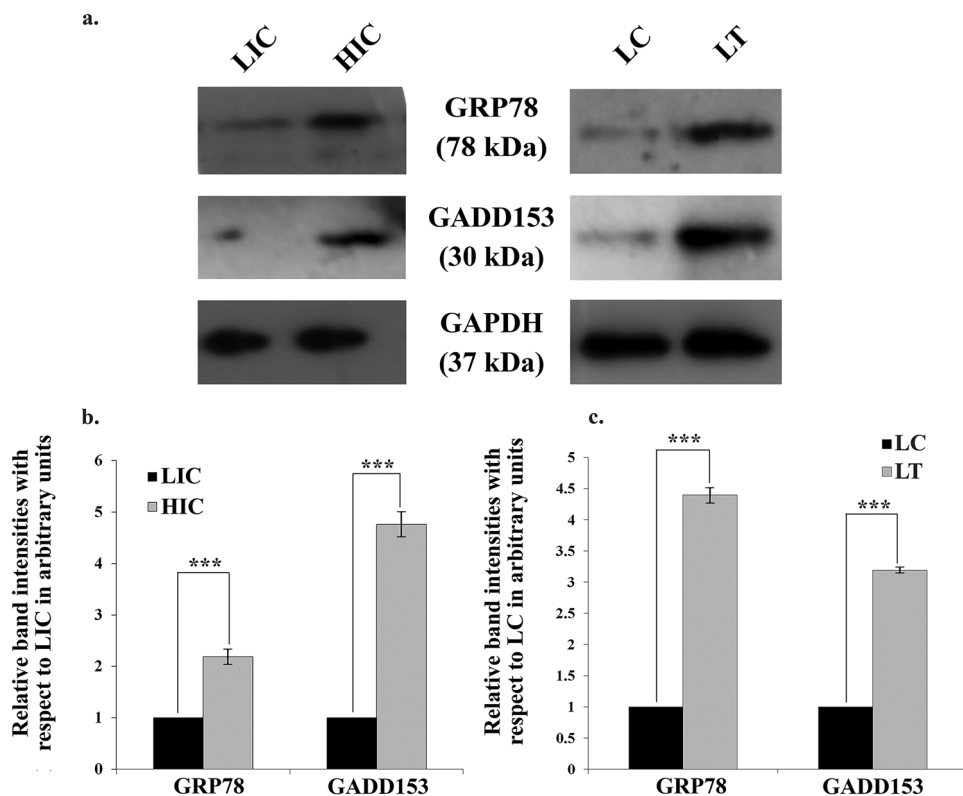


Figure 3. Expressions of ER-mediated apoptotic markers in the whole body in different groups of honey bees. **a** Representative blots showing the expressions of the proteins – GRP78 and GADD153. **b** Graph showing the relative fold change in the expressions of the marker proteins in the whole body of the bees collected from field as compared to LIC group. **c** Graph 603 showing the relative fold change in the expressions of the marker proteins in the whole body of the honey bees collected from 604 laboratory treatment as compared to LC group (***p* < 0.001 and *n* = 3).

3.6. Induced activity of caspase-3 in the pesticide-exposed honey bees

Caspase3 activity from the whole-body tissue lysates of honey bees collected from the field showed significant upregulation in caspase3 activity in HIC (3.37 ± 0.56 folds) as compared to LIC (Figure 6a). Similarly, caspase3 activity in LT groups showed a significant upregulation of caspase3 activity (6.25 ± 1.68 folds) as compared to LC group (Figure 6b). Estimation of caspase3 activity in the tissue lysates from the heads and the abdomens exhibited significant upregulation of caspase3 activity in the HIC samples (5.6 ± 0.06) when compared to LIC. Moreover, caspase3 activity was significantly upregulated in

HIC heads (12.5 ± 0.03) when compared to HIC abdomens (Figure 6c). Also, LIC heads showed significant induction in caspase3 activity as compared to respective abdomens (5.04 ± 0.3). A similar trend in caspase 3 activity was also observed in bee heads of LT samples compared to LC samples (data not shown).

All statistical values for the aforementioned results are presented in Tables II and III.

