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Original article (Orijinal araştırma)

Expression profiles and possible functions of the ecdysone-related genes in the midgut stem cells of the silkworm, *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae)[1]

İpekböceği, *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) orta bağırsak kök hücrelerinde ekdizonla ilişkili genlerin ekspresyon profilleri ve olası rolleri

Ebru GONCU[2*] **Osman PARLAK[2]**

Abstract

The insect midgut has remarkable similarities with the vertebrate intestine especially concerning controlling cell regulation by the stem cells. While the formation of the pupal midgut from stem cells is regulated by ecdysone, it is inhibited by juvenile hormone via suppression of ecdysone release. This study investigated the possible functions of ecdysone-related genes in the stem cells of *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) during larval-pupal metamorphosis. The study was conducted in the Ege University silkworm culture laboratory and insect physiology research laboratory during the years between 2014-2018. Juvenile hormone analogue, fenoxycarb was applied to the fifth instar *Bombyx* larvae to delayed or inhibit the formation of the pupal midgut. Morphologic observations were performed by hematoxylin plus eosin staining; the proliferation rate of stem cells was analyzed by bromodeoxyuridine cell proliferation assay and expression patterns of ecdysone-related genes were detected by quantitative real-time reverse transcriptase-polymerase chain reaction. Expression states of genes and developmental events of the midgut were differently affected by fenoxycarb treatment in an application time-dependent manner. According to results, genes were firstly classified based on their sensitivity of fenoxycarb, then grouped according to their expression profile in connection with morphological evaluations of stem cells.

Keywords: *Bombyx mori*, ecdysone-related genes, midgut, stem cell

Öz

Böcek orta bağırsağı, özellikle kök hücreler tarafından hücre düzenlemesinin kontrol edilmesiyle ilgili olarak omurgalı bağırsağıyla dikkate değer benzerliklere sahiptir. Pupa orta bağırsağı, kök hücreler tarafından oluşturulur ve ekdizon, orta bağırsağın yeniden şekillenme olaylarını düzenlerken, juvenil hormon, ekdizon salınımını baskılayarak engeller. Bu çalışma, larva-pupa metamorfozu sırasında *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae)'nin kök hücrelerinde ekdizonla ilişkili genlerin olası işlevlerini araştırmıştır. Bu çalışma 2014-2018 yılları arasında Ege Üniversitesi ipek böceği kültür laboratuvarı ve böcek fizyolojisi araştırma laboratuvarında yapılmıştır. Juvenil hormon analoğu fenoksikarb, pupa orta bağırsak oluşumunu geciktirmek veya engellemek için 5. dönem *Bombyx* larvalarına uygulanmıştır. Morfolojik gözlemler hematoksilen ve eozin boyama ile gerçekleştirilmiş; kök hücrelerin proliferasyon hızı, bromodeoksiüridin hücre proliferasyon analizi ile analiz edilmiş ve ekdizon ile ilgili genlerin ekspresyon durumları kantitatif gerçek zamanlı ters transkriptaz-polimeraz zincir reaksiyonu ile tespit edilmiştir. Genlerin ekspresyon durumları ve orta bağırsaktaki gelişimsel olaylar, uygulama zamanına bağlı olarak fenoksikarb tedavisinden farklı şekilde etkilenmiştir. Sonuçlara göre, genler önce fenoksikarb duyarlılıklarına göre sınıflandırılmış, daha sonra kök hücrelerin morfolojik değerlendirmeleri ile bağlantılı olarak ekspresyon profillerine göre gruplandırılmıştır.

Anahtar sözcükler: *Bombyx mori*, ekdizon bağlantılı genler, orta bağırsak, kök hücre

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Introduction

In holometabolous insects, midgut stem cells perform different functions throughout the insect life. They proliferate and differentiate before each molt for maintaining the existing larval midgut epithelium. During larval-pupal metamorphosis, the pupal midgut is developed by stem cells suited to the feeding regime of adult insect (Baldwin et al., 1996; Tettamanti et al., 2007) and ecdysone is the main regulator of this process.

