# Sublethal Doses of Essential Oils Impact Growth and Esterase Levels in German Cockroach (Blattodea: Ectobiidae) with and without Symbiotic Bacteria[1]

Ping You[2], Jun-Qing Jia[2], and Zhi-Qiang Li[3]

Guangdong Key Laboratory of Animal Conservation and Resource Utilization, Guangdong Public Laboratory of Wild Animal Conservation and Utilization, Guangdong Institute of Applied Biological Resources, Guangzhou 510260, China

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**Abstract** The inhibition by three essential oils of the growth and esterase production of German cockroaches, *Blattella germanica* (L.) (Blattodea: Ectobiidae), with and without symbiotic bacteria, was determined in laboratory assays. Essential oils extracted from *Lilium brownii* var. *viridulum* Baker, *Croton tiglium* L., and *Lonicera japonica* Thunberg were mixed with a powdered rat food at a 10% concentration and fed to cockroach nymphs. Body mass and body length were measured in adults in all three treatments. Acetylcholinesterase and nonspecific esterase activities were compared between the *Lilium* and *Croton* essential oil treatments. The nonlethal doses of these essential oils reduced cockroach growth, while the growth of the females lacking symbiotic bacteria was significantly reduced when fed the diet containing *Lilium* essential oil. *Lilium* and *Croton* essential oils significantly inhibited esterase activity. Our results indicate that symbiotic gut bacteria might be involved in mediating detoxification in the German cockroach.

**Key Words** German cockroach, symbiotic bacteria, esterase, inhibition, growth

The continuous use of synthetic conventional insecticides against the German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae), has resulted in the development of insecticide resistance in populations of this pest (Ko et al. 2015, Wu and Appel 2017). Alternative management approaches that are environmentally friendly are needed. Natural products that may have insecticidal activity are an attractive option (Dhang 2011, Oi 2011).

Plants and plant-derived secondary metabolites are considered to be safe, environmentally friendly natural products, and many have displayed biological activity (Adler and Uebel 1985, Phillips et al. 2010, Yeom et al. 2013). Essential oils extracted from plants show potential as cockroach control agents for fumigation, contact insecticides, repellents, and growth inhibitors (Alzogaray et al. 2011, Yeom et al. 2015). Some essential oils may be useful in cockroach baiting technology (Canale et al. 2013, Mansour and Abdel-Hamid 2015). Although use of essential oils as a control tactic in integrated pest management is generally recognized (Oi

[1]Received 04 May 2019; accepted for publication 04 August 2019.
[2]College of Life Science, Shaanxi Normal University, Xi'an 710062, China.
[3]Corresponding author (email: lizhiqiang61@163.com).

2011), toxicity and interaction of essential oils in cockroach management remains poorly understood.

Furthermore, symbiotic gut bacteria play a pivotal and positive role in host physiology, behavior, and health of cockroaches (Gontang et al. 2017, Wadakatsumata et al. 2015). Esterases (EC 3.1) are often reported to regulate the development of detoxification in German cockroach (Chai and Lee 2010, Kim et al. 2017). As an example, acetylcholinesterase (AchE, EC 3.1.1.7) is an important resistance-related target enzyme that can be inhibited by plant essential oils (Yeom et al. 2012, 2013). The current understanding of the combined effects of symbiotic gut bacteria and essential oils is limited. Plant essential oils of *Croton tiglium* L. (Malpighiales: Euphorbiaceae), *Lilium brownii* Baker (Liliales: Liliaceae), and *Lonicera japonica* Thunberg (Dipsacales: Caprifoliaceae) have not been examined for their potential in German cockroach management. We hypothesized that symbiotic gut bacteria would mediate the growth and detoxification of sublethal doses of essential oils in German cockroaches.

Our objective in this study was to compare the growth and esterase levels of German cockroaches, with and without symbiotic bacteria, in response to exposure to sublethal doses of three essential oils. We hoped that from these results we could determine the role, if any, of the gut symbiotic bacteria in detoxification of these oils.

