INVESTIGATION OF THE EFFECTS OF LITHIUM ON LOCOMOTOR ACTIVITY, CIRCADIAN RHYTHM, AND LEARNING USING COMPUTATIONAL METHODS IN HONEY BEES

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INVESTIGATION OF THE EFFECTS OF LITHIUM ON LOCOMOTOR ACTIVITY, CIRCADIAN RHYTHM, AND LEARNING USING COMPUTATIONAL METHODS IN HONEY BEES

submitted by **BABÜR ERDEM** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** i**n Biology, Middle East Technical University** by,

Prof. Dr. Naci Emre Altun
Dean, **Graduate School of Natural and Applied Sciences** _____

Prof. Dr. Mesut Muyan
Head of the Department, **Biology** _____

Prof. Dr. Ayşe Gül Gözen
Supervisor, **Biology, METU** _____

Assoc. Prof. Dr. Hande Alemdar
Co-Supervisor, **Computer Engineering, METU** _____

Examining Committee Members:

Prof. Dr. Ergi Deniz Özsoy
Biology, Hacettepe University _____

Prof. Dr. Ayşe Gül Gözen
Biology, METU _____

Assoc. Prof. Dr. Tülin Yanık
Biology, METU _____

Assoc. Prof. Dr. Aslı Özkök Saban
HARÜM, Hacettepe University _____

Assit. Prof. Dr. Murat Perit Çakır
Cognitive Sciences, METU _____

Date: 31.10.2024

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Last name, Name : Babür Erdem

Signature :

ABSTRACT

INVESTIGATION OF THE EFFECTS OF LITHIUM ON LOCOMOTOR ACTIVITY, CIRCADIAN RHYTHM, AND LEARNING USING COMPUTATIONAL METHODS IN HONEY BEES

Erdem, Babür
Doctor of Philosophy, Biology
Supervisor: Prof. Dr. Ayşe Gül Gözen
Co-Supervisor: Assoc. Prof. Dr. Hande Alemdar

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This thesis investigates the multifaceted effects of lithium chloride on honey bees (*Apis mellifera*), focusing on locomotor activity, circadian rhythm, and learning behaviors. Lithium is recognized for its potential as an acaricidal agent against the *Varroa* mite and its well-documented use in treating bipolar disorder in humans.

Acute treatments revealed that lithium significantly reduced light-induced high locomotor activity but had no effect in constant darkness. Chronic exposure similarly decreased the high locomotor activity under constant light. Furthermore, chronic lithium treatment disrupted circadian rhythmicity, especially under constant darkness, while lengthening the circadian period under constant light conditions.

The research also developed a novel video-tracking system to assess bee learning in an electric shock avoidance assay. This system enabled precise tracking of individual bees and their exposure to shocks. The effect of lithium on the learning success of honey bees, specifically affecting performance in aversive learning accompanied by reversal learning paradigm, was investigated using an electric shock conditioning assay. The results showed that while lithium did not affect initial learning

(acquisition phase), it impaired learning during the reversal phase, indicating a dose-dependent reduction in adaptive behavior.

Overall, the study improves our understanding of lithium's multifaceted effects on honey bee behavior and physiology. These findings have important implications for its use in apiculture, particularly in *Varroa* mite control. Moreover, the study suggests honey bees as a valuable non-mammalian model for studying the behavioral effects of lithium, paralleling its role in treating bipolar disorder.

Keywords: Honey Bee, Lithium, Locomotor Activity, Circadian Rhythm, Learning

ÖZ

LİTYUMUN BAL ARILARINDA LOKOMOTOR AKTİVİTE, SİRKADYEN RİTM VE ÖĞRENME ÜZERİNE ETKİLERİNİN HESAPLAMALI YÖNTEMLER KULLANILARAK ARAŞTIRILMASI

Erdem, Babür
Doktora, Biyoloji
Tez Yöneticisi: Prof. Dr. Ayşe Gül Gözen
Ortak Tez Yöneticisi: Doç. Dr. Hande Alemdar

Ekim 2024, 87 sayfa

Bu tez, lityum klorürün bal arıları (*Apis mellifera*) üzerindeki çok yönlü etkilerini, özellikle lokomotor aktivite, sirkadiyen ritim ve öğrenme davranışlarına odaklanarak araştırmaktadır. Lityum, *Varroa* akarına karşı akarisidal bir ajan olma potansiyeliyle ve insanlarda bipolar bozukluğun tedavisindeki iyi belgelenmiş kullanımı ile tanınmaktadır.

Akut uygulama, lityumun ışık ile tetiklenmiş yüksek lokomotor aktiviteyi önemli ölçüde azalttığı, ancak sürekli karanlıkta bir etkisinin olmadığını ortaya koyulmuştur. Benzer şekilde, kronik lityum maruziyeti de sürekli ışık altında artan lokomotor aktiviteyi azaltmıştır. Ayrıca, kronik lityum uygulaması özellikle sürekli karanlıkta sirkadiyen ritmi bozmuş, sürekli ışık altında ise sirkadiyen periyodu uzatmıştır.

Araştırmada ayrıca, elektrik şoku kaçınma deneyinde arı öğrenmesini değerlendirmek için yenilikçi bir video izleme sistemi geliştirilmiştir. Bu sistem, arı bireylerinin şoklara maruz kalma anlarının hassas bir şekilde izlenmesini sağlamıştır. Lityumun, tersine öğrenme paradigmasıyla birlikte kaçınma öğrenmesi üzerindeki

etkileri, elektrik şoku koşullandırma deneyi ile incelenmiştir. Sonuçlar, lityumun başlangıç öğrenmesini (edinim aşaması) etkilemediğini, ancak tersine öğrenme aşamasında öğrenmeyi bozduğunu ve bu durumun doza bağlı olarak uyum sağlayıcı davranışta azalmaya yol açtığını göstermiştir.

Genel olarak, çalışma, lityumun bal arısı davranışı ve fizyolojisi üzerindeki çok yönlü etkilerini detaylı bir biçimde açıklamaktadır. Tezde elde edilen bulgular, özellikle *Varroa* akarı kontrolünde, lityumun arıcılıkta kullanımı açısından önemli sonuçlar içermektedir. Ayrıca, bal arılarını, lityumun davranışsal etkilerini incelemek için memeli olmayan değerli bir model organizma olarak önerilmekte ve bu etkilerin, bipolar bozukluğun tedavisindeki rolüyle paralellikler taşıdığını ortaya koymaktadır.

Anahtar Kelimeler: Bal Arısı, Lityum, Lokomotor Aktivite, Sirkadiyen Ritim, Öğrenme

To my little sister, mom, and dad.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

Api-TRACE	Honey Bee Tracking in Constrained Environments
BeeTMA	Bee Tracking and Motion Analysis Algorithm
CCD	Colony Collapse Disease
CS	Conditioned Stimulus
CS+	Conditioned Stimulus associated with Unconditioned Stimulus
CS-	Conditioned Stimulus not associated with Unconditioned Stimulus
DAVM	Api-TRACE Data Analysis and Visualization Module
ESAA	Electric Shock Avoidance Assay
LAM	Large Activity Monitors
Li	Lithium
LiCl	Lithium chloride
LMA	Locomotor Activity
US	Unconditioned Stimulus
VPM	Api-TRACE Video Processing Module

LIST OF SYMBOLS

SYMBOLS

A	Amplitude
D	Center amplitude
f	Frequency
φ	Phase

CHAPTER 1

GENERAL INTRODUCTION

1.1 Honey bee and its importance

The Western honey bee, scientifically classified as *Apis mellifera* L., is a member of the eusocial insect group within the order *Hymenoptera*, alongside sawflies, wasps, bees, and ants (Danforth et al., 2013). Within the broader *Apidae* family, the genus *Apis* encompasses a variety of species, including bumblebees, stingless bees, carpenter bees, orchid bees, and cuckoo bees. The *Apis* genus is divided into three subgenera: *Micrapis*, *Megapis*, and *Apis*. The Western honey bee exhibits significant taxonomic diversity, with 28 subspecies distributed globally (Michael, 1999).

Recent research indicates that ecosystems worldwide may face a loss of up to 75% in biomass and species diversity (Hallman et al., 2017). The Living Planet Index reports a substantial 68% decline in populations of 4392 species of mammals, birds, reptiles, fish, and amphibians between 1970 and 2016 (Almond et al., 2020). The ongoing ecological crisis is expected to profoundly impact human society in the presumed sixth mass extinction (Ceballos et al., 2017). That issue stems from various factors, including climate change, habitat fragmentation, pollution, and the use of harmful chemicals that lead to rapid ecosystem collapse.

Honey bees are pivotal in our current ecosystem, performing a significant portion of pollination, particularly in commercial agriculture. However, their populations are facing significant declines due to adverse environmental conditions. Rapid colony collapse disease (CCD) is a major contributor, causing losses of up to approximately 35% in bee colonies (Johnson, 2013). CCD results from complex interactions among pathogens, parasites, and other stressors rather than a single cause (van Engelsdorp

et al., 2009). Notably, the interplay between *Varroa* parasites and viruses has been identified as a significant factor in colony losses (Le Conte et al., 2010; Martin et al., 2012).

1.2 Lithium

Lithium (Li) is a trace metal with the lowest density of solid elements. It has the lowest atomic mass and the smallest atomic number among metals (6.941 u and 3, respectively). Lithium is highly reactive; because of that reason, it is not found freely in nature. Metallic lithium is isolated electrolytically from lithium chloride (LiCl) salt. The most known industrial application of lithium is lithium-ion batteries. Also, lithium is used for medical purposes. It is quietly known that it is used as a mood stabilizer for bipolar disorder. However, despite decades of research, the mechanism of action of lithium in preventing bipolar disorder is only partially understood. Research on lithium is complicated by the lack of suitable animal models for bipolar disorder (Alda, 2015). Lastly, the use of lithium against the bee parasite *Varroa* has come forward (Ziegelmann, et al. 2018).

1.3 Varroa

Varroa destructor, an Acarid species, is the world's most harmful honey bee parasite that causes colony losses (Figure 1.1). The Korean genotype of *Varroa destructor* is especially the most common and damaging (Zhang, 2000). It self-feeds with the fat tissue of honey bees (Ramsey et al., 2019).

It is well-known that various chemicals such as fluvalinate, flumethrin, coumaphos, and amitraz are widely used against *Varroa* (Bahreini et al., 2020). Frequent use of these substances has led to resistance to these chemicals in *Varroa* populations (Pettis, 2004; Girisgin et al., 2019; Rinkevich, 2020). Another problem is that these chemicals leave residue in honey. These fat-soluble synthetic miticides accumulate in the beeswax and propolis (Bogdanov, 2006; Wallner, 1999). Because of that,

organic acids (formic, lactic, oxalic acid), thymol-containing fragrant oils, and various herbs have become widespread (Bogdanov, 2006). New solutions are being sought to be more effective in the fight against *Varroa*.

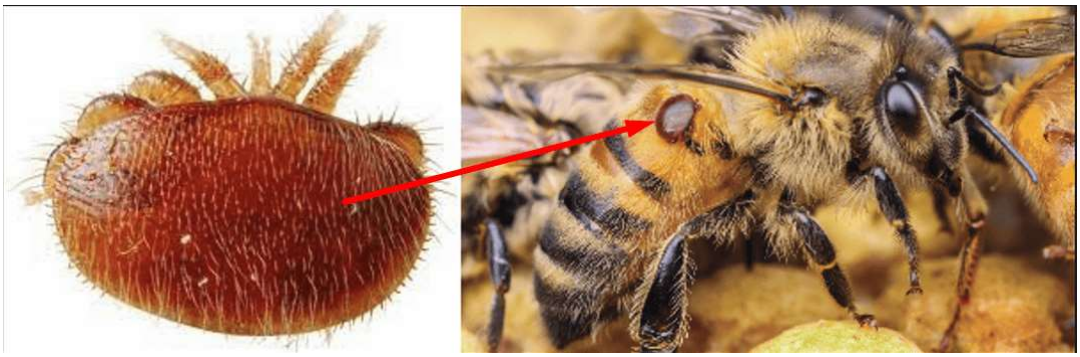

Figure 1.1 *Varroa destructor* and an infected honey bee

1.4 Bipolar disorder

In bipolar disorder, also known as manic depression, periodic mood swings occur. The elevated mood is defined as mania or hypomania. According to DSM-5-TR, mania is defined as a distinct period of abnormally and persistently elevated, expansive, or irritable mood with increased goal-directed activity or energy. Hypomania has a similar definition but is a milder form of mania. At least three symptoms are observed in mania or hypomania, which are inflated self-esteem or grandiosity, decreased need for sleep, more talkativeness than usual or pressure to keep talking, flight of ideas or subjective experience that thoughts are racing, distractibility, increase in goal-directed activity or psychomotor agitation, and excessive involvement in activities which have a high potential for painful consequences (APA, 2022). By contrast in elevated mood, symptoms in the depressive episode are persistent feelings of sadness, irritability or anger, loss of interest, excessive or inappropriate guilt, hopelessness, excessive sleeping or not

enough, changes in appetite, fatigue, self-loathing, or feelings of worthlessness, and thoughts of death or suicidal ideation are observed (Muneer, 2013).

Bipolar disorder is divided into 3 types, and the intensity of these symptoms varies across these types.

- Bipolar 1 disorder

Bipolar 1 disorder is the occurrence of manic and major depressive episodes. At least one manic episode must be observed and described as euphoric, excessively cheerful, and high. The prevalence of bipolar 1 disorder is 1.5% in the USA, and it is up to 0.6% across 11 countries. Cognitive impairments are observed in individuals with bipolar disorder. Approximately 30% of the affected people show severe impairment in work role functions. The risk of suicide is estimated to be at least 20 - 30 times that of the general population (APA, 2022).

- Bipolar 2 disorder

In bipolar 2 disorder, hypomania is observed instead of mania, and major depressive episodes must be found. Fluctuations between the depressive episodes and hypomania can cause significant distress or impairment in social, occupational, or other important areas of functioning. The prevalence of bipolar 2 disorder is 0.8% in the USA and 0.3% internationally. Like bipolar 1 disorder, individuals with bipolar 2 disorder perform more poorly than healthy individuals on cognitive tests, except memory and semantic fluency (APA, 2022).

- Cyclothymic disorder

Instead of major depressive episodes, mild depression is observed besides hypomania in cyclothymic disorder. The lifetime prevalence of cyclothymic disorder is approximately 0.4% - 2.5%. It is not as severe as the other bipolar disorders. The suicide risk is also lower than the major depression cases (APA, 2022).

1.5 Lithium studies on *Drosophila* as an insect model

Several studies have been conducted on the effect of lithium on *Drosophila.* Studies about circadian rhythm are found. According to Dokucu et al., 20 and 30 mM LiCl significantly increase period, 24.02 h and 24.4 h, respectively, according to the control group 23.7 h. Also, Lithium increases arrhythmicity, especially in doses higher than 5 mM, and it exceeds %50 at 60 mM application (2005). Another study indicates that the period of circadian rhythm also increases in GSK-3 loss of function mutants (Martinek et al., 2001). It is quite known that disruption of circadian rhythm is frequently observed in bipolar disease (Melo et al., 2017). Also, circadian preference, a subjective preference for activities in the morning or evening, appears on the eveningness chronotype. Alteration in sleep latency and reversal of sleep/wake period have been observed in bipolar disorder (Giglio et al., 2010). Anomalies in-phase and circadian rhythms are modified with lithium treatment acting as a GSK-3 inhibitor (Escamilla & Zavala, 2008). In a study on longevity and locomotion, it was determined that the life expectancy of the fruit fly increased through GSK-3 inhibition due to the application of 1–25 mM LiCl. In addition, it has been observed that it reduces the decrease in LMA due to aging (Castillo-Quan et al., 2016). Also, beta-amyloid production is reduced in Alzheimer's disease *Drosophila* models with lithium treatment. As a result, LMA problems and lifespan reductions rescued by 10 mM LiCl treatment act on GSK-3 inhibition (Hayward et al., 2004). As a result, Lithium lengthens the period, increases longevity, and positively affects locomotion via GSK-3 inhibition (Jans et al., 2021). Ries et al. developed a depression-like state on *Drosophila* using constant vibration for 3 days. They measured their motivation using a gap-climbing assay. Vibration-treated flies significantly lost their motivation. The 50 mM LiCl treatment highly elevated motivation in vibration-treated and untreated flies. The motivation was higher than

controls. This elevation is stated as similar to manic behavior. However, 5 mM LiCl treatments only recover the motivation, the same as control groups (2017).