4. DISCUSSION

We report for the first time that mitochondria, as well as ER stress-mediated apoptosis, act as the intrinsic mechanism for pesticide-induced

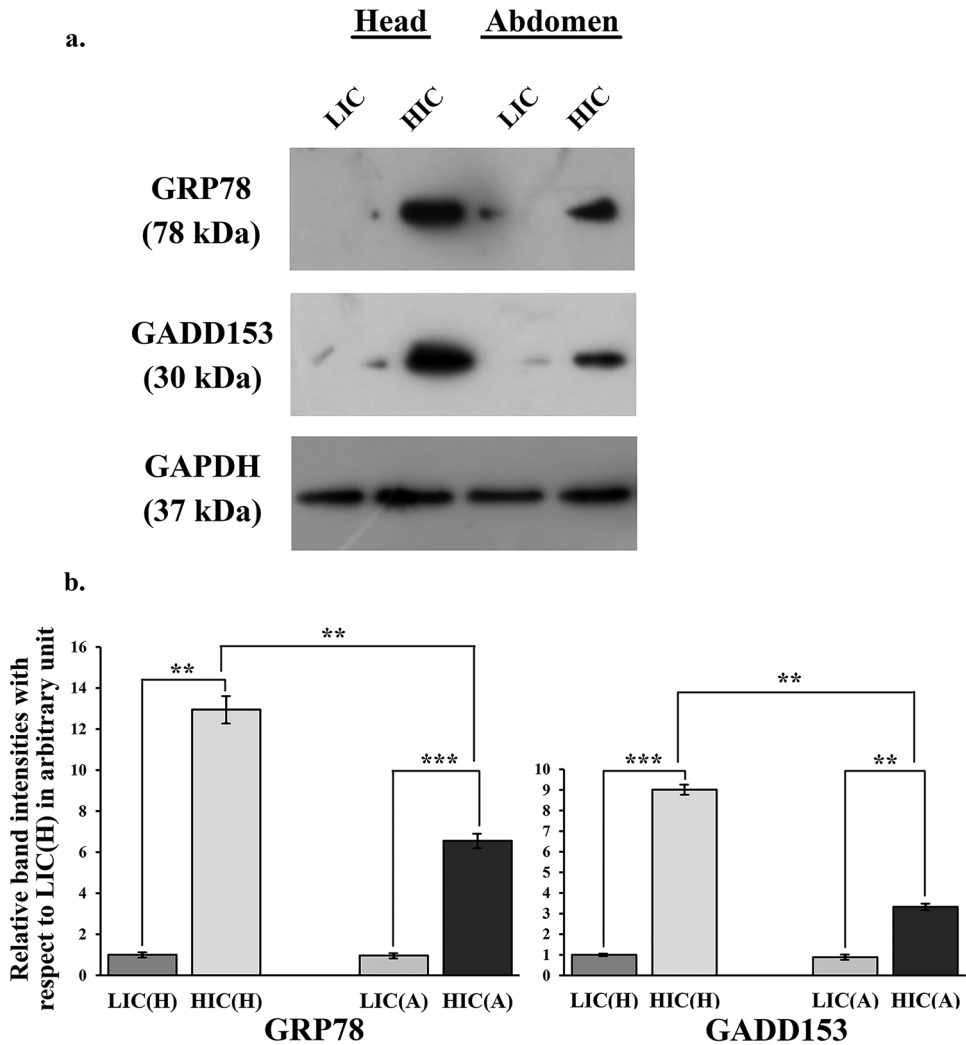


Figure 4. Differential expressions of ER-mediated apoptotic markers in head and abdomen of the honey bees. **a** Representative blots showing the protein expressions of the proteins- GRP78 and GADD153, normalized by GAPDH expression in the heads and abdomens of different groups of bees collected from field. **b** Graph showing the relative fold change in the expressions 609 of the proteins in the heads (H) and abdomens (A) of honey bees collected from field as compared to LIC(H) group. (** $p < 0.01$; *** $p < 0.001$; and $n = 3$).

morbidity in the bee populations in a pesticide-laden intensive agricultural landscape. The study further showed that pesticide exposure particularly leads to apoptosis in the head which might explain previous studies that showed deranged visual acuity (Chakrabarti et al. 2019) and olfactory abnormalities (Williamson and Wright 2013; Chakrabarti et al. 2015b; Goñalons and Farina 2018). Although earlier

studies showed altered RNA expression and downregulation of anti-apoptotic proteins at tissue levels induced by laboratory pesticide exposure in bees (Roat et al. 2014; Wu et al. 2015; Chakrabarti et al. 2020), this study for the first time establishes apoptosis in field populations exposed to pesticide cocktails applied in an agricultural landscape. Controlled laboratory studies further corroborated these findings

