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TOXIC EFFECTS OF LEMON PEEL CONSTITUENTS ON *Ceratitis capitata*

A. SALVATORE,[1] S. BORKOSKY,[2] E. WILLINK,[1] and A. BARDÓN[2,*]

[1]*Estación Experimental Agroindustrial Obispo Colombres*
Av. William Cross 3150, Las Talitas
Tucumán 4101, Argentina

[2]*Instituto de Química Orgánica, Facultad de Bioquímica*
Química y Farmacia, Universidad Nacional de Tucumán
Ayacucho 471, Tucumán 4000, Argentina

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Abstract—A series of experiments were conducted to evaluate the toxicity of lemon peel extracts incorporated into mediterranean fruit fly *Ceratitis capitata* diet. Extracts were obtained with different solvents: diethyl ether, ethyl acetate, and methanol. All three extracts were toxic to some extent; the diethyl ether extract was selected for further studies. Ether extracts of lemon peel were prepared weekly over a 2-month period, from fruits collected on the 1st d of the bioassay. Weekly GC-MS and UV analyses of the extracts demonstrated that the concentration of citral and coumarins decreased in the peel after harvest. We conducted a series of bioassays to evaluate the toxicity of the ether extract, and mixtures of this extract with citral, 5,7-dimethoxycoumarin, and linalool incorporated to *C. capitata* larvae's natural diet (lemon slices endocarp) at a concentration of 250 μg/g of diet. Significant larvicidal activity can be obtained from a fresh lemon peel extract; however, when the extract was obtained from stored lemons, toxicity decreased. Addition of small amounts of citral or 5,7-dimethoxycoumarin, and linalool to the stored lemon peel extract would bring back the toxicity to the rates of fresh lemons extracts. Finally, female adults of *C. capitata* fed on diets containing additional amounts of ether extract, 5,7-dimethoxycoumarin, and linalool, were exposed to different photoperiods to test for phototoxicity. The treatment was toxic and affected the oviposition capacity of females depending on photoperiod.

Key Words—*Ceratitis capitata*, lemon peel volatiles, citral, 5,7-dimethoxycoumarin, linalool, toxicity, oviposition inhibition.

* To whom correspondence should be addressed. E-mail: alisan@fbqf.unt.edu.ar

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata*, attacks a wide variety of hosts in subtropical and temperate regions of Argentina, causing serious economic damage and preventing fruit export. Back and Pemberton (1918) and Quayle (1914, 1929) reported that lemons were not attacked by *C. capitata* until the fruit was overripe or partially decayed. These authors attributed the resistance of lemons, among other reasons, to the peel oil. Greany et al. (1983) found that lemon peel oil was toxic to the fruit fly *Anastrepha suspensa* and contributed to making lemons immune from attack. Oxygenated monoterpene aldehydes, like citral, are reported to be responsible for the chemical resistance of lemons to attack by *C. capitata* (da Silva Branco et al., 2000). Additionally, Spitler et al. (1984) considered as extremely low the probability of an infestation of lemons with *C. capitata* in a commercial shipment. Although these reports suggest that lemon is a resistant fruit, systematic research has not been performed to determine which lemon peel compounds are responsible for resistance, or to evaluate the chemical changes in the fruit following harvest (resistance declines significantly after harvest). Identification of toxic lemon peel constituents could be the first step in the investigation of a natural insecticide based on lemon volatiles.

Continuing with our search for natural insecticides (Bardón et al., 1999), we conducted a series of experiments to evaluate the toxicity of lemon peel extracts with different solvents that were incorporated into the insect diet. The ether extract was chosen as an effective insecticide model and its toxicity was evaluated in detail. Finally, on the basis of previous studies (Ashwood-Smith et al., 1983; Nigg et al., 1993) that pointed out the phototoxicity of coumarins to insects, we conducted a second set of experiments to evaluate the effects of different photoperiods on *C. capitata* female adults.

METHODS AND MATERIALS

Chemicals. 5,7-Dimethoxycoumarin was purchased from Aldrich Chemical Company, linalool and citral from Dragoco Chemical Company, and autolyzed brewers yeast from ICN Biomedicals, Inc. All chemicals were used without further purification.

Insects. A colony of several hundred Mediterranean fruit fly adults was initiated with pupae obtained from infested oranges collected from different sites in the Northwest of Argentina. *C. capitata* was reared at Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina, according to the method of Tanaka et al. (1970). Diet consisted of a 3:1 mixture of sugar and yeast hydrolysate suspended in water. Adults were maintained in the laboratory with a photoperiod 12L:12D at 24 $\pm$ 2°C and an RH of 60 $\pm$ 10%. Eggs were

collected during a 1-hr period when females were 7–10-d old (Wong and Nakahara, 1978).