1.6 Lithium studies in honey bees

Following the discovery of lithium's lethal effect on Varroa, a parasitic threat to honey bees, researchers began considering its potential use in combatting this parasite (Ziegelmann et al., 2018). Subsequent studies have revealed the multifaceted effects of lithium treatment. While it has been shown to reduce viral load and alleviate oxidative stress through alterations in gene expression (Jovanovic et al., 2022), other research has signified its residual toxicity within honey bee colonies (Kolics et al., 2021a; Kolics et al., 2021b) and its propensity to cause brood damage (Rein et al., 2022). Furthermore, lithium has been found to accumulate in various compartments and members of the hive (Presern et al., 2020).

Behavioral studies have highlighted the impact of lithium on honey bee activity. Research indicates that lithium exposure decreases walking and increases stillness, suggesting malaise behavior (Hurst et al., 2014). Additionally, investigations into the acute effects of lithium salts on locomotor activity (LMA) have demonstrated dose and salt-specific differences (Sevin et al., 2022). Such findings underscore the importance of evaluating lithium's broader physiological effects on honey bees, including its potential influence on learning and circadian rhythms (Erdem et al., 2023).

1.7 Computational studies on honey bees

There are computerized studies on bee health and behavior in the academic literature. These studies were carried out using sensor data in the field or computer-aided imaging in the laboratory environment.

A field study was established to collect sound data from the hives monitored with thermal cameras (Edwards-Murphy et al., 2015). It has been tried to predict the development of the offspring from the vibration data obtained by recording the sound inside the hive (Bencsik et al., 2007). Another study tried to predict the swarming time by monitoring the temperature in the hive (Zhu et al., 2019). Marchal et al. manipulated and monitored the temperature of the hives. They tried to find correlations with temperature, humidity, light, rain, and wind. They also examined the bees' behavioral changes, such as foraging activities and bee dancing (2020). In another study on foraging activity, RFID tags were attached to the bee individuals. The entries and exits of the individuals were counted with the reader located at the entrance of the hive (Banaets et al., 2017). Also, systems were created to identify the colored tags attached to the individuals treated with different chemicals or diseases (Alaux et al., 2014).

The changes in nursing behavior were found to be due to the neonicotinoid pesticide application using semi-automatic systems. They recorded longitudinal truncated cells in the brood area on a honeycomb section. Nursing behavior is measured with the duration and frequency of bees visiting the larval cell. Then, they found reduced larval care under the effect of clothianidin and thiacloprid (Siefert et al., 2020). Blut et al. (2017) determined behavioral patterns among bee individuals on a computer-based system by barcoding a small number of bees. They determine antennation, begging, offering behavior, and trophallaxis behavior using machine learning implemented in the JAABA program (Kabra et al., 2013). In a later study, many bees were barcoded, and their social behavior patterns changed under the influence of a viral disease. According to this study, significantly fewer trophallaxis interactions and significantly greater distance moved (in millimeters) per hour were observed in Israeli acute paralysis virus-infected bees. Thus, social interaction decreases in the colony under the infection of this virus (Geffre et al., 2020). In a recent study, individuals could be tracked without barcoding (Bozek et al., 2021). Another study solves the question that, while it is unknown how thousands of worker bees can follow a single queen and establish communication. They found that worker

individuals can direct the airflow and deliver pheromones, which mediates communication among workers (Nguyen et al., 2021).

1.8 Aim of the study

In this thesis, it was hypothesized that lithium may influence the LMA, circadian rhythm, and learning in honey bees.

Several studies have demonstrated the diverse effects of lithium on various aspects of behavior and physiology. Lithium has been recognized for its ability to mitigate excessive activity, a hallmark symptom of mania in bipolar disorder (APA, 2022; Alda, 2015).

First, drawing parallels to human behavior, exposure to light induces heightened activity in honey bees (Spangler, 1973), prompting the prediction that lithium could similarly suppress induced activity in this organism. This prediction posited that lithium reduces the elevated light-induced LMA in honey bees.

Furthermore, research suggests that lithium treatment can modulate circadian rhythm parameters, including period length and light sensitivity of the biological clock (Dokucu et al., 2005; Smietango & Engelmann, 1989; Kavaliers, 1981; Welsh & Moore-Ede, 1990; Hofmann et al., 1978; Iwahana et al., 2004; Padiath et al., 2004; Hallam et al., 2005; Karolina et al., 2023). The second prediction is that lithium may influence the light input to the circadian clock in honey bees, thereby regulating circadian activity. According to this prediction, lithium alters circadian rhythm parameters under chronic conditions in a light-dependent manner in honey bee models.

Moreover, despite the extensive research on lithium's effects, uncertainties persist regarding its impact on learning and cognitive function across species. Existing studies on humans, rodents, and other organisms have reported both positive and negative effects on cognitive functions (Kroph & Müller-Oerlinghausen, 1979; Judd et al., 1977; O'Donnell & Gould, 2007; Wu et al., 2001; Vasconcellos et al., 2003;

Richter-Levin et al., 1992; Cappeliez & Moore, 1988; Xia et al., 1997; Hines & Poling, 1984; Tsaltas et al., 2007). Third, it was predicted that lithium would influence the learning success of honey bees, specifically affecting performance in aversive learning accompanied by reversal learning paradigm using electric shock conditioning assay. This prediction will be supported if lithium alters learning performance in the acquisition and/or reversal phases of the electric shock conditioning assay.

Thus, the aim of this study is to answer the following questions are investigated:

1. Does lithium affect the LMA and circadian rhythm in honey bees?
    a. Does lithium affect the LMA under the dark condition?
    b. Does lithium affect the LMA under the light condition?
    c. Does Lithium affect the periodicity of the circadian rhythm?
    d. Does Lithium affect the rhythmicity of circadian rhythm?
2. Does Lithium affect the learning performance of honey bees via aversive learning?

In this study, a computer vision algorithm was created to analyze electric shock conditioning experiments, the experiment method used in avoidance learning, to evaluate learning performance.

CHAPTER 2

MATERIALS AND METHODS

2.1 Sampling

Honey bee (*Apis mellifera*) hives in the apiary of Ankara University's Veterinary Faculty sources were used in this study. Hive entrances were blocked with plastic wire meshes. Returning forager bees that could not enter the hive congregated on the wire meshes and were collected into small containers (Scheiner, et al., 2013). Samples were from at least three hives and pooled for each analysis to mitigate the colony effect.

Although scientific experiments on honey bees in Türkiye are not subject to specific regulations, this study was conducted with the commitment to minimize the harm and ethical concerns.

2.2 Methods of LMA and circadian rhythm experiments

2.2.1 Acute experiments

A mortality rate of around %50 was observed in the group of bees fed one time 10 µl sucrose solution with 450 mM LiCl after 24 hours. Then, the highest dose was determined, and other doses were adjusted by reducing them by one-third of the highest dose. Sucrose solution (50% w/v) containing 50, 150, and 450 mM LiCl for treatments and a pure sucrose solution (50% w/v) for the control group were prepared at room temperature. In addition, the 450 mM NaCl treatment was used as a control for the hyper-osmotic stress that the 450 mM salt solution may cause. Bees were fed

individually 10 µl of solutions. The 10 µl solution was dropped into a small-sized stainless steel laboratory spoon via a micropipette. A cotton swab dipped in the sucrose solution was touched on the honey bee's antenna for the bee to elongate its proboscis. Then, bees were fed with the solution that was found inside the laboratory spoon. Thus, it was made sure that the bee drank all the solution.

The LMA experiment was performed as described in previous studies (Tackenberg et al., 2020;, Giannoni-Guzman et al., 2014). After treatments, honey bees of experimental groups were individually transferred into perforated 15 ml falcon tubes. A piece of fondant sugar was placed into the cap of each tube as food and covered with two layers of cheesecloth to prevent the sticking of bees to the fondant sugar. The LMA monitoring device (Figure A.1) produced by Trikinetics Inc (TriKinetics Inc, Waltham, MA, USA) was used in this study. Four modules were used with 24 holes to hold a single 15 ml falcon tube. Each falcon tube includes a single bee. Thus, the sample size for each experiment group was 24. The three infrared sensors around each hole gave a positive signal when the bee in the Falcon tube moved through the section with the beams (Rosato & Kyriacou, 2006). An environmental monitor was also attached to the system, constantly measuring and recording temperature, light, and humidity levels. The LMA monitoring modules were incubated at 33 °C and 65% humidity.

The LMAs of bees in treatment and control groups were measured for 24 hours in two experiments, one in constant darkness and another in a constant light environment.

2.2.2 Chronic experiments

The chronic LMA experiment was performed according to previous studies (Tackenberg et al., 2020; Giannoni-Guzman et al., 2014). LiCl was delivered to the bees in a piece of custom-made bee candy. The bee candy was placed into the cap of each perforated 15 ml falcon tube and covered with two sheets of cheesecloth. Bee

candy consisted of 10 parts of honey, 54 parts of powdered sugar, and 2 parts of distilled water in grams. Designated amounts of LiCl were dissolved in the water fraction of treatment groups (1, 5, and 10 mmol/kg of LiCl). Collected forager honey bees were put into falcon tubes individually. For each group, 32 bees were used. The falcon tubes were put into the modules of the LMA monitoring device. A water system was assembled into the modules of the LMA monitoring device. The water system consisted of 30-centimeter PVC (Polyvinyl chloride) pipes filled with water. The plastic straws inserted into these pipes entered the falcon tubes' rear end. The water was delivered to the bees through the filter papers placed in the plastic straws. Previous studies also used the same system (Tackenberg et al., 2020; Giannoni-Guzman et al., 2014). The chronic LMA experiment was performed under the same conditions (33 °C, 65% humidity) for 15 days with 5 days in a 12-hour light and dark cycle (light: dark, LD) followed by 5 days in constant darkness (dark: dark, DD) and the final 5 days under continuous illumination (light: light, LL).

2.2.3 Statistical analysis

All statistical analyses were conducted in RStudio with the R 4.3 version (R Core Team, 2020).

The normality of the sample distribution was checked with the Shapiro-Wilk test.

In acute experiments, in the case of the distributions that did not follow the normal distribution, the activity differences across the groups were compared by the non-parametric Kruskal-Wallis test followed by a post-hoc Dunn test. The relationship between the dosages and LMA was examined through a regression analysis. Group differences in mortality were checked via Chi-Square analysis.

In chronic treatment experiments, a survival analysis was done by log-rank test to compare mortality differences. Alteration of activities under the effect of different lithium doses in different light exposure conditions was examined via repeated measure ANOVA tests with eliminated random effects. Then, pairwise comparisons

were applied using the Wilcoxon rank-sum exact test with the Holm adjustment method. Also, a permutation test was used to verify the repeated measure ANOVA test results. Circadian rhythm analysis determining the periodicity and rhythmicity was conducted with a set of R packages called "Rethomics", available at https://rethomics.github.io (Geissmann et al., 2019). The Lomb–Scargle periodogram analysis was used to determine the rhythmic individuals and lengths of the circadian periods. Then, the logistic regression model was used to determine the association between rhythmicity and lithium treatment. The Spearman correlation test was performed to determine the association between the circadian period and lithium treatment for each condition.

2.3 Methods of electric shock avoidance learning experiments

2.3.1 Experiment setup

The experiment setup consisted of a photo studio shooting tent (diffusion softbox), laboratory stand, flat screen, webcam (mobile phone camera or any video recorder may be used), 6 V DC adapter, and electric shock apparatus. The electric shock apparatus was designed based on the apparatus used by Dinges et al. (2013). 25 M3 threaded rods, 48 M3 nuts, two meters of copper wire, transparent acetate papers, threaded rod holders, and the shuttle boxes were used to produce the electric shock apparatus.

The production stages of the electric shock apparatus was as follows (Figure 2.1):

1. Print rod holders and shuttle boxes (3D models was available in the Zenodo repository: https://doi.org/10.5281/zenodo.13375378).
2. Cut the rods using side cutters according to the screen size.
3. Insert the rods into the rod holders.

4. Place the nuts on the end of the rods, skipping the rods one by one. Do not place any nut on the middlemost rod. Make sure only one end of each rod is nutted.
5. Cut the copper wire into four half-meter pieces. Wrap it around the ends of the nutted rods. Ensure that the copper wire does not come into contact with rods that do not have nuts on the end.
6. Place another set of nuts on the ends of the nutted rods and turn the nuts so that the copper wire is tightly sandwiched between the two nuts.
7. Cut the acetate paper to 17 x 170 mm and insert it into the slide of the shuttle box.
8. Apply a thin layer of Vaseline to both the inside surface of the shuttle box and the detachable acetate paper roof. (Vaseline prevents bees from climbing to the walls and losing contact with the electric grid).

Configuration of experiment setup (3D illustrations of experiment setup was available in the Zenodo repository: https://doi.org/10.5281/zenodo.13375378):

1. Mount the webcam to the laboratory stand.
2. Place the flat screen parallel to the floor.
3. Move the lab stand close to the screen. The camera should see the entire screen surface. It should be centered and perpendicular to the screen.
4. Put the electric shock apparatus on the screen. The middlemost rod of the shock grid should align with the separation of the colors representing the shock and safe area reflected on the screen.
5. Line up the shuttle boxes on the electrical grid.
6. The experiment setup should be placed inside the photo studio shooting tent (diffusion softbox) to ensure homogeneous light conditions and prevent reflections and shadows.
7. Connect the positive and negative outputs of the adapter to the copper wires on the side of the electrical grid. A DC jack to alligator clip power adapter cable may also be used.

Transfer the bees into the shuttle boxes. Each shuttle box should contain a single bee that could freely traverse between the shock and safe areas. Then, plug the adapter into the socket.

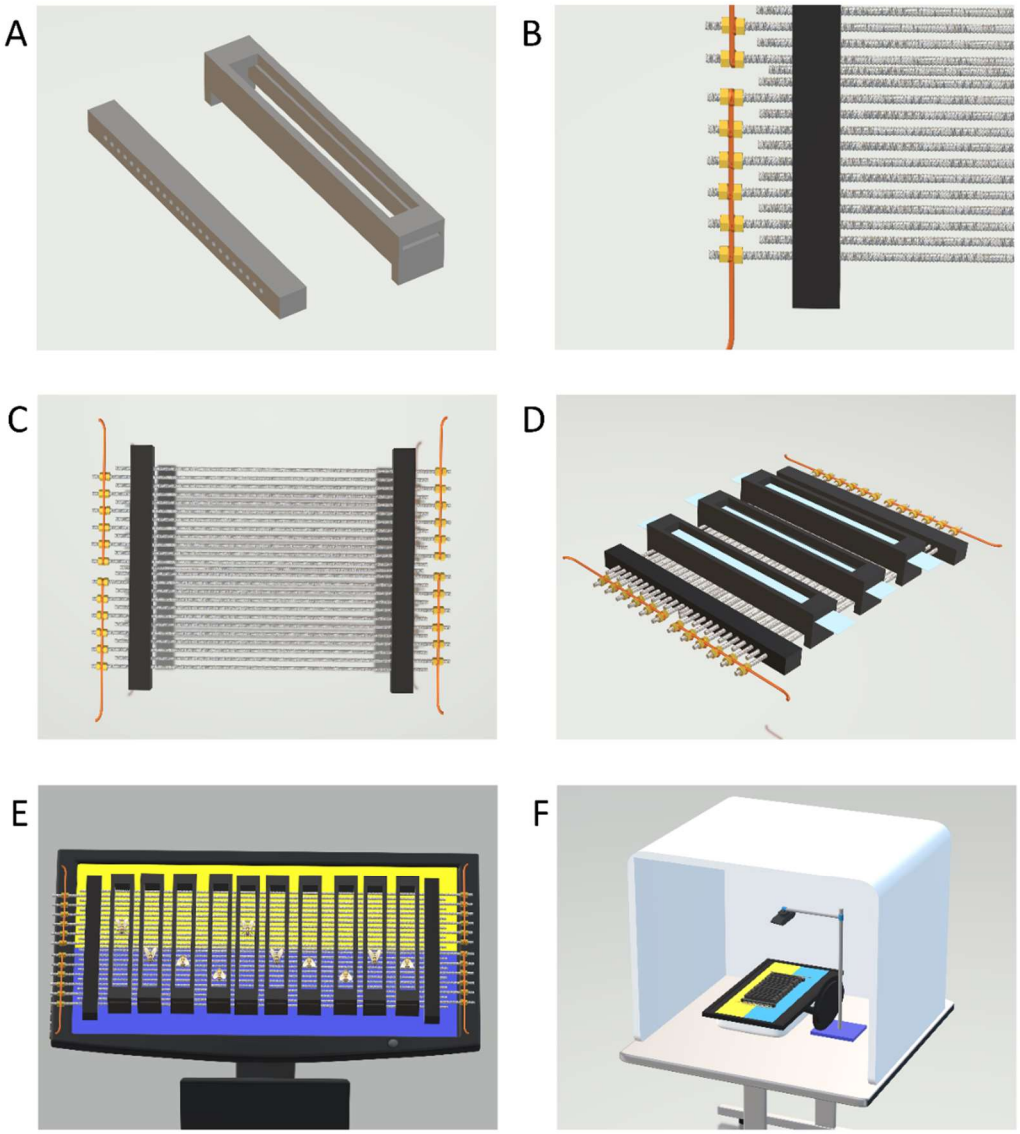

Figure 2.1 3D-printed components for the electric shock apparatus, including rod holders and shuttle boxes (A). Close-up view of the rod and copper wire assembly to build the electric grid (B). Top view of the assembled electric shock grid (C). Perspective view of the completed electric shock apparatus with shuttle boxes

aligned on the grid (D). The electric shock apparatus was placed on the flat screen display showing the delineation of shock and safe areas (E). The fully assembled experiment setup was placed inside a photo studio shooting tent to ensure homogeneous lighting conditions (F).