Ecdysone-related genes have functions during midgut remodeling in insects (Lee et al., 2002; Nishiura et al., 2005; Goncu et al., 2016). Previous studies have mostly focused on ecdysone-related gene activation involved in programmed cell death events (Goncu & Parlak, 2011; Franzetti et al., 2012), whereas our knowledge of the expression patterns of ecdysone-related genes in insect stem cells is insufficient. Through the few studies on this subject evidence has been obtained regarding the possible functions of ecdysone in the proliferation and differentiation processes of stem cells in different insect species. Inhibition of the ecdysone receptor (EcR) signal in the wing imaginal disc causes a decline in the progression of S phase and mitosis (Cranna & Quinn, 2009) and the ultraspiracle (USP) is involved in the regulation of cell cycle and differentiation in the developing eye imaginal disc of *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) (Quinn et al., 2012). Parthasarathy & Palli, (2007) showed that the EcR-A/USP-B heterodimer regulates the development of the pupal/adult midgut in *Aedes aegypti* L., 1762 (Diptera: Culicidae). Lee et al. (2002) reported that mutations in broad impact cell death in the larval midgut but do not affect the development of adult midgut in *D. melanogaster*. Our previous study showed that EcR-A and USP2 mRNAs are produced in the midgut stem cells of *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) during remodeling processes but EcR-B1, USP1 and E74B transcripts are detected during the initial phase of metamorphic events (Goncu et al., 2016).

The aim of this study was to investigate possible functions of certain genes in the formation of the pupal midgut. For this purpose, fenoxycarb administration was made to manipulate developmental events in the midgut via suppression of ecdysone release. Fenoxycarb mimics the juvenile hormone action in *B. mori* and delays or inhibits metamorphic events depending on the time of administration. Treatment with fenoxycarb on day 0 causes delay of ecdysone release but day 3 treatment inhibits it completely (Kamimura & Kiuchi, 1998). After the applications, changes in expression profiles of ecdysone-related genes, midgut morphology and proliferation patterns of stem cells were evaluated together, and information was obtained on the critical expression timing of the studied genes and their sensitivity to fenoxycarb.

Materials and Methods

Insect rearing and Fenoxycarb application

This study was conducted in the Ege University silkworm culture laboratory and insect physiology research laboratory during the years between 2014-2018. Hybrid races (Japanese × Chinese) of the silkworm, *B. mori* larvae were reared on fresh mulberry leaves at 25 ± 1ºC, 75-85% RH and 12:12 h L:D photoperiod. Insects were topically treated with 1 ng/10 µL of fenoxycarb dissolved in acetone immediately after the fourth larval ecdysis (day 0) and day 3 of the fifth instar. Throughout the article insects treated with fenoxycarb on day 0 and day 3 are named as group 1 and 2, respectively. Experiments were repeated every 12 h from day 7 larvae of the fifth instar to 24 h after pupation.

Stem cell isolation

Stem cells were isolated from the midgut according to Hakim et al. (2009). Ten insects were used for stem cell isolation every 12 h and midgut cells were classified according to their morphological features (Cermenati et al., 2007; Goncu et al., 2016). Stem cells were observed as clear and round shapes cells and counted by using a hemocytometer slide under an inverted microscope. They were counted in three

arbitrary fields each measuring 1 mm^2. Every calculation was repeated three times and high percentages (>92%) of stem cells were obtained by this method (Hakim et al., 2009). Due to the separation of the larval midgut from the basal lamina into the lumen during pharate pupal stage, the content of the midgut lumen including yellow bodies was discarded, and the remaining tissues were used as stem cell fractions.

Preparation of midgut sections for staining

Midguts were obtained every 12 h from day 7 of fifth instar larvae to the early pupal stage then, they were fixed in Bouin's solution for 5-6 h at 4ºC. Following the fixation, tissue pieces were dehydrated through a graded series of ethanol and processed for embedding in paraffin wax. For morphological evaluation, 5-µm-thick sections were stained with hematoxylin and eosin using the routine protocol. Sections were examined under a Leica DM3000 microscope and photographed with a digital camera.

Bromodeoxyuridine labeling

For in vivo labeling with (bromodeoxyuridine) BrdU, 10 µl of the labeling reagent per 1 g body weight was injected into the hemocoel of staged larvae/pupae. Midguts were dissected 4 h later and then fixed in 10% neutral buffered formalin for 8 h. Paraffin sections of midgut samples were prepared as described above for histological analysis. The sections were processed using In Situ Cell Proliferation Kit, Fluos (Roche, Penzberg, Germany) according to manufacturer's instructions. The percentage of BrdU positive cells was assessed by Image J analysis (National Institutes of Health, Bethesda, MD, USA). Automated particle counting analyze was performed for all images and percentage of positive cells were calculated according to obtained results.

RNA isolation and cDNA synthesis

Stem cell fractions were pooled and collected in Tripure Isolation Reagent (Roche) for every 12 h. Samples were homogenized in Tripure reagent and total RNA was isolated according to the manufacturer's instructions. Total RNA concentration and purity were evaluated by using Nanodrop UV/VIS spectrophotometer. cDNA synthesis by reverse transcription was performed using 1 µg of RNA and High-Fidelity cDNA Synthesis Kit (Roche) in a 20 µl reaction volume.