Extraction. Eighty ripe lemons were collected in the middle of the autumn and treated with a 0.3% fungicide (Imazalil) solution by hand spray. Lemons were maintained at 25 ± 2°C and an RH of 60–70% over 2 months. Once a week, five of the collected lemons were selected randomly, and their peels removed from the fruits. Ground peel (200 g) was further extracted with diethyl ether using an ultrasonic bath at 20°C for 20 min. After filtering, the solvent was evaporated to dryness at reduced pressure. The residue was analyzed in triplicate by GC-MS and then bioassayed.

Identification and Quantification of Ether Extract Constituents by GC-MS. Mass spectrometry was carried out by electron impact at 70 eV and 220°C. An HP 6890 Series II chromatograph linked to an HP 5972 mass selective detector with a 30 m × 0.25 mm i.d. HP-5MS 5% phenyl methyl siloxane column was employed. Temperature program: from 50 to 100°C at a rate of 1.5°C/min, from 100 to 160°C at a rate of 3°C/min, and finally from 160 to 280°C at a rate of 10°C/min.

UV Spectroscopy. Coumarins were present in small amounts in citrus peel and detected by UV spectroscopy. Coumarins display strong absorptions in the UV region (λ_{max}-225, 250, 325 nm) and exhibit fluorescence in the visible region of the electromagnetic spectrum. We measured the UV absorption of ethanolic solutions of ether extracts (0.5 mg/ml) weekly at 327 nm with a Shimadzu UV-VIS 160 A spectrophotometer.

Larval Toxicity Bioassay.

Step 1: Portions of 200 g of ground lemon peel were extracted with different solvents (diethyl ether, ethyl acetate, and methanol). After solvent evaporation, an aliquot of each extract (250 μg/g of diet) was added to *C. capitata* diet made of 4 cm diam pulp fruit slices of fresh lemons. They were placed into clear glass Petri dishes (9 cm diam × 1.5 cm tall). Immediately, 20 eggs were placed onto each treated slice and kept at 25 ± 2°C and 60–70% RH for 5 d. On the 5th d of the bioassay, the percentages of hatching and larval mortality were recorded. The experiment was conducted in 10 replicates.

Step 2: The diethyl ether extract containing the volatiles was chosen for further studies on toxicity in relation to chemical composition. Consequently, a second set of bioassays was conducted as follows: weekly, five different treatments (**T1**, **T2**,**T3**,**T4**, and **T5**) were incorporated into *C. capitata* larvae diet (4 cm diam pulp fruit slices of fresh lemons). One of them (**T1**) was the ether extract of lemon peel (obtained with the procedure described above). The extract was dissolved in acetone, and added to slices (15 replicates) at a concentration of 250 μg/g of diet. Slices were placed into clear glass Petri dishes (9 cm diam × 1.5 cm tall) and, after solvent removal, 20 eggs were placed onto each treated slice and kept at 25 ± 2°C and an RH of 60–70% for 5 d. On the 5th d of the bioassay, percentages of hatching and larval mortality were recorded. An identical procedure was followed

with the second treatment (**T2**) that contained 250 μg of ether extract with 1.25 μg of 5,7-dimethoxycoumarin per gram of diet. The third treatment (**T3**) consisted of 250 μg of diethyl ether extract, 1.25 μg of 5,7-dimethoxycoumarin, and 1.25 μg of linalool per gram of diet. The fourth treatment (**T4**) was made of 250 μg of ether extract mixed with 7.5 μg of citral per gram of diet. The fifth treatment (**T5**) consisted of 250 μg of ether extract, 2.5 μg of citral, and 1.25 μg of 5,7-dimethoxycoumarin per gram of diet. Lemon slices treated only with acetone were employed as control after solvent evaporation.

Bioassay of Adult Insects. Three groups of newly emerged *C. capitata* adults were selected from our colony. Each group, consisting of five normal male–female pairs, was placed for 35 d in a small cage to mate and lay eggs. The number of eggs and the mortality of adults were recorded weekly.

The cages were exposed to three different photoperiods; 12L:12D, 6L:18D, and 24 hr (shade), respectively. Adults subjected to treatment fed on a diet containing 100 μg of ether extract, 0.5 μg of 5,7-dimethoxycoumarin, and 0.5 μg of linalool (**T6**) per gram of dry food. Control couples fed on untreated diets. Each experiment had five replicates.