2.3.2 Experimental protocol

Collected forager bees were individually placed into separate hoarding cages. Each cage received sustenance from an overhead syringe filled with a 50% (w/v) sucrose solution containing a predetermined quantity of LiCl. Four experimental groups were formed: the low-dose group (5 mM LiCl), the medium-dose group (25 mM LiCl), the high-dose group (125 mM LiCl), and the control group (sucrose-only). The doses were determined by taking the square and square root of 25 mM, used in previous studies (Ziegelmann et al., 2018; Kolics et al., 2021b; Rein et al., 2022). The feeding regimen was upheld overnight for a total of 16 hours. The hoarding cages were kept in an incubator at a constant temperature of 33 °C and a relative humidity of 65%, all within a controlled dark environment. The experiment groups were then subjected to the ESAA.

An LG Flatron W2243S computer monitor displayed one side as blue (hex code: #376092) and the other as yellow (hex code: #FFFF00). A Logitech C920 webcam captured experiment video records, and a Godox DF-01 portable diffusion box (70x70x70 cm) was used to place the experimental setup (Figure A.6).

Experiments were conducted under ambient light and room temperature. The experimental procedure was as follows:

1. Habituation: Bees were introduced to the experimental setup, and the computer monitor was turned off for an initial habituation period of 10 minutes.

2. Acquisition phase: One of the colors was designated as the shock side (CS+), and the electric current was channeled to the CS+ side for 5 minutes.
3. Interval between phases: The electric current was deactivated, and the monitor was closed to allow the bees to rest for 10 minutes.
4. Reversal phase: The shock sides were switched, with the opposite color as the new CS+. This reversal phase also persisted for 5 minutes.

To ensure counterbalancing, the color assignments of CS+ and CS- were reversed for subsequent batches of bees. Multiple batches of bees were analyzed, with each group comprising at least two counterbalanced batches. The number of bees at the beginning of the experiment was 33, 36, 36, and 32 in the control, low-dose, medium-dose, and high-dose groups, respectively. If a bee was completely motionless throughout the experiment or the tracking code incorrectly pointed to the bee, those bees were not included in the statistical analysis. As a result of these eliminations, the sample sizes were 27, 33, 31, and 28 in the control, low-dose, medium-dose, and high-dose groups, respectively.

2.3.3 Statistical analysis

Experiment videos were processed using the Api-TRACE system (see Chapter 4.2), was developed to obtain the data required for statistics. All statistical analyses were conducted in RStudio with the R 4.3 version (R Core Team, 2020). The normality of the sample distributions was checked with the Shapiro-Wilk test. The electric shock experiment results were evaluated with a linear mixed-effects model (LMEM), which allows within-group errors, using the "*lme*" function from the "*nlme*" package. In addition, the Spearman correlation test was used to determine the association between the shock duration and increasing doses of lithium for the acquisition and reversal phases.

CHAPTER 3

EFFECTS OF LITHIUM ON LOCOMOTOR ACTIVITY AND CIRCADIAN RHYTHM OF HONEY BEES

This chapter was previously published as an article titled 'Lithium on Locomotor Activity and Circadian Rhythm of Honey Bees' in Scientific Reports in 2023.

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3.1 Introduction

Varroa is a devastating parasite of honey bees. The lethal effect of lithium on *Varroa* was surprisingly discovered by a research group while attempting to find an iRNA-based treatment for *Varroa* control (Ziegelmann et al., 2018). The lethal effect of lithium on *Varroa* was considered promising since when bees fed on a 10 µl sucrose solution with a concentration of at least 25 mM LiCl, a 24-hour application had no effect on bee survival. However, chronic feeding results in reduced honey bee lifespan (Ziegelmann et al., 2018). Lithium is not yet used as an anti-*Varroa* agent, and further studies on lithium's effect on honey bees are warranted.

Studies on lithium's effect on bee health demonstrated positive and negative impacts. Lithium treatment reduced viral load and mitigated oxidative stress by altering gene expression (Jovanovic et al., 2022). Other studies reported toxicity of the residual lithium on honey bee colonies (Kolics et al., 2021a, Kolics et al., 2021b) and significant brood damage (Rein et al., 2022). Also, lithium applied to honey bee colonies was found to accumulate in all compartments and members of the hive (Presern et al., 2020). It is important then, to consider the sublethal effects of lithium, for instance, on honey bee behavior. Currently, few reports are available on the behavioral effects of lithium applications on honey bees. One behavioral study

investigated lithium-induced malaise behavior in honey bees: injection or ingestion of LiCl decreased walking and increased stillness (Hurst et al., 2014). Only one study examined the acute effect of lithium salts on locomotor activity (LMA), demonstrating differences based on the dose and type of lithium salt used (Sevin et al., 2022).

Studies on the behavioral effects of lithium on bees may also be informative for lithium's actions as a drug. In medicine, lithium is known as the first-line treatment for bipolar disorder because of its mood-stabilizing activity and low toxicity (Alda et al., 2015). In this study, the effect of lithium on LMA and honey bees' circadian rhythm were examined. It was hypothesized that the lithium effect on LMA and circadian rhythms will depend on the light environment and reflect the moderating effect of lithium on bipolar disorder symptoms.

Repetitive shifts between unusual mood levels, such as depression and mania, characterize bipolar disorder. Irritable mood, abnormally and persistently above-average activity or energy, and reduced need for sleep are observed in the manic episode (APA, 2022). In addition to spikes of activity, circadian rhythm abnormalities accompany bipolar disorder (McClung et al., 2007). Lithium stabilizes activity levels in individuals with bipolar disorder. Furthermore, lithium treatment decelerates the fast circadian clock observed in individuals with bipolar disorder (Atkinson et al., 1975; Kripke et al., 1978). However, lithium's action mechanism remains only partially understood, and the studies investigating lithium's effect on LMA and circadian rhythm provide limited answers (Dokucu et al., 2005). That limitation is partly due to the lack of suitable animal models for bipolar disorder (Alda et al., 2015).

At times, honey bees and fruit flies serve as insect models for human disorders. Multiple models, due to nuances of molecular, neural, and developmental substrates, result in improved fundamental understanding. For instance, in the case of the circadian clock, the honey bee insect model has a circadian rhythm ontogeny that is similar to the humans. Post-embryonic development of the circadian clock has been

observed in bees, as in human infants (Meshi & Bloch, 2007). In addition, bees have a protein called pteropsin. This protein is more closely related to vertebrate opsins than to invertebrate opsins. Vertebrate opsins are involved in circadian rhythm regulation (Velarde et al., 2005). Also, bees lack *Drosophila* cryptochrome; instead, bees encode an ortholog of the two mammalian cryptochromes (Velarde et al., 2005). These studies suggest bees are more similar to vertebrates than other insects in terms of ontogeny and molecular mechanisms of circadian rhythms.

Until now, no studies have been conducted on the effects of lithium on the circadian rhythms of honey bees. For lithium, research on the fly model indicated lengthening of the circadian period, increasing longevity, and positive effects on locomotion via *Shaggy* (*Sgg*, insect orthologue of vertebrate *Gsk-3*) inhibition (Jans et al., 2021). According to a study conducted on fruit flies, concentrations of 20 mM and 30 mM LiCl significantly extended the period of circadian rhythm to 24.02 hours and 24.4 hours, respectively, when compared to the control group, which maintained a period of 23.7 hours. Additionally, lithium increased arrhythmicity, particularly at doses above 5 mM, and the arrhythmicity ratio exceeded 50% at 60 mM application (Dokucu et al., 2005). Another study on fruit flies indicated that the period of the circadian rhythm increased *Sgg* loss of function mutants (Martinek et al., 2001). In a study examining longevity and locomotion, the fruit fly life expectancy increased with *Sgg* inhibition following the application of 1–25 mM LiCl. In addition, the treatment ameliorated the decrease in aging-dependent LMA (Castillo-Quan et al., 2016). It is known that the disruption of circadian rhythm is frequently observed in humans with bipolar disorder (Melo et al., 2017). Bipolar disorder-driven sleep latency alterations and a reversal in the sleep/wake period were observed in humans (Giglio et al., 2010). As in fruit flies, circadian rhythm phase anomalies in humans were shown to be modified with lithium treatment acting as an *Sgg* inhibitor (Escamilla & Zavala, 2008). It is important to contrast the effects of lithium on flies to the effects of lithium on bees.

Honey bees have already provided valuable insights into various complex behaviors with parallels to human behavior. For instance, cocaine affected the reward

perception and withdrawal-like responses, reducing the learning ability of honey bees during the withdrawal period as in humans (Barron et al., 2009). In another example, honey bees were successfully employed as an ethanol-abuse model (Mixson et al., 2010). Dopamine, a biological amine common to humans and bees, was found to affect learning and motivation in honey bees (Agarwal et al., 2011). A remarkable study showed mechanistically relevant behavioral similarities between humans and honey bees. The study indicated that socially unresponsive worker bees had similar gene expression patterns for the homologous versions of the autism-related genes in humans (Shpigler et al., 2019). These diverse neural genetic substrates of behavior make the honey bee a good model animal for lithium research on circadian rhythm.

Honey bees were suggested as an informative and relevant insect model for investigating lithium's effects on LMA, circadian rhythm, and behavior. The bee model is informative because circadian rhythm regulation has become a prominent research focus within the field of bee studies. These studies show honey bee circadian rhythms and locomotion are sensitive to light regimes and other time givers (Meshi & Bloch 2007; Velarde et al., 2005; Spangler, 1973). The model is relevant because circadian regulation of bee activity is important for foraging and pollination. It is known that foraging activity is modified based on the time flowers provide nectar during the day (Buttel-Reepen, 1915), resulting in the maximization of collected resources. Daylight is also significant for accurately navigating to and from nectar and pollen resources and communicating their location to nest mates using the sun-compass orientation that is tuned to the intrinsic circadian clock of the bee (Bloch et al., 2017). Lastly, the circadian rhythm of honey bees is susceptible to chemical treatments (Tackenberg et al., 2020; Chicas-Moiser et al., 2018) bees face in their environment and due to human activities.

It is known that the increase in excessive activity, an important component of mania, which is the most prominent symptom of bipolar disorder, is balanced by lithium (APA, 2022; Alda, 2015). In the honey bee model, exposure to light leads to higher activity (Spangler, 1973). It is hypothesized that lithium will inhibit induced activity

also in the honey bee model. This suppression of induced activity hypothesis will be supported if lithium reduces the elevated LMA induced by light in honey bees. Secondly, previous studies have shown that lithium treatment changes circadian rhythm parameters such as period length (Dokucu et al., 2005; Smietango & Engelmann, 1989), rhythmicity, and light sensitivity of the clock (Kavaliers, 1981; Welsh & Moore-Ede, 1990; Hofmann et al., 1978; Iwahana et al., 2004; Padiath et al, 2004; Hallam et al., 2005; Karolina et al., 2023). It is hypothesized that lithium will affect the light input to the biological clock in the honey bee also. This regulation of circadian activity hypothesis will be supported if lithium alters the parameters of circadian rhythm under chronic conditions in a light-dependent manner in the honey bee model.

In order to test the hypotheses, light conditions were manipulated in acute and chronic lithium exposure experiments to investigate light-induced LMA and circadian rhythm changes. The results were discussed in relation to suppression of induced activity and regulation of circadian rhythmicity hypotheses and compare the results to data from bipolar disorder models and humans.

3.2 Results

3.2.1 Acute effects of lithium on locomotor activity

In acute LMA trials, three doses of LiCl (low, medium, and high) and one dose of NaCl with a control group were used. The low, medium, and high doses of LiCl were 50 mM, 150 mM, and 450 mM, respectively. The concentration of the NaCl applied in the acute LMA experiment was 450 mM.

The LMA measurements did not follow the normal distribution (Shapiro-Wilk test, $p < .05$). A non-parametric Kruskal-Wallis test was used to compare the treatment groups for light and dark environment experiments. In addition, descriptive statistics are found in Table A.1.

First, the LMA differences were compared in the dark environment experiment. There were no significant differences among groups (Kruskal-Wallis test: H (4) = 9.11, p = .060; Figure 3.1.a).

Next, the LMA of the groups was compared in the light environment. Significant differences were not found according to Kruskal-Wallis test (H (4) = 16.86, p < .001; Figure 3.1.b). A post-hoc Dunn test indicated that the control group differed from the medium (p = .004) and high-dose groups (p < .001); similarly, the NaCl group varied from the medium (p = .004) and high-dose groups (p < .001), and there was no difference observed between control and NaCl groups (p = .486).

Then, the relationship between doses of LiCl (the NaCl group was excluded) and LMA was investigated using regression analysis. In dark environment experiments, a significant association between dosages of lithium and activity was not found (R^2 = .0001, F (1, 92) = .01, p = .916) (Figure 3.1.a). However, the regression analysis indicated a significant association between dosages of lithium and activity (R^2 = .1, F (1, 84) = 9.18, p = .003) in the light environment experiments (Figure 3.1.b). As a result, increasing the dosage lowered the activity in the light environment but did not affect activity in the dark environment.

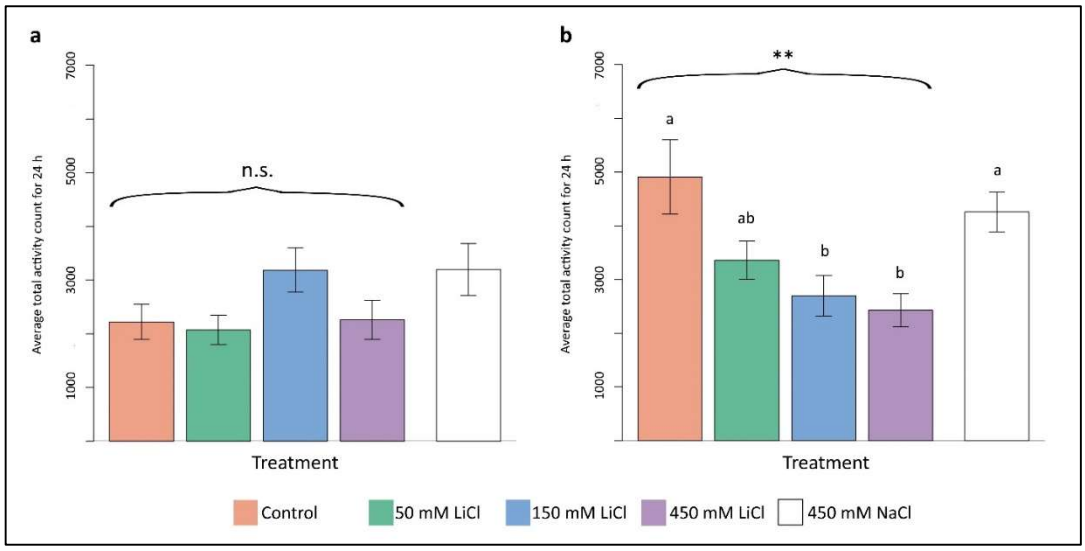

Figure 3.1 The figure illustrates the comparisons of LMA in response to acute lithium administration experiments conducted in both dark (a) and light (b) environments. In cases where a significant difference was detected among the groups according to the Kruskal - Wallis test followed by a post-hoc Dunn test, letters were added to the bars. When the same letters are present, it signifies that there is no statistically significant difference between the groups. Curly brackets are employed to convey the results of regression analysis. Asterisks denote the statistical significance level with the following thresholds: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and n.s. indicating that it is not significant. The data is presented as the mean of the total activity count with ± standard error.

