Innate Immune Response of *Galleria mellonella* (Lepidoptera: Pyralidae) Larvae to Lemongrass Essential Oil and Citral[1]

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Abstract The innate immune response in insects relies on cellular responses. In most lepidopteran species, challenging the immune system causes an increase in hemocyte numbers and promotes encapsulation and melanization of invading objects, such as parasitoid eggs or artificial objects (e.g., nylon implants). We studied the effects of carbon particles (Sumi ink) on the immune response of *Galleria mellonella* L. (Lepidoptera: Pyralidae) last-instar larvae. Ink injections increased total hemocyte counts in a dose-dependent manner, with the 40 and 80% concentrations being most effective in eliciting hemocyte response. Hemocyte numbers increased significantly at 4 and 6 h after ink injection. Lemongrass essential oil (0.18, 0.36, and 0.72 μg/larva) and citral (0.12, 0.24, and 0.48 μg/larva) reversed the mobilization of hemocytes by the ink. Melanization of nylon larvae implants started as early as 15 min after implant insertion and reached maximum observed levels within 1 h. The degree of melanization also was higher in larvae challenged with ink 1 h prior to implant removal, which suggests initialization of the immune response. Citral injections inhibited implant melanization both in unchallenged and ink-challenged larvae. In the former case, citral concentrations needed for effective inhibition were less (0.3 μg/larva) than in ink-challenged larvae (1.2 and 2.4 μg/larva).

Key Words hemocyte counts, encapsulation, melanization, ink, nylon implants

The innate immune response in insects is via cellular and humoral modes. Cellular immunity is mediated by hemocytes, and most lepidopteran larvae possess five types of hemocytes: granulocytes, plasmatocytes, oenocytoids, spherulocytes, and prohemocytes. Granulocytes and plasmatocytes can engulf and eliminate pathogens in the process of phagocytosis. In addition to phagocytosis, hemocytes can enclose foreign organisms in multicellular structures called nodules or bind to large foreign bodies (e.g., eggs of parasitic wasps) forming capsules (Lavine and Strand 2002). This encapsulation prevents pathogen growth and kills it by suffocation or free radicals formed during the melanization process of the capsule (Wojda 2017).

Hemocyte numbers in insect hemolymph can rapidly change in response to infection (Lackie 1988; Ratcliffe et al. 1985). The character of this change depends

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on insect species and the intruder. For example, *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae react to infection with the bacterium *Bacillus cereus* Frankland and Frankland by lowering the total numbers of circulating hemocytes (Ratcliffe and Walters 1983). Grizanova et al. (2014) reported the same for *Bacillus thuringiensis* Berliner. Infection of *Trichoplusia ni* larvae with *Nosema* sp. also resulted in a reduction of the total circulating hemocyte numbers (Laigo and Paschke 1966). A similar response was observed in larval *G. mellonella* and *Heliothis zea* (Boddie) infected with a nucleopolyhedrosis virus (Shapiro 1968a; Shapiro et al. 1969). Infection with *Pseudomonas aeruginosa* (Schroeter) Migula lowers total hemocyte counts in *Apis mellifera macedonica* L. (Papadopoulou-Karabela et al. 1993), and inoculation with *Candida albicans* (Robin) Berkhout does the same in *G. mellonella* larvae (Rajendran et al. 2015).

On the other hand, bacterial infection of *Manduca sexta* Johannsen larvae caused rapid elevation of total circulating hemocyte counts (Horohov and Dunn 1982). Increased total hemocytes counts also were observed in *Anopheles gambiae* Giles challenged with *Escherichia coli* (Migula) Lehmann and Neumann (King and Hilyer 2013) and in *Bombyx mori* L. inoculated with *Beauveria bassiana* (Balsamo-Crivell) Vuillemin (Mallikarjuna et al. 2002).