## Materials and Methods

**German cockroach rearing.** A population of German cockroach susceptible to insecticides was provided by Guangdong Provincial Center for Disease Control and Prevention in China. This colony had not been exposed to insecticides for >27 yr. Symbiotic gut bacteria were eliminated from one group of these cockroaches by provision of water supplemented with 500 μg/ml gentamicin (Cisen Pharmaceutical Co., Ltd., Shandong, People's Republic of China) for 1 mo as outlined by Liu (2013). A control group which retained the symbiotic bacteria was provided water without gentamicin.

Both groups of cockroaches were reared on a powdered rat feed (SPF level, Guangdong Medical Laboratory Animal Center) at 25 ± 3°C, 60–70% relative humidity and a photoperiod of 12:12 h (light:dark). The removal efficiency of the intestinal flora was evaluated by the 16S rRNA gene. Two universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTAC GACTT-3′), were used to amplify the 16S rRNA coding region by polymerase chain reaction (PCR) (Lane 1991). The amplification reactions were performed in a total volume of 25 μl containing 250 ng of DNA extracted from each cockroach gut, 10 μl of TaKaRa Premix Ex Taq, and 2 μl of each primer. The reaction mixtures were amplified with the following program: 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1.5 min; and a final extension period of 72°C for 10 min. The PCR products were examined by 1% agarose gel electrophoresis. The control with bacteria showed a bright band between 1,200 and 2,000 bp, whereas that treated with the antibiotic displayed a very weak band.

**Essential oils and bioassay.** Three plant essential oils from the dried fruit of *C. tiglium* and the dried flowers of *Lilium brownii* and *Lonicera japonica* were purchased from Shaanxi Sunrun Bio-technology Co. The *Croton* essential oil was

obtained using petroleum ether extraction. Major components identified were linoleic acid (55.9%), oleic acid (25.9%), erucic acid (7.4%), hexadecanoic acid (2.4%), and arachidic acid (1.4%) (Hu et al. 2008). The *Lilium* and *Lonicera* oils were obtained by traditional distillation with the major components of *Lilium* as di-isobutyl phthalate (59.1%), dodecanoic acid (12.4%), tetradecylenic acid (6.0%), dibutyl phthalate (4.2%), and 2-tetradecanol (4.1%),while the main components of the essential oil from *Lonicera* were n-hexadecanoic acid (29.1%), methyl hexadecanoate (9.3%), thymol (8.7%), 9,12,15-octadecatrienoic acid methyl ester (5.8%), and 9,12,15-octadecatrien-1-ol (5.1%) (Di et al. 2003, Hui et al. 2003).

Given that these essential oils had no clear repellent action against cockroaches, we combined each with the rat feed diet. At least 20 third-instar nymphs (10 males, 10 females) were placed in a plastic container (20.2 cm by 13.6 cm by 7.5 cm) in which the upper inside surface was coated with a petroleum jelly–mineral oil mixture to prevent cockroach escape. For treatments with the essential oils, the extracted oil was mixed with the powdered rat feed diet at a concentration of 10%. A control group was provided with rat feed powder containing no essential oil. Diets were supplied daily during the study. Once cockroaches molted to the adult stage, individual body length and body mass were measured. Each treatment was repeated three times.

**Esterase assay.** For topical application, adult males and females, with and without symbiotic bacteria, were treated with *Lilium* and *Croton* essential oils diluted with acetone to a concentration of 10% using a microdroplet method. Aliquots of 1 μl of the essential oil solution were applied to ventral surface of the thorax of cockroaches exposed to $CO_2$ to reduce activity. Activity of AChE and nonspecific esterases was determined on days 1, 2, 5, 7, and 9 after application. Controls were treated with acetone only.

Adult cockroaches were rinsed with distilled water and dried with filter paper on ice. Heads were excised from one male and one female per replicate and immersed in 1/15 M phosphate buffer (pH 8.0) containing 0.5% Triton X-100 in an ice bath and homogenized. The supernatant was used as the source of acetylcholinase by centrifugation at 4,000 × *g* for 15 min. German cockroach guts were dissected and washed, and intestinal material was removed at 4°C. The midguts isolated were homogenized in an ice bath with 0.04 M phosphate buffer (pH 7.0) before being centrifuged at 3,000 × *g* for 10 min at 4°C. The supernatant was transferred to fresh tubes and used as crude enzyme for measuring nonspecific esterase activity.