In addition, a chi-squared test was applied to find any difference across all groups, including both dark and light environment experiments regarding the mortality ratio in the groups. The test indicated a difference across the groups (χ^2 (9, 263) = 66.40, $p < .001$). The high-dose group in the dark environment had a higher death ratio (0.48 in the dark condition and 0.17 in the light condition). This higher death ratio was an outlier when the mortality ratios of all experimental groups in both dark and light conditions were compared (Figure A.2, Figure A.3). It has been difficult to causally explain high mortality only in this one group.

3.2.2 Chronic effects of lithium on locomotor activity and circadian rhythm

In chronic LMA experiments, three doses were compared: low (1 mmol/kg), medium (5 mmol/kg), and high (10 mmol/kg), and a control group (no lithium administered).

First, the effect of lithium on survival was analyzed. According to the survival analysis, the high-dose group had a lower survival rate (0.34) than other groups (control: 0.63, low: 0.56, medium: 0.63) on the 15th day. The difference between the groups was indicated by a log-rank test ($\chi 2 = 7.9$, $p = 0.05$) (Figure A.4). Still, according to pairwise comparisons, none of the lithium groups differs from the control ($p > .05$) (Table A.3).

Second, the alteration of locomotor activities was compared under the effect of different doses in different conditions (Figure 3.2.a). A repeated measure ANOVA test was used with eliminated random effects. Significant results were found for conditions (F (2, 34) = 8.54, $p < .001$), doses (F (3, 138) = 4.15, $p = .008$), and interaction effect (F (6, 138) = 2.69, $p = .017$). A permutation test was applied to the model because, according to the Shapiro-Wilk test, the distribution was not normal ($p < .05$). The permutation test confirmed the repeated measures ANOVA test results for conditions (B = 5000, $p = .004$), doses (B = 5000, $p = .041$), and interaction effect (B = 5000, $p = .009$). Then, pairwise comparisons using the Wilcoxon rank-sum exact test with the Holm adjustment method were performed. No difference ($p > .05$) was observed among the low and medium-dose groups across LD, DD, and LL conditions. A slight decrease was observed in the DD condition compared to the LD condition for the high-dose group ($p < .05$). An apparent increase was found in the control group in the LL condition. The LMA measured for the control group in the LL condition, and it was significantly higher than all other LMA measures of all conditions and all doses ($p < .05$) except the low dose LMA during the LL condition ($p > .05$) (Table A.4, Figure 3.2.b) (Exact p values of the comparison for all pairs according to Wilcoxon test were given in supplement file, Table A.5).

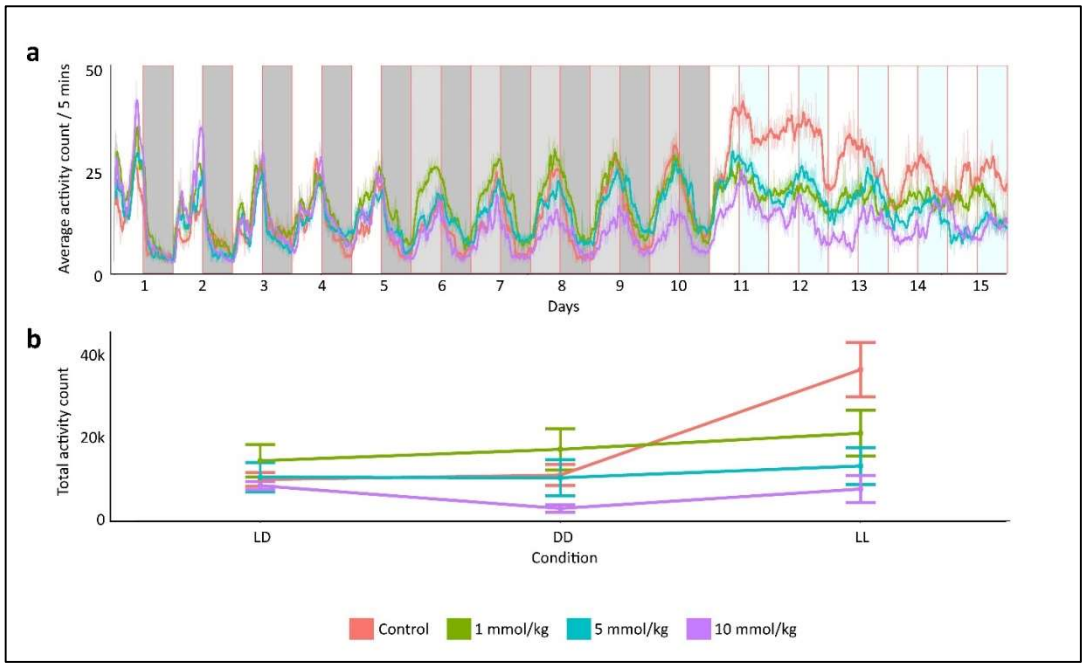

Figure 3.2 The activity graph illustrates fluctuations in LMA throughout the chronic lithium administration experiment. Dark or light grey shading indicates dark conditions, while white or light blue shading represents light conditions. These shaded areas divide the plots into 12-hour intervals. A moving average filter was applied to smoothen the data, which considered 12 data points at a time as input, resulting in an average activity computed for every 60-minute interval (a). The total activity count is depicted for LD, DD, and LL, each lasting 5 days. The data is presented as the mean of the total activity count along with the ± standard error (b).

Third, the rhythmicity of circadian rhythm ratios for each condition was compared according to the Lomb–Scargle periodogram analysis result. The ratios of rhythmic individuals were found in the LD condition for control, low, medium, and high dose groups to be 1, 0.97, 0.90, and 1, respectively. In the DD condition, 0.96, 0.96, 0.93, and 0.75. In the LL condition, 0.85, 0.83, 0.70, and 0.73, respectively (Table A.6, Figure 3.3.a). The association between rhythmicity and lithium treatment was inspected in each condition using the logistic regression model. Each bee's rhythmicity is measured as a binary indicator (the value is 1 if the bee is rhythmic

and 0 otherwise). A significant association in the DD condition ($\chi 2$ (3, 93) = 5.21, p = .023) was found but it was not in LD ($\chi 2$ (3, 110) = 0.001, p = .974) and LL ($\chi 2$ (3, 67) = 1.20, p = .273). Thus, rhythmicity significantly decreased in the DD condition with increasing dose. In addition, although not statistically significant, a noticeable decrease was observed in the LL condition with increasing doses of LiCl. (Table A.6, Figure 3.3.a).

Lastly, a Lomb–Scargle periodogram analysis was applied to determine the statistics of rhythmic individuals. According to periodogram analysis, the median of the periods of the circadian rhythm with an interquartile range (Q1 – Q3) were determined in hours. In the LD condition, the periods were 24.31 (23.90 – 24.84) for control, 24.36 (23.76 – 25.05) for low dose, 24.31 (23.95 – 24.39) for medium-dose, and 24.34 (24.08 – 24.57) for high dose. In the DD condition, the periods were 23.75 (23.65 – 24.41) for control, 23.80 (23.58 – 24.68) for low dose, 23.80 (23.17 – 25.03) for medium dose, 24.05 (23.74 – 24.41) for high dose. In the LL condition, the periods were 24.15 (22.62 – 25.05) for control, 25.00 (24.26 – 27.20) for low dose, 26.42 (24.56 – 27.20) for medium dose, and 27.77 (27.12- 27.96) for high dose (Figure 3.3.b). Then, the relationship between the periodicity and lithium treatment was investigated in each condition. A non-parametric Spearman correlation test was used because the normal distribution was not met according to the Shapiro-Wilk test ($p < .05$). There was no correlation in LD (r (106) = .003, p = .974), and DD (r (84) = .01, p = .908) conditions. However, a positive correlation was found in the LL condition (r (58) = .47, $p < .001$). Lithium affected the periodicity under the constant light condition (Table A.6, Figure 3.3.b).

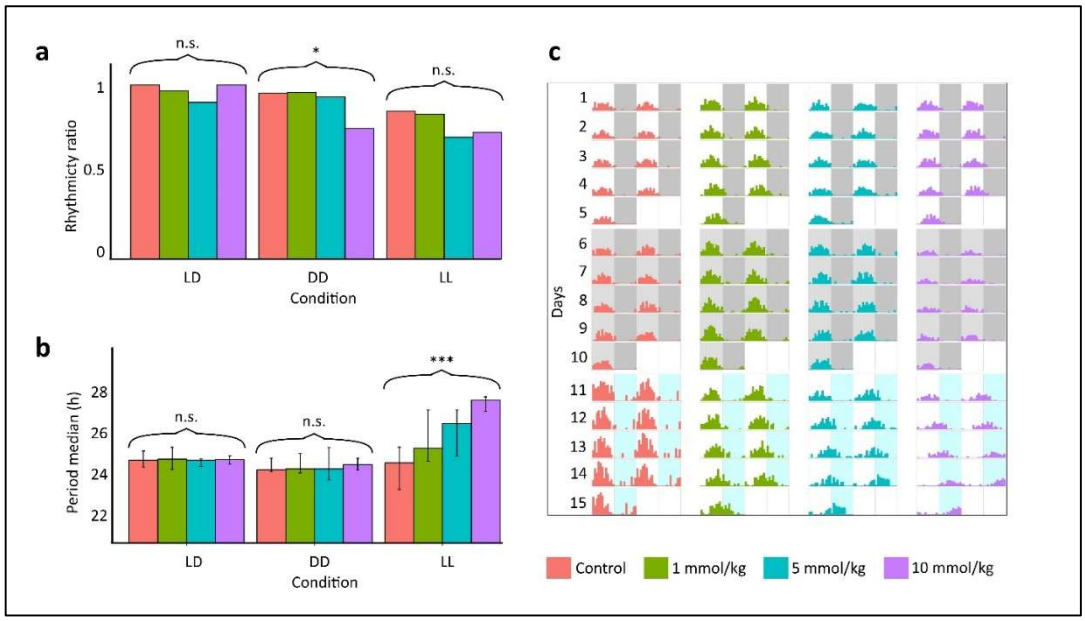

Figure 3.3 The plots represent the ratio of rhythmic individuals (a) and the period (in hours) of the circadian rhythm (b). Double-plotted actograms of representative individuals were generated using the sine wave function (Equation 1). Dark or light grey shaded as indicate darkness, white or light blue shaded areas indicate light periods, and the areas divide the plots into 12-hour intervals (c). Curly brackets were used to represent the result of the logistic regression model for rhythmicity and the Spearman correlation for periodicity in the different conditions. Asterisks indicate the level of statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$, n.s. not significant. Error bars showing interquartile range.

Additionally, the circadian period length was compared for each dose across conditions. According to the Kruskal-Wallis test, the light condition was not effective on the length of the circadian period for the control (H (2) = 0.98, p = .61) and low dose group (H (2) = 4.18, p = .12). Effect of the light condition on circadian period length appeared in medium (H (2) = 11.40, p < .001) and high dose groups (H (2) = 19.70, p < .001). Post-hoc Dunn test indicated the length of the circadian period in LL condition differed from LD and DD conditions for both medium (p = .002 and p < .001) and high dose (p < .001 and p < .001) groups. Thus, period length is only increased by the constant light condition for medium and high doses (Figure A.5).

Double-plotted actograms of representative individuals were generated for visualization of the LMA of each group in all conditions. They are created with the *sine wave* function (Equation 1):

$$y(t) = A * \sin(2\pi f + \varphi) + D$$

Amplitude (*A*), center amplitude (*D*), and frequency (*f*) variables were tuned according to the LMA averages and lengths of the circadian period of each dose group in each condition, also, arrhythmicities were added as noise (Figure 3.3.c).

3.3 Discussion

The principal finding of this study was that lithium altered honey bees' LMA and circadian rhythms as predicted by the suppression of induced activity and regulation of circadian activity hypotheses. According to the acute lithium administration experiment results, lithium treatment lowered the light-induced high activity in honey bees yet did not affect the low activity observed under dark conditions (Figure 3.1). Similarly, in the chronic experiment, lithium decreased the elevated locomotor activity in the constant light condition. (Figure 3.2). The effect of lithium on rhythmicity and the circadian period was also examined in the chronic lithium administration experiment. The lithium treatment disrupted rhythmicity significantly by lowering the rhythmicity ratio in the DD condition. Additionally, a decreasing trend in the rhythmicity ratio under the LL condition was observed. The circadian period was not affected by different light conditions in the control group, yet it lengthened with lithium treatment under the constant light condition.

In this study, constant light triggered a higher activity, as reported before (Spangler, 1973). It was likened to mania-like behavior. In other bipolar disorder models, different methods were applied to increase the activity to develop a mania-like state:

paradoxical sleep deprivation (Armani et al., 2012), drugs (Smith, 1980), and mutations in the *Clk* gene, which is important for circadian rhythms (Roybal et al., 2007) have been used to trigger mania-like activity. Lithium decreased the elevated activity in all three studies (Armani et al., 2012; Simith, 1980; Roybal et al, 2007). In this study, similar to previous studies, lithium treatment reduced bees' elevated activity under acute and chronic light conditions when compared to control. In contrast, control and lithium-treated bees exhibited similar, lower locomotor activity under dark conditions. This observation is consistent with lithium maintenance trials in patients with bipolar disorder, where lithium is primarily effective against mania (Tondo et al., 2019; Bowden et al., 2003; Calabrese et al., 2003).

In addition to effects on locomotor activity levels, lithium also impacts the strength of circadian rhythms and the length of the circadian period. The lithium studies have shown that lower rhythmicity was observed in fruit flies in DD (Dokucu et al., 2005) and house flies (*Musca domestica*) under low light (Smietanko & Engelmann, 1989). In this study, the disruptive effect of lithium on bees' rhythmicity was also observed. The possible molecular mechanism underlying this observation may also be attributed to lithium inhibition of the activity of vertebrate *Gsk-3* ortholog *Sgg*, which is related to regulating signal transduction, xenobiotic stress resistance, and neuronal health as it was proposed for fruit flies (Jans et al., 2021; Stoleru et al., 2007).

Results of long-term experiments on diurnal vertebrates such as goldfish (*Carassius auratus*), and squirrel monkeys (*Saimiri sciureus*) have found a lengthening of the circadian period with lithium treatment, and these were measured in the LL condition (Kavaliers, 1981; Welsh & Moore-Ede, 1990). In this study, an increase in the circadian period was observed only in the LL condition. In contrast to this study, the period has been shown to lengthen under the DD condition in other insects subjected to lithium treatment. In previous investigations, lithium has been shown to affect the circadian period length in various organisms, including nocturnal mammals such as mice and hamsters (*Mesocricetus auratus*), as well as insects such as cockroaches (*Leucophaea maderae*), fruit flies, and house flies, under DD or weak red light

conditions (Dokucu et al., 2005; Smietanko & Engelmann, 1989; Hoffman et al., 1978; Iwahana et al., 2004; Padiath et al., 2004).

The lengthening of the circadian period under constant light in insects is widely accepted (Aschoff rule) (Aschoff, 1979). Surprisingly, a statistically significant increase in the period under the LL condition (24.15 hours) was not observed when compared to the DD condition (23.8 hours) in the control group. The results suggest that honey bees may differ from other insects in their circadian period regulation. Honey bees carry the mammalian-type *Cryptochrome* gene *Cry2* but lack the *Cry1* and *Tim1* genes, which have been shown to regulate circadian rhythm in other insects (Beer & Bloch, 2020). The absence of these interactions in honey bees may be why a lengthening in the circadian period in DD condition with lithium treatment was not observed. However, instead of light-sensitive *Cry1*, vertebrate-like opsin called pteropsin was found to be predominant in honey bees (Velarde et al., 2005).

In fact, similar to bees, *Cry1* loss-of-activity mutant fruit flies did not exhibit period lengthening in the LL conditions (Emery et al., 2000). The absence of *Cry1* in honey bees may explain why period lengthening in the control group under constant light conditions (LL) was not observed. The inactivation of *Sgg* was reported to cause circadian period lengthening under DD conditions (Martinek et al., 2001), and *Sgg* was also found to regulate the *Cry1* and *Tim1* genes in fruit flies (Smith, 1980). Therefore, it was proposed that, as observed in diurnal vertebrates, the interactions between SGG and pteropsin might cause the circadian period lengthening with lithium treatment under the LL condition in bees.

