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Original article

Effects of coumaphos on locomotor activities of different honeybee (*Apis mellifera* L.) subspecies and ecotypes

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Abstract – The effects of acute sublethal doses of coumaphos, an acaricide used against *Varroa destructor* infestation in beekeeping, on the locomotor activities of four native honeybee subspecies of Türkiye including two ecotypes (Carniolan honeybee -*A. m. carnica*, Syrian honeybee -*A. m. syriaca*, Caucasian honeybee- *A. m. caucasica*, and Muğla and Yığılca ecotypes of Anatolian honeybee *A. m. anatoliaca*) were investigated using an individual locomotor activity monitoring system. Analysis of locomotor activity data in the first 12-h, last 12-h, and 24-h time periods showed that bees from *caucasica* and *carnica* subspecies were not affected by coumaphos at all three acute doses (1, 2, and 5 µg coumaphos in 10 µl sucrose syrup for each bee). In contrast, bees from *A. m. syriaca* subspecies showed significantly elevated locomotor activity levels at 2 and 5 µg coumaphos doses within the first 12 h. Bees from both Muğla and Yığılca ecotypes of *anatoliaca* subspecies also showed elevated locomotor activity levels at 5 µg coumaphos dose but the magnitude of increase was lower in these ecotypes compared to that seen in *syriaca* subspecies in the first 12-h period. In general, increasing doses of coumaphos resulted in increased locomotor activity (locomotor activity), with differences in sensitivity across honeybee populations. Possible mechanisms underlying this variance and suggestions for further studies are discussed.

Honey bee (*Apis mellifera*) / *Varroa destructor* / Coumaphos / Learning / Acaricides / Acetylcholinesterase (AChE)

1. INTRODUCTION

The mite *Varroa destructor* is one of the most harmful pests of *Apis mellifera* colonies. Varroa infestation and viruses vectored by this parasite are one of the main reasons for colony losses (Le Conte et al. 2010; Martin et al. 2012). Numerous types of pesticides with synthetic or natural ingredients are applied to beehives to control Varroa infestation (Bogdanov 2006). Coumaphos is a synthetic pesticide of the organophosphate group. It is sold by Bayer in two formulations, Check mite strips and Perizin (solution containing 3.2% coumaphos as active ingredient) and applied to honeybee colonies for *Varroa* control. Coumaphos is distributed through trophallaxis and contact among nestmates (Van Buren et al. 1992, 1993). Coumaphos has a systemic action as the *Varroa* mites that feed on bees that have ingested coumaphos die due to irreversible inhibition of their acetylcholinesterase enzyme (Johnson et al. 2010). Coumaphos has been used as an in-hive antiparasitic agent first against *Varroa* and later also against the small hive beetle *Aethina tumida*, due to its low toxicity to honeybees compared to target organisms (Johnson et al. 2010). However, sublethal doses of coumaphos are reported to exert negative effects on honey bee behavior. Williamson et al. (2013a) reported a moderate

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disruption of olfactory learning in honeybees treated with sublethal doses of coumaphos. Bevk et al. (2012) treated worker bees with Perizin (a commercial 3.2% solution of coumaphos produced by Bayer) and found that coumaphos significantly disrupted food transfer between bees by reducing trophallaxis behavior. Synthetic pesticides like coumaphos applied to beehives tend to leave residues in beeswax and propolis due to their lipid-soluble properties (Bogdanov 2006; Wallner 1999).

Beyond the effects of coumaphos on bee physiology and activity, studies have shown that the toxicities of some pesticides can be variable among different honeybee subspecies (Suchail et al. 2000; Laurino et al. 2013; Rinkevich et al. 2015), which may be due differences in the detoxification system (Zhu et al. 2020).

The genus *Apis* includes 10 species, and among these, the Western and Eastern honeybees, *A. mellifera* and *A. cerana*, are the ones domesticated by humans (Thakar 1973). These two species have been described as sister taxa (Arias and Sheppard 2005). *A. cerana* is distributed in south and southeast Asia, including China, and it is represented by 8 subspecies. *A. mellifera*, on the other hand, is distributed throughout the rest of the world thanks to human intervention and has 28 designated subspecies (Engel 1999).

Türkiye has a considerably rich diversity in terms of honeybees represented by 5 subspecies: *A. m. caucasica* in northeast Anatolia, *A. m. syriaca* in southeastern Anatolia near the Syrian border, *A. m.meda* in southeastern Anatolia, *A. m.anatoliaca* in western and central Anatolia and, an ecotype of *A. m. carnica* in Thrace region of northeast Anatolia (Ruttner 1988; Kandemir et al. 2000, 2005). This diversity is mostly believed to be shaped by Anatolia acting as a geographical refuge for many species during Glacial Maxima (Hewitt 1999). Kükrer et al. (2021) showed that Anatolia still conserves high genetic diversity and the presence of distinct subspecies in their native areas despite the genetic admixture and homogenization effects caused by migratory beekeeping, as well as unregulated queen and colony trade prevalent in Turkish beekeepers today. Importantly, honeybee subspecies of Türkiye have been reported to display differences in terms of Varroa infestation levels, hygienic and grooming behavior rates (Kence et al. 2013), foraging preferences (Çakmak et al. 2010), appetitive and reversal conditioning, and daily locomotor activity (Perez-Claudio et al. 2018; Erdem 2018).

