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Effects of Five Pesticides on Toxicity, Detoxifying and Protective Enzymes in *Phauda flammans* Walker (Lepidoptera: Zygaenidae)

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ABSTRACT

Effects of beta-cypermethrin, abamectin, chlorpyrifos, thiamethoxam and bisultap on toxicity against 1st-6th instar larvae in *Phauda flammans* Walker (Lepidoptera: Zygaenidae) were tested using the leaf-dipping method. Meanwhile, effects of these pesticides on detoxifying and protective enzymes in *P. flammans* larvae were also measured. Results showed that the LC_{50} value of beta-cypermethrin, abamectin, chlorpyrifos, thiamethoxam and bisultap against *P. flammans* larvae were related to the instar and pesticide. Control efficacy of beta-cypermethrin was the best among of these pesticides, and the LC_{50} value of which against 1st instar larvae in *P. flammans* reached 2.038 mg/L at 8 h after treatment, and reached 6.416-48.764 mg/L for 2nd-6th instar larvae at 24 h after treatment. Control efficacy of abamectin was less than beta-cypermethrin, and the LC_{50} value of which against 1st instar larvae in *P. flammans* reached 241.953 mg/L at 8 h after treatment, and reached 19.285-266.207 mg/L for 2nd-6th instar larvae at 24 h after treatment. Control efficacy of chlorpyrifos, thiamethoxam and bisultap was less than the first two pesticides. Changes of detoxifying and protective enzymes in *P. flammans* larvae were related to the kinds of enzyme and its sensitivity to pesticide after treatment with different pesticides. Compared with control, effect of beta-cypermethrin on Carboxylesterase (CarE) was the most obvious than other four pesticides; activity of glutathione-s transferase (GST) was inhibited by abamectin, while the trend was in the opposite direction for other four pesticides; activity of superoxide dismutase (SOD) was promoted by beta-cypermethrin and chlorpyrifos, and inhibited by abamectin and bisultap; Activity of catalase (CAT) was promoted by beta-cypermethrin, chlorpyrifos and bisultap, inhibited the influence of promotion by thiamethoxam. Results suggested that *P. flammans* larvae could be effectively controlled by beta-cypermethrin 10 mg/L and abamectin 25 mg/L with alternate spray, which could prevent the resistance to pesticides in this pest.

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Authors' Contribution
XLZ conducted this research. ALH, LYM, WZ, GYL and HHT designed and implemented the experiment. JYL, HHT and WL analyzed the data. ALH, LYM and XLZ wrote the article.

Key words
***Phauda flammans*, Pesticides, Toxicity, Detoxifying enzymes, Protective enzymes.**

INTRODUCTION

Ficus (Urticales: Moraceae) are important decorative trees which used to line avenues in the urban landscapes of China and Southeast Asian countries (Liu *et al*., 2016). However, biodiversity descended obviously in an ecosystem or landscape with the increasing of the cultivated area of *Ficus* resulting in a sharp rise of pest species (Arthurs *et al*., 2016; Bhandari and Cheng, 2016).

Phauda flammans Walker (Lepidoptera: Zygaenidae) is one of the notorious defoliating pests of *Ficus* in southern China (Liu *et al*., 2015a), India (Nageshchandra *et al*., 1972; Verma and Dogra, 1982), Vietnam (FOF, 2015) and Tailand (Anonymous, 2017). *P. flammans* develops 2-3 generations per year in Nanning City, Guangxi, southern China. Adults fly in the daytime. Eggs are aggregated on leaves. The larval peaks of the first and second generations occur from mid-May to late June and early August to mid-October, respectively. Most larvae prefer to pupate near roots and tussocks exposed on the ground, and only a few individuals pupate in the topsoil. Larvae of this species can be found on *F. microcarpa* leaves even during winter (Liu *et al*., 2014, 2015a). *P. flammans* is a chill-intolerant species, which larvae can live safely on *F. concinna* and *F. benjamina* leaves during winter (Liu *et al*., 2015b; Zheng *et al*., 2017).

Although four species of insect parasitoids were identified from *P. flammans*, chemical control is one of the main ways for the management of *P. flammans* due to the lower parasitoid rates of their enemies (Zheng *et al*., 2015). However, much less is known of the effective pesticides and its concentrations to control them, especially during

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the period of outbreak.