As a caveat, the results of any study on bees in isolation may be confounded by the absence of social interactions. However, using control groups under similar isolation conditions leads to useful mechanistic insights in honey bee studies. For instance, research has shed light on the development of circadian rhythms (Bloch, 2010), the role of temperature in training the circadian clock (Giannoni-Guzman et al., 2014), and the toxicity of particular compounds (Tackenberg et al., 2020; Chicas-Moiser et al., 2018). This study employed the same LMA monitoring device that had

demonstrated reliability in previous research (Tackenberg et al., 2020; Giannoni-Guzman et al., 2014). In the acute lithium administration experiment, the duration of social isolation was only one day. Under different light conditions, controls and salt treatment groups exhibited similar LMA to each other and differed from the lithium treatment. To conclude that increased LMA under constant light and its suppression by lithium is independent of any potential social isolation effects. In addition, the outcome of the chronic lithium administration experiment (15-day social isolation) is consistent with the outcome of the acute administration (1-day social isolation), even though the social isolation periods were very different. In both experiments, an increase in LMA under constant light were observed. Additionally, upon the start of the LL condition in the chronic experiment, a sharp increase in LMA was observed (Figure 3.2.a). Possibly, LMA was affected by constant light and lithium, and the effects were not related to social stress.

This study also provides data on the safety of the recommended acaricide dose of LiCl. A significant increase in the mortality of *Varroa* was observed in the group of bees that were individually fed 10 µl sucrose solution with 25 mM LiCl (Ziegelmann et al., 2018). Even though the low dose (50 mM) in acute lithium administration experiments was twice the effective dose on *Varroa*, it did not affect LMA. In the chronic lithium administration experiments, even at the highest dose (10 mmol/kg corresponds to about 70 ppm), there was no observable effect either in LMA or in the rhythmicity and periodicity of the circadian rhythm for the first five days under the LD condition. The highest dose in the chronic lithium administration experiment was 3.5 times the effective dose for managing *Varroa* (25 mM LiCl in 1:1 sucrose syrup corresponds to 19.88 ppm), and it was administered to the bees *ad libitum*. Thus, the results support the suggested safe LiCl dose to combat *Varroa*. Although conclusive data on LMA and circadian rhythm in adult bees, there is still a need to further determine the effect of lithium on bee behavior. For future research on honey bees, it will be important to consider the unique circadian rhythm ontogeny of bees, driven by age and social environment. In honey bees, the rhythmicity development depends on the worker bees' roles in the colony. Young nurse bees do

not display circadian rhythms when in contact with the brood. In contrast, older forager bees exhibit robust circadian oscillations in the expression of circadian-related genes such as *Per*, *Cry2*, *Tim2*, and *Clk* (Bloch, 2010).

In conclusion, this research highlights that lithium's impact on honey bees may have significant implications for bee health. The safe acaricidal use of lithium is especially important for the long-term sustainability of global food production. Lastly, based on the findings and previous reports by other researchers, it was proposed the honey bee as an experimental animal model for lithium effects in bipolar disorder due to similarities between bees and humans in related responses, genes, and behavior (Barron et al., 2009; Mixson et al., 2010; Agarwal et al., 2011; Shpigler et al., 2019).

CHAPTER 4

API-TRACE: A SYSTEM FOR HONEY BEE TRACKING IN A CONSTRAINED ENVIRONMENT TO STUDY BEE LEARNING PROCESS AND THE EFFECT OF LITHIUM ON LEARNING

This chapter was previously published as a pre-print titled “Api-TRACE: A System for Honey Bee Tracking in a Constrained Environment to Study Bee Learning Process and the Effect of Lithium on Learning.” In BioRxiv.

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4.1 Introduction

Honey bees are crucial in agriculture and essential pollinators that significantly enhance crop yields and biodiversity. Determining learning success is essential because associative learning is key in bee foraging behavior, dance communication, and predator avoidance (Hammer & Menzel, 1995; Ings & Chittka, 2008). Learning-related behaviors are vital not only for the individual but also for the colony (Menzel & Müller, 1996). The passive avoidance task is a behavioral experiment that measures an organism's ability to learn to avoid a negative stimulus. The avoidance learning task has been used in various studies on honey bees (Abramson, 1986; Agarwal et al., 2011; Dinges et al., 2013; Avalos et al., 2017). Electric shock avoidance assay (ESAA) is a fundamental experiment method for passive avoidance tasks. The assay has been used to investigate the effect of environmental conditions and pesticide applications on learning.

Furthermore, through the assay learning pathways have been clarified. To quantify the results of this assay, researchers view experiment videos and record the time the bees stay in the shock area individually. In other words, this quantification process

takes time per observer equal to the number of bees in samples multiplied by the duration of the experiment. That becomes highly labor-intensive and introduces possible errors due to observer fatigue. In order to automatize the process, the Api-TRACE: Honey Bee Tracking in Constrained Environments was created to analyze ESAA videos. The Api-TRACE is a computer vision (CV) aided system to track honey bees in a constrained environment. This novel system eliminates labor and possible measurement errors.

Prior research on bee behavior and bee health employed computer-assisted techniques. CV methods were used to determine foraging activity (Ngo et al., 2019; Ngo et al., 2021), behavioral patterns (Blut et al., 2017; Kabra et al., 2013; Bozek et al., 2021), social behavior changes under an infection (Geffre et al., 2020) and nursing behavior affected by neonicotinoid pesticides (Siefert et al., 2020). Thus, the CV algorithms have been a valuable help for the researchers. The Api-TRACE system was suitable, especially for analyzing avoidance learning assays in bees and other animals (Tsaltas et al., 2007; Richter-Levin et al., 1992; Cappeliez & Moore, 1988; Xia et al., 1997; Hines & Poling, 1984; Agarwal et al., 2011; Avalos et al., 2017; Avalos et al., 2021).

In addition, the production of the experiment apparatus has limited the widespread application of ESAAs because it requires professional help. To simplify this, an electric shock apparatus was designed with rods and nuts that can easily be obtained from any hardware store or online shopping site and created 3D printing models for the plastic components. As a result, this learning test can now be easily implemented in related laboratories.

The easy-to-produce experimental setup and the new software were used to evaluate the effect of lithium on learning in bees. The use of lithium in the fight against *Varroa*, a harmful ectoparasite affecting honey bees, began to be considered (Ziegelmann et al., 2018). However, lithium treatment has multifaceted effects on bee health. For example, while lithium reduced viral load and mitigated oxidative stress (Jovanovic et al., 2022), it caused toxicity (Kolics et al., 2021a; Kolics et al.,

2021b) and significant brood damage (Rein et al., 2022). Moreover, lithium treatment affected behaviors such as movement (Hurst et al., 2014), locomotor activity, and circadian rhythms (Sevin et al., 2022; Erdem et al., 2023). Incidentally, in humans, lithium treats bipolar disorder, marked by mood shifts, increased activity, distractibility, and heightened goal-directed behavior (Alda, 2015; American Psychiatric Association, 2022). Lithium also affects learning. In humans, lithium impairs memory in free-recall tasks (Kroph & Müller-Oerlinghausen, 1979), visual-motor function, and processing speed (Judd et al., 1977). In rodents, lithium causes cognitive deficits (Wu et al., 2001), memory issues (Vasconcellos et al., 2003), and learning impairments (Richter-Levin et al., 1992). Furthermore, it was recorded that lithium impaired passive-avoidance learning in rats (Cappeliez & Moore, 1988; Hines & Poling, 1984). On the contrary, another study indicated lithium enhanced long-term retention in passive-avoidance tests (Tsaltas et al., 2007). Concerning insects, in the fruit flies (*Drosophila melanogaster*), lithium abolished memory in avoidance learning tasks (Xia et al., 1997). It was found that it is appropriate to study the effect of lithium on bee learning because lithium may be used in hives against mites.

This study investigates whether lithium affects the avoidance learning and reversal learning success of honey bees. The reversal learning paradigm allows the measurement of adaptive behavior and cognitive flexibility (Izquierdo et al., 2017; Claudio et al., 2018) because plasticity in learning is crucial for adapting to a constantly changing environment (Seeley, 1994; Ferguson et al., 2001). The hypothesis is that lithium negatively affects avoidance learning or its plasticity in honey bees. To test the hypothesis, the learning performance in the acquisition and reversal phase in the ESAA was measured, which has also been used in several studies previously (Avalos et al., 2021; Dinges et al., 2013).

4.2 Api-TRACE: Honey Bee Tracking in Constrained Environments

4.2.1 Api-TRACE Video Processing Module

A code The video processing module (VPM) was developed with Python (>=3.6) to track the movement of bees in video footage and analyze whether they were exposed to the stimulus, which was the electric shock in the experiments, during a specific time interval. CV techniques were used to track the bees' positions and did geometric calculations to determine whether the bees were within a defined exposure area (Figure 4.1). "*OpenCV",* "*NumPy*", "*Shapely*", and "*MoviePy*" libraries were used. In order to achieve accurate tracking and analysis, the following stages were used:

Video Processing:

The recorded video was processed to extract a subclip containing the desired trail of the experiment. The *ffmpeg_extract_subclip* function from the "*moviepy*" library was employed. The subclip spanned from the stimulus initiation time to a defined duration of the trial of the experiment.

Bee Size Measurement:

The user was prompted to measure the length of a bee in the video. The program allowed the user to draw a line on the first frame of the cut video frame via a graphical user interface (GUI) using the *OpenCV* library to measure the bee's size (Figure 4.2.a). The minimum and maximum sizes of a bee were calculated based on the user's measurement. These measurements were then used in the “Bee Tracking and Motion Analysis” stage for thresholding and dilation operations.

Defining Regions of Interest (ROIs):

Two types of ROIs were defined on the video frames for subsequent analysis:

1. Shuttle Boxes: Areas of interest where bee movement was tracked. These were manually defined by drawing polygons around specific regions of the

video frame via GUI. The polygons served as borders of the shuttle boxes to track bee movement within the bordered area (Figure 4.2.b).

2. Exposure Area: The specific region where exposure event occurred. This area was manually drawn as defined for the shuttle boxes by drawing a polygon on the video frame via GUI (Figure 4.2.c).

Bee Tracking and Motion Analysis Algorithm (BeeTMA):

Bee movement was tracked and analyzed within the defined shuttle boxes. The process involved the following steps:

1. A frame was read (Figure 4.2.d) and converted to grayscale (Figure 4.2.e).
2. Background subtraction was performed using the Gaussian Mixture Model algorithm (*cv2.createBackgroundSubtractorMOG2*) to identify the region of motion in the video frames.
3. The motion mask obtained from the background subtraction was processed using thresholding and dilation operations to extract motion areas.
4. Contours were identified within the motion areas using the *cv2.findContours* function. Bees were identified based on their size. In the beginning, proper minimum and maximum sizes were assigned as variables in pixels (Figure 4.2.f).
5. Bounding boxes were drawn around the detected bees, and their center coordinates were recorded for further analysis (Figure 4.2.g).

Detection of the Bees Exposed to Stimulus:

To determine whether individual bees were exposed to a stimulus, the "*Shapely*" library is used for geometric calculations. The positional information of bees within the exposure area was compared against the defined exposure polygon. Bees whose center coordinates fell within the exposure area were considered as receiving the stimulus.

Data Output and Visualization:

The following outputs were generated for analysis and visualization purposes:

1. A text file was created to record exposure event data. Each row of the file represented a video frame, and columns indicated whether each bee was exposed to the stimulus (True) or not exposed (False) during that time frame.
2. A video was generated, displaying the tracked positions of the bees within the shuttle boxes throughout the experiment. Each bee's position was represented by a single dot (Figure 4.2.h).

A video tutorial on using the algorithm is provided as supplementary material (A tutorial video and the VPM script were available in the Zenodo repository: https://doi.org/10.5281/zenodo.13375378).

4.2.2 Api-TRACE Data Analysis and Visualization Module

In the Data Analysis and Visualization Module (DAVM), R scripts were provided to facilitate the statistical analysis of the exposure event data obtained from the VPM. DAVM served to calculate exposure durations, export the refined data to a tab-delimited text file for further analysis, and generate accurate visualizations (Figure 4.1). This module commenced by loading essential R packages, namely "*dplyr*", "*ggplot2*", and "*ggpubr*", which enabled efficient data manipulation, visualization, and exporting capabilities. It had the following steps:

Data Preprocessing:

The script read the exposure event data. The categorical exposure responses ("True" and "False") were transformed into a numeric format (1 for "True" and 0 for "False"). This conversion laid the foundation for subsequent calculations.

Construction of Structured Data Frame:

A new exposure response data frame was created to structure the processed data. This data frame was designed to store essential information. The data frame included Bee ID, Time, and Exposure Duration. Also, it contained independent variables and

experiment-related information, such as treatment, dose, subspecies, phase, and replicate, which came from the metadata file. Each column was populated with relevant details to enable organized analysis.

Calculation of Exposure Duration:

Exposure durations were calculated by summing exposure responses over defined time intervals. The cumulative sum of exposure responses was computed by iterating through the exposure response data, providing a quantitative representation of exposure duration for each interval.

Data Export:

The processed exposure duration data was exported to a tab-delimited text file. These refined data were then used for further statistical analysis.

Data Visualization:

Two types of plots were generated to represent the exposure duration patterns visually. The first type involved generating line plots for individual bees, illustrating each bee's exposure duration over time (Figure 4.2.i). The second type employed grouped line plots to compare exposure duration trends across independent variables (Figure 4.2.j). These plots showcased the mean and standard error of exposure duration over time, aiding in identifying potential treatment effects. (The DAVM script was available in the Zenodo repository: https://doi.org/10.5281/zenodo.13375378).

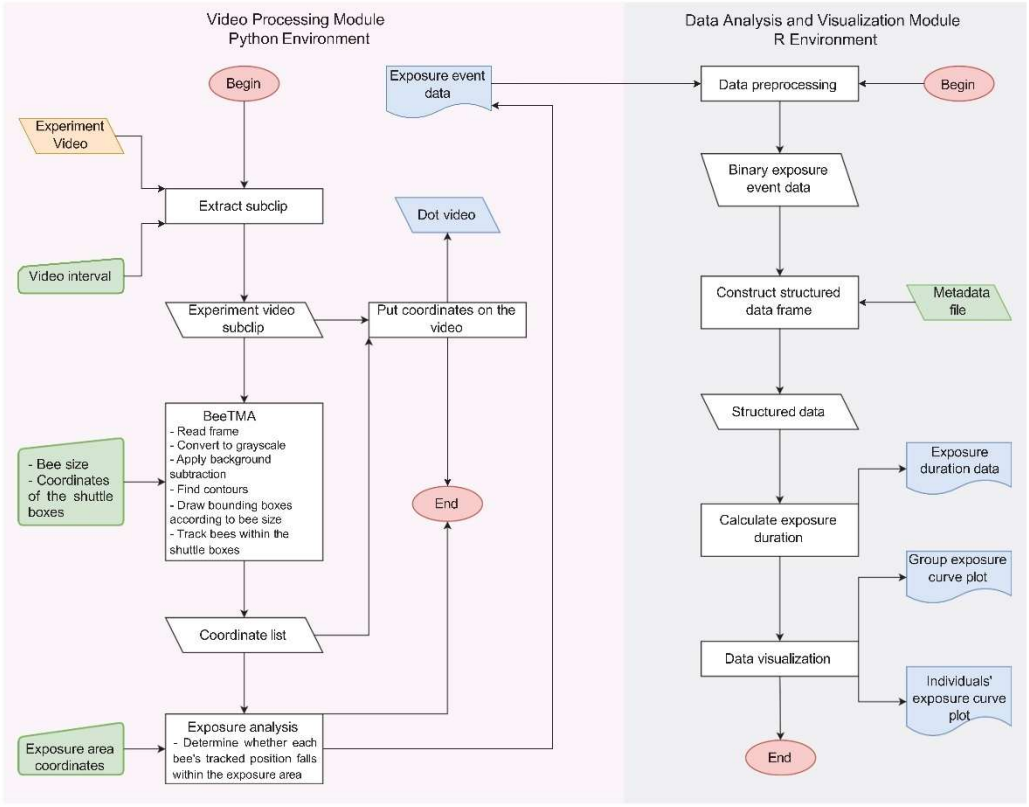

Figure 4.1 Flowchart of video processing module and data analysis and visualization module.