Besides the presence of high genetic diversity among bees in Türkiye, there also exists large variation in beekeeping applications throughout the country. Outside of the limited regions which have been designated as conservation areas for *caucasica* and for *carnica* (within their native range), there exists a mixture of local stationary and country-wide migratory beekeeping activities. Queen bee and colony trade is also widespread (Kükrer et al. 2021). Because of these aspects, a diverse array of beekeeping methods and agents, especially in Varroa control, is present among Turkish beekeepers. For example, coumaphos is widely used by beekeepers in *caucasica conservation area* in the Artvin province, while flumethrin and amitraz-based acaricides imported from Bulgaria are frequently used by beekeepers in the Thrace region which hosts *carnica* conservation areas. Given this variable environment, including both Varroa levels and treatment intensity, whether different honeybee subspecies in Türkiye vary genetically in their responses to acaricides has remained unknown. We hypothesized that this diversity in honeybee genetic backgrounds and beekeeping practices in Türkiye may shape variations among honeybee subspecies of Türkiye regarding their susceptibility to sublethal effects of acaricides used against Varroa. Besides, known toxicity symptoms of AChE inhibiting compounds such as coumaphos include agitation, muscle weakness, involuntary convulsions, and paralysis (Colović et al. 2013), which directly affect movement and locomotor activity of an organism. Therefore, we measured the effects of different doses of acute coumaphos administration on locomotor activities of four native Turkish honeybee subspecies including two ecotypes using Trikinetics locomotor activity monitoring system to test our hypothesis.

2. MATERIAL AND METHODS

2.1. Honeybees

Our study includes the following subspecies native to Türkiye: *A. m. caucasica* (from Borçka, Artvin Province in Northeast Anatolia), *A. m. syriaca* (from Arsuz, Hatay province in South Anatolia), *A. m. carnica* (from Kırklareli province in Thrace Region), and Muğla (from Muğla province in southwestern Anatolia) and Yığılca (from Düzce province in southwestern Anatolia) ecotypes of *A. m. anatoliaca* (Fig. 1). The distinctiveness' of these subspecies was confirmed by RAPD (Tunca and Kence 2011) and microsatellite (Kükrer et al. 2021) methods in their source areas and by the inter-SSR method in a common garden (Arslan 2020). Two colonies for each subspecies and ecotypes were used in subsequent studies. All colonies were kept and maintained in a common garden apiary near the Biology Department at the Middle East Technical University in Ankara, Türkiye (39° 53′ 45.7″ N 32° 46′ 45.9″ E).

2.2. Coumaphos treatment

Acute coumaphos administration was performed based on Bevk et al. (2012) using the commercial formulation, Perizin (3.2% coumaphos as the active ingredient). Perizin is applied to the hives by trickling a 50-ml solution of 1/50 diluted Perizin over the bees using a syringe. However, this method results in an uneven distribution of the pesticide in the hive (Van Buren et al. 1993). Consequently, the bees in the hive may be exposed to variable acute doses of coumaphos (Bevk et al. 2012). In our experiments, we attempted to replicate this situation. For each dose group, a specific amount of Perizin was added to 1000 µl 50% sucrose solution (*w*/*v*) in a microtube and was vortexed until a white homogenous emulsion was formed. The amounts of Perizin were adjusted such that each microtube assigned to a dose group contained 1, 2, or 5 µg of coumaphos per 10 µl of sucrose solution. Worker bees were randomly collected from the brood combs located in the middle of the hives for sampling bees (OECD 1998; Suchail et al. 2000). Although bees were not collected based on chronological age, the collection location, near the brood, likely resulted in a sample of bees doing in-hive tasks such as nursing. These bees were chosen since they were the most likely to be exposed to chemicals applied directly to a hive. After sampling, worker bees were brought to the laboratory and were starved for 2 h in a free

Figure 1. Locations of the common garden apiary and source colonies in a map of Türkiye.

flying cage of 25 × 25 × 25 cm with a cloth tunnel for hand access. Then, each bee was removed from the cage by holding its wings by thumb and index fingers, and a 10-µl droplet of sucrose solution only (control group), or sucrose solution containing 1, 2, or 5 µg of coumaphos (treatment groups), was dropped on the bee's mouth with a micropipette. Bees which did not extend their proboscis or did not completely consume the droplet were discarded (Williams et al. 2013).

2.3. Locomotor activity monitoring assay

Various methods are utilized to assess the effects of pesticides on the locomotion parameters of honeybees. The simplest method is the observation of honeybees in a petri dish or a translucent chamber. Duration of walking behavior during the whole observation time or calculated walking length of the subject bee using a surface divided by squares and grids are common parameters to evaluate locomotor activity (El Hassani et al. 2008; Williamson et al. 2013b, 2014; Bartling et al. 2019). Digital video tracking is also used as a more sophisticated method (Charreton et al. 2015; Teeters et al. 2012; Tosi and Nieh 2017). Special chambers equipped with sensors that can detect the movements of individual bees are also used (Bloch et al. 2003; Harano et al. 2007; Giannoni-Guzmán et al. 2014). We used this last method (see Giannoni-Guzmán et al. 2014) for locomotor activity monitoring to measure the locomotor activities of individual bees. This system was originally designed for *Drosophila* and later modified to be used in honeybees, wasps, and other insects of similar size.

After coumaphos treatment, each bee was individually put into a 15-ml perforated falcon tube whose cap contains a small amount of (approx. 0.85 g) commercial fondant sugar (Konya Seker Inc.) covered with a layer of cheesecloth to prevent bees from getting stuck to the food. Falcon tubes were then placed individually into the chambers of the Large Activity Monitor (LAM) system built by Trikinetics Inc. based in Waltham, MA, USA. This device consists of four monitoring modules, containing 32 holes each. These holes were large enough to accommodate a 15-ml falcon tube and were equipped with three infrared beam sources and their corresponding sensors, which encircled the middle of the falcon tube (Fig. 2). Whenever a honey bee in motion passed through the falcon tube and disrupted one or more of the three beams, a signal indicating the presence of movement was transmitted to the computer that was linked to the system. The total number of these positive signals in a designated time interval was analyzed as locomotor activity for that interval. Modules were kept in an incubator (33 °C and 55% (± 5) humidity) for 24 h in constant darkness. Locomotor activity experiments began at 19:00 PM and lasted for 24 h until 19:00 PM the next day.