The detoxifying and protective enzymes of insects, removing or dissolving toxic substances in the body for self-protection, mainly include carboxylesterase (CarE), glutathione-s transferase (GST), and superoxide dismutase (SOD), catalase (CAT) (Bolter and Chefurka, 1990; Zou *et al*., 2017). The activities of detoxifying and protective enzymes varied after different pesticides act on insects due to the different mechanisms (Desneux *et al*., 2007). Thus, study on the effects of different pesticides on detoxifying and protective enzymes in insects can provide theoretical basis for rational using of pesticides (Zhang *et al*., 2009; Allen` and Balin, 1989).

The purpose of this study is to explore the effects of several pesticides on toxicity against *P. flammans* larvae, and their effects on detoxifying and protective enzymes. Addressing this issue is conducive to provide reasonable suggestion to manage the *P. flammans* population.

MATERIALS AND METHODS

Insects

Larvae of 1st - 6th instar were collected from Guangxi University (108°29'E, 22°85'N) in Nanning City, Guangxi, southern China. Three *P. flammans* larvae were reared with fresh leaves of *F. microcarpa* on each petri dish (d = 9 cm) in laboratory with a light photoperiod of L16:D8 h and 70 ± 5% relative humidity maintained at 27 ± 1 °C. Leaves were renewed daily. Larval instars were determined according to Liu *et al*. (2015a).

Pesticides

Five pesticides were tested: beta-cypermethrin 3% EW, abamectin 16% SW, chlorpyrifos 30% EW, thiamethoxam 25% SC and 36% bisultap mother liquor. All of the pesticides were provided by Guangxi Tianyuan Biochemical Co., Ltd. (Guangxi, China).

Chemicals

Carboxylesterase (CarE) activity determination kit, glutathione-s transferase (GST) assay kit, superoxide dismutase (SOD) test box, catalase (CAT) test box, coomassie brilliant blue G-250 were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Bovine serum albumin, dibasic sodium phosphate (Na_2HP0_4), sodium dihydrogen phosphate (NaH_2PO_4), phosphoric acid (analytical reagent, purity ≥ 95%), ethanol (analytical reagent, purity ≥ 95%), acetic acid (analytical reagent, purity ≥ 95%), acetone (analytical reagent, purity ≥ 95%) were obtained from Tianjin Fuyu Fine Chemical Co. Ltd. (Tianjin, China).

Toxicity of pesticides against P. flammans *larvae*

The leaf-dipping method was used to determine the toxicity of five pesticides to *P. flammans* larvae. Based on the preliminary experiments, pesticides were diluted to a series of concentrations as the experimental treatment and the distilled water as the control (Wang *et al*., 2012; Rong *et al*., 2016). The leaves of *F. microcarpa* without any pesticides were punched by puncher (Φ = 2cm), and immersed in the diluted pesticides for 10 s. These leaves were used to rear 1st - 6th instar larvae (3 larvae / petri dish) after natural drying, and maintained at 27 ± 1 °C placed with a light photoperiod of L16: D8 hr and 70 ± 5% relative humidity in laboratory.

This experiment was three replications with total of 60 per instars in each concentration and pesticide. After treatment of 8 h, 12 h, 24 h, 48 h, the number of dead larvae of *P. flammans* were counted. In this experiment, the volume concentrations of beta-cypermethrin, abamectin, chlorpyrifos, thiamethoxam and bisultap were 6, 7.5, 10, 15 and 30 mg/L; 32, 40, 53.3, 80 and 160 mg/L; 60, 75, 100, 150 and 300 mg/L; 50, 62.5, 83.3, 125 and 250 mg/L; 72, 90, 120, 80 and 360 mg/L.

Effects of pesticides on detoxifying and protective enzymes

According to the results of toxicity, the LC_{50} of each pesticide was selected as sub-lethal metrology to study the effects of five pesticides on detoxifying and protective enzymes in 4th instar larvae of *P. flammans*. The result of this experiment was expressed as the enzyme specific activity (Rumpf *et al*., 1997; Quan *et al*., 2016). The leaf-dipping method was used as mentioned above, and the 4th instar larvae after treatments for 24 h and 48 h were collected as experimental materials.