Many abiotic agents (among them ink and nylon thread or monofilament) may act as immunomodulators in insects (Shaurub 2013). Effects of ink on insect hemocytes were studied in *G. mellonella* by Cameron as early as 1934, although literature on this topic is scarce. Most authors concentrated on the phagocytosis of ink particles in *B. mori* (Akai and Sato 1973), *G. mellonella* (Cameron 1934; Yokoo et al. 1995), *Hyalophora cecropia* L. (Lea and Gilbert 1966), or *Drosophila* sp. (Lanot et al. 2001). However, changes in hemocyte counts after ink injections were studied as well. Wigglesworth (1956) reported an "enormous increase" in the numbers of *Rhodnius prolixus* Stål hemocytes in response to injected India ink. India ink also increased granulocyte counts in *Dermatobia hominis* L. (Faraldo and Lello 2003). Implants made of nylon thread were used to demonstrate encapsulation and melanization in *B. mori* larvae (Akai and Sato 1973). The encapsulation response is a general measure of immune function requiring the coordination of many cellular and humoral immune effector systems into a single, easily measured response (Dubovskiy et al. 2016). Failure to encapsulate equates to failure of the immune system (Webb and Luckhart 1996). Historically, the presence of hemocyte layers covering the implants, visualized by transmission electron microscopy, was evidence of encapsulation (Akai and Sato 1973). Later, video imaging systems measuring grey values of light passing through the implants were introduced (Köning and Schmid-Hempel 1995). More recently, digital videography analyzed with computer-aided image analysis was used for measuring grey values (Rantala et al. 2002), and this technique is used presently in works ranging from evaluating encapsulation rates in insects upon bacterial infection (Dubovskii et al. 2010) to modulation of immune response to plant volatiles (Ghosh and Venkatesan 2019).

It is recognized that insects lack the acquired immune response characteristic of vertebrates. They possess only innate immune systems, providing responses to foreign invaders that are only temporary. However, there are reports showing that insects exposed to infection with low doses of microorganisms became more resistant to a future infection. This phenomenon is called immune priming or trained

immunity (Chambers and Schneider 2012). Priming may be observed upon second exposure to the same (homologous priming) or a different (heterologous priming) pathogen (Cooper and Eleftherianos 2017; Wojda and Vertyporokh 2017). In *G. mellonella*, priming was achieved after injection of yeasts, glucan, laminarin, lipopolysaccharides, or heat-killed bacteria (Bergin et al. 2006; Mowlds et al. 2010; Vertyporokh et al. 2019; Wu et al. 2015). We are not aware of any works on priming insect immune systems with ink.

During the last decade, progress has been made in studies on the effects of plant-derived products on insect hemocytes. An extensive review by Ghoneim (2018) lists 29 contributions, 15 devoted to the effects of products from *Azadirachta indica* A. Jus and 14 reporting the effects of various plant extracts or essential oils. Total hemocyte counts may increase or decrease in response to plant-derived chemicals. However, a decrease in the numbers of hemocytes was reported more often (e.g., 21 reports). For instance, a lowering of the total hemocyte counts were reported in *Helicoverpa armigera* (Hübner) treated with essential oils from *Artemisia annua* L. and *Artemisia conyzoides* (L.) (Padmaja and Rao 2000). Essential oil from *Acorus calamus* L. lowered total hemocyte counts in *Spodoptera litura* (F.) larvae (Sharma et al. 2008). The same was observed in *Ephestia kuehniella* Zeller larvae treated with essential oil from *Ferula gummosa* Boissier (Ghasemi et al. 2014). We are not aware of any reports on the effects of lemongrass essential oil or extracts on insect immune systems.

We found several reports on only the effects of plant-derived secondary metabolites on encapsulation/melanization of nylon implants. Abscisic acid enhances immune response against nylon implants in *A. mellifera* (Negri et al. 2015). Larvae of *Lymantria dispar* L. react to elevated levels of volatiles such as geraniol, linalool, eugenol, or hexadecanol by a slight increase in total hemocyte counts and encapsulation of nylon implants (Martemyanov et al. 2012). (E)-β-ocimene markedly increased encapsulation of latex beads in *S. litura* larvae (Ghosh and Venkatesan 2019).

Given the extent of recent research on botanicals (Isman 2006, 2020), reports on impairing insect immune systems with plant-derived substances remain scarce. We undertook the study reported herein to determine the effects of carbon particles (Sumi ink) on the immune response of *G. mellonella* last-instar larvae and the effects of lemongrass essential oil and citral on the mobilization of hemocytes by the injected ink.

