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Letter

Mitochondrial Dysfunction: A Plausible Pathway for Honeybee Colony Collapse Disorder (CCD)

Chensheng Lu,* Chi-Hsuan Chang, Bernardo Lemos, Quan Zhang, and David MacIntosh

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ABSTRACT: Neonicotinoids, the most widely used systemic insecticides in the world, have been shown to be one of the risk factors to colony collapse disorder (CCD). Although the exact underlying mechanisms of honeybee (*Apis mellifera*) CCD have not yet been confirmed, we postulated that mitochondrial DNA (mtDNA) damage in honeybees resulting from sublethal neonicointoids exposure could be a plausible cause leading to malfunctioning thermoregulation. In this study, we used the relative mitochondrial DNA copy number (RmtDNAcn) as an early biomarker to elucidate the association between chronic sub-lethal neonicotinoids exposure and mtDNA damage in honeybees. We collected adult worker bees from hives that were either served as controls or exposed to a sub-lethal level of imidacloprid or clothianidin in three different brood generations that coincided with before, during, and after neonicotinoids treatments. Although there were no apparent adverse health effects in individual bees or the whole colony during and after neonicotinoids administration, the cumulative mtDNA damage in winter bees might have negatively impacted their hives' survival over winter due to the perturbation of energy metabolism. These results contribute to the explanation of a potential mechanism of CCD.

INTRODUCTION

Although the exact cause of the decline of honeybee (*Apis mellifera*) population remains controversial, neonicotinoids (neonics) have been implicated in the disappearance of honeybees from healthy hives in winter, a phenomenon known as colony collapse disorder (CCD).[1−6] Neonics, the most widely used systemic insecticides in the world, have been shown to impair reproductive and behavioral abilities in honeybees, affect foraging activities, inhibit respiration, influence the electron transit chain (ETC), reduce the level of generation of ATP in the thorax and head of honeybees, and cause rapid nicotinic acetylcholine receptor (nAChR)-dependent mitochondrial depolarization in bumblebees (*Bombus terrestris*).[7−13] Mitochondria are eukaryotic organelles and are often present in cells with higher energy demands, such as the thoracic muscle of honeybees where legs and wings are connected. Mitochondrial DNA (mtDNA) is known to be more susceptible to environmental toxicants than nuclear DNA (nDNA) with its proximity of nucleoids to ETC and limited repair pathway.[14] Under normal conditions, reactive oxidation species (ROS) generated during oxidative phosphorylation, the pathway for the production of energy by mitochondria, can cause damage to mtDNA, although it could be gradually repaired. In contrary, ROS generated under ROS-stress conditions could damage mtDNA persistently, leading to dysfunction of oxidative phosphorylation.[15] This chronic oxidative stress resulting from damaged mitochondria could increase the rate of mtDNA replication and repair that leads to different states of mtDNA.[16]

Honeybees have evolved to become a perennial pollinating insect because of their ability to live through winter. Honeybees crowd tightly together by forming a cluster inside the hive and shivering their thoracic flight muscle constantly throughout the winter, to maintain an optimal hive temperature for the survival of the whole colony during winter, which is a highly energy dependent activity for individual bees.[14] Considering the essential thermoregulation mechanism in honeybees and the occurrence of CCD in winter in which massive numbers of adult bees suddenly disappear from their hives,[4,5,17] it is logical to speculate mitochondrial dysfunction, or specifically mtDNA damage, a plausible pathway leading to the onset of honeybee CCD. As the increasing relative mtDNA copy number (RmtDNAcn) has been shown to be related to mtDNA damage[18] and knowing the mechanism of mitochondrial heteroplasmy,[19,20] we used RmtDNAcn as an inter-

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mediate biomarker for mtDNA damage at an earlier stage under sublethal neonic exposure conditions.

We evaluated the study hypothesis on the basis of (i) whether neonic-treated hives have a higher risk of honeybee disappearance and mortality over winter, (ii) whether later brood generations of honeybees from neonic-treated hives would have a level of mtDNA damage higher than the level of those from control hives, and (iii) whether mtDNA damage could be inherited from queen to worker bees raised from later brood generations.