2.4. Statistical analysis

Statistical analyses were performed with SPSS. Bee samples for each subspecies were randomly collected from two colonies to compensate for colony effects. The sample size was 24 for all experimental groups (i.e., each combination of 4 dozes × 5 subspecies/ecotypes) except the 1 µg and 2 µg dose groups of *caucasica* subspecies which contained 23 bees each and 5 µg dose groups of *syriaca* subspecies and Yığılca and Muğla ecotypes which contained 21, 23, and 23 bees, respectively. The total number of bees monitored in the experiment was 474. The Shapiro–Wilk test was used to check the normality of locomotor activity data. When comparing control and treatment groups among subspecies and ecotypes, the Student's *t*-test was used for normally distributed data, and log or square root transformation was used if one or both groups were not normally distributed. In case where transformation could not normalize the data, a nonparametric Mann–Whitney *U* test was applied. The Bonferroni correction was used to adjust *p* values. This was done by multiplying *p* values by 5, which is the number of control vs treatment group comparisons for each coumaphos

Figure 2. One of the activity monitors used in the experiments. The activity monitor has 32 cells, and each cell can take 15-ml (dimensions: 17 mm O.D., 120 mm length) falcon tube with single honeybee in it.

dose. For further comparison of the effects of coumaphos on subspecies and ecotype levels, treatment groups of each subspecies were normalized to their controls to determine possible differences among subspecies in terms of their response to Perizin administration. This was done by dividing each variable of a subspecies or ecotypes' treatment group data by the mean of its respective control group data. This new set of ratio data for each subspecies and ecotypes were log-transformed and compared by one-way ANOVA.

To find out if there were similarities in locomotor activity patterns between groups in each subspecies and ecotype, collinearity analysis was used. The analysis is based on *cosine* similarity principle, and it defines the *cosine* of the angle between two vectors. Thus, the value varies between 0 and 1, with values closer to 1 indicating higher similarity (Han et al. 2011). The statistic is calculated by the following formula:

$$\cos\theta_{ij} = \frac{v_i.v_j}{||v_i||||v_j||}$$

3. RESULTS

Line graphics showing hourly total locomotor activities of each dose group for each subspecies and ecotypes are presented in Fig. 3. Visual examination of the graphics showed visible locomotor activity differences between dose groups of *syriaca* in the first half of the assay period. Therefore, we decided to analyze the first 12-h, second 12-h, and total 24-h post exposure periods of locomotor activities of experimental groups for each subspecies and ecotypes. We selected the first 12-h period to investigate whether there were any significant differences in early onset locomotor activity following the administration of acute coumaphos. The second 12-h period was chosen to analyze any possible activity differences that may have occurred during this time period and/or continued from the first 12 h. Finally, we studied the total 24-h period to determine whether these activity differences were strong or persistent enough to significantly reveal themselves throughout the entire assay time.

Furthermore, there was a pattern of gradual increase in locomotor activities from morning to afternoon, then a gradual decrease from late afternoon to night (Fig. 3). The lowest locomotor activities were approximately at 5:00 to 8:00 AM for all experimental groups in each subspecies and ecotypes.

3.1. Analysis of the first 12-h period post exposure to coumaphos

Normality tests and statistical comparisons of the first 12-h data are given in Supplementary Tables S1, S2, and S3. No significant difference was found between control and treatment groups of subspecies and ecotypes in control vs. 1 µg coumaphos dose group comparisons ($p = 1.00$; Fig. 4a). On the other hand, the 2 µg treatment group of *syriaca* subspecies showed a significant increase in locomotor activity compared to its control ($p = 0.015$) while no significant difference was found in control and treatment group comparisons of other subspecies and ecotypes for this dose (Fig. 4b). In control vs. 5 µg comparisons (Fig. 4c), significant locomotor activity increase in the treatment groups of the *syriaca* ($p < 0.001$) subspecies, as well as Muğla ($p < 0.01$) and Yığılca ($p = 0.04$) ecotype of the *anatoliaca* subspecies, was found compared to their respective control groups while no significant difference was observed in other subspecies and ecotypes ($p = 1.00$ for *caucasica* and $p = 0.15$ for *carnica*). Because control vs. 5 µg treatment group comparisons gave significant results in multiple subspecies and ecotypes, we further investigated possible differences in response magnitude among the populations, by comparing 5 µg treatment/control mean ratios of subspecies and ecotypes (Fig. 7a). One-way ANOVA showed a significant difference ($F_{4,\ 110} = 6.222$, $p < 0.001$), and the post hoc Tukey test showed that *syriaca* has a significantly higher locomotor activity ratio compared to *caucasica*, *carnica* ($p < 0.001$ and $p < 0.01$ respectively) and Yığılca ecotype of *anatoliaca* ($p = 0.013$), while no significant difference ($p > 0.05$, Table S3) was observed in other paired comparisons between groups.

3.2. Analysis of the second 12-h period post exposure to coumaphos

Normality tests and statistical comparisons of the second 12-h data are provided in Supplementary Tables S4 and S5. No significant difference ($p > 0.05$, Table S5) was found between control and treatment groups of any subspecies and ecotypes in any coumaphos doses (Fig. 5a–c). In the direct comparisons among groups using 5 µg treatment/control mean ratios, the treatment/control ratios of *syriaca* did not conform to a normal distribution after log transformation ($W_{21} = 0.565$, $p < 0.001$), and therefore, the non-parametric Kruskal–Wallis test was utilized. The results showed no significant difference ($H_4 = 8.026$, $p > 0.05$) between subspecies and ecotypes (Fig. 7b).