The specific activity was determined in accordance with a nitrophenol standard curve and the protein concentration of the crude enzyme. Protein concentrations were determined by the Bradford (1976) method.

AChE inhibition activity was evaluated using the improved Ellman method (Gorun et al. 1978). The reaction mixtures consisting of 50 μl of crude enzyme, 50 μl of S-acetylthiocholine iodide, and 100 μl of phosphate buffer (0.007 M, pH 8.0) were incubated at 30°C for 15 min. Then, 1.8 ml of 5,5′-dithiobis (2-nitrobenzoic acid)–phosphate–ethanol reagent was added to terminate the reaction, and a colorimetric determination was performed at 412 nm using a Victor 3 Multi-label Microplate Reader (Perkin Elmer, Waltham, MA).

Nonspecific esterase activity tests referred to the method of metabolic enzyme activity determination (Valles et al. 1999). A total of 0.03 ml of crude enzyme was mixed with 1.2 ml of 1-naphthyl acetate (0.0003 M) and 0.3 ml phosphate buffer (pH

7.0) for 15 min. The reaction was terminated with the addition of 0.33 ml of a solution of two parts 1% tetrazotized o-dianisidine and five parts 5% sodium dodecyl sulfate. After 15 min, the optical density was measured at 600 nm.

**Data analysis.** All statistical analyses were conducted using and SPSS 22.0 (IBM Corp., Chicago, IL). Mean body masses, body lengths, and enzyme activity data were analyzed with one-way analysis of variance (ANOVA) and Scheffé correction for multiple comparisons. Tukey's multiple comparison tests were used to separate significant treatment means. The inhibition rate of enzyme activity was evaluated according to the following formula, where inhibition rate (%) = ([control enzyme activity − treated enzyme activity]/control enzyme activity) × 100.

## Results

**Body mass response.** Treatment with the essential oils reduced the body mass of cockroaches (Table 1). In both groups, with and without symbiotic bacteria, there was a significant difference in body mass between essential oil treatment and the control, except for male cockroaches in the control (symbiotic bacteria) group. Body masses of female cockroaches treated with *Lilium* and *Croton* essential oils were significantly different between the groups with and without the symbiotic bacteria. Female cockroaches from which gut bacteria had been removed exhibited a significantly lower body mass after feeding on both *Lilium* and *Croton* essential oils.

**Body length response.** Except for the treatment of *Lonicera* in cockroach males without symbiotic bacteria, all three essential oils significantly reduced cockroach body length relative to the controls with and without bacteria (Table 2). Male body length was significantly different between those with and without bacteria. When fed essential oils, this difference disappeared. The body length of female cockroaches without bacteria treated with *Lilium* essential oil was decreased compared with the control.

**AChE inhibitory activity.** AChE enzyme activities and inhibition rates for *Lilium* and *Croton* essential oils are shown in Figs. 1 and 2, and Table 3. AChE activities in all essential oil treatments were significantly lower than activity in the control. Compared with the groups without bacteria, inhibitory rates had distinct fluctuations in the control. The AChE inhibitory rate of *Lilium* essential oil on the control cockroach group was higher than that of the corresponding group without bacteria at 1 d and continued to increase. However, the AChE inhibitory rate of the cockroaches with gut bacteria was obviously lower than that of those without bacteria at 9 d. The AchE inhibitory rate of *Croton* essential oil was the same in the two cockroach groups at 1 and 2 d, but the AChE inhibitory rate on the control with bacteria also clearly decreased and differed from that of group without bacteria at 9 d.

**Nonspecific esterase inhibitory activity.** Activities the nonspecific esterases in the two groups of cockroaches treated with *Lilium* and *Croton* essential oils were significantly different from those of the control, except for *Lilium* at 5 d and *Croton* at 7 d (Figs. 3, 4). Esterase activity was clearly inhibited by the essential oils, but esterase activity in the control group was higher than that of the cockroaches without bacteria. The inhibitory rates decreased over time, but the inhibitory rates of the control cockroach group treated with the essential oils were clearly lower than