MATERIALS AND METHODS

We set up a split-plot open field study with experimental hives at three apiaries with six honeybee hives per site, for a total of 18 experimental hives. At each apiary, two hives were exposed to either imidacloprid (IMI), clothianidin (CLO), or no pesticide (control) weekly for 13 consecutive weeks started from early July to mid-September 2012. The estimated daily dosage in sugar water that we administered for 13 consecutive weeks (or 91 days) was 37 μg/colony. Assuming each hive contained 50000 honeybees during the active foraging season from April to September, the estimated daily dosage of either IMI or CLO would be 0.74 ng per bee per day. This dosage was substantially lower than those reported in a field study in which total neonics, expressed as $\text{Imidacloprid}_{\text{RPF}}$, in pollen that are collected monthly from April to August from 62 hives established across the Commonwealth of Massachusetts were in the range from nondetected to 276.1 ng/g.[21] No adverse effects were observed in any of the 18 hives during and right after the administration of neonics. We kept track of the well-being of hives by measuring the brood counts and numbers of frames containing adult bees biweekly during and after the administration of neonics from October 27, 2012, to April 4, 2013, using the methods described previously.[22] Detailed information about the field study is available in the Supporting Information.

We collected adult worker bees from each hive by intercepting them at the entrance of their respective hives upon returning from foraging. Because of the average life span (25−35 days) of worker bees and their age eligibility for foraging (approximately ≥21 days),[22−24] those bees that we collected were from different brood generations of the same queen. They represented the pre-treatment (F0 collected on July 2), during- (F2 collected on August 20), and post-treatment groups (F4 collected on October 15). We quantified mtDNAcn in the thoracic muscle (middle) section by dissecting individual bees immediately after removing them from the −80 °C freezer. We then extracted the total gDNA following the instructions of the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). We used the QuantiTect SYBR Green PCR Kit (Qiagen) for the q-PCR analysis (Applied Biosystem StepOne Plus Real-time PCR). We selected the *Cyt b* gene (11004−12155 bp) and *18s rRNA* to represent mtDNA and the nuclear gene, respectively. The thermal cycling conditions for *Cyt b* and *18s rRNA* PCR are as follows; 15 min at 95 °C to activate the HotStarTaq DNA Polymerase and 40 cycles comprised of denaturation for 15 s at 95 °C, annealing for 30 s at 58 °C, and extension for 30 s at 72 °C. Each run is completed by melting curve analysis to confirm the amplification specificity and the absence of primer dimers. All Ct values are listed in Table S1.

We normalized mtDNAcn by the copy number of a selected nuclear DNA (nDNAcn) as the internal reference gene to ΔCt using eq 1

$$\Delta\text{Ct} = \text{Ct}_{(Cyt\ b)} - \text{Ct}_{(18s\ rRNA)} \quad (1)$$

where Ct is the threshold cycle number at the determined level of log-based fluorescence and therefore inversely proportional to the log-based initial copy number of the target gene. *Cyt b* and *18s rRNA* were used to measure the Ct for mtDNA and nDNA, respectively. The following primers were used for *Cyt b* and *18s rRNA* genes for q-PCR: *Cyt b*, 5′ AGC AGC TGC ATT TAT AGG ATA TG 3′ (forward) and 5′ AGA TTA GTA ATA ACT GTT GCA CCT C 3′ (reverse); *18s rRNA*, 5′ GTG GGC CGA TAC GTT TAC TT 3′ (forward) and 5′ CCG AGG TCC TAT TCC ATT ATT CC 3′ (reverse).

To ensure similar amplification efficiencies for the designated primers of *Cyt b* and *18s rRNA*, we calculated the amplification efficiencies for those primers by running five serial dilutions of DNA samples on q-PCR using eqs 2 and 3

$$\text{Ct} = \alpha - \beta \log_{10}[X_0] \quad (2)$$

$$E = 10_{-1/\beta} - 1 \quad (3)$$

where $[X_0]$ is the initial DNA concentration of targeted genes and E is the amplification efficiency. Results from the five serial dilutions of DNA samples via q-PCR showed that the amplification efficiencies (E) of the designated primers for *18s rRNA* and *Cyt b* genes were about the same (−3.41 and −3.37, respectively). The measurements for Ct of the *18s rRNA* and *Cyt b* genes were within 18−24 and 15−23 of the coefficients of variations (CV) across samples of 6.9% and 9.6%, respectively. Those results showed the stable Ct measurements.