Enzymatic preparation

Larvae (n = 9) treated by different pesticides and times were selected, respectively. All selected larvae were placed in precooled mortar, and then 2 mL phosphate buffer (0.1 mol/L, pH = 7.0) were put to mill in the ice bath. The mixture were centrifugalized with TGL-16 high-speed refrigerated centrifuge (Hunan Xiangyi Laboratory Instrument Development Co. Ltd., Hunan, China) in the 2 ml centrifuge tube (4 °C, 10000 r/min) for 15 min after milling. The supernatant stored at -20°C for the following experiments.

Determination of protein content

According to Bradford (1976) method, 0.1 ml enzyme, 0.9 ml distilled water and 5 ml Coomassie Brilliant Blue G-250 were added to a test tube and the contents mixed either by inversion or vortexing. The control group without enzyme was added in the same reagents as the experiment group. The absorbance at 595 nm was measured with visible spectrophotometer (Spectrumlab 722 sp, Shanghai

Jiguang Technology Co. Ltd., Shanghai, China) after 2-3 min, and this experiment was three replications. The protein content was calculated according to the standard curve of bovine serum protein.

Table I.- Toxicity of five pesticides to 1st - 6th instar larvae in *Phauda flammans.*

Insecticides	Instar	Treatment time (h)	Regression equation	χ^2	P	LC_{50} (mg/L)	95% Fiducial limit
Beta-cypermethrin	1	8	y=-0.967+3.130x	0.335	0.953	2.038	-
	2	24	y=-2.437+3.019x	3.835	0.28	6.416	5.057-7.475
	3	24	y=-0.888+1.065x	0.563	0.905	6.82	2.885-9.572
		48	y=-0.853+1.664x	0.253	0.969	3.256	1.055-4.944
	4	24	y=-1.113+1.254x	4.391	0.222	7.719	4.547-10.228
		48	y=-1.298+1.926x	0.398	0.941	4.722	2.620-6.255
	5	24	y=-1.230+1.241x	0.383	0.944	9.799	6.727-13.000
		48	y=-0.952+1.346x	1.364	0.714	5.097	2.190-7.166
	6	24	y=-1.356+0.028x	4.909	0.179	48.764	33.601-149.585
		48	y=-2.145+1.955x	0.705	0.872	12.511	10.313-15.450
Abamectin	1	8	y=-4.352+1.826x	0.521	0.914	241.953	163.273-580.977
	2	24	y=-1.880+1.463x	0.135	0.987	19.285	6.568-28.902
	3	24	y=-4.459+3.018x	1.404	0.705	30.034	22.473-35.582
		48	y=-3.930+3.315x	1.085	0.781	15.324	1.860-23.278
	4	24	y=-3.565+2.213x	1.229	0.746	40.849	32.486-48.141
		48	y=-2.778+2.094x	0.116	0.99	21.205	11.142-28.549
	5	24	y=-2.850+1.626x	2.669	0.445	56.645	44.475-70.568
		48	y=-2.340+1.671x	0.351	0.95	25.127	13.129-33.896
	6	24	y=-1.944+0.802x	0.828	0.843	266.207	131.157-36274.553
		48	y=-2.295+1.502x	1.859	0.602	33.672	20.307-43.711
Chlorpyrifos	1	8	y=-4.507+2.476x	9.27	0.026	66.046	2.299-102.087
	2	24	y=-3.758+2.735x	0.569	0.904	23.665	1.846-39.591
	3	24	y=-1.260+0.838x	0.36	0.948	31.956	0.484-60.031
		48	y=-1.700+1.310x	0.98	0.806	19.846	1.390-38.629
	4	24	y=-4.234+2.105x	6.23	0.101	102.666	55.268-165.564
		48	y=-1.895+1.196x	1.638	0.651	38.486	10.151-59.701
	5	24	y=-3.337+1.634x	2.591	0.459	110.155	87.865-136.959
		48	y=-2.650+1.496x	0.945	0.814	59.046	34.405-77.293
	6	24	y=-4.170+1.578x	2.506	0.474	438.66	290.298-1113.188
		48	y=-3.461+1.745x	0.159	0.984	96.299	76.144-117.040
Thiamethoxam	1	8	y=-2.580+2.029x	1.603	0.659	18.684	2.669-32.036
	2	24	y=-3.302+2.011x	3.292	0.349	43.773	27.803-55.724
	3	24	y=-2.592+1.055x	0.77	0.857	286.866	180.019-1245.596
		48	y=-4.322+2.207x	1.942	0.584	90.758	76.629-107.144
	4	24	y=-2.915+1.029x	0.097	0.992	680.492	300.340-49718.394
		48	y=-2.354+1.137x	1.242	0.743	117.768	85.174-191.653
	5	24	y=-1.861+0.574x	0.33	0.954	1754.739	-
		48	y=-1.975+0.910x	0.599	0.897	148.041	102.677-385.708
	6	24	y=-1.891+0.482x	0.143	0.986	8319.048	-
		48	y=-2.729+1.083x	0.436	0.933	331.013	200.551-1631.084
Bisultap	1	8	y=-3.645+2.123x	1.231	0.746	52.096	23.481-71.487
	2	24	y=-4.125+2.280x	4.586	0.205	64.472	38.039-82.536
	3	24	y=-3.961+1.711x	0.989	0.804	206.475	166.847-285.195
		48	y=-3.522+1.642x	0.936	0.817	139.884	111.079-177.169
	4	24	y=-4.171+1.744x	0.546	0.909	246.348	195.658-361.874
		48	y=-3.788+1.706x	0.579	0.901	168.891	135.276-214.532
	5	24	y=-4.024+1.659x	2.613	0.455	266.572	208.327-411.650
		48	y=-3.470+1.531x	0.871	0.832	184.82	148.113-255.082
	6	24	y=-3.349+1.357x	5.109	0.164	293.475	215.709-563.172
		48	y=-4.090+1.783x	2.900	0.407	196.482	161.407-260.268