Materials and Methods

Insects. *Galleria mellonella* larvae were obtained from Knutson's Live Bait (Brooklyn, MI) and maintained in glass jars with ventilated lids on an artificial diet consisting of 400 ml of Gerber multigrain cereal (Fremont, MI), 37 ml of glycerin, 25 ml distilled water, and 25 ml of cane sugar. The Gerber multigrain cereal provided vitamins (A, B1, B2, B3, B6, B12, C, and E) and minerals (calcium, iron, phosphorus, and zinc). The food was stored at 4°C for up to 1 mo. Larvae were maintained in a dark incubator (Model 2005, VWR Scientific Products, Radnor, PA) at 30°C and 80% relative humidity. For all experiments, larvae that reached day 3 of the last instar and weighing 125 ± 10 mg were used.

Chemicals. An anticoagulant buffer (98 mM NaOH, 18 6mM NaCl, 17 mM Na_2EDTA, 41 mM citric acid [pH 4.5]), and neutral red stain (2 mg bacto-neutral red in 1 ml anticoagulant buffer) were prepared prior to experimentation. NaCl and NaOH were purchased from Sigma-Aldrich, St. Louis, MO; Na_2EDTA and citric acid from Thermo Fisher Scientific, Pittsburgh, PA; and bacto-neutral red from BD, Franklin Lakes, NJ. Black Sumi ink was from Yasutomo, San Francisco, CA. Lemongrass essential oil was purchased from NOW Foods, Bloomingdale, IL. Citral was acquired from Santa Cruz Biotechnology, Dallas, TX. For experimentation, lemongrass essential oil and citral were dissolved to desired concentrations (v/v) in cholesterol-free soybean oil (Cal Western Packaging Corporation, Memphis, TN).

Challenging larvae with ink. Before each experiment, large ink particles were eliminated by centrifuging 1 ml of Sumi ink at 2,000 × *g* for 10 min. The supernatant was then collected and diluted in double-distilled water to the desired concentration(s). Additionally, the ink was vortexed for 5 s before injections. *Galleria mellonella* larvae were anesthetized by submersion in water for 10–15 min at room temperature. The larvae were then dried on paper towels and injected through a proleg with 5 μl of ink solution using a Hamilton microliter syringe, 26G/2"/30° (Reno, NV). Double-distilled water (5 μl/larva) was injected into the control insects. The optimal time of incubation was delineated in pilot experiments. Treated insects were kept in a dark incubator at 30°C and 80% relative humidity.

Dose- and time-dependent response of *G. mellonella* hemocytes to Sumi ink. For studying dose-dependent effects, Sumi ink was diluted (v/v) with double-distilled water to obtain 10, 20, 40, and 80% concentrations. *Galleria mellonella* larvae were challenged with ink and maintained at 30°C and 80% relative humidity for 4 h. Next, to extract the hemolymph, a proleg was cut off with micro iris scissors, the larva was bled onto a piece of parafilm, and 5 μl of hemolymph was quickly transferred into a microcentrifuge tube containing 40 μl anticoagulant buffer and 4 μl neutral red stain. The hemolymph solution was vortexed and incubated for 10 min at room temperature to stain the hemocytes.

When staining was complete, the sample was vortexed, 10 μl of hemocyte suspension was pipetted to the upper and lower chamber of an improved Neubauer hemocytometer and covered with a 22 × 22 mm Corning cover glass. The samples were inspected using a Galen III optical microscope (Cambridge Instruments, Cambridge, Madison, CT). Hemocytes were counted boustrophedonically using the outside four corner quadrants. This procedure was used for 13–21 larvae per concentration of ink. Results from this experiment indicated that Sumi ink produces the greatest hemocyte counts in *G. mellonella* larvae at a concentration of 40%.

In subsequent experiments, the time-dependent effects of 40% Sumi ink on *G. mellonella* hemocyte counts were examined for up to 6 h after injection. Hemolymph was extracted for hemocyte counting at 30, 120, 180, 240, and 360 min postinjection. Hemocytes were subsequently counted using an improved Neubauer hemocytometer for each time interval. This procedure was applied to 15–21 larvae per each time interval. Results from this experiment indicated that Sumi ink produces the greatest hemocyte counts in *G. mellonella* larvae 4 h after injection.