Statistical Analyses. The results are reported as mean $\pm$ SEM. Differences in the mean values were evaluated by analysis of variance (ANOVA). The Tukey test was used for all pairwise multiple comparisons of groups. In all statistical analysis, $P > 0.05$ was not considered significant.

RESULTS AND DISCUSSION

Lemon peel extracts obtained with different solvents contain compounds of different polarities. The methanol and ethyl acetate extracts contain, among others, nonvolatile aromatic compounds like psoralens, coumarins, and flavonoids (Berhow et al., 1998), while the diethyl ether extract contains mainly volatile monoterpenic hydrocarbons (Figure 1), alcohols, and aldehydes Limonene (**1**, CAS# 138-86-3) is the major monoterpene constituent (64–70%).

The diethyl ether extract obtained from fresh lemon peel was the most toxic to mediterranean fruit fly larvae. At a concentration of 250 μg/g of diet, this extract caused 98.8 $\pm$ 3.7% (mean $\pm$ SEM) larval mortality, as shown in Table 1. The diethyl extract was chosen as an insecticide model, and further studies on its chemical composition and bioactivity were performed once a week over a 2-month period with ether extracts obtained from fruits collected on the 1st d of the bioassay. GC-MS analyses indicated that monoterpene alcohols account for approx 1–1.3%, while aldehydes, mainly geranial (**2**, CAS#141-27-5) and neral (**3**, CAS#106-26-3), were 2.5–3% of the ether extract (Marty Klyver et al., 1992, 2000). The natural mixture of the two isomeric aldehydes geranial and neral is known as citral. Rapid

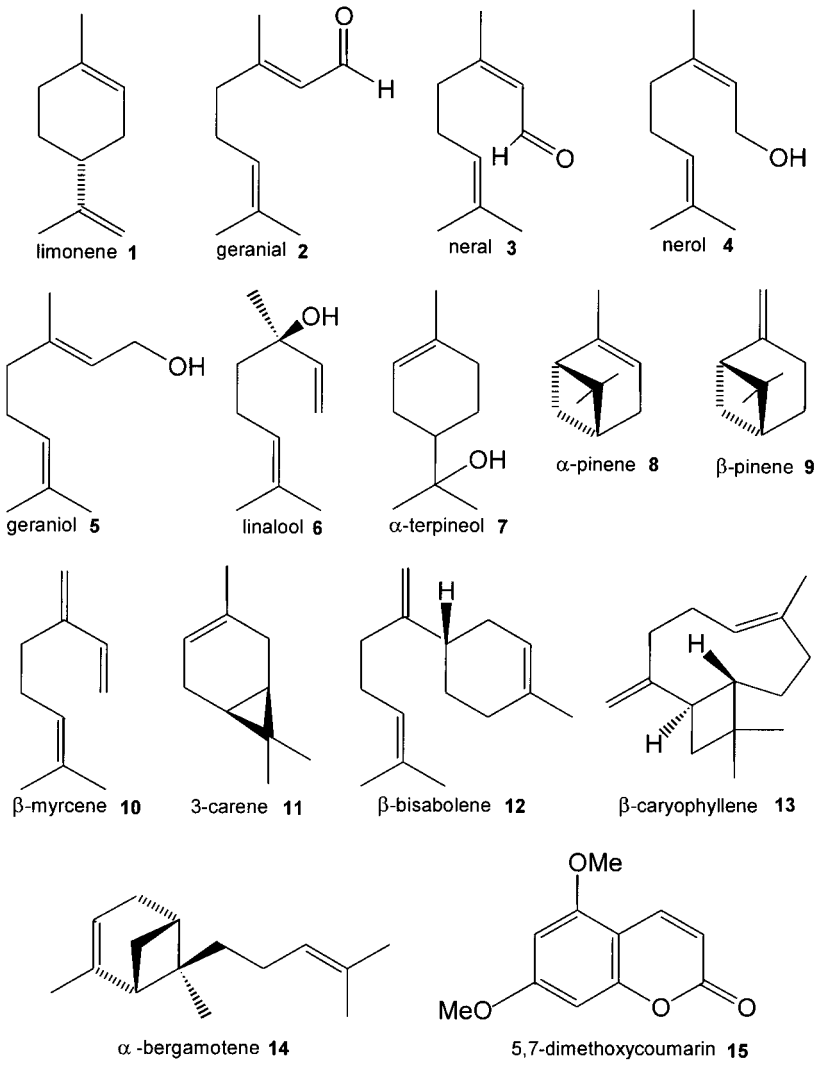

FIG. 1. Ether extract constituents of lemon peel identified by GC-MS.