Determination of detoxifying and protective enzymes activities

CarE, GST, SOD and CAT activities were determined by using assay kit (Nanjing Jiancheng Biotechnology Institute, Nanjing, China), and the tests were carried out strictly with the assay kit directions. The experiments were replicated three times.

Data analysis

Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA). Duncan's multiple range tests was used for significance test ($P \leq 0.05$).

RESULTS

Effect on P. flammans *larvae*

The LC_{50} values of the same pesticide increased gradually with increasing of the larval instar of *P. flammans*. The LC_{50} values of the same pesticide decreased gradually with increasing of the treatment time (Table I). Beta-cypermethrin showed the highest toxicity for *P. flammans* larvae in the five pesticides. Abamectin had no significant lethal effect for *P. flammans* 1st instar larvae at 8 h after treatment in the five pesticides. Bisultap had the lowest toxicity for *P. flammans* 2th instar larvae at 24h after treatment and 3th - 5th instar larvae at 48h after treatment, while the lowest toxicity for *P. flammans* 6th instar larvae at 48h after treatment was thiamethoxam. The order of toxicity against *P. flammans* larvae from high to low for the five pesticides was as follows: beta-cypermethrin, abamectin, chlorpyrifos, thiamethoxam and bisultap.

Effects on detoxifying enzymes

There were different effects of five pesticides on detoxifying enzymes in *P. flammans* larvae (Fig. 1A, B). Compared with control, CarE activity in *P. flammans* 4th instar larvae at 24h ($F = 55.938$, $df = 5, 17$, $P < 0.001$) and 48h ($F = 15.148$, $df = 5, 17$, $P < 0.001$) after treatment were significant difference in the five pesticides. Activity of CarE was promoted by beta-cypermethrin, the effect of which on CarE activity was the most obvious (Fig. 1A). Compared with control, GST activity in *P. flammans* 4th instar larvae at 24h ($F = 6.926$, $df = 5, 17$, $P < 0.01$) and 48h ($F = 21.771$, $df = 5, 17$, $P < 0.001$) after treatment were significant difference in the five pesticides. Beta-cypermethrin, thiamethoxam and bisultap played a significant role in promoting GST activity. Beta-cypermethrin and bisultap promoted the activity of GST, and weakened slowly, but thiamethoxam always enhanced the activity of GST (Fig. 1B). As the results showed, effects of beta-cypermethrin on the CarE and GST activities were the most obvious among the five pesticides.