Chromatographic fingerprinting of lemongrass essential oil. Chromatographic fingerprinting of lemongrass essential oil was done using High Performance Thin Layer Chromatography (HPTLC). HPTLC plates (10 × 10 cm; Silica gel 60 F_{254}, purchased from EMD, Gibbstown, NJ) were washed in methanol and dried

under a fume hood. Lemongrass essential oil was spotted using a CAMAG Nanomat 4 dispenser (Camag Scientific Inc., Wilmington, NC) at 0.3 μg or 0.15 μg per lane. Citral standards were spotted at 0.075 μg or 0.0375 μg per lane. The plates were allowed to air dry for 5 min and developed in a CAMAG Horizontal Developing Chamber using a mobile phase consisting of toluene and ethyl acetate (93/7 v.v.) The plates were then air-dried for 5 min, derivatized in vanillin reagent, and heated for 10 min on a CAMAG TLC Plate Heater III at 100°C. The plates were then scanned in white light using a Canon CanoScan 5200F flatbed scanner for documentation.

Effects of lemongrass essential oil and citral on total hemocyte count. Lemongrass essential oil diluted in cholesterol-free soybean oil was applied at doses of 0.09, 0.18, 0.36, and 0.72 μg/larva. For citral diluted in soybean oil, the doses were equal to 0.06, 0.12, 0.24, and 0.48 μg/larva. The larvae were anesthetized by submersion in water for 10–15 min at room temperature, dried on paper towels, and injected through a proleg using a Hamilton microliter syringe, 26 G/2"/30°, with 5 μl of ink solution in double-distilled water (5 μl/larva), followed by second injection (5 μl) of lemongrass essential oil or citral solution. Cholesterol-free soybean oil (5 μl) was injected into the control insects. Treated insects were maintained in a dark incubator at 30°C and 80% relative humidity. Hemocytes were counted after 4 h of incubation. These procedures were applied to 15–33 larvae per concentration of lemongrass essential oil or citral.

Melanization response. To further investigate the effects of citral on *G. mellonella* immune response, additional experiments involving the melanization of nylon implants were conducted. Sections of nylon monofilament (Spiderwire, Spirit Lake, IA) 5 mm long and 0.2 mm in diameter served as implants. The surface of the nylon monofilament was scratched with P600 sandpaper. The implant was inserted into the hemocoel of each larva through a puncture in the cuticle.

First, to delineate the optimal time of implant incubation in the larval hemocoel, we studied time-dependent dynamics of implant melanization. To that end, the larvae were anesthetized by submersion in water for 10–15 min at room temperature, dried on paper towels, implanted with one implant, and returned to a dark incubator maintained at 30°C and 80% relative humidity. The implants were removed after 15, 30, 60, or 120 min, placed in 96% isopropanol, and stored at room temperature. This procedure was repeated using larvae injected with 5 μl of ink prior to implant insertion into the hemocoel. A total of 13–25 larvae were used for each time interval.

In the second series of experiments, dose-dependent effects of citral on implant melanization were studied. Here, citral was dissolved in cholesterol-free soybean oil to provide doses of 0.075, 0.15, 0.3, 0.6, 1.2, and 2.4 μg/larva, based on prior results. Water-anesthetized larvae were injected through a proleg and implanted. Each experimental larva was injected with 5 μl of cholesterol-free soybean oil containing a tested dose of citral and received one nylon implant. Control larvae received 5 μl of cholesterol-free soybean oil without citral and one nylon implant. Based on the results of the experiment with time-dependent melanization, the implants were removed after 60 min and stored in 96% isopropanol at room temperature. This procedure was repeated using larvae injected with 5 μl of ink prior to the citral injection and implant insertion into the hemocoel. In either case, we used 12–23 larvae per treatment.