Quantitative real-time reverse transcriptase-polymerase chain reaction

Relative expression of selected genes was detected by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) using light cycler 480 real-time PCR detection systems (Roche) as described (Gibson et al., 1996). qRT-PCR was performed in a total reaction volume of 10 µl containing 2 µl of cDNA, 0.5 µl each of forward and reverse sequence-specific primers, 2.7 µl H_2O, 0.2 µl tProbe, and 5 µl enzyme. PCR conditions were 95ºC for 10 min followed by 45 cycles of 95ºC for 10 s, 60ºC for the 30 s, 72ºC for 1 s, 40ºC for 30 s. The primers used for PCR that were derived from the sequences of the *Bombyx* genes and references are listed in Table 1. Primer probe design was performed by using Clustal W and Oligo 7 software. The specificity of obtained primers and probes was controlled by using the blast program. *Bombyx mori* Actin 3 was used as an endogenous control. Mean and standard errors were obtained from the averages of three independent sample sets. For each gene, we tested to examine gene expression by using a One-way analysis of variance, SPSS statistics software (IBM, Armonk, NY, USA).

Table 1. Forward and reverse primers used in quantitative real-time reverse transcriptase-polymerase chain reaction

Gene no	Forward primer Reverse primer	Accession no	Upl prob no
Bombyx mori Actin 3	5′- GCTCCCTCGAGAAGTCTTACG-3′ 5′- CTGGGCAACGGAATCTTTC -3′	U49854	9
EcR A	5'-CATCCGGTCAACGGACAC-3' 5'- ACCGTAGCTGCCTGAGGATA-3'	D87118	141
EcR B1	5'- ACTTGGCAGTCGGATGAAG - 3' 5′- CGTCATCTCCGTGATCTGG -3′	L35266	153
USP1	5′- TCAAATAGGCAACAAACAGATAGCCGCTC-3′ 5′- CAGGAACTCCATAGACCG -3	U06073	150
USP2	5′- CAGTGTCACATGTAGAGTGCAAAGA -3′ 5′- CCACTTTCATAGAACAGTTCAGTTGC -3′	AB182582	FAM- GTTCAACGACCTTGTTGCTGACA GGTTC-Tamra- Taqman probe
E74A	5′- CCACTTTCATAGAACAGTTCAGTTGC -3′ 5′- CCACCTATCGAGATAAAGCAAGA -3′	Q1KLS0	141
E74B	5′- ACCCGAGTGACTACGTGAGG -3′ 5′- CGGAGTCTGTGCCTGAGTCT -3′	Q1KLR9	95
E75A	5′- TCGGTCGAGCTTGAGTGAG -3′ 5′- GATGAAGGTCGCTTGTCTCG -3′	AB024904	59
E75B	5′- GGACAGCTCTCAAAGACGTGA -3′ 5′- CGCACCATTCACTACACTCG -3′	AB024905	91
BRC-Z1	5′- TCTGCAGAGTCCTCTCGCTTC -3′ 5′- TACACGCGCTGGCAAATG -3′	AB166725	36
BRC-Z2	5′- TCTGCAGAGTCCTCTCGCTTC -3′ 5′- GTGTATATGTGCGTCATCAGGGA -3′	AB166726	99
BRC-Z4	5′- TCTGCAGAGTCCTCTCGCTTC -3′ 5′- TCTTGTGGTTGTTGAGCGAGTT -3′	AB166727	56
βFTZ-F1	5′- TTCCGCAAGTATCATCATTGAC -3′ 5′- CTTGTCGTGAGTTGGTGGTG -3′	10953	141
BHR3	5′- GGGATGCAAAGGATTCTTCA -3′ 5′-GCGAGGACACTGGTAGTTCAC -3′	AB024902	99

Results

Juvenile hormone analogue, fenoxycarb, delays or inhibits the formation of the pupal midgut epithelium

In the control group, the feeding activity lasted until the end of day 7 of the fifth instar. After the gut purge, larvae were actively spun cocoon on day 8 and 9 of the fifth instar, and these days were considered as the early prepupal stage. Larvae became pharate pupae on day 10 of the fifth instar in which the spinning activity was almost finished and larvae became quiescent. Therefore, this day was determined as the late prepupal stage and larval-pupal ecdysis occurred at the end of day 10. Fenoxycarb treatment in group 1 extended the feeding stage to the end of day 10. Since gut purge was retarded until day 10 of the fifth larval stage, days 11 and 12 were determined as the early pupal stage and day 13 as the pharate pupal stage. Larval-pupal ecdysis occurred at the end of day 13 in this group. Fenoxycarb treatment in group 2 produced dauer larvae with feeding activity until day 13 of the fifth larval stage. Gut purge, spinning activity, and larval-pupal ecdysis did not occur in this group of animals.