RESULTS AND DISCUSSION

Mitochondria have been proposed to be a molecular clock that can reflect biological aging via either molecular changes in mtDNA or alterations in the nDNA encoding mitochondrial proteins.[19,25−27] To compensate for mtDNA damage, cells need to generate more copies of mtDNA, thus leading to a simultaneous increase in mtDNA abundance and oxidative damage, a hallmark feature of mitochondrial aging.[26] In the study presented here, we did not find significant differences in RmtDNAcn measurements in individual honeybees across the18 newly established experimental hives prior to the administration of neonics, indicating the similar mtDNA status among honeybees and their queens. However, a significant increase (one-way analysis of variance, $p < 0.001$) of RmtDNAcn was found in honeybees collected from later brood generations of control colonies, suggesting a non-constant mtDNA status but reflecting the possible biological aging of queen bees (Table 1). A similar upward trend but with a larger magnitude increase in RmtDNAcn was seen in honeybees exposed to IMI or CLO versus control bees in the F2 generation (Figure 1A), and even higher in the F4 generation in the absence of exposure to neonics (Figure 1B). Those results demonstrated that while we could observe the normal aging effect via mtDNA damage in the control honeybees from F0 to F4, the additional increase in RmtDNAcn measured in honeybees of generations F2 and F4 is very likely associated with sublethal exposure to IMI or CLO. Because we observed almost identical baseline mtDNAcn levels measured among honeybees prior to the

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Table 1. Relative Mitochondrial DNA Copy Number (RmtDNAcn) Ratios[a] in the Thoracic Muscles of Honeybees Collected before (F0), during (F2), and after the Administration (F4) of Control, Imidacloprid (IMI), or Clothianidin (CLO)

treatment	sampling generation	no. of hives	no. of bees	mean (95% confidence interval)
control	F0	6	16	1.00
	F2	6	17	2.29 (1.64, 3.18)
	F4	6	18	5.23 (2.70, 10.12)
IMI	F0	6	16	1.11 (0.57, 2.16)
	F2	6	18	3.50 (1.87, 6.55)
	F4	6	18	11.06 (5.06, 24.15)
CLO	F0	6	15	1.07 (0.55, 2.09)
	F2	6	18	2.80 (1.50, 5.23)
	F4	6	18	7.31 (3.35, 15.97)

[a]The RmtDNAcn ratio is defined as the relative mitochondrial DNA copy numbers with respect to the nuclear DNA copy number of a specific group compared to that of the June control group [$2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{(TRT,\,j)} - \Delta Ct_{(CON,\,j)}$].

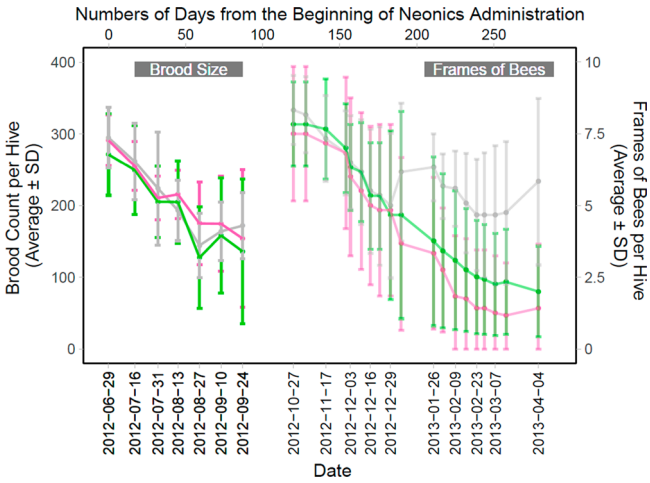

Figure 2. Brood counts (y-axis) and frames of adult honeybees that remained per hive before (z-axis), during, and after the administration of neonics. Gray, pink, and green points and error bars show the mean and 95% confidence interval of the RmtDNAcn levels we observed from the control, IMI, and CLO treatment groups, respectively. Error bars denote the standard error, and lower bounds below zero were set to zero.

beginning of the dosing regime, it is highly likely that the additional variations in mtDNAcn that we measured in honeybees after the administration of neonics originated from the effects of sublethal exposure to neonics, and to a less degree from normal aging of the queen bees.

