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Araştırma
(Research)

Bal Arılarının (*Apis mellifera*) Şeker Tüketimi, Sağkalım Oranı ve *Nosema ceranae* Duyarlılığı Üzerine Coumaphos ve Lityum Klorür Maruziyetinin Etkileri

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Öz

Amaç: Bu çalışmanın amacı, Varrao kontrolünde kullanılan coumaphos ve lityum klorür maruziyetinin bal arısı işçilerinin (*Apis mellifera*) yaşam süresi, sukroz tüketimi ve *Nosema ceranae* duyarlılığı üzerine etkilerini değerlendirmektir.

Materyal ve Yöntem: Bu araştırmada, üç Kafkas bal arısı kolonisinden alınan iki çerçeve kapalı yavrulu çıta, %80 bağıl nem ve 35°C sıcaklıkta inkübe edildi. Petek gözünden çıkan işçi arılar deneysel kafeslere her bir kafeste 50 arı olacak şekilde doldurduldu. *Ad libitum* şeker şurubu ve polen ile beslendi. Arılar kontrol, coumaphos'a maruz kalanlar, lityum klorüre maruz kalanlar, ve acetona (coumaphos'un çözücüsü) maruz kalanlar; bu dört gruba ek olarak, aynı grupların *N. ceranae* sporları ile enfekte edilmiş olanları olmak üzere sekiz gruba ayrıldı. Şeker şurubu tüketimi ve ölüm oranları günlük olarak kaydedildi, *N. ceranae* spor sayımları deneme sonunda yapıldı.

Araştırma Bulguları: Gruplar arasındaki ölüm oranları ve sukroz solüsyonu tüketimi arası farklılıkların istatistiksel olarak anlamlı olmadığı ($p>0.05$) belirlenmiştir. Nosema spor sayımı bakımından ise; lityum klorür, lityum klorür *Nosema* ve coumaphos grubu istatistiki olarak diğer gruplardan önemli ölçüde daha az *Nosema* sporuna sahip olduğu belirlenmiştir ($p<0.05$).

Sonuç: Lityum klorür maruziyeti, *Nosema ceranae* ile enfekte edilmiş arı gruplarında, spor sayılarını önemli ölçüde azaltmıştır ($p<0.05$). Sukroz tüketimi açısından, gruplar arasında istatistiksel olarak anlamlı bir fark bulunmamıştır. Bu durum uygulamanın arıların beslenme alışkanlıkları üzerinde önemli bir etkisi olmadığını göstermektedir. Sonuç olarak, lityum klorürün varroa akarlarının kontrolünde kullanımının ötesinde, *N. ceranae* enfeksiyonlarına karşı potansiyel faydaları üzerine araştırmalar yapılmalıdır.

Anahtar Kelimeler: Bal arısı (*Apis mellifera*), Lityum klorür, Coumaphos, *Nosema ceranae*, Sukroz tüketimi

The Effects of Coumaphos and Lithium Chloride Exposure on Honey Bees' (*Apis mellifera*) Sugar Consumption, Survivability and *Nosema ceranae* Susceptibility

Abstract

Objective: The main objective of this study was to assess the effects of coumaphos and lithium chloride exposure used in Varroa control on the life span, sucrose consumption, and *Nosema ceranae* susceptibility of worker honey bees (*Apis mellifera*).

Materials and Methods: In this research, two frames of capped brood were taken from three Caucasian honey bee colonies and incubated at 35°C and 80% relative humidity. Newly emerged worker bees were collected into experimental cages, with 50 bees per cage, and fed *ad libitum* with sugar syrup and pollen. Bees; the control group consisted of those exposed to coumaphos, those exposed to lithium chloride, and those exposed to acetone (coumaphos solvent). In addition to these four groups, the same groups were divided into eight groups, including those infected with *N. ceranae* spores. Sugar syrup consumption and

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mortality rates were recorded daily, while. *ceranae* spore counts were conducted at the end of the trial.

Results: The study found that there were no statistically significant variations in mortality rates and sucrose solution consumption among the groups ($p>0.05$). The groups treated with lithium chloride, lithium chloride *Nosema*, and coumaphos showed considerably lower *Nosema* spore counts compared to the other groups ($p<0.05$).