In the control group, healthy larval midgut epithelium consisted mainly of mature columnar epithelial cells and goblet cells (Figure 1a-b). After the cessation of feeding, several conical or spindle-shaped stem cells located in the basal region of epithelium enlarged and began to proliferate in the early prepupal stage (Figure 1c-d). Proliferating stem cells were observed under the larval epithelium on day 9 (Figure 1e-f). In the late prepupal stage (day 10), when the degenerated larval midgut detached from the basal membrane

stem cells formed a multilayer epithelial structure surrounding the lumen (Figure 1g-h). Stem cells started to differentiate into columnar epithelial cells with brush border membrane to constitute pupal midgut structure in the early pupal stage (Figure 1i-k).

In group 1, pupal midgut formation was temporarily inhibited until the end of feeding on day 11 of the fifth larval stage, and very few stem cells were detected in the basal region of the larval midgut epithelium (Figure 2a-h). Stem cells began to enlarge and proliferate as similar to the control group during the early pupal stage on days 11 and 12 (Figure 2i-l). The mature larval midgut cell layer was detached from the basal membrane at the end of day 13 (Figure 2m-n). The formation of the monolayer columnar epithelium of the pupal midgut continued in the early pupal stage (Figure 2o-q).

Larval midgut structure continued to exist in group 2 until the end of day 13 of the fifth instar. Healthy larval midgut epithelium was observed on days 7, 8 and 9 (Figure 3a-f). Stem cells beneath the larval midgut epithelium begun to enlarge and proliferate at the beginning of day 10 (Figure 3g-h). In addition to the reduction of the connection with the basal lamina in some regions, various morphological changes such as condensation in the cytoplasm and nucleus were indicative of degeneration in the mature larval midgut cells on day 11 (Figure 3i-j). Especially on day 12, degenerated old larval epithelial remnants and undigested mulberry leaves as feeding material were detected in the midgut lumen (Figure 3k-l). On day 13, newly formed larval epithelium from stem cells showed an abnormal multilayered pattern in some regions (Figure 3m-n). The reduced feeding activity observed in these insects was probably related to this abnormal midgut epithelium.