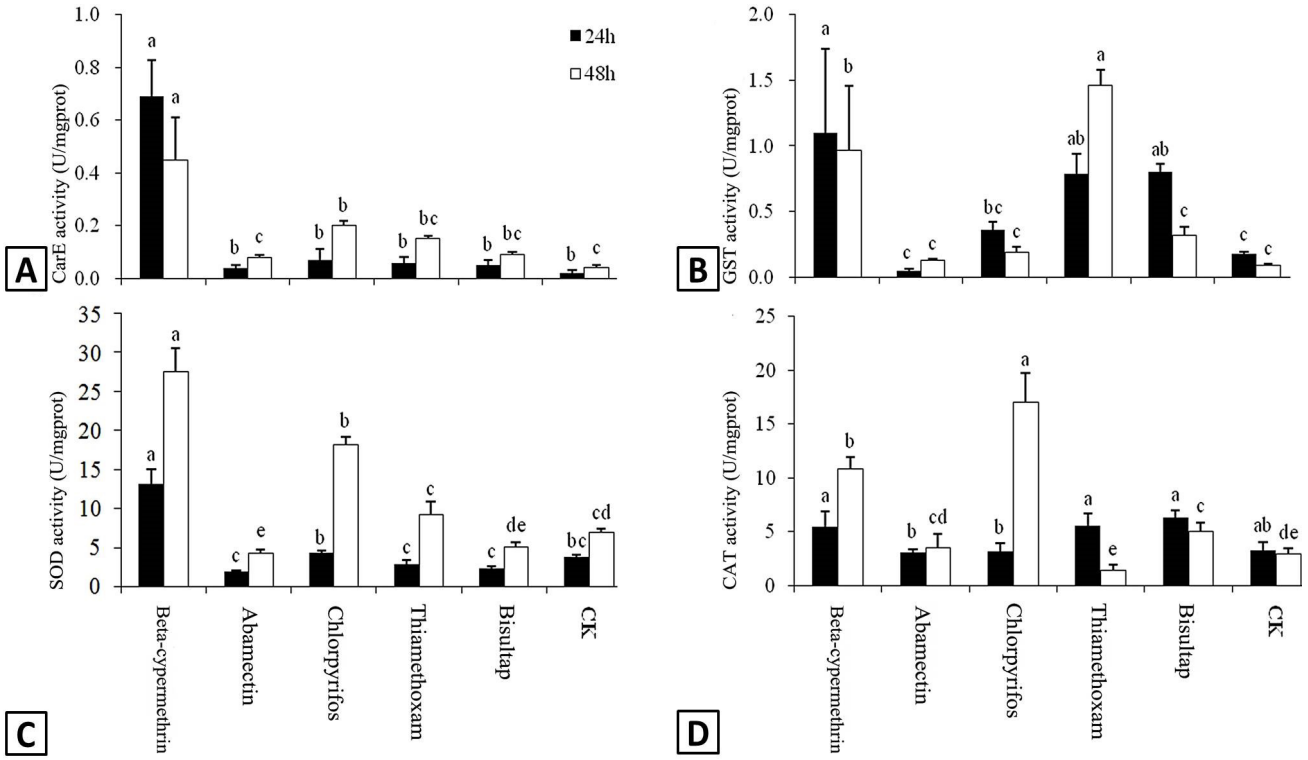

Fig. 1. Effects of five insecticides on the activities of CarE (A), GST (B), SOD (C) and CAT (D) in *Phauda flammans*.