Conclusion: Exposure to lithium chloride significantly reduced the spore counts in groups infected with *N. ceranae* ($p<0.05$). No statistically significant differences were found in terms of sucrose consumption among the groups, indicating that exposure did not have a significant effect on the feeding habits of the bees. Consequently, research should be conducted on the potential benefits of lithium chloride against *N. ceranae* infections, beyond its use in controlling varroa mites.

Keywords: Honey bees (*Apis mellifera*), Lithium chloride, Coumaphos, *Nosema ceranae*, Sucrose consumption

Introduction

Honey bees, *Apis mellifera* are one of the most prominent pollinators of agricultural crops (Klein et al., 2006). Even though the domesticated honey bee population have elevated worldwide over the last 50 years, colony populations have dropped off dramatically in North America and many European countries considering pesticide use, habitat destruction, pathogens and climate change (Aizen and Harder 2009; National Research Council 2007). Nutritional limitation and sub-lethal dose of pesticides exposure may alter the susceptibility of bee parasites and pathogens (Foley et al., 2012; Wu et al., 2012; Alaux et al., 2010). Recent studies show that sub-lethal effects of pesticides on bees. Insecticides and fungicides can affect development, laying behaviours, mating ratio, mobility, direction findings, feeding behaviour and immunity functions of insects (Wu et al., 2012; vanEngelsdorp et al., 2009).

Interaction between pesticides and *Nosema* have greater impacts on bee health (Wu et al., 2012 and decrease honey bee lifetime (Alaux et al., 2010). In particular, pesticide exposure to honey bees increases gut pathogens *Nosema* (vanEngelsdorp et al., 2009). The other stress factor of the honey bees is an ectoparasite *Varroa* which considering a major threat to the apiculture sector. For controlling varroa mites, some acaricides have been used such as organophosphate coumaphos (Checkmite®, Asuntol®, Perizin®) and pyrethroids Flumethrin (Bayvarol®), synthetic acaricides against *V. destructor*, are used over 15 years (Pettis et al., 2012). However, these miticides possess some negative aspects such as being stored in wax Boncristiani et al. (2012) and *Varroa* mites can develop resistance to miticides (Vidau et al., 2011). Otherwise, there are researches about lithium chloride which effectively kills *Varroa* mites without doing residues problems (Rosenkranz et al., 2010). However, the varroa against effects of lithium chloride previously discovered, and bee scientists have little knowledge about its effects on honey bee health.

The main goal of this study is to address two significant questions. The effects of coumaphos and lithium chloride on honey bees, and answering the question whether there are any synergistic effects between *Nosema* and each of these two varroacide chemicals.

Material and methods:

Experimental procedures

We used three Caucasian honey bee colonies and we confirmed that these colonies were *Nosema* free confirmed by PCR (Utuk et al., 2019). Two frames of sealed brood were taken from each colony and incubated in 35°C and %80 relative humidity. Exposed to a TempQueen (queen mandibular pheromone) released. Emerged bees were collected into disposable experimental cages (7.5 X 7.5 X 6 cm). Each cage accumulated with 50 newly emerged worker bees were fed with candy sugar and water *ad libitum.* Caged bees were also fed pollen patties to supply protein, simulating colony rearing circumstances as closely as possible. Following the day of caging bees were randomly separated into eight experimental groups; 1st control group, 2nd exposed coumaphos chronically, 3rd exposed lithium chloride, 4th acetone (solvent of coumaphos), 5th infected with *N. ceranae*, 6th infected with *N. ceranae* and exposed coumaphos chronically, 7th infected with *N. ceranae* and exposed lithium chloride, 8th infected with *N. ceranae* and acetone (solvent of coumaphos). Honey bees were infected individually and we ensure that each bee in the consumed same amount of *Nosema* spores (see below *Nosema* infection). Every day the feeders were scaled, and sucrose solution consumption was quantified. After 3 days of infection, the coumaphos and lithium

chloride exposure was started in *ad libitum* sugar syrup 50% (w/v). In this experiment acetone was used as solvent of coumaphos in acetone group worker bees were exposed to %0,3 acetone *ad libitum*. Through the experiment every morning the feeders were scaled and sucrose, pesticides, lithium chloride and acetone consumption was quantified.