**Table 1. Effects of essential oils on body mass of German cockroach.***

| | No Bacteria (mg) | | Control with Bacteria (mg) | | Male, NB versus Con | | Female, NB versus Con | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Male** | **Female** | **Male** | **Female** | ***t*** | ***P*** | ***t*** | ***P*** |
| *Lilium* | 32.4 ± 3.4b | 47.7 ± 6.5c | 37.8 ± 3.3a | 58.0 ± 14.5b | −2.07 | 0.051 | −3.03 | 0.005 |
| *Croton* | 35.0 ± 3.6b | 47.0 ± 12.5c | 37.6 ± 4.1a | 55.0 ± 10.5b | −1.65 | 0.133 | −2.31 | 0.027 |
| *Lonicera* | 34.9 ± 4.1b | 60.0 ± 1.5b | 37.4 ± 3.8a | 60.0 ± 10.9b | −1.56 | 0.133 | −0.11 | 0.912 |
| Control | 41.8 ± 4.0a | 85.9 ± 13.7a | 42.7 ± 3.8a | 86.1 ± 15.2a | −0.59 | 0.561 | −0.05 | 0.964 |
| ANOVA | $F = 16.462$; df = 3,50; $P < 0.001$ | $F = 41.271$; df = 3,75; $P < 0.001$ | $F = 3.192$; df = 3,48; $P = 0.032$ | $F = 22.633$; df = 3,75; $P < 0.001$ | — | — | — | — |

* Mean ± SE values with the same letter are not significantly different within a column (Scheffé's test) at $P < 0.05$. NB indicatesithout bacteria; C, control with bacteria.

**Table 2. Effects of essential oils on body length of German cockroach.***

| | No Bacteria (mg) | | Control with Bacteria (mg) | | Male, NB versus Con | | Female, NB versus Con | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Male** | **Female** | **Male** | **Female** | ***t*** | ***P*** | ***t*** | ***P*** |
| *Lilium* | 11.40 ± 0.38b | 10.72 ± 0.38bc | 11.57 ± 0.67b | 11.02 ± 0.71b | −0.45 | 0.660 | −2.12 | 0.041 |
| *Croton* | 11.40 ± 0.77b | 10.40 ± 0.56c | 11.58 ± 0.56b | 10.72 ± 0.68b | −0.66 | 0.513 | 0.28 | 0.138 |
| *Lonicera* | 11.85 ± 0.57ab | 11.25 ± 0.89bc | 11.58 ± 0.53b | 10.97 ± 0.89b | 1.20 | 0.242 | 0.95 | 0.348 |
| Control | 12.16 ± 0.57a | 12.19 ± 0.61a | 12.54 ± 0.38a | 12.15 ± 1.01a | −2.06 | 0.049 | 0.12 | 0.909 |
| ANOVA | $F = 4.795$; df = 3,51; $P = 0.005$ | $F = 26.372$; df = 3,74; $P < 0.001$ | $F = 9.805$; df = 3,48; $P < 0.001$ | $F = 10.854$; df = 3,75; $P < 0.001$ | — | — | — | — |

* Mean ± SE values with the same letter are not significantly different within a column (Scheffé's test) at $P < 0.05$. NB indicatesithout bacteria; C, control with bacteria.

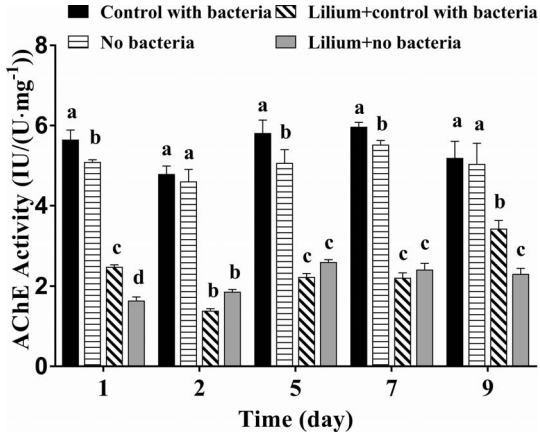


**Fig. 1. German cockroach acetylcholinesterase activity following *Lilium* essential oil treatments.**

those without bacteria at the termination of the experiments (Table 4). For both *Lilium* and *Croton* essential oils, the inhibition rate in the cockroaches without bacteria was significantly higher than that of the control group after 1 d.