In this study, the significant increase in RmtDNAcn in F4 bees exposed to neonics may represent accelerated aging in the winter bee generations, which in turn could not meet their energy demands to maintain the optimal temperature inside the hive throughout the winter.[28] We linked mtDNA damage in worker bees to the survival probabilities of the whole colony over winter. Figure 2 shows the overall decreasing trends of brood counts in colonies regardless of treatment group during the 13-week dosing period, suggesting that neonics may have no adverse effects on brood rearing of queen bees. However, as the observation period extended beyond the completion of the administration of neonics in early September, we observed a clear separation of the numbers of frames that remained with adult honeybees in control hives from those treated with neonics (Figure 2). The point of departure occurred when winter arrived. Hives treated with IMI showed the greatest disappearance of adult bees with an average of 1.4 frames of honeybees left at the end of the winter, followed by 2 frames of adult honeybees left in the CLO-treated hives. Control hives, on average, were left with 5.8 frames of adult honeybees. Overall, we observed a higher survival probability of control hives compared to IMI- or CLO-treated hives. At the end of the field experiment, five of six control hives were still alive; however, four and two of the six CLO- and IMI-treated colonies still included adult honeybees, respectively. It is also true that the timing of empty hives occurred earlier for neonic-treated hives than the controls in which IMI-treated colonies had an earlier onset of adult honeybee disappearance followed

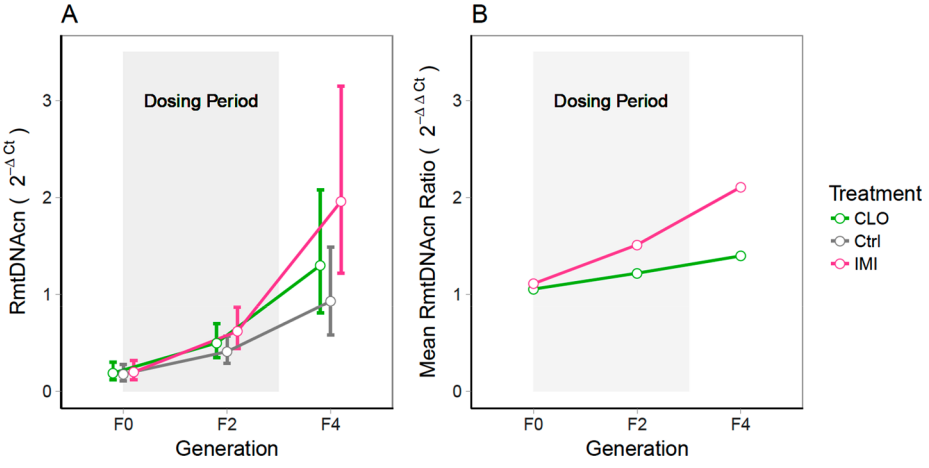

Figure 1. (A) Relative mtDNA copy number (RmtDNAcn) in adult honeybees collected from different brood generations of the same colony (the same queen). The dosing period (13 consecutive weeks) is shaded, which started immediately after baseline (F0) measurements on July 2 and ended September 17. F2 generation collected in June and F4 generation collected in October. (B) Magnitude of RmtDNAcn across different brood generations, representing before (F0), during (F2), and after administration (F4) of neonics. Gray, pink, and green points and error bars show the mean and 95% confidence intervals of the RmtDNAcn levels we observed from the control, IMI, and CLO treatment groups, respectively. Increasing average RmtDNAcn ratios in subsequent brood generations resulted from sublethal IMI and CLO administration.

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by CLO-treated colonies. The only dead control hive was due to disease, with vast numbers of dead bees found at the bottom of the hive, but not caused by the disappearance of adult honeybees.