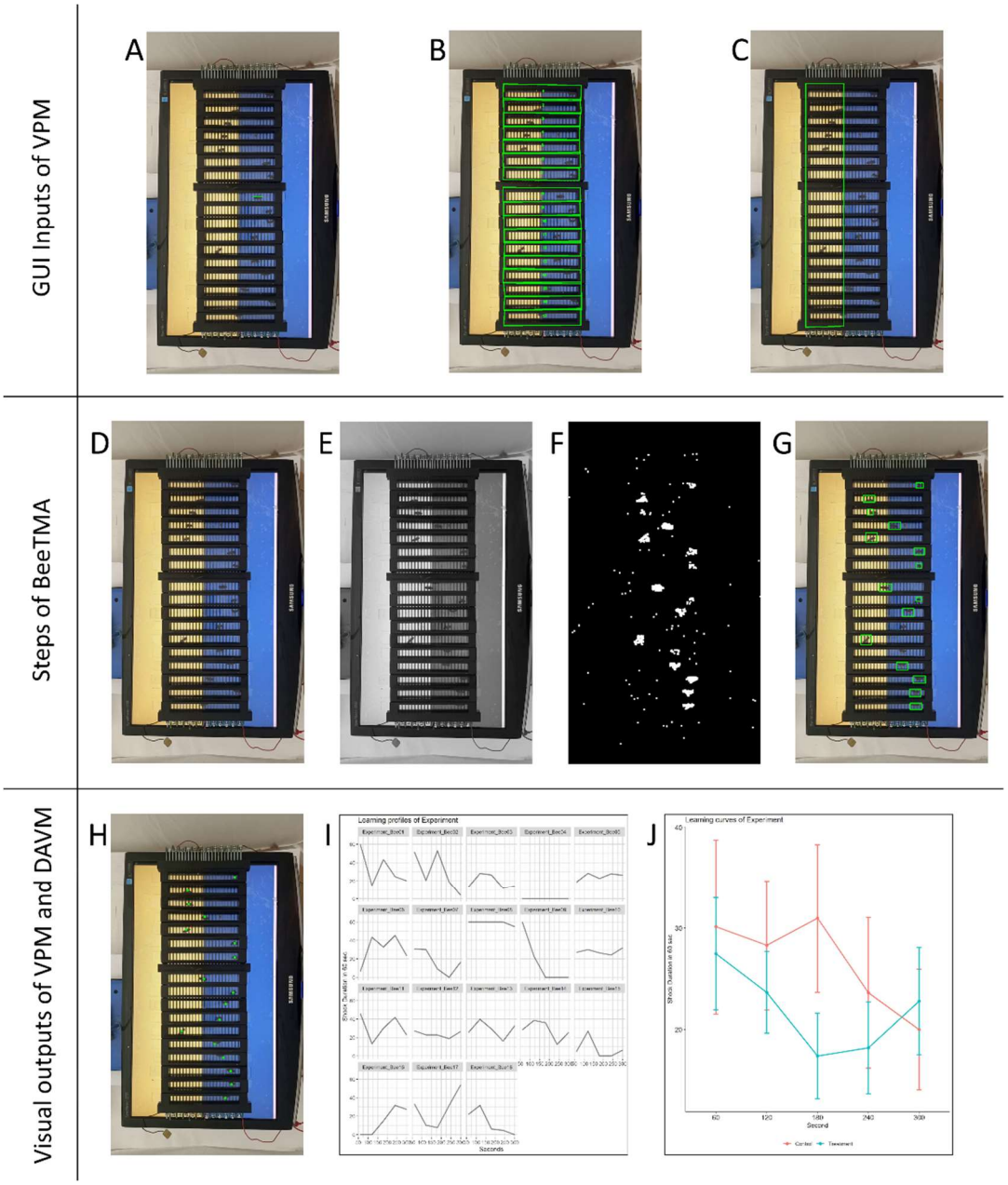

Figure 4.2 Images of the video processing module and data analysis and visualization module.

4.3 Results

In BeeTMA algorithm processed 16 videos of the experiment to track bee motion in 274 ROIs that define shuttle boxes. The output videos displaying the bees' tracked positions were visually examined. The visual examinations indicated that the algorithm could not track the bees in only 8 ROIs. Thus, the algorithm's performance was 97.08% in terms of successfully tracked ROIs.

Moreover, the run time of the BeeTMA algorithm were determined on computers with different configurations. An experiment video (720 p and 30 fps) was used that included 18 bees and lasted 5 minutes. The average processing time obtained from the randomly selected computers was 8.20 minutes, the slowest processing time was 15.60 minutes, and the fastest was 3.61 minutes (Table A.7). If an observer examined this video, the observer would have to spend 5 minutes on each bee, and the time spent on this video would be 90 minutes.

Then, lithium's effect on bees' learning was determined using the algorithms. In the acquisition phase, significant results were found in the time effect (β = -0.015, F (1, 474) = 10.721, p = 0.001). However, the group effect (β = -0.011, F (1, 117) = 0.324, p = 0.570) and interaction effect (β < 0.001, F (1, 474) = 2.128, p = 0.145) were not significant according to LMEM (Figure 4.3.a). Then, a permutation test was also applied to the model because the distribution was not normal according to the Shapiro-Wilk test (p < 0.05). The permutation test confirmed the significant LMEM results for the time effect (B = 1000, p = 0.001). The results suggested that in the acquisition phase, all groups learned to avoid the color associated with electric shock over the 5-minute. Thus, lithium did not affect learning in the acquisition phase. In the reversal phase, significant results were not found in the time effect (β = -0.003, F (1, 474) = 0.353, p =0.553), group effect (β = -0.020, F (1, 117) = 0.956, p = 0.330), and interaction effect (β < 0.001, F (1, 474) = 0.038, p = 0.845) according to LMEM (Figure 4.3.b).

The median and interquartile range (Q1 – Q3) of shock duration of doses for both phases were calculated. There were 140.700 (115.333 – 156.350) for control, 138.567 (114.833 – 156.367) for low-dose, 147.200 (141.767 – 150.650) for medium-dose, and 148.950 (129.550 – 160.567) for high-dose groups in the acquisition phase. In the reversal phase, there were 129.967 (74.350 – 149.267) for control, 134.733 (118.633 – 146.667) for low-dose, 147.067 (139.267 – 152.317) for medium-dose, and 149.300 (131.625 – 162.133) for high-dose groups (Table A.2). Then, the relationship between the duration of exposure to electric shock and lithium doses in each phase was determined. A non-parametric Spearman correlation test was used because the normal distribution was not met according to the Shapiro-Wilk test ($p < .05$). There was no correlation found in the acquisition phase (r (117) = 139, p = 0.131) (Figure 4.3.c). However, a positive correlation was found in the reversal phase (r (117) = 0.282, p = 0.002) (Figure 4.3.d). According to this result, it has been observed that increased lithium doses negatively affect learning only in the reversal phase.

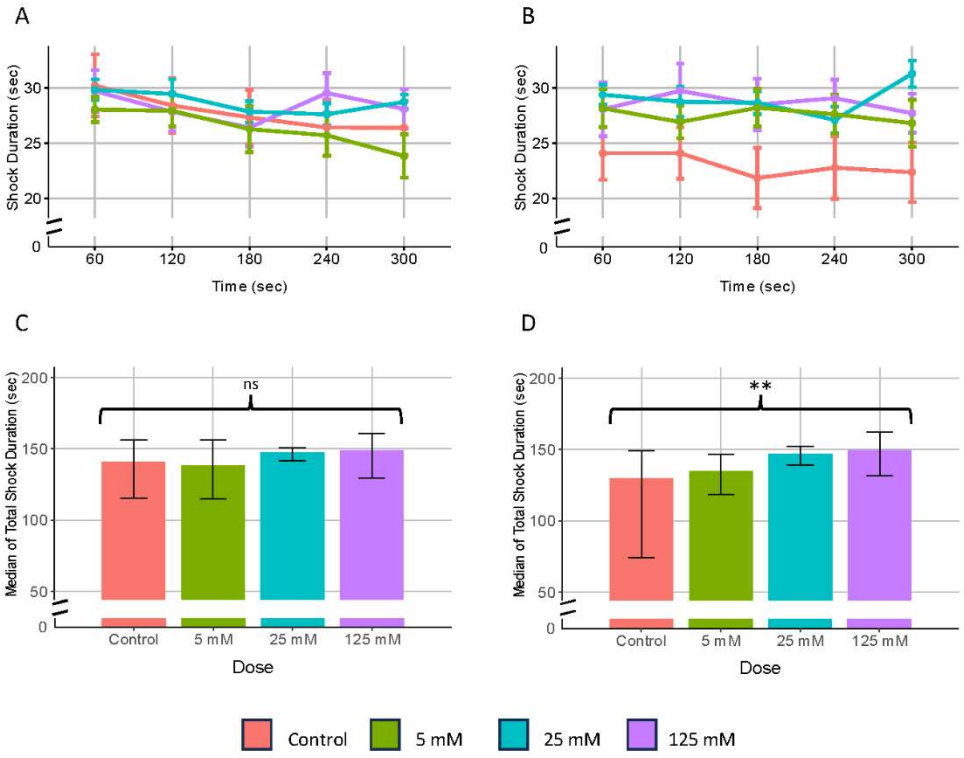

Figure 4.3 Comparison of spatial-avoidance learning rate across the groups during the acquisition phase (a) and reversal phase (b) of ESA assay. Each data point shows the mean (± standard error) of the time honey bees spent on the shock side in a minute. The bars represent the median of the total duration of exposure to electric shock groups during the acquisition phase (c) and reversal phase (d). Error bars showing interquartile range. Curly brackets were used to represent the result of the Spearman correlation for determining the relationship between the duration of exposure to electric shock and lithium doses. Asterisks indicate the level of statistical significance: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, n.s. not significant.

4.4 Discussion

In this study, the Api-TRACE system was developed and successfully validated to analyze the ESAA in honey bees. By automating the tracking of individual bees and the detection of stimulus exposure, the labor and potential observer errors typically associated with manual analysis were significantly reduced. The results

demonstrated the BeeTMA algorithm's performance in terms of correctly tracked ROIs as 97.08%. In the broader sense, the ability to accurately measure and analyze learning success through automated means allows for more detailed and large-scale investigations into various behaviors.

In this study, the potential impact of lithium on honey bee learning was investigated through passive avoidance and reversal learning paradigms through an electric avoidance assay using the experimental setup and algorithms. The results of LMEM revealed a significant time effect in the acquisition phase but not in the reversal phase of the experiment. Thus, the duration of stay in the electric area decreased over time at the acquisition phase, indicating that learning could occur. However, this was not the case for the reversal phase. A positive correlation was found between lithium doses and shock duration in the reversal phase, suggesting that increased lithium doses negatively affected learning during the reversal phase.

In explaining the results, it is essential to consider previous research on lithium's effects on avoidance learning. Lithium has exhibited both favorable (Tsaltas et al., 2007) and adverse effects (Richter-Levin et al., 1992; Cappeliez & Moore, 1988; Xia et al., 1997; Hines & Poling, 1984) on avoidance learning. These studies also showed the dual effect of lithium.

One of the molecular pathways regarding the effect of lithium on learning can be established through the relationship between lithium and GSK-3 protein. The inhibitory effect of lithium on GSK-3 (ortholog of the Shaggy found in insects) activity has been demonstrated in studies on many organisms (Klein & Melton, 1996; Stambolic et al., 1996; Hedgepeth et al., 1997; Hong et al., 1997; Williams & Harwood, 2000; Shaldubina et al., 2001; Jope, 2003; Zhan et al., 2003; Castillo-Quan et al., 2016). It was later determined that GSK-3 activation had a detrimental effect on learning, memory, signal transduction, and caused habituation (Grigor'yan, 2014; Storozheva et al., 2015; Jope et al., 2017; Franciscovich et al., 2008; Beurel et al., 2015; Wolf et al., 2007). The impairment of long-term potentiation caused by GSK-3 is recovered by lithium (Hooper et al., 2007). Thus, the positive impact of lithium

on learning may be elucidated by its capacity to inhibit GSK3, given that the detrimental effects of GSK-3 activity on learning have been established.

Nevertheless, an alternative pathway should be considered to elucidate the negative consequences of lithium. In this context, the impact on dopamine pathways becomes pertinent. Lithium inhibits dopamine-mediated behaviors, such as motor activity, by interfering with GSK-3-mediated pathways in mice (Beaulieu et al., 2004). Dopamine is essential for learning olfactory-electric shock conditioning in fruit flies (Zhou et al., 2019). It is shown that in fruit flies, the unexpected uncoupling of an olfactory cue from electric shock punishment activated reward-encoding dopaminergic neurons through reduced punishment-encoding dopaminergic neuron activity in a reversal learning experiment (Davoudian & Nitabach, 2021). Also, dopamine release was documented in the brains of honey bees receiving electric shocks (Jarriault et al., 2018). It is shown that blocking dopaminergic receptors had a detrimental effect on learning in ESAA in honey bees (Vergoz et al., 2007). Lastly, dopamine inhibitors decreased learning success in a dose-dependent manner in honey bees during a spatial avoidance conditioning assay, the same passive avoidance task was used in the current study (Agarwal et al., 2011). These findings suggest that the dual nature of lithium's effects on avoidance learning involves intricate interactions with both GSK-3 and dopamine pathways. In this case, it is possible to explain the negative effect of lithium on learning, which was observed in the reversal phase of the ESAA, by the dominance of the dopamine mechanism in reversal learning. Such dominance was exemplified in a related study (Costa et al., 2015).

An unexpected situation encountered in the experiments was that the shock duration in the control group was lower in the reversal phase than in the acquisition phase. Initially, it was predicted that learning to avoid the other color associated with the electric shock during the reversal phase would take more time. This situation can be explained via a "rule learning" concept. The term "rule learning" in the context refers to the honey bees' ability to understand general principles or rules from their environment. Bees can go beyond basic learning and comprehend broader concepts

such as categorization, contextual learning, and abstract rules (Giurfa, 2007). In this case, instead of associating a color with an electric shock and being conditioned to avoid that color permanently, the bees may have learned the rule of positioning themselves opposite the color that caused the electric shock. This interpretation suggests a higher cognitive flexibility and adaptability level in honey bee behavior.

CHAPTER 5

CONCLUSION

This thesis has systematically explored the effects of LiCl on the behavioral and physiological aspects of honey bees, with a particular focus on locomotor activity, circadian rhythms, and learning behaviors. The findings significantly contribute to lithium's impact not only on honey bees but also on broader implications in entomological research and practical apiculture.

The experiments demonstrated that lithium can significantly alter locomotor activity and disrupt circadian rhythms in honey bees. Acute and chronic administration of lithium under constant light conditions led to a marked reduction in light-induced locomotor activity. Also, chronic treatment of lithium under constant darkness disrupted the rhythmicity of the circadian rhythm and caused extension of the circadian period under constant light conditions. These results suggest that lithium influences the internal clock of honey bees.

Assessing learning success is crucial since associative learning underpins bee foraging, dance communication, and predator avoidance, all vital for the colony's survival. The passive avoidance task, which measures an organism's ability to avoid negative stimuli, is a key experiment for studying honey bee behavior. The ESAA is a fundamental method in investigating those tasks and is useful for exploring the effects of many independent variables on learning. In this study, an easily constructible experimental apparatus and an algorithm were developed to analyze the experiment videos. Then, these were used to determine the effects of lithium, which is recommended as an acaricide, on learning.

The Api-TRACE system provides precise and efficient tracking of bee movement during the avoidance assay. This tool enhances the accuracy of behavioral data

collection and analysis, offering a significant improvement over traditional manual observation methods.

The design of the experimental apparatus allowed us to use standard hardware and 3D-printed components. By lowering the cost and technical barriers associated with setting up the ESAA, laboratories conducting related research can adopt and implement this method, leading to a broader application of avoidance learning studies in honey bees. Moreover, applying the algorithm and apparatus can be extended to other organisms and experimentally achieved in a constrained environment. Exploring the use of the system in studying learning in other insect species or small animals could broaden the scope of behavioral research.

The results of this study proved a negative impact of lithium on honey bee learning. Since lithium affects the learning and cognitive abilities of bees, it could lead to detrimental consequences for the overall health and productivity of the colony. Therefore, the results emphasize the importance of careful consideration and monitoring when using lithium as an acaricide in honey bee colonies.

The insights derived from these experiments underscore the sensitivity of honey bees to lithium and highlight the necessity for careful consideration in lithium use. Moreover, this research underlines the importance of selecting appropriate doses and exposure durations to mitigate adverse effects on bee health and behavior.

This thesis contributes to the field of computational behavioral analysis by developing and implementing a novel computer vision algorithm capable of detailed tracking and behavioral analysis. This methodology enhanced the precision of the experiments and provided a template for future behavioral studies.

In conclusion, the findings of this research provide crucial information into the effects of lithium on honey bees, highlighting risks and considerations for its use in apicultural practices. It also opens avenues for further research into the mechanisms by which lithium affects insect physiology and behavior, which could have significant implications for managing bee populations and the broader ecological

impacts of lithium usage. Future studies might explore alternative acaricidal treatments that minimize negative impacts on bee health, thus enhancing the sustainability of beekeeping industries globally by applying the computational system developed in this study.