## Discussion

The use of Lamiaceae essential oils mixed with food bait was effective against the olive fruit fly, *Bactrocera oleae* (Rossi), in laboratory conditions and in semifield conditions (Canale et al. 2013). Baits containing plant essential oils and commercial insecticides against the desert locust, *Schestocerca gregaria* (Forskäl), have been

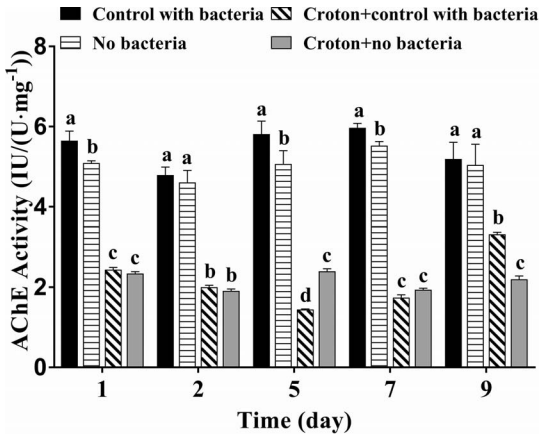


**Fig. 2. German cockroach acetylcholinesterase activity following *Croton* essential oil treatments.**

**Table 3. Acetylcholinesterase inhibition rates for essential oils of German cockroach.***

| Treatment | Inhibition Rate (%) | | | | |
|---|---|---|---|---|---|
| | 1 d | 2 d | 5 d | 7 d | 9 d |
| *Lilium* + control with bacteria | 56.03 ± 1.74a | 70.97 ± 2.18a | 61.52 ± 3.29b | 62.99 ± 2.20a | 33.67 ± 3.75b |
| *Lilium* + no bacteria | 33.67 ± 3.75b | 59.45 ± 3.06b | 48.23 ± 4.66c | 56.33 ± 2.65c | 53.88 ± 5.11a |
| *Croton* + control with bacteria | 56.78 ± 2.27a | 58.15 ± 1.55b | 75.08 ± 1.59a | 70.84 ± 1.39a | 35.74 ± 5.58b |
| *Croton* + no bacteria | 54.00 ± 0.77a | 58.37 ± 3.84b | 52.48 ± 3.72c | 64.95 ± 1.24b | 55.92 ± 6.31a |
| ANOVA | $F = 63.54$; df = 3,19; $P < 0.001$ | $F = 24.45$; df = 3,19; $P < 0.001$ | $F = 57.24$; df = 3,19; $P < 0.001$ | $F = 46.49$; df = 3,19; $P < 0.001$ | $F = 24.59$; df = 3,19; $P < 0.001$ |

* Mean ± SE values with the same letter are not significantly different within a column (Scheffé's test) at $P < 0.05$.

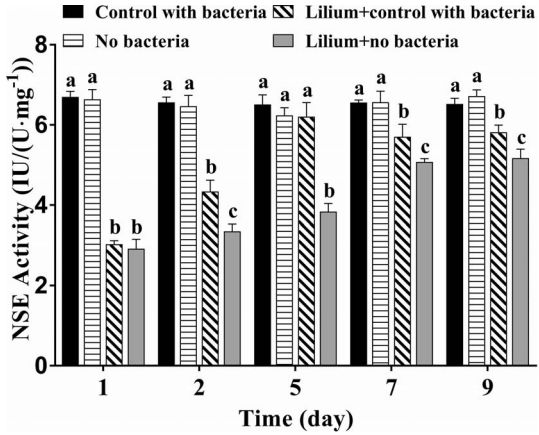


**Fig. 3. German cockroach nonspecific esterase activity following *Lilium* essential oil treatments.**

shown to be potent biopesticides (Mansour and Abdel-Hamid 2015). Although baiting technology has gained widespread acceptance for the control of cockroaches, the focus of essential oil research has generally been on repellents, fumigants, and contact properties against the German cockroach (Lee et al. 2017, Liu et al. 2015, Phillips and Appel 2010, Yoem et al. 2013). Our results reveal that, when incorporated into feed as bait, *Lilium* and *Croton* essential oils demonstrated a degree of biological activity against German cockroaches, with reduced growth and esterase activity. Previous studies have also shown a significant difference in body mass between laboratory-reared and field-collected German cockroaches (Wu and Appel 2017). Kim et al. (2017) also reported lower variations in body length of