decay in the concentration of citral (2.6–1.41%) was observed in peels during the 1st month after harvest (Figure 2). The concentration of monoterpenic alcohols (nerol, **4** CAS# 106-25-2; geraniol, **5** CAS# 106-24-1; linalool, **6** CAS# 78-70-6; and α-terpineol, **7** CAS# 98-55-5) was slightly reduced (1.09–0.92%) after

TABLE 1. TOXICITY OF LEMON PEEL EXTRACTS OBTAINED WITH DIFFERENT SOLVENTS AGAINST *C. capitata*

Extracts	% Hatch[a,b]	% Larval Mortality[a,c]
Ethyl acetate	85.5 ± 5.0d	90.9 ± 7.6f
Methanol	85.0 ± 4.1d	94.1 ± 5.5g
Diethyl ether	86.0 ± 3.9d	98.8 ± 3.7h
Control	93.0 ± 5.9e	2.6 ± 3.7i

Note. Means within a column followed by the same letter are not significantly different ($P > 0.05$, Tukey multiple range test).
[a] Numbers in columns represent mean ± SEM; $N = 10$.
[b] Percentage calculated on the basis of number of eggs placed on diets.
[c] Percentage calculated on the basis of number of hatched eggs.

2 months of storage. The concentration of hydrocarbon (limonene, 1 CAS# 138-86-3; α-pinene, **8** CAS# 80-56-8; β-pinene, **9** CAS# 127-91-3; myrcene, **10** CAS# 123-35-3; 3-carene, **11** CAS# 13466-78-9; β-bisabolene, **12** CAS# 495-61-4; β-caryophyllene, **13** CAS# 87-44-5; and α-bergamotene, **14** CAS# 73127-38-5) did not change during the 2 months. Decay in the concentration of total coumarins was detected at λ 327 nm. The concentration decreased 35% over 2 months. The content of 5,7-dimethoxycoumarin (**15** CAS# 487-06-9) was quantitatively determined by GC-MS and found to be reduced by a 59% over 2 months of postharvest storage.

Weekly evaluation of larval toxicity caused by the different treatments is described in Table 2. The diethyl ether extract (**T1**) was responsible for 97.9 ± 2.7% larval mortality compared with the control. Larval mortality decreased significantly to 20.5 ± 6.6% after 2 months ($F = 156.95$; $df = 7, 14$; $P < 0.001$).

Since the coumarin content decreased after harvest, a second treatment, **T2,** was added to the larval diet and was composed of the extract plus an additional amount of the natural lemon peel constituent 5,7-dimethoxycoumarin. The treatment also caused high mortality during 5 weeks, which dropped in the last 2 weeks of the bioassay. In fact, **T2** made with fresh lemon extract caused a 96.2 ± 4.1% decrease in larval mortality, while **T2** prepared with lemon extract obtained 7 weeks after harvest resulted in a 54.6 ± 9.1% decrease in the larval population ($F = 46.79$; $df = 7, 14$; $P < 0.001$).

The third treatment (**T3**) contained the extract, 5,7-dimethoxycoumarin, and the monoterpene alcohol linalool-a potential synergist with insect repellent properties (Assabgui et al., 1997). **T3** affected the larvae during the 2-month bioassay period (Table 2). Mortality increased to 97.2 ± 3.8% with a 2-month-old extract.

Treatments **T4** and **T5** were as toxic to larvae as **T3** during the 2 months of the bioassay. Mortality remained over 90.1% with no significant differences during the 2-month bioassay period.