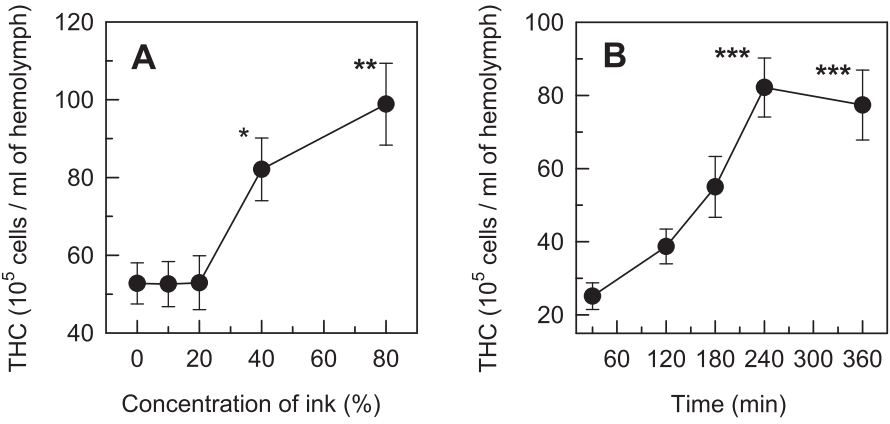

Fig. 1. Effects of Sumi ink on total hemocyte count (THC) in *G. melonella* last-instar larvae. (A) Dose-dependent response. (B) Time-dependent response. N = 13–21 larvae per data point. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in analysis of variance followed by a Tukey comparison of means.

After removal from the hemocoel, implants were photographed under a stereomicroscope, and the resulting digital images were evaluated using ImageJ (National Institutes of Health, Bethesda, MD). The degree of the melanization was quantified by measuring the coloration (gray value) of all areas of each implant and comparing obtained values with those of an intact implant (Dubovskiy et al. 2013; Rantala and Roff 2006).

Statistics. All data sets were tested with GraphPad Software Inc., San Diego, CA. Sample means ± standard error measure (SEM) were compared among control and experimental groups with analysis of variance, followed by a Tukey comparison of means, and considered significantly different at $P < 0.05$. Additionally, the means ± SEM for time-dependent melanization of nylon implants were compared with a Student *t*-test, pairwise, in unchallenged and ink-challenged last instar larvae, for the same times of incubation. Here, the means were also considered significantly different at $P < 0.05$.

Results

Dose- and time-dependent response of *G. mellonella* hemocytes to Sumi ink. Sumi ink injections increased total hemocyte counts from approximately 50×10^5 cells per milliliter of hemolymph to approximately 33×10^5 cells per milliliter of hemolymph in a dose-dependent manner (Fig. 1A). At 40 and 80% ink concentrations, the increase of hemocyte numbers was statistically significant ($F = 7.3$; df = 8.6; $P < 0.05$). Also, *G. mellonella* larvae responded to Sumi ink injections in a time-dependent manner (Fig. 1B). Hemocyte numbers increased significantly at 4 h and 6 h after ink injection ($F = 11.19$; df = 88; $P < 0.05$).

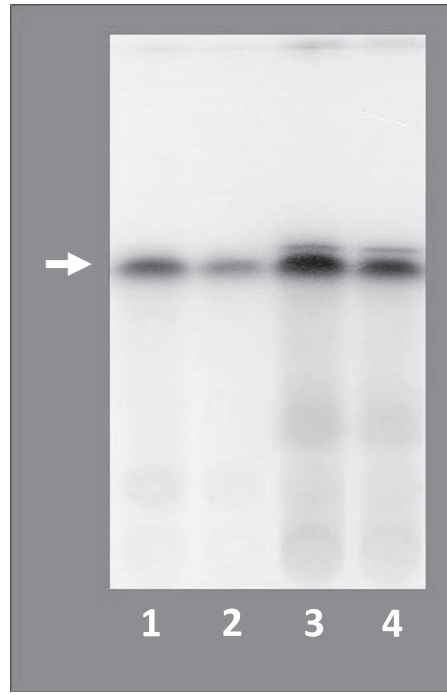

Fig. 2. High Performance of Thin Layer Chromatogram of citral (lanes 1 and 2) and lemongrass essential oil (lanes 3 and 4). Lane 1 = 0.075 μg citral; lane 2 = 0.0375 μg citral; lane 3 = 0.3μg lemongrass oil;lane 4 = 0.15 μg lemongrass oil. Citral standards collocate with the main ingredient of lemongrass essential oil (arrow).