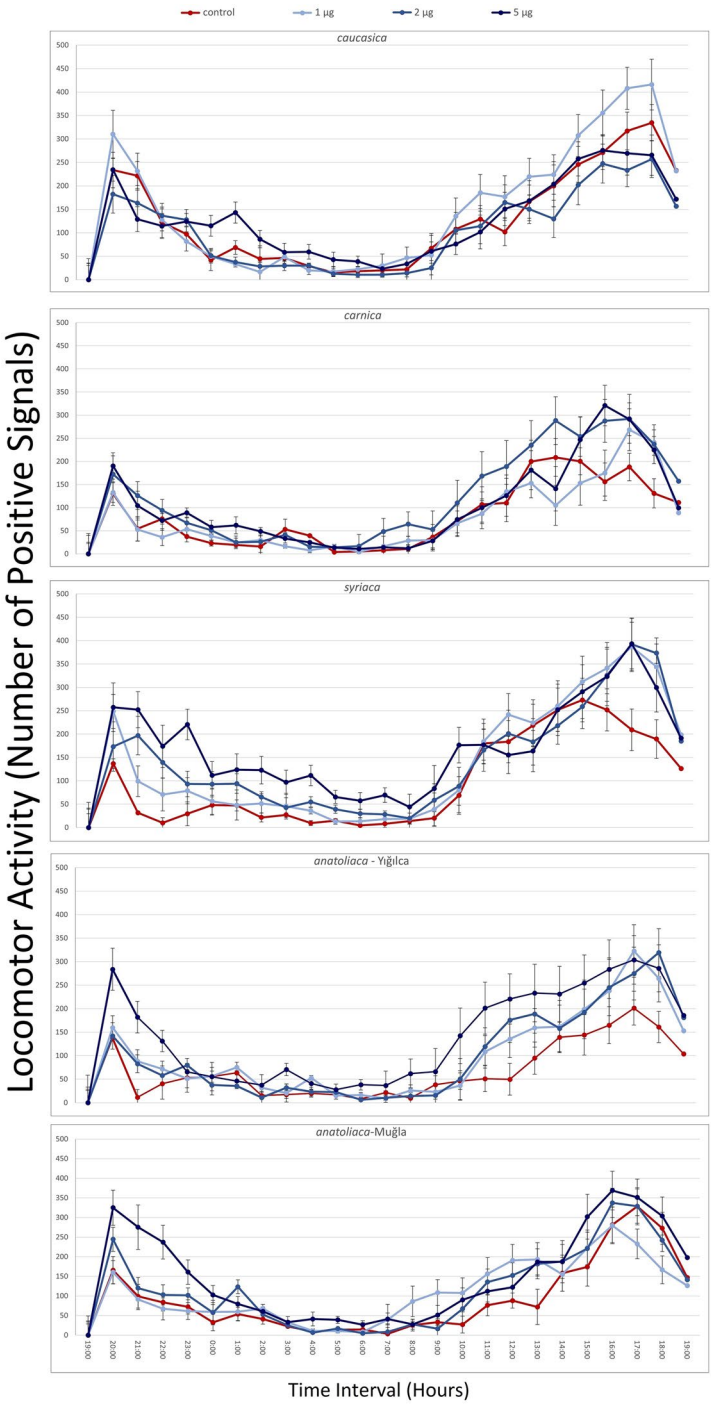

Figure 3. Means and standard errors of locomotor activities at each hour in control and treatment groups of bee subspecies and ecotypes.

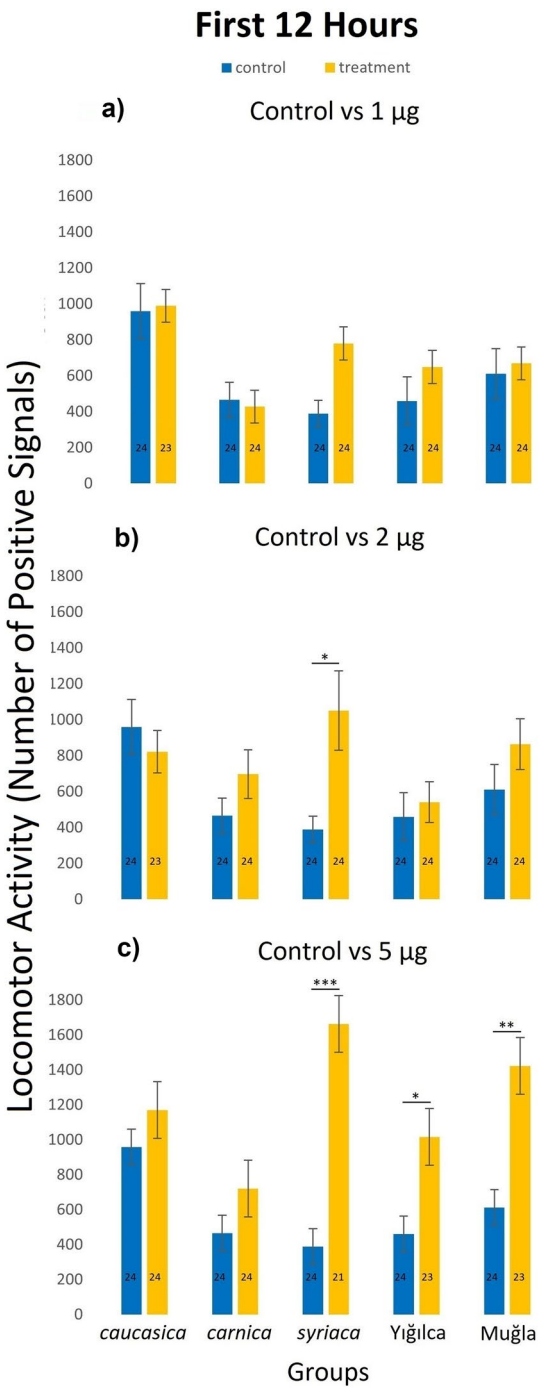

Figure 4. Locomotor activities of subspecies and ecotypes over the first 12 h after acute exposure to 1 µg (**a**), 2 µg (**b**), and 5 gμ (**c**) coumaphos. Sample sizes are in the bars (mean ± SE). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