Nosema infection

Nosema spores were isolated from *Nosema* positive colonies, and infection was done the protocol developed by (Elzen and David, 2002). The worker bees were collected from *Nosema* positive colonies and their guts were macerated into 10 ml of water. Then spore water suspension was centrifuged at 3000 rpm for 10 minutes. The supernatant was separated, and the *Nosema* pellets were resuspended 50% (w/v) sugar syrup. The spore concentration was counted by haemocytometer. The species of Nosema was determined by PCR according to (Utuk et al., 2019) and PCR products were shown in Figure 1. The worker bees in Nosema infected groups were infected individually with 2µl 50% (w/v) sugar syrup containing 200 000 *Nosema* spores once only (Elzen and David, 2002). *Nosema* free experimental groups were similarly treated without *Nosema* spores sugar syrup to be exposing the same stress.

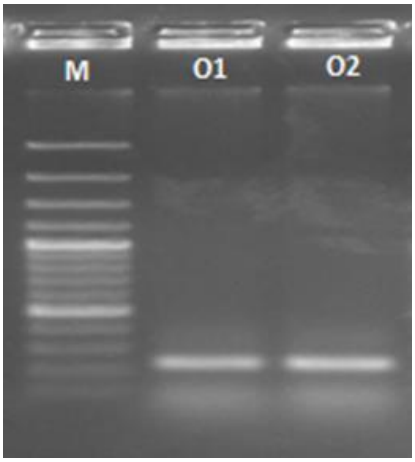

Figure 1. PCR products of *N. ceranae*. Lane M control, lane O1 and O2 *N. ceranae* positive sample

Exposure to coumaphos and lithium chloride

The sucrose solution 50 % (w/v) was used for feeding bees containing coumaphos, lithium chloride or acetone. The LD_{50} of coumaphos were calculated at 6,23 µg per bee, and LD_{05} (0,347 µg per bee) was used as a sublethal dosage in our experiment as considering (Ziegelmann et al., 2018). The first feeder; sucrose solution 50 % (w/v). The second experimental feeder; 9,51 mg coumaphos were scaled and solved in 3 ml acetone by doing vortex. Then 9,51 mg/L coumaphos sucrose solution was prepared including % 0,3 acetone. The third experimental feeder; 1 liter sucrose solution was included only % 0,3 acetone. The fourth experimental feeder; 1,06 gr lithium chloride scaled and solved in 1 liter sucrose solution. Thus, 1 liter 25 millimole lithium chloride solution was prepared. Nosema infection was completed on the first day and following 3 days all groups were fed with the first feeder. After those different feeders were applied to experimental groups.

Statistical analysis

The normality of the data was checked using the Shapiro-Wilk's test. The assumption of the equal variances of groups was checked using the Levene's test. A two-way repeated measures ANOVA with a Greenhouse-Geisser correction (sphericity not assumed) was used for daily sucrose solution consumption. In the analysis, the assumption of the equivalence of covariance matrix was tested by Box's Test. A one-way ANOVA was used to compare the cumulative percent mortality of groups after the data was transformed by Box-Cox transformation. A Kruskal-Wallis test followed by Dunn's multiple comparison test was used for Nosema counts of groups. The level of statistical significance was set at $p<0.05$. The statistical analyses were performed using SPSS v26 (IBM Inc., Chicago, IL, USA) and Minitab 19 (Minitab, LLC, State College, Pennsylvania) statistical software.

Results and discussion

The variation of the daily average sucrose solution consumption amounts per bee (µl/bee/day) according to time and groups was investigated by two-way repeated measures analysis of variance. As a result of the analysis of variance, both the Group×Time interaction and the main effects of the factors were not found to be statistically significant ($p>0.05$).