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APPENDICES

Effects of Lithium on Locomotor Activity and Circadian Rhythm of Honey Bees

The datasets generated during and/or analyzed during the current study are available in the Zenodo repository, https://doi.org/10.5281/zenodo.7939046

Figure A.1 Locomotor activity monitoring device.

Table A.1 Descriptive statistics for acute LMA experiments. Sample sizes (n), mean of the total activity count for 24 hours, standard deviations (Std. Dev.), and standard errors (Std. Err.) are given in the table.

		n	Mean	Std. Dev.	Median	Std. Err.
Dark Environment Experiments	Control	24	2220.46	1596.29	1913.00	325.84
	50 mM LiCl	23	2070.65	1322.75	1721.00	275.81
	150 mM LiCl	22	3184.46	1932.33	2422.50	411.97
	450 mM LiCl	25	2259.08	1829.77	1748.00	365.95
	450 mM NaCl	24	3196.58	2364.28	2480.00	482.61
Light Environment Experiment	Control	22	4908.55	3246.16	3827.50	692.08
	50 mM LiCl	24	3360.38	1739.24	3134.50	355.02
	150 mM LiCl	20	2695.30	1694.88	2509.50	378.99
	450 mM LiCl	20	2426.75	1373.42	2477.00	307.11
	450 mM NaCl	24	4259.17	1824.97	3728.00	372.52

Table A.2 Mortality ratios of the groups acute LMA experiments in the dark and light environment.

Experiment	Treatment	Dose	Mortality Ratio
Acute LMA Experiment in Dark Environment	LiCl	Control	0.00
	LiCl	50 mM	0.04
	LiCl	150 mM	0.08
	LiCl	450 mM	0.48
	NaCl	450 mM	0.00
Acute LMA Experiment in Light Environment	LiCl	Control	0.08
	LiCl	50 mM	0.00
	LiCl	150 mM	0.17
	LiCl	450 mM	0.17
	NaCl	450 mM	0.00

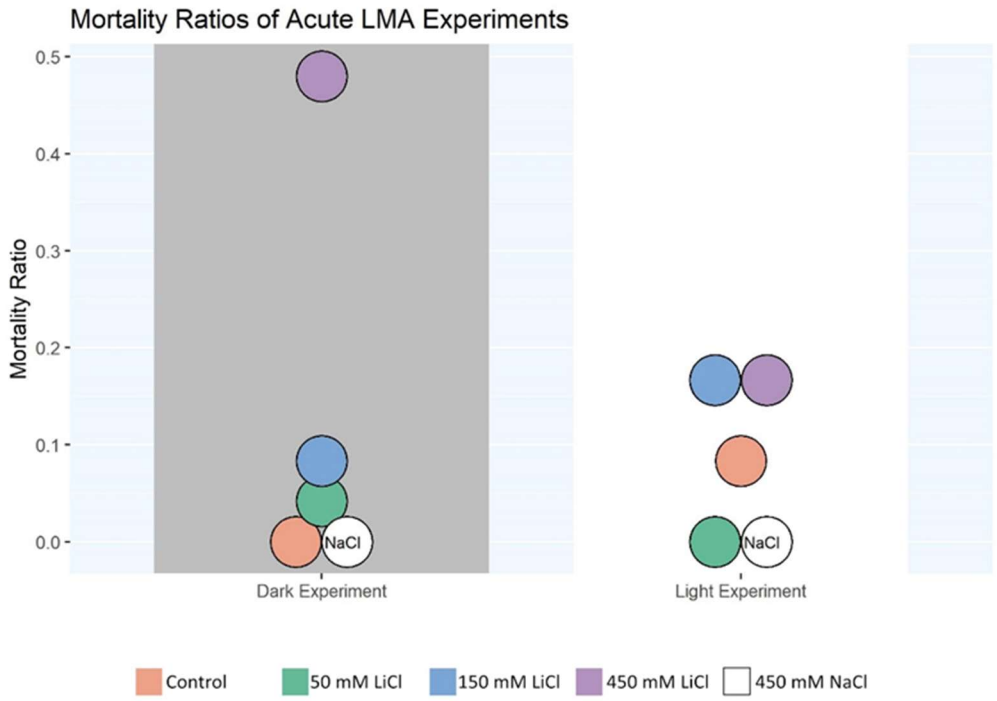

Figure A.2 Mortality ratios of the groups' acute LMA experiments in the dark and light environment.

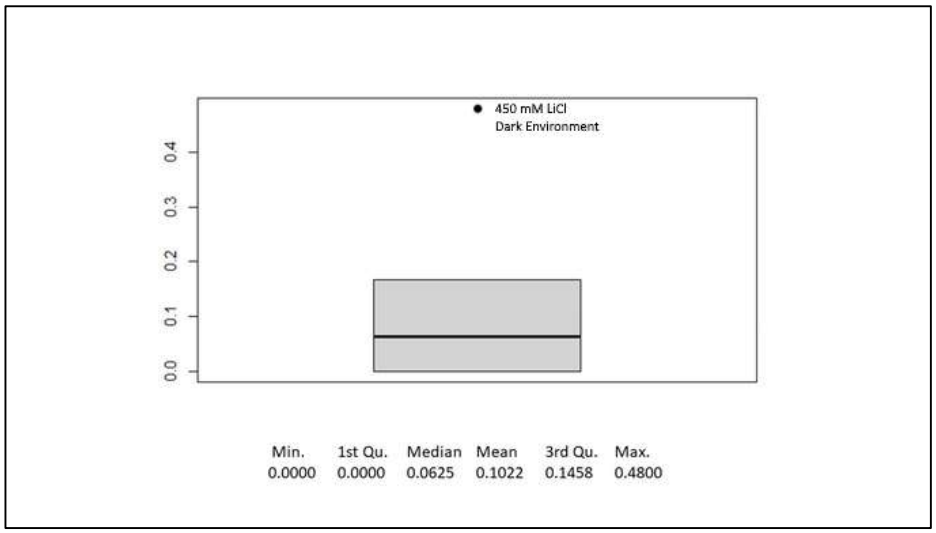

Figure A.3 Box plot and descriptive statistics of mortality ratios of all dose groups in both dark and light experiments. The death ratio of the 0.45 M group in the dark experiment is 0.48. The high-dose group in the dark environment appeared as an outlier.

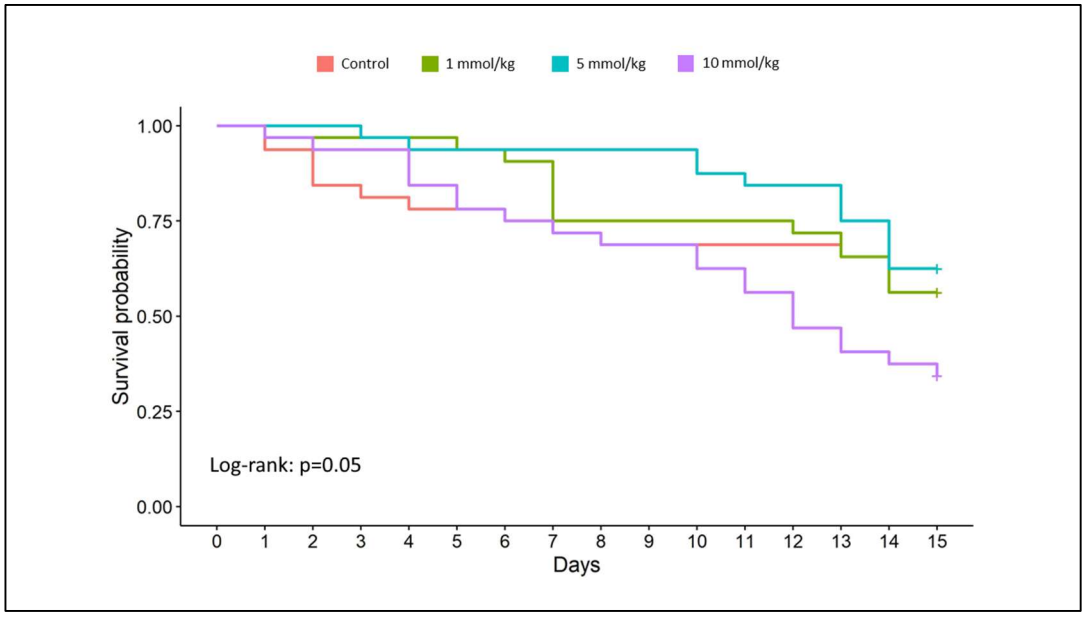

Figure A.4 Log-rank test plot. There was no difference found between the groups ($\chi 2 = 7.9$, $p = 0.05$)

Table A.3 Pairwise comparisons of the survival probabilities of the doses using Log-Rank test. P values are adjusted with BH method.

	Control	**1mmol/kg**	**5mmol/kg**	**10mmol/kg**
Control	-	0.871	0.821	0.138
1mmol/kg	0.871	-	0.751	0.138
5mmol/kg	0.821	0.751	-	0.051
10mmol/kg	0.138	0.138	0.051	-

Table A.4 Descriptive statistics for chronic LMA experiments. Sample sizes (n), mean of the total activity count in conditions as LD, DD, and LL, (total activity count in 5 days), standard deviations (Std. Dev.), and medians are given in the table.

	Condition	n	Mean	St. Dev.	Median
Control	LD	20	12445.80	6994.88	11653.00
	DD	20	13415.50	10570.17	11588.50
	LL	20	37158.90	27481.49	25793.00
1 mmol/kg	DD	18	19215.06	19739.43	9329.50
	LD	18	16655.72	15558.48	12473.50
	LL	18	22845.72	21835.67	10716.00
5 mmol/kg	LD	20	12952.80	14754.55	9651.50
	DD	20	12826.55	18271.93	7354.50
	LL	20	15472.85	18533.57	9632.50
10 mmol/kg	LD	11	11028.18	2903.24	11207.00
	DD	11	5893.27	2844.56	5100.00
	LL	11	10289.27	10167.60	5496.00

Table A.5 Pairwise comparisons of LMA levels in chronic experiment for all data points. The comparisons were achieved using the Wilcoxon rank sum exact test, and p values were adjusted with the Bonferroni method. In the table, High, Med., Low, and Cont. indicates 10 mmol/kg, 5 mmol/kg, 1 mmol/kg, and control, respectively. LD: Light/Dark, DD: constant dark, LL constant dark.

	Cont_LD	Cont_DD	Cont_LL	Low_LD	Low_DD	Low_LL	Med_LD
Cont_DD	0.93966	-	-	-	-	-	-
Cont_LL	0.00114	0.00189	-	-	-	-	-
Low_LD	0.87670	0.57117	0.00725	-	-	-	-
Low_DD	0.98848	0.87670	0.03207	0.84468	-	-	-
Low_LL	0.78234	0.52650	0.12355	0.93014	0.78234	-	-
Med_LD	0.53153	0.84468	0.00064	0.39134	0.93014	0.78234	-
Med_DD	0.27354	0.39134	0.00064	0.10341	0.41790	0.22031	0.45197
Med_LL	0.74179	0.86015	0.00438	0.41790	0.78234	0.41790	0.87670
High_LD	0.78234	0.93966	0.00189	0.78234	0.93966	0.92661	0.60251
High_DD	0.00725	0.02558	3e-05	0.00189	0.09429	0.03065	0.02558
High_LL	0.38889	0.38889	0.00466	0.11174	0.35650	0.10798	0.28666

	Med_DD	Med_LL	High_LD	High_DD
Cont_DD	-	-	-	-
Cont_LL	-	-	-	-
Low_LD	-	-	-	-
Low_DD	-	-	-	-
Low_LL	-	-	-	-
Med_LD	-	-	-	-
Med_DD	-	-	-	-
Med_LL	0.60251	-	-	-
High_LD	0.35650	0.85489	-	-
High_DD	0.27354	0.11174	0.00505	-
High_LL	0.86015	0.61916	0.35650	0.78465

Table A.6 Sample sizes (n), number of rhythmic individuals in the samples, the ratio of the rhythmic individuals to the sample, and descriptive statistics for the period (in hours) of the circadian rhythm as mean, standard deviation, and median are given in the table. In addition, the results of the Spearman correlation test for periodicity, and logistic regression test for rhythmicity are included in the table.

		n	*n* (rhythmic)	Rhythmic ratio	Period Mean	Period St.Dev.	Period Median	Spearman correlation		Logistic regression	
								r	*p*	χ^2	*p*
LD	Control	25	25	1	24.10	1.45	24.31	0.003	.97	0.001	.97
	1 mmol/kg	31	30	0.97	24.53	1.04	24.36				
	5 mmol/kg	30	27	0.90	24.21	0.64	24.31				
	10 mmol/kg	26	26	1	24.45	0.95	24.34				
DD	Control	22	21	0.96	24.09	1.04	23.75	0.01	.91	5.21	.02
	1 mmol/kg	24	23	0.96	24.03	0.75	23.80				
	5 mmol/kg	29	27	0.93	24.41	1.92	23.80				
	10 mmol/kg	20	15	0.75	24.06	1.15	24.05				
LL	Control	20	17	0.85	23.77	3.09	24.15	0.47	<.001	1.20	.27
	1 mmol/kg	18	15	0.83	25.20	3.73	25.00				
	5 mmol/kg	20	14	0.70	26.04	2.02	26.42				
	10 mmol/kg	11	8	0.73	27.74	1.43	27.77				

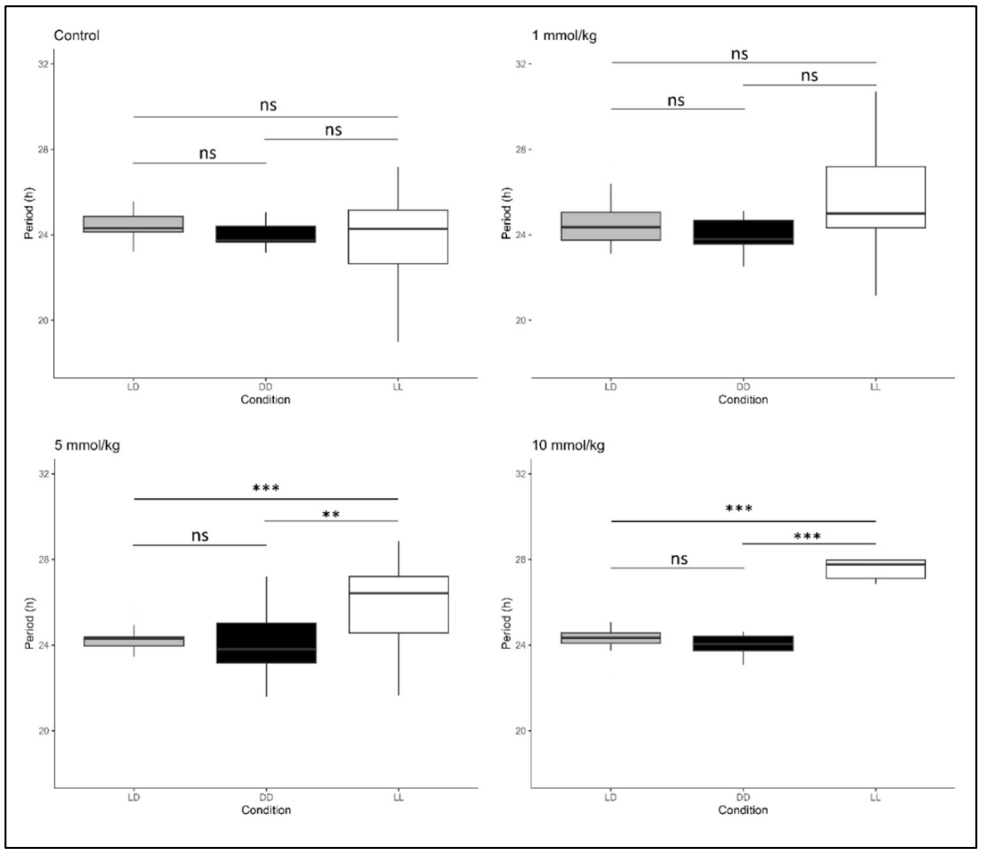

Figure A.5 Light conditions did effective on the circadian period for medium and high dose ($p < .001$) but not for control and low dose groups ($p > .05$) according to the Kruskal-Wallis test. The length of the circadian period in LL condition was significantly higher than in other conditions according to the Dunn test, * $p < .05$, ** $p < .01$, *** $p < .001$, ns: not significant. Grey boxes represent LD, black boxes are DD, and whites are LL.