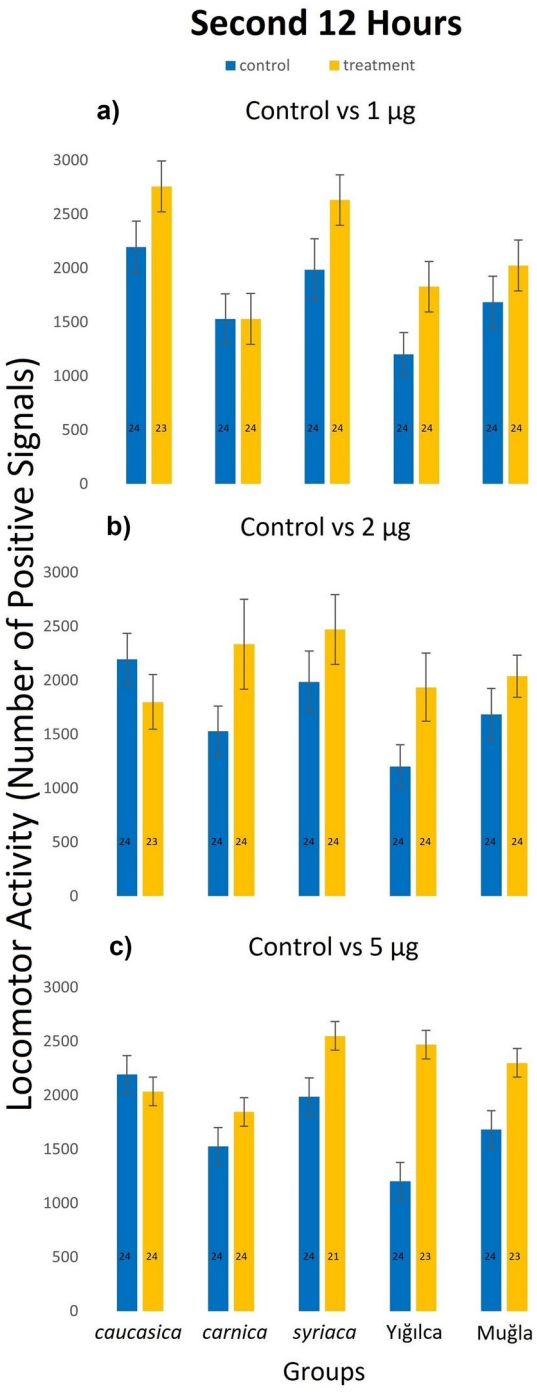

Figure 5. Locomotor activities of subspecies and ecotypes over the second 12 h after acute exposure to 1 µg (**a**), 2 µg (**b**), and 5 gμ (**c**) coumaphos. Sample sizes are in the bars (mean ± SE).

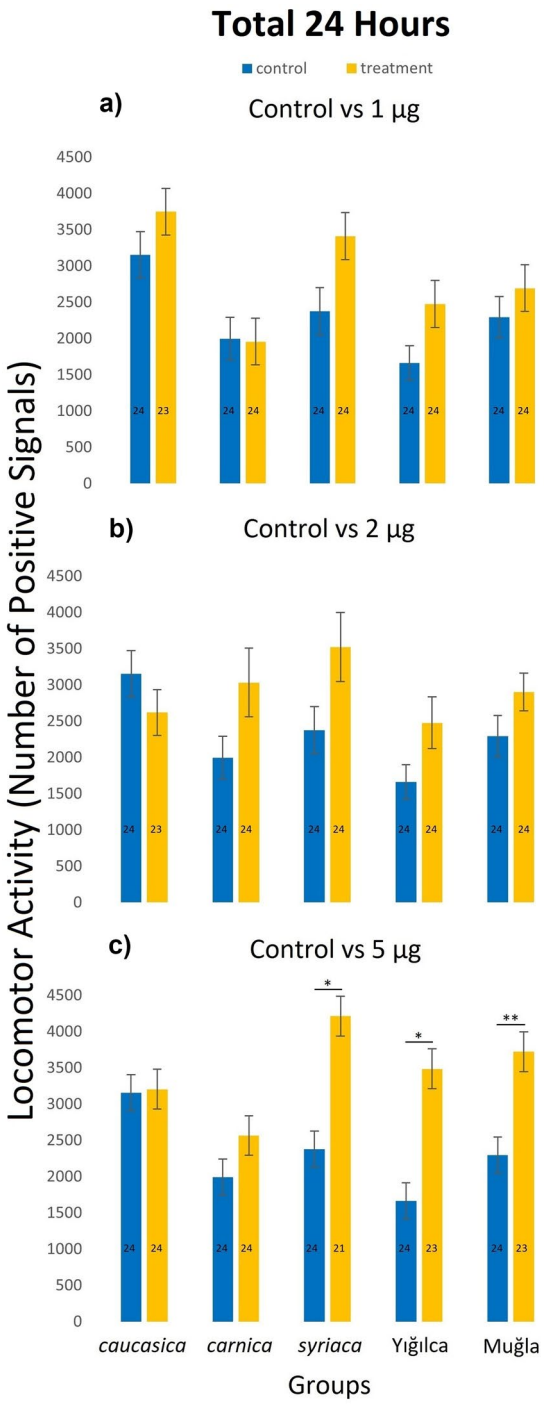

Figure 6. Locomotor activities of subspecies and ecotypes over the total hours after acute exposure to 1 µg (**a**), 2 µg (**b**), and 5 gμ (**c**) coumaphos. Sample sizes are in the bars (mean ± SE). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