The average daily consumption of sucrose solution (µl/bee/day) per bee obtained during the study period is shown in Table 1. When it is examined, it can be seen that the consumption of sucrose solution varies somewhat according to the days. The highest sucrose solution consumption was observed on the: first day (30.64±3.01), 7th day (40.14±12.59), 12th day (37.99±7.52), 13th day (46, 74±2.55) and 14th day (45.99±5.43) in the acetone *Nosema* group; 3rd day (31.68±5.77), 4th day (31.21±9.26), 5th day (29.26±5.98), 8th day (37.81±0.56), 9th day

(39.42±3.82), 10th day (44.24±4.70) and 11th day (35.52±2.07) in the control group; 2nd day (28.77±5.19) and 6th day (33.57±4.35) in the acetone control group. The lowest sucrose solution consumption was observed in the first day (22.12±2.51) and second day (20.56±3.70) coumaphos *Nosema* group, on the 3rd day (21.73±9.99) coumaphos group, and on the 4th day (23.04±1.86) acetone *Nosema* group. After the 5th day (19.57±5.12), the lowest sucrose solution consumption was observed in the lithium chloride *Nosema* group: 6th day 18.49±8.67), 7th day (13.53±12.38), 8th day (17.54±11.59), 9th day (15.79±15.93), 10th day (15.44±14.41), 11th day (10.78±16.98), 12th day (10.25±15.02), 13th day (10.23±17.73), and 14th day (9.68±16.77). However, all these differences were not found to be statistically significant (p>0.05).

When Table 1 are examined, in the control group, the mean consumption of sucrose, which was 27.29±6.92 µl/bee/day on the 1st day then increased gradually until the 14th day, and was measured as 38.72±0.56 µl/bee/day on the 14th day. In the acetone control group, the mean consumption of sucrose solution was 27.07±7.94 µl/bee/day on the 1st day, 25.50±6.24 µl/bee/day and 24.92±6.56 on the 4th and 5th days, respectively. Then, it continued to increase, and it was measured as 31.90±14.58 µl/bee/day on the 14th day. Similarly, in the Acetone *Nosema* group, the mean consumption of sucrose solution, which was 30.64±3.01 µl/bee/day on the 1st day, decreased until the 4th day and became 23.04±1.86 µl/bee/day on the following days, increasing gradually and reached 45.99±5.43 µl/bee/day on 14th day. In the coumaphos group, the mean consumption of sucrose solution was 24.37±7.94 µl/bee/day on the 1st day and increased gradually until the 14th day as 31.49±7.53 µl/bee/day. The amount of sucrose solution consumption showed less change in the lithium chloride group by days. The mean consumption of sucrose solution was 24.34±2.86 µl/bee/day on the 1st day, decreased to 22.06±1.93 µl/bee/day on the 5th day, then reached to 26.86±3 days on the 14th day with small increases. In the Nosema group, the mean consumption of sucrose solution was 27.86±1.46µl/bee/day on the 1st day, decreased until the 5th day 24.63±4.41 µl/bee/day, then increased gradually and reached 68±0.36 µl/bee/day. The consumption of sucrose solution in the coumaphos Nosema group showed less change compared to the days. Although it increased to 30.93±6.47 µl/bee/day on the 4th day, the average sucrose solution consumption was 22.12±2.51 µl/bee/day on the 1st day and 19.37±17.08 µl/bee on the 14th day. In the lithium chloride Nosema group, unlike the other groups, the average consumption of sucrose solution decreased gradually over the days. The mean consumption of sucrose solution was 24.01±2.80 µl/bee/day on the 1st day then it was decreasing to 9.68±16.77 µl/bee/day on the 14th day. All these differences between the groups in terms of sucrose solution consumption averages were not found to be statistically significant (p>0.05).

Table 1. Descriptive statistics of daily sucrose solution consumption (µl/bee/day)