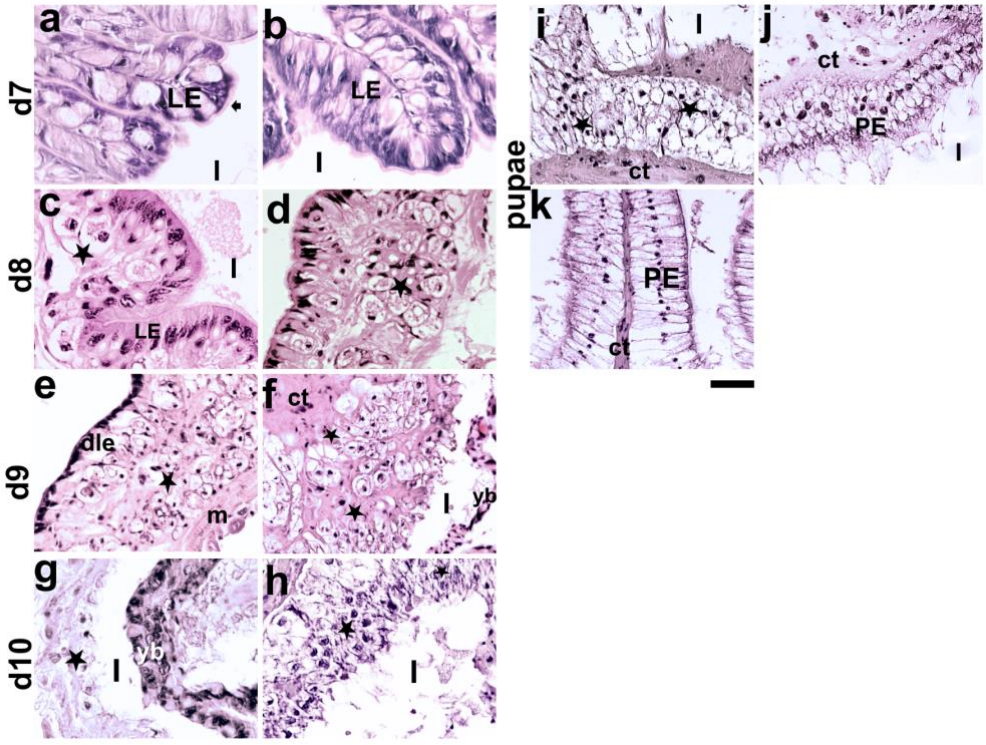

Figure 1. Morphological changes in midgut stem cells visualized by haematoxylin plus eosin staining. Control group: a) fifth larval instar day 7; b) day 7, 12 h; c) day 8; d) day 8, 12 h; e) day 9; f) day 9, 12 h; g) day 10; h) day 10, 12 h; i) pupa, 0 h; j) pupa, 12 h; k) pupa, 24 h (scale bar 50 µm).
Black star, stem cell; ct, connective tissue; dle, degenerated larval epithelium; l, lumen; LE, larval midgut epithelium; m, muscle; PE, pupal epithelium; and yb, yellow body.

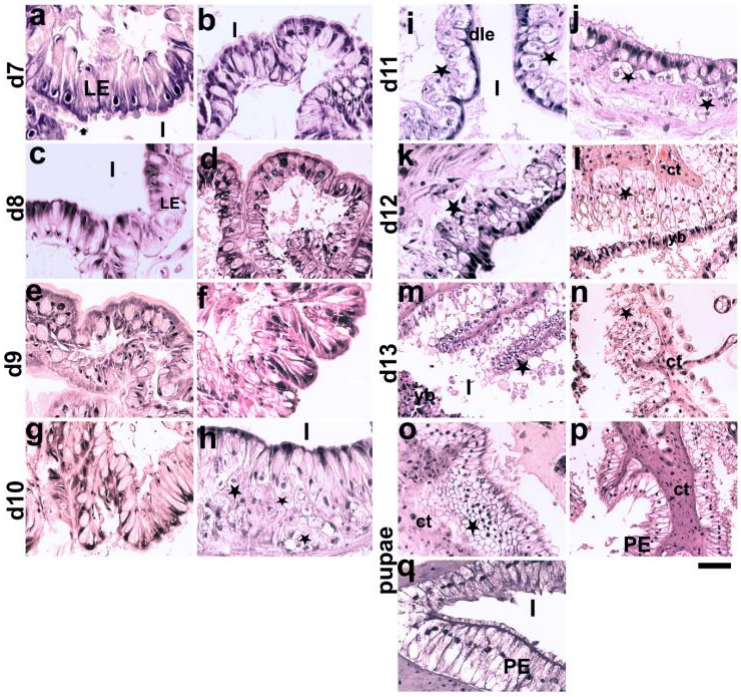

Figure 2. Morphological changes in midgut stem cells visualized by haematoxylin plus eosin staining. Group 1 (day 0 fenoxycarb treated insects): a) fifth larval instar day 7; b) day 7, 12 h; c) day 8; d) day 8, 12 h; e) day 9; f) day 9, 12 h; g) day 10; h) day 10, 12 h; i) day 11; j) day 11, 12 h; k) day 12; l) day 12, 12 h; m) day 13; n) day 13, 12 h; o) pupa, 0 h; p) pupa, 12 h; q) pupa, 24 h (scale bar 50 µm).
Black star, stem cell; ct, connective tissue; dle, degenerated larval epithelium; l, lumen; LE, larval midgut epithelium; PE, pupal epithelium; and yb, yellow body.