Effects on protective enzymes

Five pesticides had different effects on detoxifying enzymes of *P. flammans* larvae (Fig. 1C, D). Compared with control, SOD activity in *P. flammans* 4th instar larvae at 24h ($F = 76.773$, $df = 5, 17$, $P < 0.01$) and 48h ($F = 116.253$, $df = 5, 17$, $P < 0.001$) after treatment were statistical significance in the five pesticides. Beta-cypermethrin and chlorpyrifos promoted the activity of SOD, while abamectin and bisultap inhibited SOD activity; thiamethoxam inhibited the SOD activity at first and promoted it at later (Fig. 1C). Compared with control, CAT activity in *P. flammans* 4th instar larvae at 24h ($F = 3.320$, $df = 5, 17$, $P < 0.05$) and 48h ($F = 57.217$, $df = 5, 17$, $P < 0.001$) after treatment were statistical significance in the five pesticides. Effects of beta-cypermethrin, chlorpyrifos and bisultap on CAT activity had positive impacts, while abamectin and thiamethoxam showed no significant effects on CAT activity compare with the control (Fig. 1D). As the results, effect of beta-cypermethrin on the SOD activity was the most obvious among five pesticides, and chlorpyrifos had the greatest effect on CAT activity.

DISCUSSION

Beta-cypermethrin, a pyrethroid pesticide, is an efficient isomer of cypermethrin with high biological activity, which has the characteristics of wide insecticide controlling spectrum, rapid effects and high insecticidal activity (Qu *et al*., 2017). Wang *et al*. (2012) found that beta-cypermethrin had high toxicity in the toxicity test of 6 pesticides on *Histia rhodope* Ceamer. Our results also indicated that low-dose beta-cypermethrin had good control effect on 1st-6th instar larvae of *P. flammans*. Abamectin, an antibiotic pesticide, had high toxic activity to *Achelura yunnanensis* Horie&Xue (Rong *et al*., 2016). In our study, the mortality of larvae rose with treated time delay of abamectin. The changes of detoxifying and protective enzymes in insects are different under the action of pesticides (Li *et al*., 1994; Ding *et al*., 2001). In order to achieve detoxification, insects will oxidize, restore, hydrolyze or combine the toxic compounds entered into the body from natural environment by increasing the activity of their detoxifying enzymes, so as to enhance the water-solubility of the toxic compounds, and make it easy to eliminate toxins from the body or turn them into low-toxic or non-toxic substances (Xing *et al*., 2011; Zhang *et al*., 2015; Jia *et al*., 2016a). The enhancement of detoxifying enzymes activity is one of the mechanisms for insects to improve the resistance against pesticides. For example, abamectin and beta-cypermethrin could induce the increasing of GST activity in *Plutella xylostella* L. (Liang *et al*., 2003). However, some pesticides killed insects by inhibiting the activity of detoxifying enzymes. For example, sub-lethal concentration of abamectin could obviously inhibit the GST activity of *Diadegma semiclausum* Hellen (Jia *et al*., 2016b). Results from the current study showed that beta-cypermethrin promoted the activities of both CarE and GST in *P. flammans* larvae, while abamectin promoted CarE activity but inhibited GST activity. It indicated that the sub-lethal concentration of beta-cypermethrin induced the increase of detoxifying enzymes activity to enhance the detoxification ability of *P. flammans* larvae. Therefore, we speculated that *P. flammans* might resist beta-cypermethrin when it used for a long time. The sub-lethal concentration of abamectin inhibited detoxifying enzymes activity in *P. flammans*, which reduced the ability of larvae to degrade pesticides and enhanced its toxicity. Furthermore, effect of beta-cypermethrin on SOD activity in *Blattella germanica* appeared a trend of "rise-decrease-rise", which was reported by Ma *et al*. (2009). However, it was different from the positive effects of beta-cypermethrin on SOD and CAT activities in *P. flammans* larvae. It hinted that *P. flammans* larvae resisted beta-cypermethrin mainly by enhancing the protective enzymes activity. Abamectin could inhibit SOD activity, but effect of abamectin on CAT activity was not significant, which suggested that abamectin could destroy protective enzymes when it acted on *P. flammans* larvae for killing them.

Although the toxicity of beta-cypermethrin was the best in this study, it significantly activated the activity of detoxifying enzymes in *P. flammans* which may lead to insect resistance. Effect of abamectin on toxicity was slightly inferior than beta-cypermethrin, but it effectively inhibited detoxifying and protective enzymes activities. Therefore, we recommend that *P. flammans* larvae could be effectively controlled by beta-cypermethrin 10 mg/L and abamectin 25 mg/L with alternate spray, which could prevent the resistance to pesticides in this pest.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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