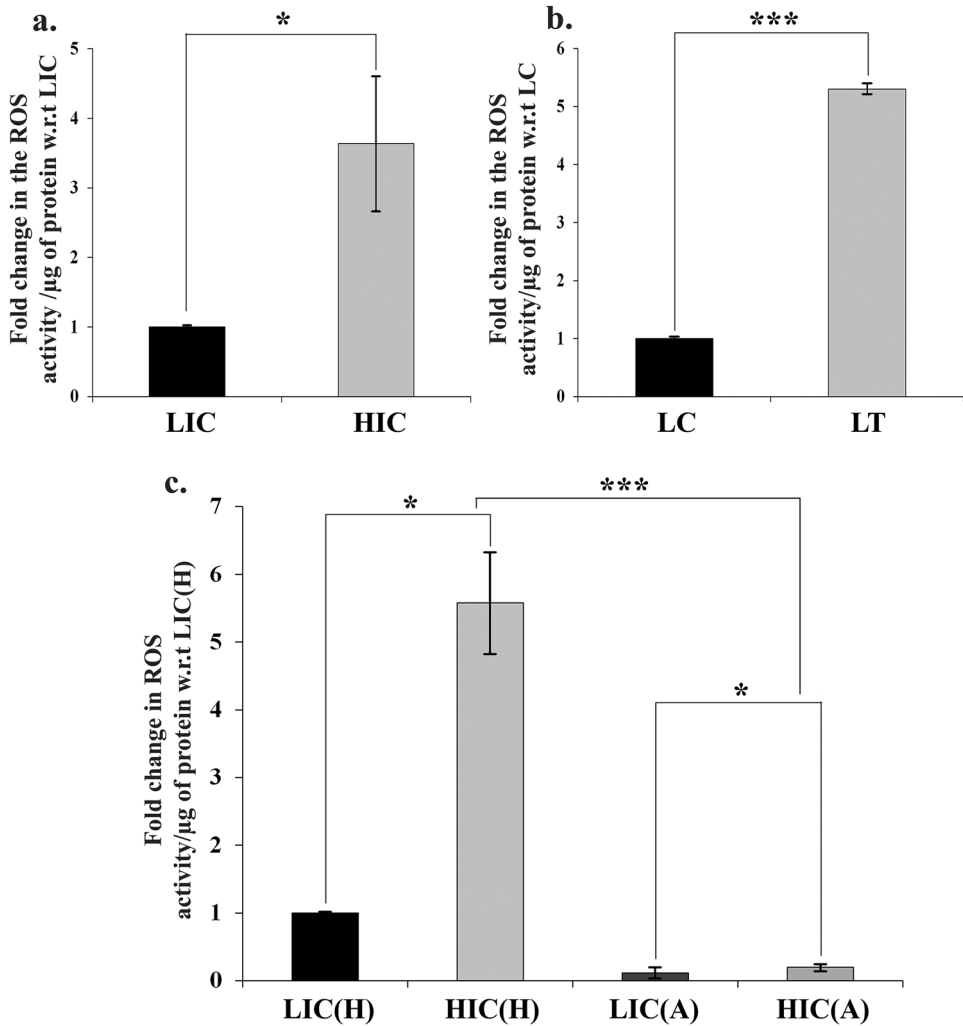


Figure 5. Representative graph showing the ROS activity in the a whole body tissue lysates of the honey bees collected from the LIC and HIC sites of the field. **b** in the tissue lysates from bees treated in laboratory **c** head (H) and abdomen (A) tissue lysates of honey bees collected from field (* $p < 0.05$, *** $p < 0.001$, and $n = 3$).

– which not only reiterates the importance of understanding the molecular underpinnings to pesticide toxicity in bees but also further establishes the differential impact of pesticide in different body parts. Chakrabarti et al. (2019) using the same pesticide exposure regime observed visual impairments in pesticide-exposed field *A. cerana* samples.

There was a confounding difference in expression of four important mitochondrial apoptotic markers, e.g., p53, Cyt C, Bax, and Bcl2

proteins between the HIC and the LIC areas. Increased expressions of these markers were found in both the head and abdominal tissues in HIC samples compared to LIC. For HIC samples, the expression was significantly more in the head tissues compared to the abdominal tissues. Same patterns were also observed for the ER-mediated apoptotic markers, e.g., GRP78 and GADD153/CHOP. A significant increase in these markers was found in HIC and LT whole-body tissue samples and also in the HIC heads