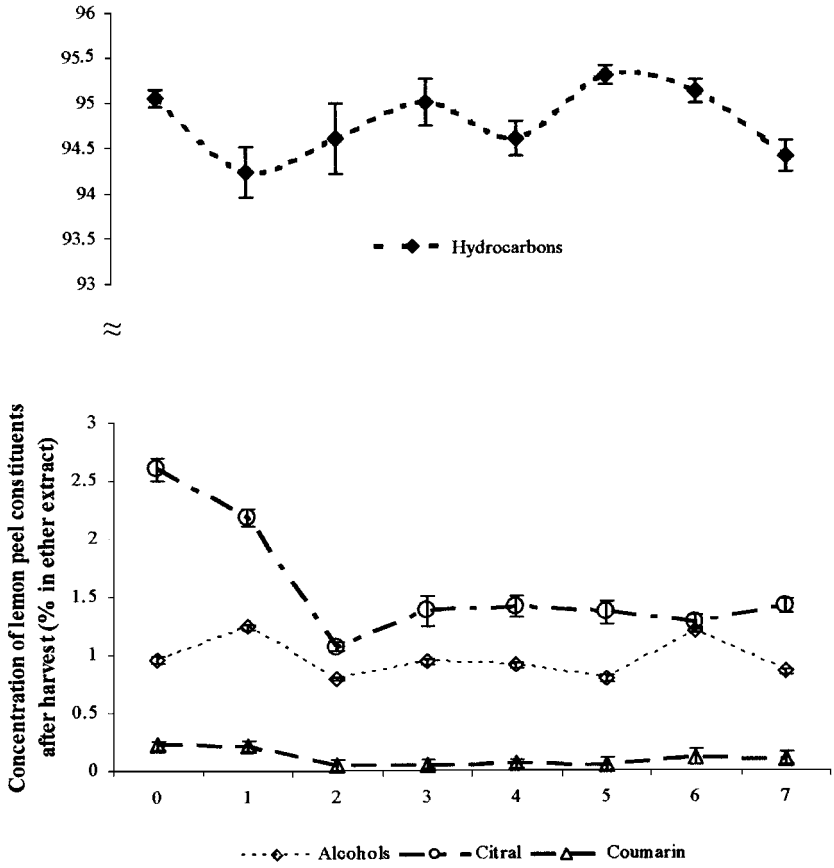

FIG. 2. Variation in the concentration of lemon peel constituents after harvest.

None of the treatments affected the eggs, with no significant differences in the percentage of hatching observed between controls and treatments.

Our results indicate that a potent larvicide can be obtained from fresh. diethyl ether extract of lemon peel. However, if the larvicide is obtained from stored lemons, only the addition of small amounts of citral (**T4**) or citral and 5,7-dimethoxycoumarin (**T5**) can bring the toxicity of the extract back to the levels of fresh lemons. As shown in Table 2, the association of the diethyl ether extract with small amounts of 5,7-dimethoxycoumarin and linalool (**T3**) has the larvicidal effects of the extract of fresh lemon, even when the extract is from stored lemons.

The coumarin 5,7-dimethoxycoumarin induces mutagenesis in bacteria (Ashwood-Smith et al., 1983) as well as being a phototoxic (Nigg et al., 1993). Because of the phototoxic nature of this coumarin, we evaluated the effects of our

TABLE 2. EFFECTS OF TREATMENTS **(T1, T2, T3, T4** AND **T5)** INCORPORATED IN LARVAL DIET OF *C. capitata*[a]

Weeks after harvest	Ether extract **(T1)** (% mortality)[b]	Ether extract + coumarin **(T2)** (% mortality)[b]	Ether extract + coumarin + linalool **(T3)** (% mortality)[b]	Ether extract + citral **(T4)** (% mortality)[b]	Ether extract + coumarin + citral **(T5)** (% mortality)[b]	Control (% mortality)[b]
0	97.9 ± 2.7cA	96.2 ± 4.1cA	98.9 ± 2.2cA	95.3 ± 4.4cA	98.2 ± 2.5cA	3.3 ± 3.9i
1	95.8 ± 5.3cA	93.6 ± 6.8cA	97.5 ± 3.4cA	93.5 ± 5.4cA	96.5 ± 2.6cA	2.4 ± 2.9i
2	79.9 ± 10.4dB	93.0 ± 4.5cA	98.2 ± 3.9cA	93.1 ± 3.8cA	98.2 ± 2.5cA	2.8 ± 3.3i
3	63.9 ± 10.0eD	88.5 ± 7.5cC	98.2 ± 2.6cC	92.3 ± 7.1cA	98.5 ± 2.4cA	2.3 ± 3.4i
4	66.7 ± 8.6eC	78.6 ± 9.0dB	98.9 ± 2.2cA	93.8 ± 5.6cA	97.5 ± 2.3cA	2.7 ± 3.1i
5	50.9 ± 12.4fC	77.8 ± 8.2dB	98.6 ± 3.1cA	91.4 ± 7.2cA	97.2 ± 3.2cA	3.1 ± 2.8i
6	36.2 ± 8.7gC	68.6 ± 8.7eB	98.5 ± 3.2cA	92.9 ± 4.8cA	96.1 ± 5.1cA	3.1 ± 3.6i
7	20.5 ± 6.6hC	54.6 ± 9.1fB	97.2 ± 3.8cA	89.9 ± 5.0cA	95.6 ± 4.3cA	2.7 ± 3.2i

[a] Numbers in columns represent mean ± SEM; $N = 15$.