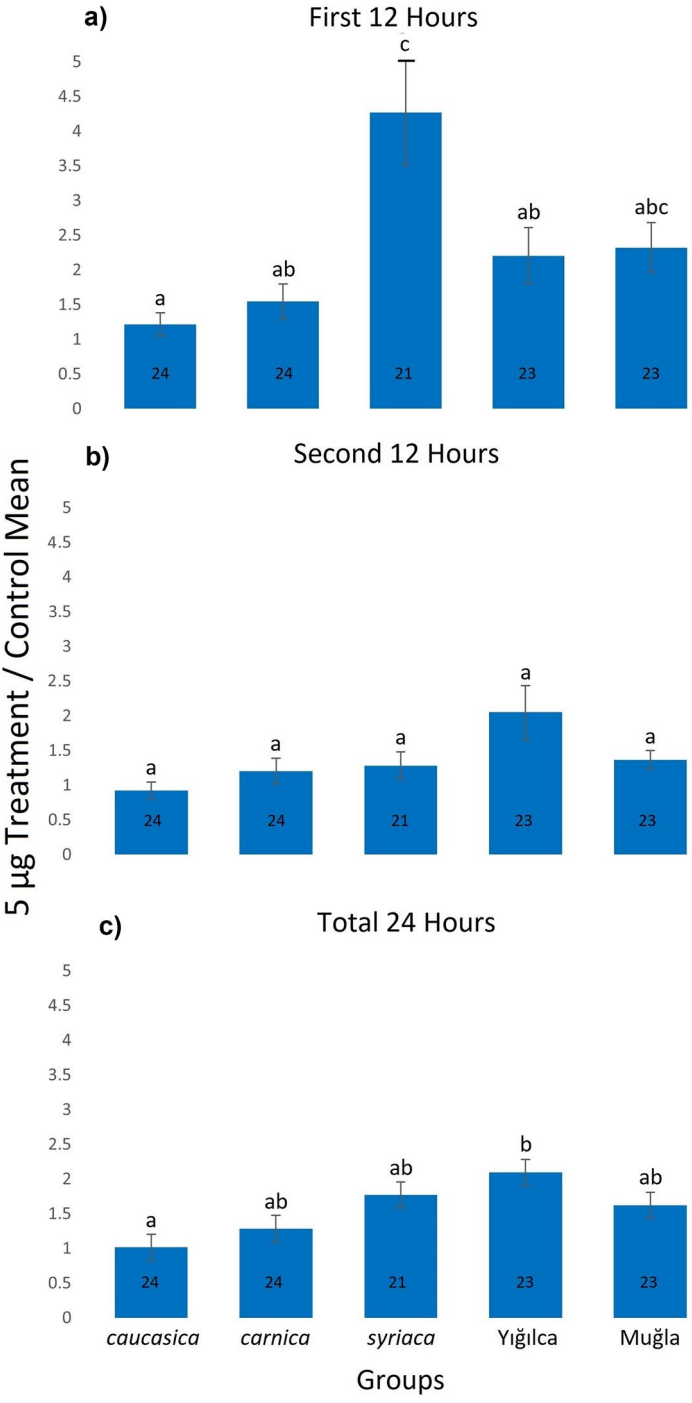

Figure 7. Normalized locomotor activities of subspecies and ecotypes over the first 12 h (**a**), second 12 h (**b**), and total 24 h after acute exposure to 5 gµ of coumaphos (**c**). Activity for the treatment bees was divided over the average activity of the control group from the same population to obtain normalized locomotor activity measures (*Y*-axis). Sample sizes are in the bars (mean ± SE).

Table I Collinearity comparison matrices of experimental groups for each honeybee subspecies and ecotype

caucasica					*carnica*				*syriaca*	
a)	Control	1 µg	2 µg	5 µg	Control	1 µg	2 µg	5 µg	Control	1 µg
Control	1.00	0.96	0.94	0.93	1.00	0.86	0.92	0.86	1.00	0.94
1 µg		1.00	0.95	0.92		1.00	0.90	0.91		1.00
2 µg			1.00	0.92			1.00	0.90		
5 µg				1.00				1.00		

syriaca		*anatoliaca* Yığılca					*anatoliaca* Muğla			
2 µg	5 µg	b)	Control	1 µg	2 µg	5 µg	Control	1 µg	2 µg	5 µg
0.89	0.82	Control	1.00	0.96	0.94	0.93	1.00	0.86	0.92	0.86
0.94	0.88	1 µg		1.00	0.95	0.92		1.00	0.90	0.91
1.00	0.91	2 µg			1.00	0.92			1.00	0.90
	1.00	5 µg				1.00				1.00

3.3. Analysis of the total 24-h period post exposure to coumaphos

Normality tests and statistical comparisons of the total 24-h data are provided in Supplementary Tables S6, S7, and S8. No significant difference ($p > 0.05$, Table S7) was found between control vs. 1 µg treatment groups and control vs. 2 µg treatment groups in any of the tested populations for 24-h data (Fig. 6a, b). In control vs. 5 µg treatment group comparisons (Fig. 6c), a significant locomotor activity increase in treatment groups of *syriaca* and the Yığılca and Muğla ecotypes of *anatoliaca* ($p = 0.02$, $p = 0.01$ and $p < 0.01$, respectively) was found compared to their respective controls, while no significant difference was observed in the *caucasica* and *carnica* ($p < 0.05$, Table S7). One-way ANOVA comparison of the 5 µg treatment data/control mean ratios showed a significant difference ($F_{4, 110} = 3.991$, $p < 0.001$) among subspecies and ecotypes (Fig. 6c), and post hoc Tukey comparisons showed that the Yığılca ecotype of *anatoliaca* has a significantly ($p = 0.013$) increased locomotor activity ratio compared to *caucasica,* while Muğla ecotype of *anatoliaca* and *syriaca* subspecies also show tendencies towards elevated locomotor activity ratios compared to *caucasica* ($p = 0.057$ for both). No significant difference ($p > 0.05$) was observed in other comparisons between groups (Fig. 7c; Table S8).