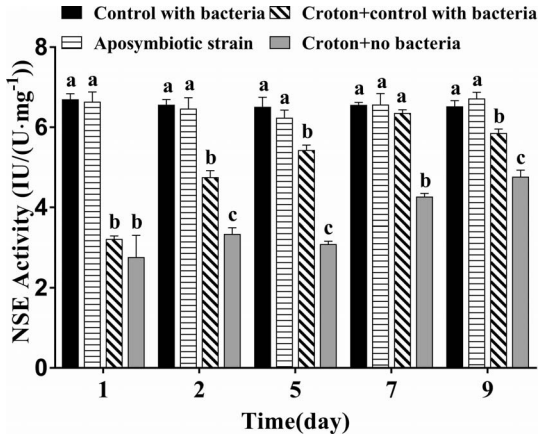


**Fig. 4. German cockroach nonspecific esterase activity following *Croton* essential oil treatments.**

**Table 4. Inhibition rates of essential oils on nonspecific esterases of German cockroach.***

| Treatment | Inhibition Rate (%) | | | | |
|---|---|---|---|---|---|
| | 1 d | 2 d | 5 d | 7 d | 9 d |
| *Lilium* + control with bacteria | 54.70 ± 1.83a | 33.95 ± 3.23b | 4.46 ± 8.32d | 13.03 ± 4.92c | 10.78 ± 3.24b |
| *Lilium* + no bacteria | 56.05 ± 2.74a | 48.02 ± 3.95a | 38.48 ± 2.10b | 22.59 ± 3.99b | 23.04 ± 3.11a |
| *Croton* + control with bacteria | 51.92 ± 0.87a | 27.48 ± 3.04c | 16.47 ± 3.13c | 3.05 ± 1.60d | 10.14 ± 3.42b |
| *Croton*+no bacteria | 58.30 ± 7.80a | 48.23 ± 2.08a | 50.45 ± 0.90a | 34.88 ± 3.38a | 29.00 ± 3.50a |
| ANOVA | $F = 1.95$; df = 3,19; $P = 0.163$ | $F = 54.21$; df = 3,19; $P < 0.001$ | $F = 198.54$; df = 3,19; $P < 0.001$ | $F_9 = 68.19$; df = 3,19; $P < 0.001$ | $F = 39.19$; df = 3,19; $P < 0.001$ |

* Mean ± SE values with the same letter are not significantly different within a column (Scheffé's test) at $P < 0.05$.

female than male German cockroaches. Our results also showed less variation in female than male body length in the respective treatment groups with and without symbiotic bacteria. However, after cockroach nymphs were fed *Lilium* essential oil, the two cockroach groups showed significant differences in both body mass and body length for adult females rather than for adult males. *Lilium* essential oil did not show any significant antifeedant effect on the cockroaches at low concentrations.

Esterase often has the function of regulating development and detoxification (Kim et al. 2017, Teese et al. 2010), so decreasing the effect of esterases might be one potential mechanism to inhibit the growth of German cockroaches. AChE and other esterases are related to insecticide resistance (Alout et al. 2012, Barrios et al. 2010), and the insecticidal activity of essential oils is correlated with the ability to inhibit German cockroach AChE (Yeom et al. 2015). Our results support that AChE and nonspecific esterases may be targets of *Lilium* and *Croton* essential oils. However, the bioactive composition of the each essential oil was not determined here, and further studies including effect of the essential oil or their combination with other management tactics are needed.

Symbiotic gut microbes can regulate many aspects of insect biology (Douglas 2015), and cockroaches are also nutritionally and immunologically dependent on them (Wadakatsumata et al. 2015). Removing gut symbiotic bacteria in our study produced a synergism with essential oils to inhibit the growth and esterase activity of the German cockroach in the lab, especially with the essential oil of *Lilium*. Removing symbiotic bacteria might contribute to German cockroach control strategies, but the methods of safe and efficient removal need to be studied. Plant-based insecticides can be useful for reducing the usage of conventional insecticides. Thus, the combination of removing gut symbiotic bacteria and using plant-based materials, especially in baiting approaches, may be a viable management approach for potential development for the German cockroach.

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