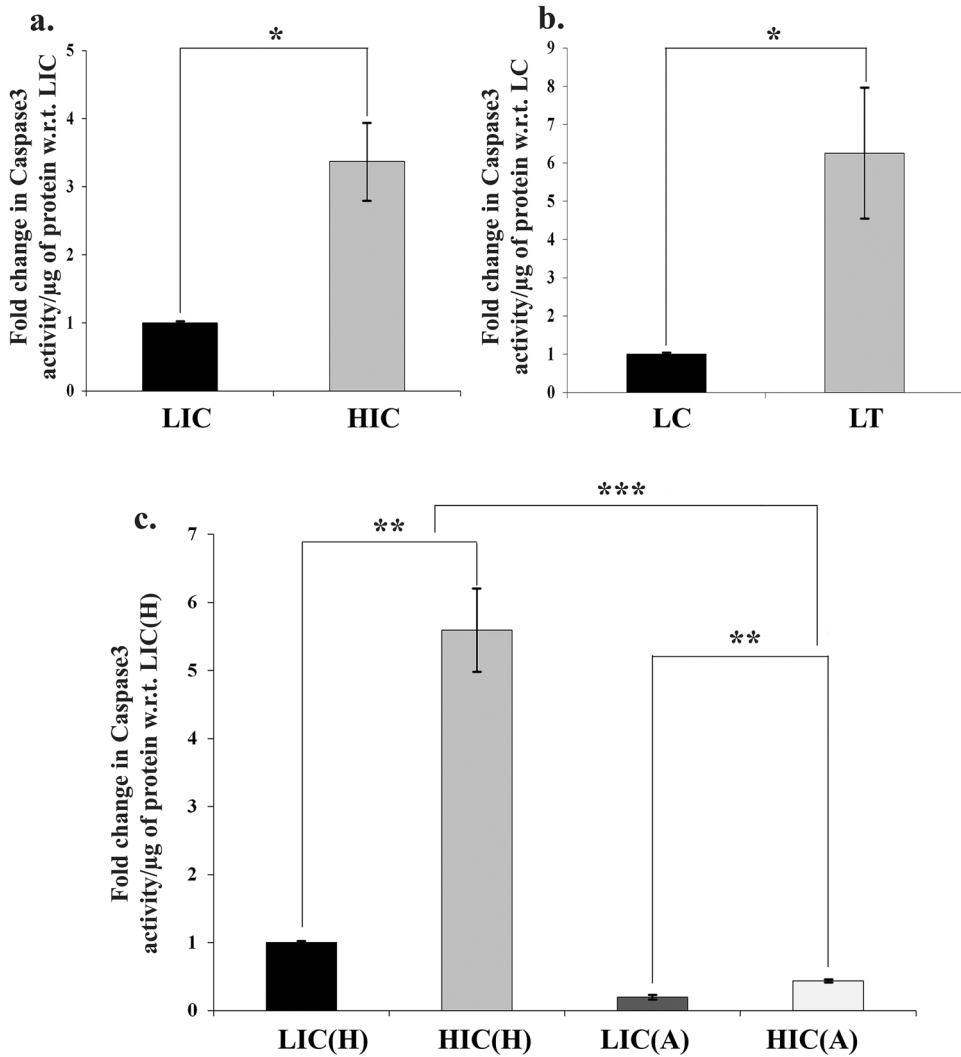


Figure 6

Figure 6. Representative graph showing Caspase3 activity in the a whole body tissue lysates of honey bees collected from different field sites **b** among the laboratory experimental groups of honey bees **c** Representative graph showing Caspase3 activity in the head and the respective abdomen tissue lysates of honey bees collected from different field sites (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 3$).

and abdominal tissues. The highest expression of the proteins was also observed to be more in the HIC head tissues. Earlier studies have shown pesticide-mediated oxidative stress to enhance ROS level in the cytosol, which in turn invokes p53 translocation to the mitochondrial outer membrane and consequent release of pro-apoptotic factors like Bax. Bax then facilitates mitochondrial Cyt-C release (Ali et al. 2018;

Ahmad et al. 2019; Shu et al. 2019) and downstream Cas-3 activity (Zhang et al. 2016; Shieh et al. 2019; Ren et al. 2018; Zhang et al. 2015; Ahmad et al. 2019) (Supplementary Table III). Interestingly, the anti-apoptotic Bcl2 protein also showed a small yet significant increase in its expression in HIC as compared to LIC. A similar difference in the expression of these markers between the Laboratory Control or LC

Table II. Table showing the fold changes in protein expressions in the whole bodies of bees in different experimental groups (df = 2)