One major limitation of this study is the numbers of hives that we collected honeybees from were not large enough to achieve sufficient statistical power. With respect to the outcomes of the model simulation shown in Figure S1, we would need to substantially increase the number of hives in the field study to 90 to achieve 80% of the statistical power for detecting the same levels of significance in the increase in mtDNA damage resulting from IMI exposure. This no doubt would add a significant burden to the research personnel and the project resources due to the nature of the field study involving intensive and frequent data and specimen collection throughout the year. Another inherited limitation for this study was that we did not directly measure mtDNA damage in queen bees in which under the perfect circumstances, it would be necessary. However, it is almost not possible to accomplish this task due to the fact that there is only one queen bee in each colony. Any minor perturbation to the queen bee would likely lead to adverse effects, other than the toxicity of neonics, to the whole colony, not to mention collecting thoracis muscle from queen bee for mtDNAcn measurements.

The damaged and dysfunctional mtDNA in honeybees' thoracic muscle may parallel the decline in energy production and biological characteristics of aged organisms.[19] Therefore, the occurrence of CCD could result from accelerated aging in winter bees induced by sublethal exposure of neonicotinoids. This hypothesis is supported by not only the magnitude of mtDNA damage as measured in bees collected in October but also the fact that honeybees normally neither die inside their hive nor abandon their hives during the winter. Maternally inherited mtDNA alterations leading to early aging and other health conditions have been discussed in recent studies.[29−32] Therefore, we surmise that the occurrence of CCD proceeds with a declining number of winter bees due to early aging as a result of sublethal neonic-induced mtDNA damage already present in early brood generations of worker bees. The findings reported here are crucial because we offer a preliminary mechanistic explanation of honeybee CCD by linking upstream exposure to neonics with honeybee colonies' overall health, including brood sizes, numbers of frames with bees, and colony winter survival. The incremental increases in RmtDNAcn among worker bees in the post-treatment generations (F4) suggest that the mtDNA damage that occurred in queen bees can be passed onto worker bees in the subsequent brood generations over time even in the absence of exposure to neonics. Such inheritance of mtDNA damage caused by very low levels of neonics exposure in honeybees has yet to be reported. Although no apparent adverse health effects in individual bees or the whole colony were observed during and after the period of administration of neonics, the persistent and cumulative mtDNA damage in winter bees might have negatively impacted their hives' survival over winter due to insufficient energy production. These results contribute to the explanation of a potential mechanism of CCD. It is therefore imperative to focus on validating our findings in future research and to fill the gap of neonics-induced mitochondrial dysfunctions and inefficient thermoregulation in honeybees, which could ultimately lead to the explanation of the onset of CCD.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.0c00070.

Materials and Methods, Figure S1, Tables S1−S3, and additional references (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Chensheng Lu − *College of Resources and Environment, Southwest University, Chongqing 400716, People's Republic of China; Department of Environmental Health, Harvard T. H. Chan School of Public Health, Boston, Massachusetts 02115, United States;* orcid.org/0000-0003-0680-2226; Phone: +86-023-68251249; Email: alexlcs@swu.edu.cn

Authors

Chi-Hsuan Chang − *Department of Environmental Health, Harvard T. H. Chan School of Public Health, Boston, Massachusetts 02115, United States;* orcid.org/0000-0002-5707-994X

Bernardo Lemos − *Department of Environmental Health, Harvard T. H. Chan School of Public Health, Boston, Massachusetts 02115, United States*

Quan Zhang − *College of Environment, Zhejiang University of Technology, Hangzhou 310032, Zhejiang, People's Republic of China;* orcid.org/0000-0001-9112-0662

David MacIntosh − *Department of Environmental Health, Harvard T. H. Chan School of Public Health, Boston, Massachusetts 02115, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.estlett.0c00070

Author Contributions

C.L. and C.-H.C. are co-first authors. C.L. contributed to study design, data collection, model interpretation, and manuscript preparation. C.-H.C. contributed to statistical analyses, model development and interpretation, and manuscript writing. B.L., Q.Z., and D.M. provided assistance in data interpretation and contributed to model interpretation.

Notes

The authors declare no competing financial interest.

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