3.4. Collinearity analysis

In collinearity analysis, we studied the similarity between time-dependent activity patterns in control and treatment conditions in each subspecies and ecotypes. The results of this analysis are given in Table I. The lowest *cosine* similarity was observed between control and 5 µg treatment groups of *syriaca* (0.82) while the highest was observed between control and 1 µg groups of *caucasica* and the Yığılca ecotype of *anatoliaca* (0.96 for both). Control vs. 5 µg treatment group comparisons had lower *cosine* similarity than other comparisons in subspecies and ecotypes except in *carnica* subspecies and the Muğla ecotype, in which control vs. 5 µg and control vs. 1 µg comparisons had equal values (0.86 for both).

4. DISCUSSION

Our experiments yielded two main observations. One was that Perizin treatment increases total activity of bees but does not appear to

change daily locomotor patterns. The second and most notable finding was that Turkish honeybee populations differ in their pesticide sensitivity, which was possible to effectively measure within our common garden setting. This variation may be expected as these populations are genetically divergent and adapted to highly variable different habitats and conditions and have further been subjected to different beekeeping practices and pest management products for long generations. Especially, the lower level of sensitivity observed in honeybee populations native to areas where Perizin application is widespread (i.e., Caucasus) invokes the idea that different beekeeping practices cause different selection pressures on honeybee populations, as we discuss in the following.

According to the results of our total locomotor activity analyses, *caucasica* and *carnica* subspecies appeared to be the least sensitive to coumaphos regarding locomotor activity as no significant difference was found in any time duration and coumaphos dose. The *syriaca* subspecies, as well as Yığılca and Muğla ecotpyes of *anatoliaca* subspecies, showed significant locomotor activity and increased responses to coumaphos administration. Dose and time effects varied, with the most prominent effects seen in *syriaca*, and smaller effects observed in other bees.

In contrast to total activity levels, with respect to temporal patterns of activity, collinearity analysis showed high levels of (more than 80%) resemblance between locomotor activity data patterns of experimental groups in all subspecies and ecotypes. This indicates that coumaphos treatment did not have apparent effects on 24-h activity patterns of honeybee subspecies and ecotypes included in this study.

The inactivation of AChE by organophosphate pesticides causes elevated levels of the excitatory neurotransmitter acetylcholine which overstimulates cholinergic receptors (Colović et al. 2013; Brown 2019) Indeed, increased agitation and restlessness are among the acute onset symptoms of organophosphate toxicity (Peter et al. 2014). Therefore, we believe that the increased locomotor activities observed in coumaphos treatment groups of *syriaca* subspecies as well as Yığılca and Muğla ecotypes of *anatoliaca* subspecies can be due to stimulative and agitative effects of sublethal acute coumaphos exposure.

Honeybees performing in-hive tasks are known to work around the clock without any circadian rhythm unlike foragers which have strong diurnal activity cycles (Moore et al.1998). However, individually isolated nurse bees in petri dishes were reported to display strong circadian rhythms (Nagari et al. 2017). So, it is not surprising that our individually isolated bees displayed time-dependent activity patterns. In addition to this, the lack of remarkable differences between the activity patterns of control and treatment groups of subspecies and ecotypes may indicate that acute coumaphos exposure have limited effects on daily rhythms of honeybees; still, this hypothesis should be further tested by future experiments with durations of longer than 24 h accompanied by periodogram and rhythmicity analyses.

Williamson et al. (2013b) fed honeybees (*A. m. mellifera*) with 1 µM, 100 nM, and 10 nM concentrations of coumaphos and 10 nM concentrations of other AChE inhibiting pesticides chlorpyrifos, aldicarb, and donepezil, for 24 h. After pesticide treatment, individual bees were put into a petri dish and observed for 15 min. Behaviors detected in these observations were classified as walking, flying, remaining still, falling upside down, grooming, and unusual abdominal spasms and movements. Walking behavior, the character also studied here, was found to be slightly decreased by coumaphos, chlorpyrifos, and aldicarb treatment in summer bees, but not in winter bees, while this decrease was statistically significant in only chlorpyrifos, but not in coumaphos and aldicarb. Grooming behavior, on the other hand, was significantly increased by all four pesticides in pooled summer and winter bee data. Coumaphos was also found to have a dose-dependent positive effect on grooming and abdominal spasms. Stürmer et al. (2014) measured locomotor activities of cockroaches *Phoetalia pallida* by putting them in a water tank and recording the duration of their swimming with a video camera, after treatments with 0.25, 0.5, and 1 µM doses of organophosphate pesticide trichlorfon.

Swimming duration rates were observed to be significantly increased compared to controls in the 1-µM dose group. In their research on the combined effects of the pesticide DDT (dichlorodiphényltrichloroéthane-AChE inhibitor pesticide) and temperature on locomotor activities of three Drosophila strains, Fournier-Level et al. (2016) used the Drosophila Activity Monitoring (DAM) System, similar to the system used in our study. Fournier-Level and colleagues identified 5 different Drosophila groups based on the displayed activity patterns and observed that increased DDT doses also increased the frequency of their 5th activity group, defined by early peak activity and high mortality. Early peak activity is similar to the 2 µg and 5 µg coumaphos treatment groups of *syriaca* in our study, although we observed negligible mortality (only seven dead bees across all subspecies and ecotypes) in our locomotor activity assays.

Overall, the latter two studies show that sublethal doses of AChE inhibitor pesticides can increase locomotor activities of insects similar to the results of our study.

The lack of significant effects of coumaphos on locomotor activity in the Williamson et al. (2013b) study may be related to the very short period of observation, limited only to a period of the day, in that study. Our method also demonstrates much reduced and similar activity levels for all bees of all populations of all treatments from 5 am till 8 am (twilight and dawn period). Although bees were kept indoors and not exposed to typical outside temperatures, circadian activity patterns remain trained to external conditions for several days (Giannoni-Guzmán et al. 2021). These are factors to consider in future studies of pesticide effects on activity. Our study indicates that full day or at least 12-h periods of measurements are necessary to be able to avoid circadian effects.