Protein	HIC vs LIC	P value	LT vs LC	P value
p53	3.08 ± 1.07	0.001	2.89 ± 0.15	0.006
Cyt C	6.03 ± 0.19	0.0001	2.22 ± 0.15	0.011
Bax	9.24 ± 1.07	0.006	2.49 ± 0.05	0.003
Bcl2	1.65 ± 0.11	0.009	1.81 ± 0.09	0.03
GRP78	2.12 ± 0.14	0.004	4.4 ± 0.11	0.002
GADD153/CHOP	4.76 ± 0.24	0.002	3.19 ± 0.03	0.001

(equivalent to LIC) and Laboratory-treated or LT (equivalent to HIC), even with shorter exposure to pesticide compared to the field colonies, corroborates the field results.

It is also to be noted that both the facultative and obligatory pesticide exposure of the field colonies and the lab-reared colonies, respectively, were enough to mount a severe stress response as evident from the upregulation of ER stress markers besides mitochondrial apoptosis. Bees belonging to the field colonies facultatively feed on the insecticide-laden resource (Chakrabarti et al. 2015a, b), whereas the laboratory-kept population obligatorily fed on the field mimicking insecticide-laden resource and hence were force-fed. The nature of this pesticide exposure is important with respect to the toxicity observed in this study since a 12-month facultative exposure in the field yielded a stress response identical to a 2-month obligatory exposure in the laboratory. Taken together, this indicates that irrespective of the duration of pesticide exposure, the mitochondria- and the ER-mediated

apoptotic pathways imply severe damage to the bee tissues both in situ and in the laboratory.

In our study, the excessive ROS generation in the pesticide-exposed population led to severe oxidative stress in the bees which was associated with an induction of both the mitochondrial and ER stress-mediated apoptotic pathways. It must be noted that beyond a certain threshold level of ROS accumulation in tissues, both these apoptotic pathways are turned on (Mitra et al. 2013, 2018; Schieber and Chandel 2014; Redza-Dutordoir and Averill-Bates 2016) and this underscores the significance of studying both the mitochondrial and ER stress markers. Interestingly, concerted upregulation of both GRP78 and GADD153/CHOP has been shown to occur only under conditions of severe oxidative stress and not under moderate stress (Chaudhuri et al. 2020). However, since it is known that lower levels of ROS content are associated with induction of only the mitochondrial pathway to apoptosis (Mitra et al. 2013, 2018), it needs to be investigated in the future to analyze

Table III. Table showing the fold changes in protein expressions in the parts of bodies of bees in different experimental groups (NS = not significant) (df = 2)

Protein	HIC (H) vs LIC (H), P value	HIC(A) vs LIC(A), P value	LIC(A) vs LIC(H), P value	HIC(H) vs HIC(A), P value
p53	2.90 ± 0.29, 0.001	1.86 ± 0.01, 0.004	0.77 ± 0.013, 0.016	2.02 ± 0.07, 0.005
Cyt C	3.42 ± 0.19, 0.045	9.31 ± 0.46, 0.001	0.35 ± 0.053, NS	2.70 ± 0.15, 0.002
Bax	1.88 ± 0.01, 0.002	1.31 ± 0.07, 0.02	0.96 ± 0.002, NS	1.50 ± 0.03, 0.002
Bcl2	4.25 ± 0.22, 0.001	1.16 ± 0.02, NS	1.45 ± 0.013, 0.008	2.50 ± 0.03, 0.001
GRP78	12.94 ± 0.28, 0.002	6.81 ± 1.84, 0.002	0.96 ± 0.35, NS	1.98 ± 0.43, 0.003
GADD153/CHOP	9.01 ± 0.23, 0.001	3.77 ± 0.30, 0.002	0.88 ± 0.06, NS	2.71 ± 0.12, 0.001

whether sub-lethal exposure to pesticide activates the mitochondrial stress pathway without causing ER stress in the bee tissues.