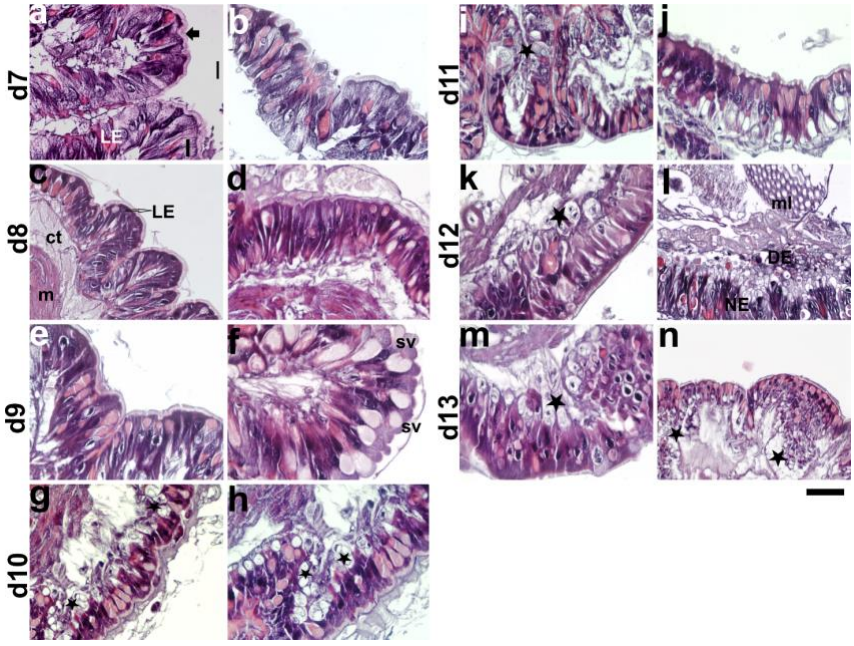

Figure 3. Morphological changes in midgut stem cells visualized by haematoxylin plus eosin staining. Group 2 (day 3 fenoxycarb treated insects): a) fifth larval instar day 7; b) day 7, 12 h; c) day 8; d) day 8, 12 h; e) day 9; f) day 9, 12 h; g) day 10; h) day 10, 12 h; i) day 11; j) day 11, 12 h; k) day 12; l) day 12, 12 h; m) day 13; n) day 13, 12 h (scale bar = 50 µm).
Black star, stem cell; ct, connective tissue; DE, degenerated epithelium; l, lumen; LE, larval midgut epithelium; m, muscle; ml, mulberry leaf; NE, newly formed epithelium; sv, secretory vesicle.

Bromodeoxyuridine (BrdU) incorporation assay was used to evaluate the stem cell proliferation rate (Figure 4-6), and the percentage of positive cells was assessed by Image J analysis (Figure 7). In the control group, a few numbers of BrdU positive cells were detected in the basal layer of the larval epithelium on day 7 of the fifth instar (Figure 4a). The number of stem cells with a positive signal increased on day 10 (Figure 4b). Image J analysis of midgut sections from control group insects revealed that the percentage of maximum BrdU incorporated cells were 26 ± 2.9% just before pupal ecdysis (Figure 7). After pupation, the number of positive cells gradually decreased (Figure 4c, d). BrdU positive cells in the midguts of group 1 were determined as 4.7 ± 0.5% on day 7 (Figures 5a and 7) then decreased to very low levels until day 12 of the fifth instar (Figures 5b and 7). The significant increase in BrdU positive cells in group 1 was detected just before pupation (Figure 5c) and 20 ± 2.4% of the cells were BrdU positive (Figure 7). As detected in the control insects, the number of cells including BrdU gradually decreased after pupation (Figure 5d, e). In the midgut of group 2, the amount of BrdU positive cells did not exhibit significant changes during the experiment (Figure 6) and the maximum BrdU positive cells were determined to be 6 ± 1.0% on day 10 of the fifth instar (Figure 7).

Expression patterns of ecdysone receptors and ecdysone-related transcription factors in the midgut stem cells

Since the application of fenoxycarb caused prolongation of the feeding stage, x-axis diagrams were prepared separately for the control and treated insects. In stem cells isolated from the control group, moderate amounts of EcR A transcripts during feeding and in the early prepupal stage increased sharply on day 10 and then remained moderate levels up to 24 h after pupation (Figure 8a). However, EcR A expression was temporarily suppressed in group 1 until day 12 of the fifth instar. The highest EcR A mRNAs were detected in the first half of day 13 and followed by a gradual decrease up to 24 h after pupation (Figure 8a). EcR A expression in the stem cells of group 2 was completely inhibited until day 13 of the fifth instar (Figure 8a).