Api-TRACE: A System for Honeybee Tracking in a Constrained Environment to Study Bee Learning Process and the Effect of Lithium on Learning

The data of the lithium experiment, the scripts of the Api-TRACE, 3D models of the parts used in the electric shock apparatus, and 3D illustrations of the experiment setups are available in the Zenodo repository: https://doi.org/10.5281/zenodo.13375378

Also, the scripts that will include possible future updates can be accessed from this repository: https://github.com/baburerdem/Api-TRACE

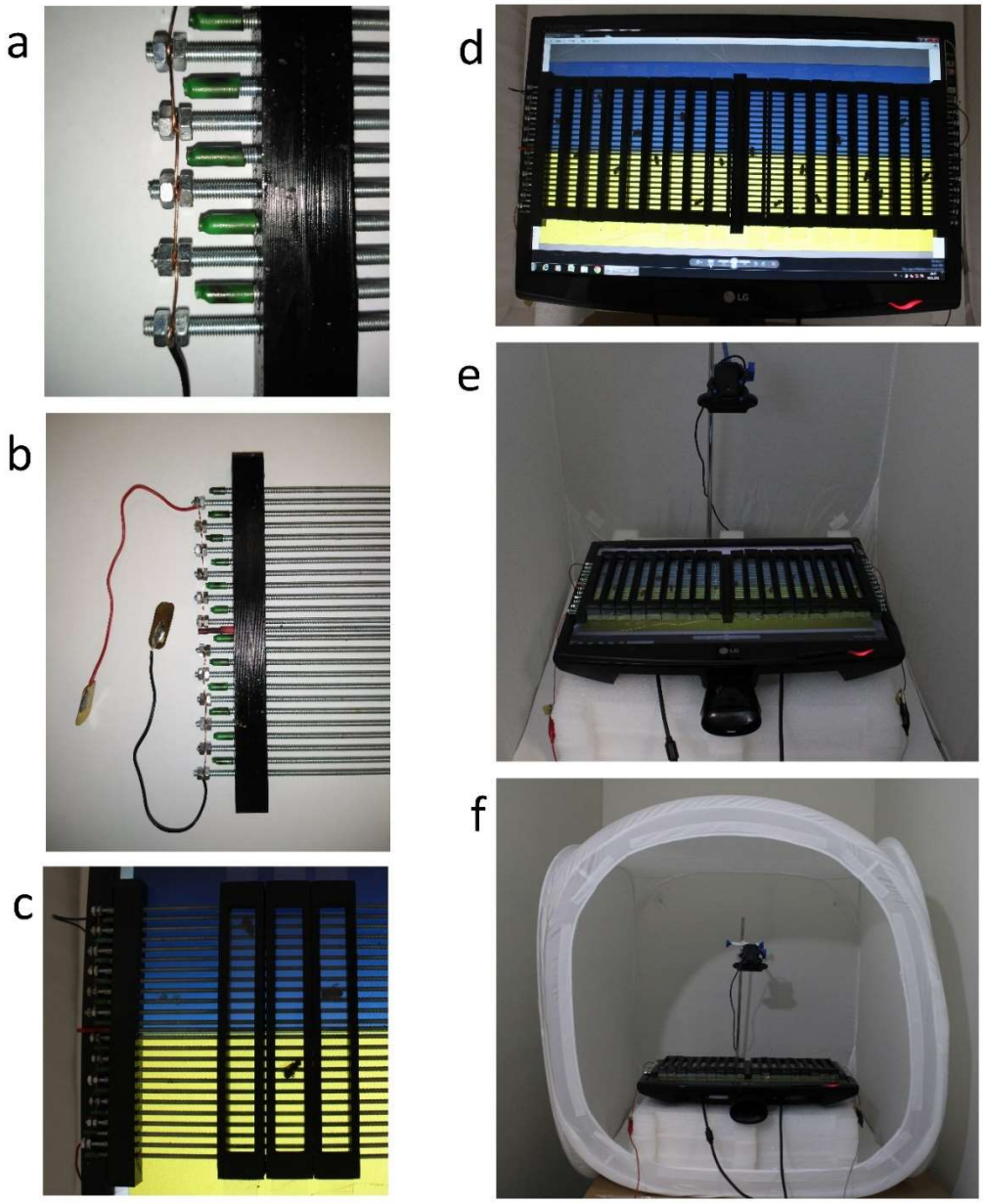

Figure A.6 Experiment setup. Details of the electric grid (a, b) and experiment setup (c). The setup was positioned on the screen (d). All components were placed in a photo studio shooting tent (e). The experiment setup was fully prepared.

Table A.7 Video processing time of our computer vision algorithm on computers with different configurations.

CPU	**Core number**	**Freq (GHz)**	**RAM (GB)**	**Avg. Duration (sec)**
AMD Ryzen 3 3100	4	3.60	16	429.31
AMD Ryzen 7 5800HS	8	2.80	16	449.26
Apple M1	8	3.20	16	452.12
Intel i3 1005G1	2	1.20	8	460.95
Intel i5 7200U	2	2.50	4	935.76
Intel i7 11800H	8	2.30	16	216.55
Intel i7 1255U	2	1.70	32	499.32

Table A.8 Descriptive statistics for ESA assay.

Reversal Phase				Acquisition Phase			
125 mM	25 mM	5 mM	Control	125 mM	25 mM	5 mM	Control	**Dose**
28	31	33	27	28	31	33	27	**n**
143.093	145.190	137.734	115.235	141.633	143.492	131.839	138.799	**mean**
37.668	20.484	36.770	53.033	34.656	19.993	38.417	45.807	**sd**
7.119	3.679	6.401	10.206	6.549	3.591	6.687	8.816	**se**
149.300	147.067	134.733	129.967	148.950	147.200	138.567	140.700	**med**
131.625	139.267	118.633	74.350	129.550	141.767	114.833	115.333	**quant25**
162.133	152.317	146.667	149.267	160.567	150.650	156.367	156.350	**quant75**
30.508	13.050	28.033	74.917	31.017	8.883	41.533	41.017	**IQR**
0.000	90.500	35.000	2.267	52.200	55.400	20.467	34.967	**min**
198.467	196.767	247.533	204.867	211.900	180.133	192.700	289.000	**max**

CURRICULUM VITAE

Surname, Name: Erdem, Babür

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biology	2018
BS	METU Biology	2015
High School	Efeler High School, Aydın	2006

FOREIGN LANGUAGES

Advanced English

WORK EXPERIENCE

1. Researcher, Middle East Technical University, Center for Robotics and Artificial Intelligence. Project: RoboRoyal: ROBOtic Replicants for Optimizing the Yield by Augmenting Living Ecosystems EU-Horizon2020-FET-Open – ID: 964492 (December 2021 – Ongoing)
2. Founder & CEO, Vitaliter Natural Sciences and Engineering R&D Ltd. (March 2016 – November 2018)

PUBLICATIONS

ARTICLES in SCI and SCI-EXPANDED JOURNALS

1. Ulrich, Ulrich, J., Stefanec, M., Rekabi-Bana, F., Fedotoff, L., Roucek, T., Gundeger, B., Saadat, M., Blaha, J., Janota, J., Hofstadler, D., Zampachu, K., Keyvan, E. E., **Erdem, B.**, Sahin, E., Alemdar, H., Turgut, A., Arvin, F., Schmickl, T., & Krajnik, T. (2024). Autonomous tracking of honey bee behaviors over long-term periods with cooperating robots. *Science Robotics, 9,* eadn6848. https://doi.org/10.1126/scirobotics.adn6848

2. Ekici, S., Bozkaya, O., Sevin, **Erdem, B.**, Arslan, O. C., , Cinar, O. O., Bozkaya, E., & Ekici, H. (2024). Investigation of the biological activity and toxicity of bioactive silver nanoparticles synthesized via Vitex agnus-castus seed extract on honey bees. *Veterinary Research Communications*. https://doi.org/10.1007/s11259-024-10550-6

3. **Erdem, B.**, Arslan, O. C., Sevin, S., Gozen, A. G., Agosto-Rivera, J. L., Giray, T., & Alemdar, H. (2023). Effects of lithium on locomotor activity and circadian rhythm of honey bees. *Scientific Reports, 13*,19861. https://doi.org/10.1038/s41598-023-46777-7

4. Arslan, O. C., **Erdem, B.**, Somel, M., Giray, T., & Kence, M. (2023). Effects of coumaphos on locomotor activities of different honeybee (Apis mellifera L.) subspecies and ecotypes. *Apidologie, 54*, 39. https://doi.org/10.1007/s13592-023-01017-9

5. Arslan, O. C., Sevin, S., & **Erdem, B.** (2023). Practical protocol for honey bee immobilization for oral administration of drugs and pathogens. *Journal of Apicultural Research; 62*(4), 932-935. https://doi.org/10.1080/00218839.2021.2014645

6. Sevin, S., Bommuraj, V., Chen, Y., Afik, O., Zarchin, S., Barel, S., Arslan, O. C., **Erdem, B.**, Tutun, H., & Shimshoni, J. A. (2022). Lithium salts: assessment of their chronic and acute toxicities to honey bees and their anti-

Varroa field efficacy. *Pest Management Science 78*(11), 4507-4516. https://doi.org/10.1002/ps.7071

7. Kirman, E.A., Asar, N. V., **Erdem, B.**, Turkyılmaz, I. (2022). Effects of abutment length and platform size on the retention of implant-supported CAD/CAM crowns using six different cements. *Journal of Dental Sciences, 17*(1), 601-603. https://doi.org/10.1016/j.jds.2021.04.013

ARTICLES in TURKISH-INDEXED JOURNALS

1. **Erdem, B.**, & Özkök, A. (2017). Can food supplement produced from apilarnil be alternative to testosterone replacement therapy? *Hacettepe Journal of Biology and Chemistry, 45*(4), 635 – 638. DOI: 10.15671/HJBC.2018.207

PREPRINTS

1. **Erdem, B.**, Ince, A., Sevin, S., Arslan, O. C., Gozen, A. G., Giray, T., & Alemdar, H. (2024). Api-TRACE: A system for honey bee tracking in a constrained environment to study bee learning process and the effect of lithium on learning. *bioRxiv.* https://doi.org/10.1101/2024.09.24.614513

2. Yapar, E., Sağlıcan, E., Dönertaş H. M., Özkurt, E., Hu, H., Guo, S., **Erdem, B.**, Rohlfs, R. V., Khaitovich, P., Somel, M. (2021). Convergent evolution of primate testis transcriptomes reflects mating strategy. *bioRxiv.* https://doi.org/10.1101/010553

PATENT

Erdem, B., Arslan, O. C., Mülayim, M. S. (2018). *Turkish Patent No. TR 2016 14275 B. "Separation of Apilarnil lipid extract and usage as testosterone booster."* Ankara: Turkish Patent and Trademark Office.

PROJECTS

PROJECT MANAGER

1. *Development of a Biosensor Using Honey Bees for Detection of Explosives and Narcotic Substances* (July 2016 – September 2017). Supported by Scientific and Technological Research Council of Turkiye (TUBITAK) under the 1512 program. (Budget: $ 5,200)
2. *Effects of Light and Serotonin on Sex Determination in Water Fleas (Daphnia magna)* (September 2013 – September 2014). Supported by Scientific and Technological Research Council of Turkiye (TUBITAK) under the 2209-A program. (Budget: $ 750)

RESEARCHER

1. *Study of Behavioral Interactions and Nutrition of Queen and Worker Bees to Determine Queen Bee Health and Needs* (June 2021 – June 2022). Supported by METU Scientific Research Projects Coordination Unit (Project Manager: Hande Alemdar / Budget: $ 1,175)

AWARD

Erdem, B., Akpolat, M. T., & Arslan, O. C. (Kovan Team) "*Biosensor development for explosives and narcotics detection using honeybees.*" Yeni Fikirler Yeni İşler (New Ideas New Businesses) technopreneurship program special prize in IDEA Category, organized by ODTÜ Teknokent & METU. December 5, 2015. Ankara, Turkiye.

SCHOLARSHIP

1. Scientific and Technological Research Council of Turkiye - TÜBİTAK BİDEB 2250 Graduate Performance Scholarship (September 2022 – Present)

2. Project assistant. Hive 4.0: Artificial intelligence-based objective approaches in honeybee monitoring. Middle East Technical University, Department of Computer Engineering, (May 2022 – November 2024) Supported by TÜBİTAK

3. The Council of Higher Education - YOK 100/2000 Doctoral Fellowship in Bioinformatics – Biostatistics field (2019 – 2021)

4. Project assistant. Investigation of the neural basis of attention in the fruit fly. TOBB Economy and Technology University, Psychology Department (December 2017 – July 2018) Supported by TÜBİTAK

TRAININGS & CERTIFICATES

1. *T-Jump Entrepreneurship Accelerator Camp*, February 9-19, 2016. METU Technopolis, San Francisco, USA

2. *New Ideas New Businesses Technopreneurship Program*, June – September 2015. METU Technopolis, Ankara, Turkiye

3. *Mt. Erciyes & Sultan Reeds Nature Education Certificate*. August 2010. Erciyes University & TÜBİTAK, Kayseri, Turkiye

CONFERENCE PAPERS

INTERNATIONAL CONFERENCE PRESENTATIONS

1. **Erdem, B.**, Arslan, O. C., Sevin, S., Gödelek, O., Gözen A. G., Agosto Rivera, J. L., Giray, T. & Alemdar, H. "*Effect of lithium on locomotor activity, circadian rhythm and social behaviors of honeybees.*" 47th Apimondia International Apicultural Congress. August 24-28, 2022. İstanbul, Turkiye. Abstract Book: p 83 (Oral Presentation)

2. Roboroyale Consortium. "*Robotic replicants for improving queen bee health and persistence of the honey bee colony.*" 47th Apimondia International Apicultural Congress. August 24-28, 2022. İstanbul, Turkiye. (Poster Presentation)

3. **Erdem, B.**, Arslan, O. C., Kence, M., Somel, M., & Giray, T. "*Determining the differences of learning abilities and behaviors between the three honey bee subspecies."* 45th Apimondia International Apicultural Congress. September 29 – October 4, 2017. İstanbul, Turkiye. Abstract Book: p 205 (Poster Presentation)

4. Sağlıcan, E., Dönertaş, M., Rohlfs, R., Özkurt, E., Hu, H., Neme, R., **Erdem, B.**, Khaitovich, P., & Somel, M. *"Mechanisms of convergent testis transcriptome evolution in primates."* 86th Annual Meeting of the American Association of Physical Anthropologists, organized by AAPA. April 19-22, 2017. New Orleans, USA. Abstract Book: p 364 (Oral Presentation) (SCI-Exp)

5. Parvizi, P., Turan, Z. G., Özkurt, E., Baloğlu, O., **Erdem, B**., Karaca, M., Erbaba, B., Burduroğlu, H. C., Doğru, E., Somel, M. *"Testing the hypothesis that chronic stress accelerates brain aging in a mouse model."* 3rd International Congress of the Molecular Biology Association of Turkiye, organized by İzmir Institute of Technology & Molecular Biology Association of Turkiye. September 10-12, 2014. İzmir, Turkiye. Abstract Book: p 186 (Poster Presentation)

NATIONAL CONFERENCE PRESENTATIONS

1. **Erdem, B**., Mandacı, B. C., Kence, M., Beklioğlu, M. "*The effect of serotonin in hypoxia induced hemoglobin producing pathway in water fleas (Daphnia magna)."* Ecology and Evolutionary Biology Symposium, organized by Ecology and Evolutionary Biology Society of Turkiye &

İstanbul Technical University. July 11-13, 2017. İstanbul, Turkiye. Abstract Book: p 40 (Poster Presentation)

2. **Erdem, B.**, Usal, K. A., & Hohenberger, A. *"Smell recognition and odor – shape matching."* 4th International Symposium on Brain and Cognitive Science, organized by Hacettepe University. April 30, 2017. Ankara, Turkiye. (Poster Presentation)

3. **Erdem, B**. *"Daphnia: a rising model organism*." Laboratory Animal Science 3rd National Congress, organized by Erciyes University. September 26-28, 2013. Kayseri, Turkiye. Abstract Book: pp 68-69 (Oral presentation in Turkish)

4. **Erdem, B**. *"Biological origins of obesity and food-based regulations for prevention."* Turkish 11th Food Congress, organized by Mustafa Kemal University & The Association of Food Technology. October 11-12, 2012. Hatay, Turkiye. Abstract Book: p 18 (Oral presentation in Turkish)

5. **Erdem, B.**, & Akyüz, C. *"Contradictions between scientific theories for obesity and government policies."* Obesity and Ethics: 3rd National Medical Ethics Project Contest, organized by Gazi University. May 23, 2012. Ankara, Turkiye. Contest Projects Book: pp 189-201 (Finalist)