[b] Means within a column or row followed by the same letter (lower and upper case, respectively) are not significantly different ($P > 0.05$, Tukey multiple range test).

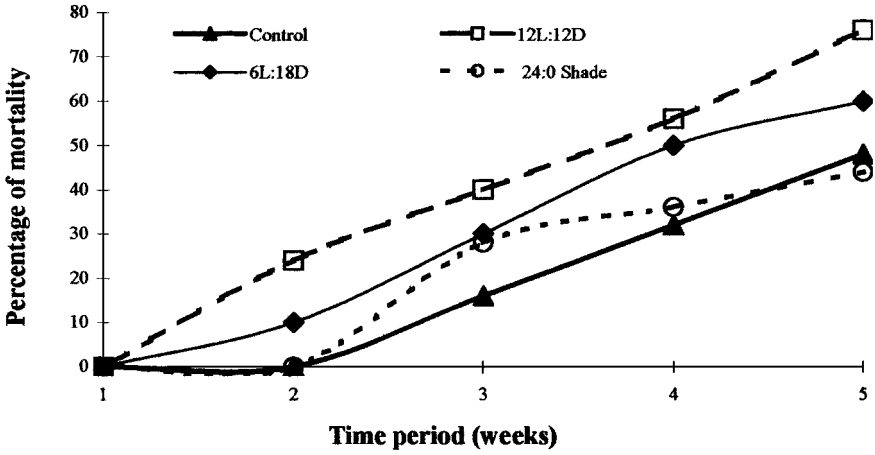

FIG. 3. Mortality of *C. capitata* adult females that fed on treated diets and were exposed to three photoperiods, 12L:12D, 6L:18D, and 24:0 (shade).

treatment (100 μg of ether extract + 0.5 μg 5,7-dimethoxycoumarin + 0.5 μg linalool per gram of diet) on adults of *C. capitata* under three different light regimes (Figure 3). Females exposed to a 12L:12D photoperiod were significantly affected by treatment ($F = 16.05; df = 3, 24; P < 0.001$). Mortality was not observed in the control during the first 2 weeks, while the mortality rose to 25% in the 12L:12D experiment. After 5 weeks, the number of dead females doubled that of the control. In addition, as shown in Figure 4, the oviposition

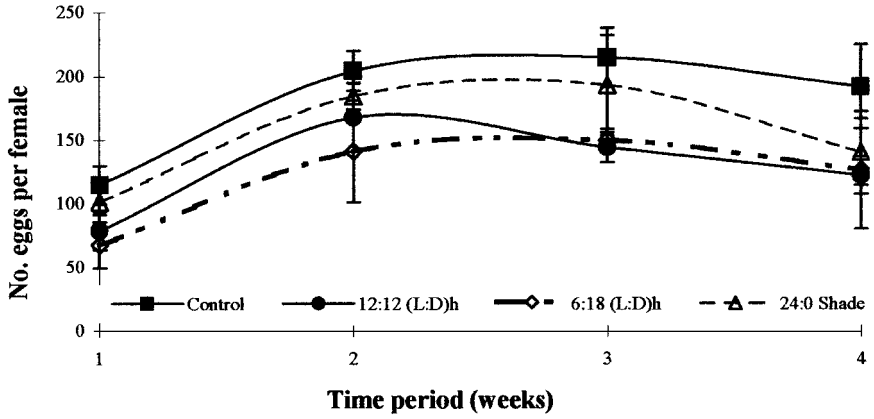

FIG. 4. Number of eggs laid by females that fed on treated diets and were exposed to three photoperiods 12L:12D, 6L:18D, and 24:0 (shade).

capacity of females in the 12L:12D photoperiod conditions was significantly altered ($F = 11.72$; $df = 3, 19$; $P < 0.001$). The number of eggs laid by the females exposed to a 12L:12D photoperiod was half the number of the control, indicating that a longer exposure to light affects oviposition capacity to a greater extent.

Our results indicate that a mixture of nonpolar constituents of lemon peel with additional amounts of citral, 5,7-dimethoxycoumarin, and linalool might be useful as a natural insecticide for treatment of larvae and adults at the concentrations tested. However, because no differences were observed in the alimentary behavior of the adults toward treated and control diets, the treatment might be added to bait with good results as a bait–pesticide mixture.

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