	Control group		**Acetone control**		**Acetone *Nosema***		**Coumaphos**		**Coumaphos Nosema**		**Lithium chloride**		**Lithium chloride *Nosema***		***Nosema***		***Overall (n=24)***	
	Mean	**SS**	**Mean**	**SS**	**Mean**	**SS**	**Mean**	**SS**	**Mean**	**SS**	**Mean**	**SS**	**Mean**	**SS**	**Mean**	**SS**	**Mean**	**SS**
1st Day	27.29	6.92	27.07	7.94	30.64	3.01	24.37	7.94	22.12	2.51	24.34	2.86	24.01	2.80	27.86	1.46	*25.96*	*4.99*
2nd Day	28.18	3.47	28.77	5.19	28.72	3.92	25.57	10.76	20.56	3.70	24.66	6.10	22.32	5.77	27.51	5.68	*25.79*	*5.80*
3rd Day	31.68	5.77	29.28	3.00	27.51	6.03	21.73	9.99	24.59	1.07	28.31	0.67	22.33	4.15	25.13	6.55	*26.32*	*5.65*
4th Day	31.21	9.26	25.50	6.24	23.04	1.86	27.78	6.05	30.93	6.47	27.60	1.76	25.64	2.68	30.32	4.46	*27.75*	*5.34*
5th Day	29.26	5.98	24.92	6.56	27.00	7.22	27.11	5.72	21.33	5.44	22.06	1.93	19.57	5.12	24.63	4.41	*24.48*	*5.57*
6th Day	31.22	9.70	33.57	4.35	32.20	8.29	25.57	4.48	21.57	8.88	25.55	6.38	18.49	8.67	27.72	3.97	*26.99*	*7.82*
7th Day	33.41	7.39	30.65	6.74	40.14	12.59	19.62	5.39	22.43	10.28	27.35	1.62	13.53	12.38	32.21	5.49	*27.42*	*10.81*
8th Day	37.81	0.56	30.38	14.30	36.64	15.58	28.92	6.24	21.63	11.80	29.01	5.95	17.54	11.59	30.36	2.75	*29.03*	*10.60*
9th Day	39.42	3.82	33.51	18.43	36.75	21.18	31.19	9.32	22.80	14.65	29.77	8.15	15.79	15.93	35.61	1.90	*30.61*	*13.43*
10th Day	44.24	4.70	36.50	15.85	35.01	16.28	35.53	12.02	27.13	17.06	28.02	4.14	15.44	14.41	40.31	3.30	*32.77*	*13.40*
11th Day	35.52	2.07	32.94	17.99	34.99	18.54	26.80	9.58	20.25	14.27	24.53	4.23	10.78	16.98	34.09	2.37	*27.49*	*13.45*
12th Day	31.02	0.96	29.82	18.00	37.99	7.52	24.44	8.33	20.87	11.65	26.67	3.25	10.25	15.02	28.26	4.05	*26.17*	*11.57*
13th Day	41.42	1.61	34.57	18.12	46.74	2.55	33.97	12.45	21.21	18.61	25.65	2.94	10.23	17.73	30.49	3.84	*30.54*	*14.96*
14th Day	38.72	0.56	31.90	14.58	45.99	5.43	31.49	7.53	19.37	17.08	26.86	3.03	9.68	16.77	36.68	0.36	*30.09*	*14.01*
p	Group: 0.065; Day: 0.061; Group x Day interactions: 0.324																	

Nosema spore numbers of the groups (spor ×105) are given in Table 2. As a result of the Kruskal-Wallis test, it was determined that there was a statistically significant difference between *Nosema* spore numbers of groups p=0.003. According to Dunn's test, there was no significant difference between control, acetone control, *Nosema,* acetone *Nosema* and coumaphos *Nosema* groups in terms of *Nosema* spore number (p>0.05). *Nosema* spore numbers of coumaphos, lithium chloride and lithium chloride *Nosema* groups lower than those of *Nosema,* acetone *Nosema* and coumaphos *Nosema* groups (p<0.05).

Table 2. The *Nosema* spore numbers (spore x 10^5) of groups

Groups	n	Mean	SS	Median	IQR	Mean Rank	p
Control	3	40.00	18.64	36.88	36.88	12.33 ^{AB}	
Acetone control	3	45.53	31.22	57.86	58.68	12.33 ^{AB}	
Nosema	3	519.03	47.02	523.33	93.75	22.00 ^{A}	
Acetone Nosema	3	307.08	137.99	362.50	258.75	18.00 ^{A}	**0.003**
Coumaphos Nosema	3	386.88	255.40	428.75	505.63	20.00 ^{A}	
Coumaphos	3	10.08	0.96	9.89	1.89	8.33 ^{B}	
Lithium chloride	3	0.00	0.00	0.00	0.00	2.00 ^{B}	
Lithium chloride Nosema	3	1.30	0.24	1.25	0.47	5.00 ^{B}	

The difference between groups without a common letter is statistically significant (p<0.05)

In the study, the average cumulative death percentages of the groups by day were calculated (Figure 2). Mortality rates of the groups were compared with one-way analysis of variance after transforming with the Box-cox transformation method. There was no statistically significant difference between the groups (p=106).