Effects of lemongrass essential oil and citral on total hemocyte counts. Citral is known to be a constituent of lemongrass essential oils (Wagner and Bladt 1996). Qualitative HTPLC analysis indicated that citral was present in the lemongrass oil used in our study. Derivatized citral produced a band that collocated at Rf 0.55 with a massive band in the lanes containing derivatized lemongrass essential oil (Fig. 2).

Lemongrass essential oil reversed the mobilization of hemocytes by Sumi ink. Sumi ink injections combined with cholesterol-free soybean oil resulted in hemocyte counts of 80×10^5 cells per milliliter of hemolymph. Lemongrass essential oil injected to ink-challenged larvae at doses of 0.18, 0.36, and 0.72 μg per larva effectively lowered hemocytes counts (Fig. 3, $F = 5.163$; df = 142; $P < 0.05$).

Citral reversed the mobilization of hemocytes by Sumi ink in a manner similar to lemongrass essential oil. In the control larvae injected with cholesterol-free soybean oil, hemocyte counts reached approximately 72×10^5 cells per milliliter of hemolymph. Citral injected into ink-challenged larvae at doses of 0.12, 0.24, and 0.48 μg/larva effectively lowered hemocyte counts (Fig. 3, $F = 6.669$; df = 73; $P < 0.05$). Our results show that citral is more effective in reversing the hemocyte

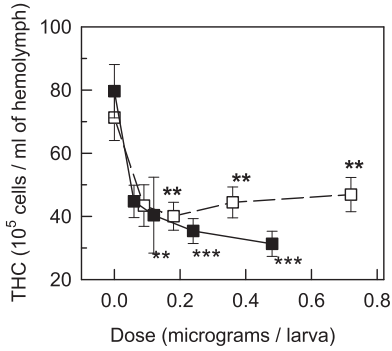

Fig. 3. Dose-dependent changes in total hemocyte count (THC) in *G. mellonella* last-instar larvae after injections of lemongrass essential oil (open squares) or citral (solid squares). N = 15–33 larvae per data point. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in analysis of variance followed by a Tukey comparison of means.

mobilizing effects of Sumi ink (reduction of hemocyte numbers occurs at lower concentrations and to a higher degree).

Melanization response. The melanization of nylon implants is a rapid event in *G. mellonella* larvae. In unchallenged larvae, it starts as early as 15 min after implant insertion into the hemocoel. Thirty minutes after implantation, the degree of melanization was already statistically significant ($F = 10.079$; $P < 0.05$; Fig. 4). Similar, time-dependent responses were observed in the larvae challenged with Sumi ink prior to implantation ($F = 30.478$; $P < 0.05$; Fig. 4). Injections of ink also

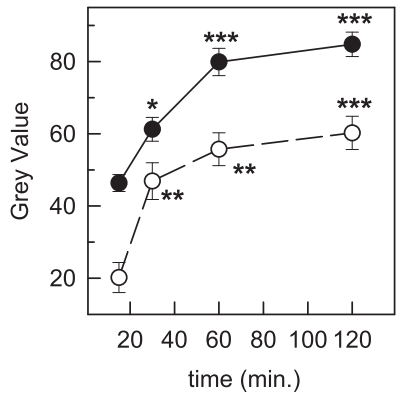

Fig. 4. Time-dependent melanization of nylon implants in unchallenged (open points) and ink-challenged (solid points) *G. mellonella* last-instar larvae. N = 13–25 larvae per data point. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in analysis of variance followed by a Tukey comparison of means. The means were additionally compared with a Student *t*-test, pairwise, for the same times of incubation ($P < 0.05$ for all pairs).