Under normal homeostatic conditions, the ER serves as a central site for quality control for protein folding, and therefore, its ability to cope with adverse conditions like pesticide toxicity is an important determinant of the stress response (Malhotra and Kaufman 2007). Misfolded proteins under stress accumulate within the ER and bind to ER-resident chaperones like GRP78 or are targeted for proteasomal destruction altogether. This initial adaptive phase of the unfolded protein response (UPR) is characterized by the activation of complex intracellular signaling pathways that lead to the upregulation of the expression of these ER chaperones to promote protein folding (Malhotra and Kaufman 2007). However, if protein misfolding persists, the same signalling pathways turn on pro-apoptotic programs, among which GADD153 plays a principal role. GADD153 acts as a transcription factor to induce the expression of genes that potentiate apoptosis (Malhotra and Kaufman 2007). Therefore, the parallel upregulation of GRP78 and GADD153 upon pesticide exposure as evident from our data indicates that these samples had been subjected to severe ER stress beyond the initial adaptive phase of the UPR. Although the UPR has been documented previously in honeybees experimentally subjected to pharmacological ER stressors (Johnston et al. 2016), our study is the first to report ER stress-mediated apoptosis in field populations subjected to pesticide toxicity.

The earlier report of behavioral and neurophysiological impairments in the brains of *A. cerana* (Chakrabarti et al. 2015a, b; Chakrabarti et al. 2019) prompted us to investigate the difference in the expression of the apoptotic markers in the head and abdominal tissues of honeybees exposed to the pesticide. The increased predominance of mitochondrial and ER stress-mediated apoptosis in the head tissues as observed in our study explains these earlier findings. It should also be noted that the upregulation of ER stress in the pesticide-exposed honeybee brains is indicative of protein misfolding that has long been established

as a hallmark of various neurodegenerative diseases caused by such proteotoxic stress in insect brains like those of *Drosophila* (Rincon-Limas et al. 2012).

A significant increase in ROS expressions, in both field (Figure 5a) and laboratory pesticide groups (Figure 5c), indicates pesticide exposure to be the cause for oxidative stress as was also indicated in previous studies (Chakrabarti et al. 2015a, 2020; Gao et al. 2020). Moreover, ROS activity was found to be higher in pesticide-exposed honey bee heads compared to the heads of the respective controls. More importantly, the HIC heads showed higher ROS levels compared to the respective abdomens (Figure 5b). This in turn signifies increased stress in the heads as compared to the other parts of the honey bee body.

Caspase-3 activation has previously been reported to cause apoptosis upon pesticide exposure in animals and particularly in bees (Wu et al. 2015; Jabłońska-Trypuć 2017; Chakrabarti et al. 2020). Our study not only observed the same phenomenon but also found prominent upregulation of caspase 3 in the heads compared to abdomens. Our finding that both fields as well as laboratory pesticide exposure leading to intrinsic stress is not only apoptotic in nature but is also more pronounced in the head regions is significant.

The link between behavioral alternations and apoptosis in the brain has been established in the *Drosophila* model (Chihara et al. 2014; Khatun et al. 2018). To draw similar conclusions, we can thus assume from our study that pesticide-induced apoptotic stress was higher in the bee heads compared to abdomens and can cause a profound impact on the intrinsic mechanisms behind the behavioral impairments in honey bees, as previously reported (de Almeida Rossi et al. 2013).

5. CONCLUSION

The present study clearly shows increased oxidative stress in honeybees at pesticide-intensive agricultural landscape and concomitantly a significant increase in cellular apoptosis. This study further shows that both the mitochondria- and the ER stress-mediated apoptotic pathways are

operational in the bee tissues. Although ER-mediated apoptosis has been previously reported in honey bees, the implication of mitochondria-mediated apoptosis needs to be understood. The upregulation of ER stress in pesticide-exposed samples indicates overwhelming injury to the tissues of the bees both from the field and in the laboratory, which can therefore be proposed to be a qualitative molecular marker to assess the severity of pesticide toxicity in honey bees. A higher level of apoptosis in the honeybee brain tissues is a significant finding and this corroborates different behavioral aberrations reported earlier in Asian honeybees suffering from oxidative stress in pesticide-intensive agricultural landscapes.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

PB and SS planned the study. DK and DB carried out field and laboratory work. DK, SS and PB wrote the manuscript.

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DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

NA.

DECLARATIONS

Ethics approval NA.

Consent to participate NA.

Consent for publication NA.

Conflict of interest The authors declare no competing interests.

Abbreviations

Cyt-C: Cytochrome C; GADD153: DNA damage-inducible gene 153; CHOP: C/EBP homologous protein; C/EBP: CCAAT-enhancer-binding protein; ROS: Reactive oxygen species; GRP78: 78-KDa glucose-regulated protein; Cas3: Caspase 3; mg: Milligram; μ l: Micro liter; BSA: Bovine serum albumin; PBS: Phosphate buffer saline; Bax: BCL2 Associated X; Bcl-2: B-cell lymphoma 2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; DCFDA: 2',7'-Dichlorodihydrofluorescein diacetate

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