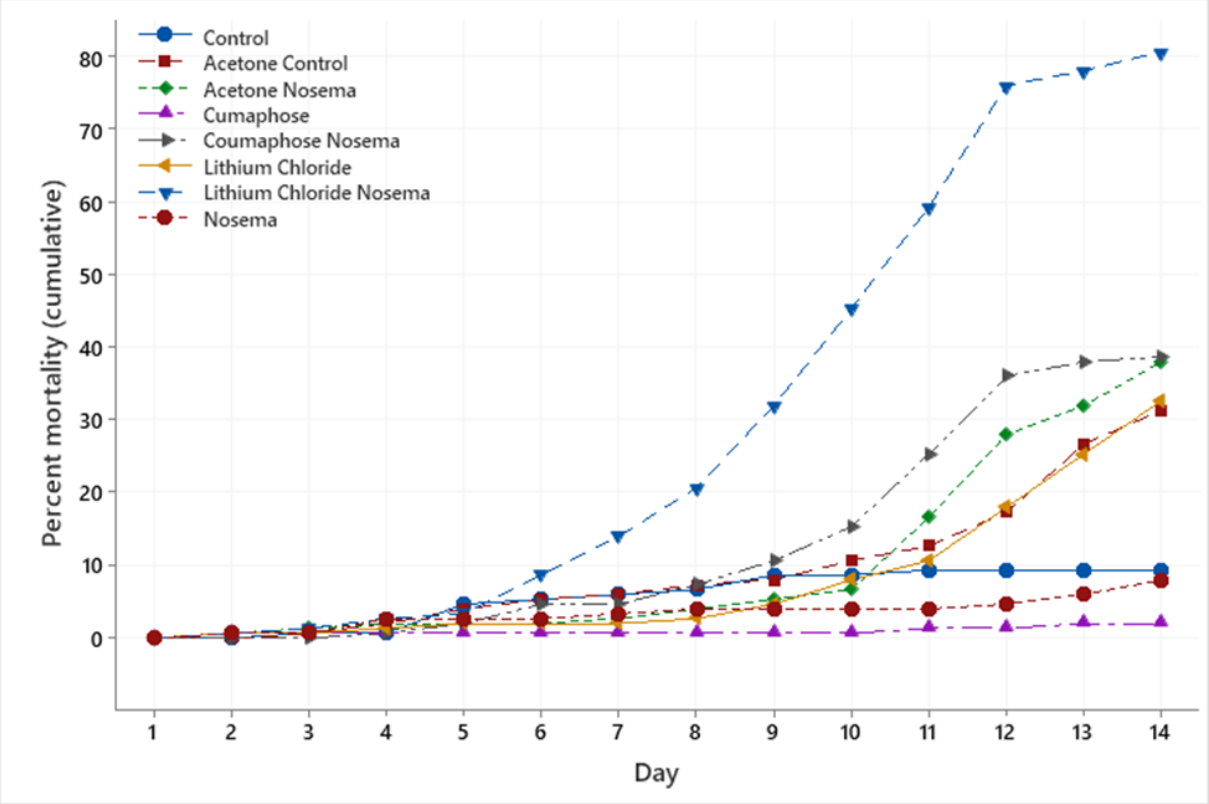

Figure 2. Percent mortality. Mortality is expressed as the mean percentage of cumulated number of dead bees per cage and per day. Three colonies were analyzed, with three cage replicates for each colony (n = 50 bees per cage)

The interaction of diseases and pesticides have stronger negative effects on honey bee colonies (Higes et al., 2006). Regarding this, pesticides exposure to honey bees increases the susceptibility of the gut pathogen *N. ceranae*. (vanEngelsdorp et al., 2009). Coumaphos were used for controlling *Varroa* mites for many years (Pettis et al., 2012). However, Rosenkranz et al. (2010) discover *Varroa* against the effects of lithium chloride which very fresh molecule in apiculture. Even though lithium chloride kills *Varroa* mite, we did not know its certain effects on honey bee health. In addition, the interaction between lithium chloride exposure and *N. ceranae* susceptibility is not clear. Our findings clearly show that lithium chloride decreases the N. *ceranae* susceptibility of worker honey bees.