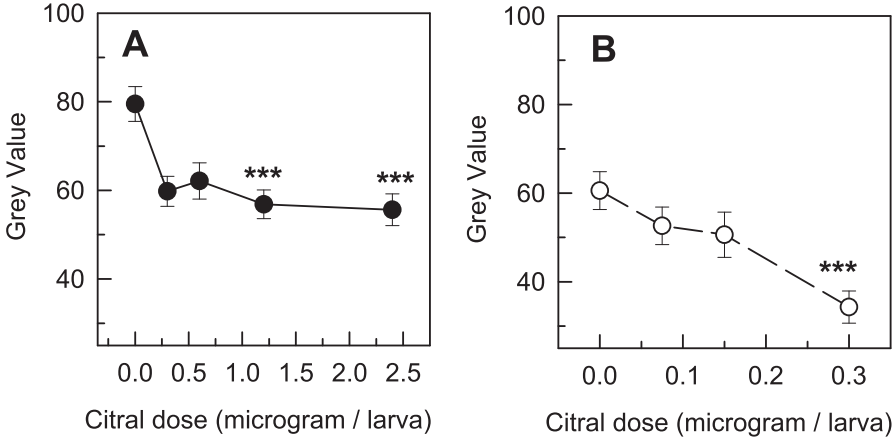

Fig. 5. Dose-dependent effects of citral injections on encapsulation of nylon implants in ink-challenged (panel A, solid points) and unchallenged (panel B, open points) *G. mellonella* last-instar larvae. N = 12–23 larvae per data point. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in analysis of variance followed by a Tukey comparison of means.

increased the degree of implant melanization—for all times after implantation, the grey values were significantly higher in ink-challenged larvae than in larvae that did not receive ink. (Student *t*-tests between means representing the same time of implant incubation in hemocoel resulted in $P < 0.05$, with $t = 5.939$, df = 36 for 15 min incubation; $t = 2.468$; df = 36 for 30 min; $t = 5.399$, df = 44 for 60 min; and $t = 4.368$; df = 42 for 120 min of incubation).

Citral injections followed with a nylon implant lowered the degree of implant melanization in a dose-dependent manner. In larvae injected with Sumi ink and citral concurrently, implant melanization was significantly reduced at 1.2 and 2.4 μg/larva ($F = 8.238$; df = 60; $P < 0.05$; Fig. 5A). When only citral was injected, the reduction of implant melanization occurred at a lower dose of 0.3 μg/larva and was more pronounced ($F = 5.991$; df = 57; $P < 0.05$; Fig. 5B).

Discussion

Our results show that dose- and time-dependent effects of ink on total hemocyte counts are in concordance with scarce literature data available on the subject. An "enormous increase" in the number of hemocytes after injection of Indian ink into the hemocoel of *R. prolixus* was reported by Wigglesworth (1956). Werner and Jones (1969) reported an increase in total hemocyte count in *G. melonella* last-instar larvae after injecting Chinese ink. Although they tested only two concentrations (less than in our study), the time-dependent dynamics of the immune response in their study was very similar to our results. Two, more-recent studies showed an increase in hemocyte counts following ink injection into larvae of *D. hominis* (Faraldo and Lello 2003).

Chromatographic fingerprinting (Fig. 2) indicated that citral was a main component of the lemongrass essential oil used in our study. This finding is consistent with the report of Wagner and Bladt (1996). Also, our results on the effects of lemongrass essential oil and citral on total hemocyte counts in *Galleria* larvae (Fig. 3) are consistent with scarce data on the effects of plant-derived chemicals on total hemocyte counts in this species as reported by other authors. In their studies, a decrease in the total hemocyte count in *G. melonella* larvae was reported after treatment with abscisic acid (Er and Keskin 2016), neem extract (Er et al. 2017), and two essential oils: *Citrus bergamia* Risso and *Boswellia carteri* Birdwood (Peterson 2019). In our study, lemongrass and citral lowered total hemocyte counts at concentrations much less (0.2 μg/ larva) than those reported by aforementioned authors for abscisic acid (100 μg / larva), neem (5 μg/larva), or *C. bergamia* and *B. carteri* oils (1,500 μg/larva in both cases).