Neonicotinoids are nicotinic ACh receptor agonists (Jeschke and Nauen 2008), and therefore, their effects on cholinergic system resemble organophosphates such as coumaphos. Tackenberg et al. (2020) investigated the chronic effects of neonicotinoid pesticides thiamethoxam and clothianidin on locomotor activities and circadian rhythms of honeybees by a Trikinetics LAM system in different illumination conditions (12-h light/dark, constant light, and constant darkness) for several days. They found that neonicotinoids significantly altered circadian rhythms in 12-h light–dark period and disrupted sleep in all light conditions but did not have significant effects on overall locomotor activity levels.

We believe that two possible aspects are affecting the variation in locomotor activity upon exposure to coumaphos among Turkish honeybee populations: differences in life-history traits (local preadaptation) and differences in beekeeping practices (novel selection). Bees of *caucasica* subspecies were adapted to a high-altitude habitat with long winters and short foraging seasons. They have gentle behavior with high honey production. Bees of the *carnica* subspecies are native to a temperate climate and are known for their gentleness and easy handling. Honeybees of *syriaca* subspecies, on the other hand, are adapted to hot and dry arid climates and are well known for their aggressive behavior due to predator wasp species present in its native region (Ruttner 1988). Muğla ecotype of *anatoliaca* subspecies is adapted to a climate with hot humid summers and mild winters while the native habitat of Yığılca ecotype has a more temperate climate with milder summers and colder winters compared to Muğla. The *anatoliaca* subspecies is also known for its aggressive behavior. Güler (1995) compared aggressiveness in several Turkish honeybee genotypes in a common garden and reported that *caucasica* colonies are the least aggressive while Muğla colonies are one of the most, and *carnica* colonies stand between these two. According to our observations in our common garden, subspecies and ecotypes of this study can be arrayed from least aggressive to most as *caucasica*, *carnica*, Yığılca, Muğla, and *syriaca*. Thus, there may be a correlation between aggressiveness, adaptation to warmer climates, and sensitivity to coumaphos, among different honeybee genotypes.

Due to its high honey yield, gentle behavior, and low swarming tendency, *caucasica* is the most popular subspecies by commercial beekeepers in Türkiye. Indeed, bees from *caucasica*

subspecies are the only commercially recognized certified honeybee breed in Türkiye. According to the survey of Karaca and Karaman (2018), which comprised 28 commercial queen bee breeding enterprises in 10 provinces, 60.7% of them bred *caucasica*, 21.4% *carnica*, 14.3% *anatoliaca* (ecotype not specified), and 3.4% the Yığılca ecotype of *anatoliaca*. There is also one breeding and artificial selection center for Muğla ecotype of *anatoliaca* which is not included in the survey. Colonies of *syriaca*, on the other hand, are largely confined to small-scale or hobbyist beekeepers who breed their queens, in its native region due to aggressive behavior, low honey yield, and high swarming tendency characteristics of this subspecies. Honeybee genotypes used in intensive commercial beekeeping are more frequently exposed to in-hive chemicals, especially in the form of acaricides, and therefore may develop an evolutionary resistance against these chemicals. This may explain the resistance of *caucasica* to coumaphos administration in terms of locomotor activity because *caucasica* is the most frequently used honeybee subspecies by commercial beekeepers, and therefore, *caucasica* colonies can be expected to have been intensely exposed to coumaphos and other commercial acaricides for decades. Coumaphos containing commercial acaricide Perizin is also frequently used in Artvin-Camili queen breeding center, where we obtained our *caucasica* colonies from. Therefore, *caucasica* subspecies may have developed resistance to coumaphos due to long-term sublethal exposure to commercial acaricides.

There may be multiple proximate or mechanistic explanations for differences across bee populations, such as detoxification metabolism, sensitivities of the target site of the AChE, or penetration barriers for the pesticide (Dahlgren 2014). Mao et al. (2011) showed that three cytochrome P450's, CYP9Q1, CYP9Q2, and CYP9Q3, are mostly involved in coumaphos detoxification in honeybee midguts. In addition to this, piperonylbutoxide (PBO), a p450 inhibiting chemical, is also found to have synergistic effects with coumaphos in honeybees (Johnson et al. 2009) indicating the importance of the cytochrome P450 system in the metabolization of coumaphos. Therefore, further studies should focus on the biochemical and genetic aspects of differences among Turkish honeybee subspecies in terms of their responses to locomotor activity increasing effects of acute sublethal coumaphos doses. Especially, AChE activity levels and expressions of CYP9Q1, CYP9Q2, and CYP9Q3 genes should be compared between the subspecies after coumaphos administrations. Furthermore, population genomic analyses may reveal selective processes across populations as demonstrated in the comparison of bee populations facing new conditions (Avalos et al. 2017).

In conclusion, we observed differences in susceptibilities of Turkish honeybee subspecies and ecotypes to acute sub-lethal coumaphos doses in terms of locomotor activity. These differences, especially those between the *caucasica* and *syriaca* subspecies, appear to broadly correlate with possible coumaphos exposure in the recent past. Although it may appear attractive to speculate that this correlation could represent recent adaption to coumaphos pressure in Caucasian honeybees, such differences could also arise as byproducts of other local physiological adaptations (local preadaptation). Evaluating the local preadaptation versus novel selection hypotheses requires a better understanding of the molecular mechanisms underlying differences in susceptibility. Nevertheless, our study brings into focus questions on human influence on natural selection through agricultural practices.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

OA, BE and TG and MK designed the study. OA and BE conducted the experiments and drafted the manuscript. OA, BE and MS performed the data analysis. OA wrote the paper. OA and other authors participated in the revisions.

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AVAILABILITY OF DATA AND MATERIAL

The datasets of this study are available at Zenodo with DOI number: https://doi.org/10.5281/zenodo.7988130.

CODE AVAILABILITY

The R code for mixed model analysis is available at Zenodo with DOI number: https://doi.org/10.5281/zenodo.7988130.

DECLARATIONS

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