In our research *N. ceranae* spores count experimentally infected groups were statistically high than uninfected groups($P<0.05$). There is also seen *N. ceranae* spores in control groups the potential reason for these newly emerged worker bees chewing some *N. ceranae* spores according to Malone and Gatehouse (1998) study. Some research has similar results that determine *N. ceranae* in the control group (Alaux et al., 2010; vanEngelsdorp et al., 2009).

Pesticide exposure and toxins increase the susceptibility of diseases including the gut parasite *Nosema* spp. (vanEngelsdorp et al., 2009; Alaux et al., 2010). In addition, the interaction of pesticides and Nosema microspores affects bee health and reduces bee longevity (Alaux et al., 2010; Vidau et al., 2011). Understanding the impact of interactions between different diseases or pesticides within hives is critical for understanding bee disease dynamics and bee mortality (Gashout 2017; Malone and Gatehouse 1998). This implication is compatible with our findings. Neither along with *N. ceranae* infection nor exposure of coumaphos, lithium chloride and acetone cause serious cumulative death ($P>0,05$). On the other hand, *N. ceranae* infected lithium chloride, coumaphos, and acetone exposure groups showed more cumulative mortality than uninfected lithium chloride, coumaphos, and acetone exposure groups.

The lowest sucrose consumption is in the lithium chloride *Nosema* group. In general trend of sucrose solution consumption is increasing day by day as (Alaux et al., 2010; Vidau et al., 2011) illustrate the same result of our sucrose consumption rates. However, sucrose consumption of lithium chloride *Nosema* group shows a drastic decrease during the experiment. *N. ceranae* can influence nutritional requirements in hosts by consuming host nutrients and causing energetic stress (Cornman et al., 2012). Microsporidia are often amitochondriate and unable to undergo oxidative phosphorylation, implying a significant reliance on host ATP, particularly during germination, which demands a high level of energy (Gashout 2017; Malone and Gatehouse, 1998). The solvent of coumaphos was acetone in our experiment. Both coumaphos and acetone did not increase food intake compared to the control group. *Nosema* infection may increase consumption reasoning that we mentioned above. Interestingly, the sucrose consumption of lithium chloride Nosema was seriously low than the lithium chloride group. Lithium chloride is very novel to use in varroa treatment. That is why there appears to be no previous research exploring about the interaction of lithium chloride and *N. ceranae*. The main symptom of *N. ceranae* infection was energetic stress Vidau et al., (2011) and infected bees consumed some more sucrose solution (Mayack and Naug, 2009). However, lithium chloride exposure was negatively affected sucrose consumption of *N. ceranae* infected worker bees.

Conclusion

Lithium chloride is a recently proposed molecule for used in control of *Varroa* infestation molecule and it is a lot hopping to varroa controlling in terms of low residues and efficiency. However, coumaphos has been used for varroa treatment for a long time. In our study, we compared coumaphos and lithium chloride and their *Nosema* interactions. Lithium chloride exposure to *Nosema* infected group showed a high mortality rate and also lithium chloride exposure decreased the *Nosema* spores counts.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Authorship contribution statement

SO: Conducted laboratory cage experiments, performed detailed observations and measurements, counted Nosema spores and manuscript writing.

SA: Conducted laboratory cage experiments, performed detailed observations and measurements, counted Nosema spores and manuscript writing.

YKA: Performed statistical analysis, contributed to manuscript drafting and revision.

GA: Conducted laboratory cage experiments, performed detailed observations and measurements, counted Nosema spores and manuscript writing.

SS: Conducted laboratory cage experiments, performed detailed observations and measurements, counted Nosema spores and manuscript writing.

SA: Conducted laboratory cage experiments, performed detailed observations and measurements, counted Nosema spores and manuscript writing.

SS: Conducted laboratory cage experiments, performed detailed observations and measurements, counted Nosema spores and manuscript writing.

AG: Conducted laboratory cage experiments, performed detailed observations and measurements, counted Nosema spores and contributed to manuscript drafting and revision.

İK: Conducted molecular identification of Nosema species.

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