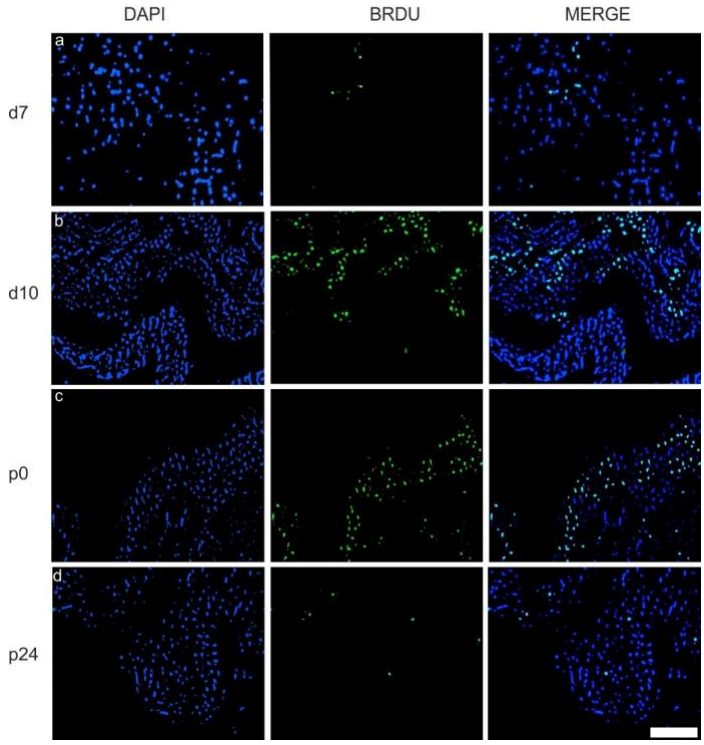

Figure 4. The proliferation rate of stem cells in the control group visualized by BrdU staining (nuclear staining was performed by DAPI): fifth larval instar a) day 7 (d7); b) day 10 (d10); c) at pupation (p0); d) pupa 24 h (p24) (scale bar 150 µm).

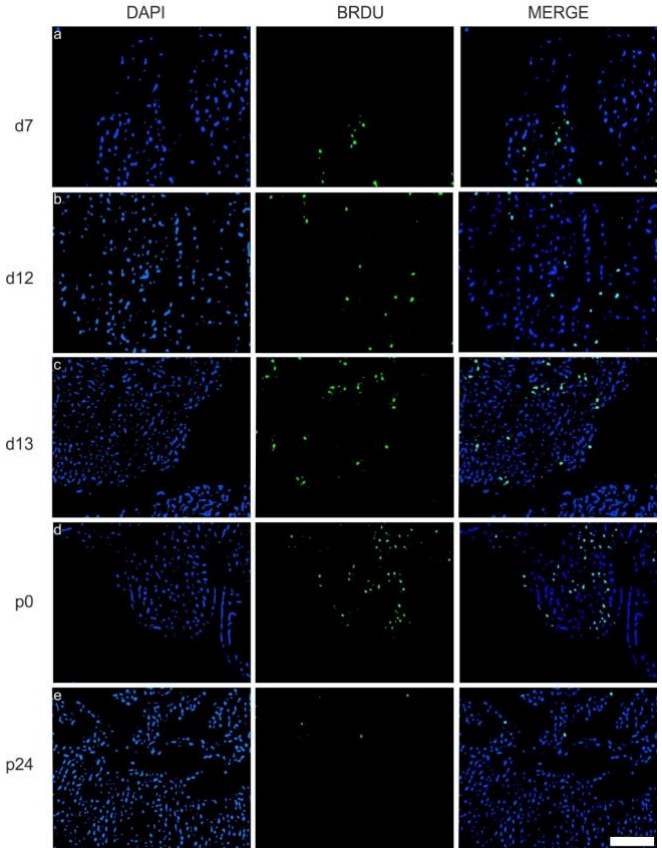

Figure 5. The proliferation rate of stem cells in the Group 1 visualized by BrdU staining (nuclear staining was performed by DAPI): fifth larval instar a) day 7 (d7); b) day 12 (d12); c) day 13 (d13); d) at pupation (p0); e) pupa 24 h (p24) (scale bar 150 µm).

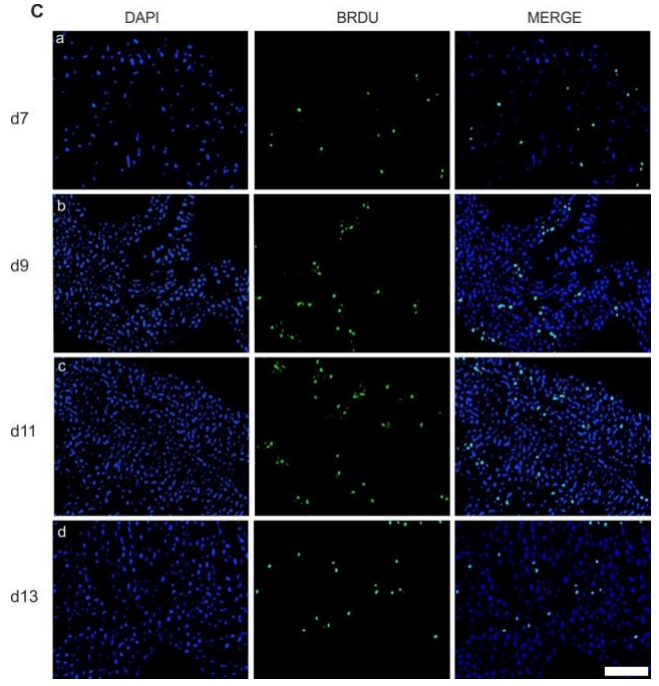

Figure 6. The proliferation rate of stem cells in the Group 2 visualized by BrdU staining (nuclear staining was performed by DAPI): fifth larval instar a) day 7 (d7); b) day 9 (d9); c) day 11 (d11); d) day 13 (d13) (scale bar 150 µm).