The dynamics of encapsulation/melanization response in larval *Galleria* to nylon implants in our experiments (Fig. 4) is in concordance with the results of Grizanova et al. (2018). In both cases, the encapsulation process starts as early as 15 min after implantation, reaching the maximum values in 2–3 h after implantation. This finding indicates that the encapsulation response in *Galleria* is a much faster process than those reported for other species—as long as 24 h (Ghosh and Venkatesan 2019) or even 4 d (Calleri et al. 2007).

The immune response to nylon implants in our experiments with ink-challenged and unchallenged *Galleria* larvae (Fig. 4) raises questions as to the nature of increased encapsulation in larvae that received ink prior to the nylon implants. Because the numbers of circulating hemocytes do not rise significantly within 1 h after ink injection (Fig. 1B), it is unlikely that the increase in implant encapsulation exhibited by ink-injected larvae (Fig. 4) is a response relying solely on an increase of hemocyte numbers. We suggest that the increase of implant melanization in ink-challenged larvae is a priming mechanism induced by ink injection. Reports on priming insect immune response to nylon implants are very scarce. We found only two reports showing a priming response to the implants in insects. In both cases, homologous priming was observed. Priming by nylon implant insertion into the pupae of *G. mellonella* resulted in a higher intensity of nylon implant encapsulation by adult males who had been reared on a poor-quality diet in their larval stage (Kangassalo et al. 2018). In *Tenebrio molitor* L. adults, priming by insertion of nylon implants and removal of the implant increased encapsulation rates to secondary nylon implants inserted after removal of the first one (Daukšte et al. 2012). Heterologous priming of the insect immune response to nylon implants by ink is a novel finding.

In our experiments, citral inhibited the encapsulation of nylon implants both in ink-challenged and unchallenged larvae (Fig. 5). These findings are difficult to discuss because publications on the effects of botanicals or plant secondary metabolites on encapsulation/melanization of artificial implants are very scarce and report an increase in encapsulation in response to tested chemicals (Ghosh and Venkatesan 2019; Martemyanov et al. 2012; Negri et al. 2015). The only report of a decrease in encapsulation of nylon implants in response to a plant-derived substance (neem essential oil) of which we are aware came from our laboratory (Haszcz 2016). Our present results are in concordance with that report. Citral also inhibited an encapsulation/melanization response in unchallenged larvae at

concentrations lower that those effective in ink-challenged larvae. This finding corresponds well with our data on the putative priming effects of ink.

Mechanisms of *G. mellonella* response to ink, lemongrass oil, and citral remain unexplained. However, even at current stage of investigation, we can suggest certain putative processes involved. First, we have shown that ink injections increase total hemocyte count (THC) counts. Because the main hemocytes involved in encapsulation in Lepidoptera are plasmatocytes and granulocytes (Strand 2008), and these two classes of hemocytes dominate in insect THC counts (Shapiro 1979), it is likely that an increase in hemocyte numbers simply translates into elevated levels of encapsulation and melanization. On the other hand, it is likely that a decrease in THC levels, caused by lemongrass and citral, leads to lowered implant melanization.

It is difficult to suggest what may be the mechanisms of lemongrass and citral inhibitory effects on THC counts and melanization. However, some speculations could be made based on earlier works on lemongrass oil and citral effects on mammalian cells. For example, citral inhibits murine cell proliferation by interacting with intracellular oxygen radicals and oxidative stress (Kapur et al. 2016; Sanches et al. 2017). In *G. melonella,* plasmatocytes and granulocytes proliferate in response to stress such as wounding, and this process contributes to an increase of hemocyte counts (Shapiro 1968b). Preliminary experiments made in our laboratory suggest that ink injections can stimulate cell proliferation in some classes of hemocytes of *G. mellonella* (data not shown). If our preliminary findings are correct, citral could inhibit ink-induced hemocyte proliferation and lower THC counts.

Noteworthy, Bachiega and Sforcin (2011) reported that both lemongrass extract and citral lower cytokines production by mice macrophages, which results in lowering of murine immune response. In insects, adhesion of hemocytes to the invader during encapsulation is mediated by cytokines (Dubovskiy et al. 2016; Pech and Strand 2000). Perhaps citral inhibits melanization process directly, in addition to lowering THC counts. Further investigations are needed to delineate how lemongrass essential oil and citral impair insect immune response.

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