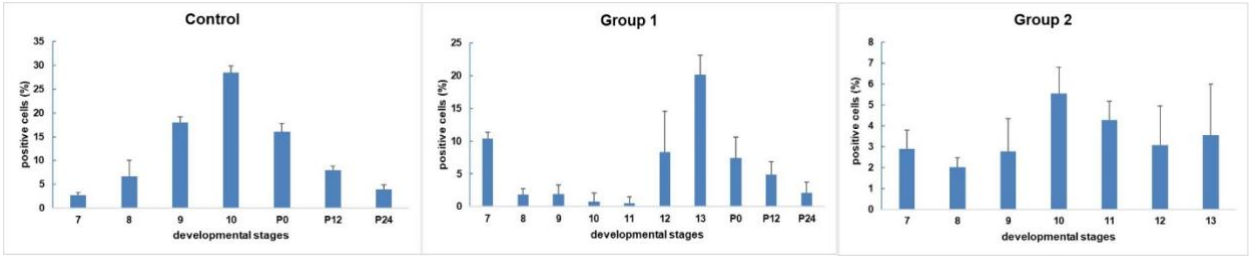

Figure 7. The percentage of BrdU positive nuclei determined using Image J software (http://imagej.nih.gov/ij) for Control, Group 1 (day 0 treated insects), and group 2 (day 3 treated insects) (mean ± SD for the three independent experiments are shown).

High amounts of EcR B1 transcripts were detected in the stem cells of the control insects on days 7 and 8, and the amount of transcripts decreased after day 9 (Figure 8b). EcR B1 transcripts in stem cells of group 1 were mostly at low levels, and a significantly higher level was detected only in the first half of day 12 (Figure 8b). In stem cells of group 2, EcR B1 mRNA levels were almost undetectable until day 11, and only a small increase occurred on this day (Figure 8b). The highest USP 1 mRNA levels in the stem cells of control group insects were detected on days 7 and 8 (Figure 8c). In stem cells of group 1, low USP1 transcripts began to increase from day 9 and peaked on day 11. Following the peak, mRNA levels decreased (Figure 8c). In the stem cells of group 2, USP1 mRNA levels were low until day 13 and then peaked sharply (Figure 8c). The USP 2 levels in stem cells of the control group were relatively high during feeding and early prepupal stage and peaked in the first half of day 10. Following this, the amount of transcripts decreased (Figure 8d). USP 2 transcripts in the stem cells of group 1 were at moderate levels until day 9 of the fifth instar and then peaked on this day. Following the peak, mRNA levels decreased to moderate levels again (Figure 8d). In the stem cells of group 2, USP2 expression was completely suppressed until the end of the day 13 of the fifth instar (Figure 8d).

Moderate E74A mRNA levels in the stem cells of the control group peaked just before pupation (Figure 8e). In stem cells of group 1, moderate levels of E74A decreased until day 10 and remained low until day 13 of the fifth instar. The most striking E74A expression were detected on day 13 just before pupation, followed by a gradual decrease in the early pupal period (Figure 8e). In the stem cells of group 2, relatively high E74A mRNA levels peaked on day 11 and maintained high levels until day 13 (Figure 8e). In the stem cells from the control group, relatively high E74B levels detected on day 8 of the fifth instar gradually increased after pupation and peaked 24 h after pupation (Figure 8f). In the stem cells of group 1, mostly suppressed E74B expression until day 11 of the fifth instar increased at the beginning of day 11 and moderate transcript levels were maintained until pupation. The highest E74B mRNA levels were detected at pupation, followed by a decrease during the early pupal period (Figure 8f). In stem cells of group 2, E74B transcripts were at almost undetectable levels until day 13 of the fifth instar (Figure 8f). In the stem cells of the control group, moderate E75A mRNA levels peaked in the second half of day 9. Although a small decrease occurred on day 10, relatively high transcript levels remained until 24 h after pupation (Figure 9a). In the stem cells of group 1, quite low E75A mRNA levels until day 13 peaked sharply on this day, and then decreasing transcripts were maintained at moderate levels throughout the early pupal stage (Figure 9a). In the stem cells of group 2, E75A levels were almost undetectable until day 13 when a small increase occurred (Figure 9a). E75B transcripts in the stem cells of the control group began to rise after cessation of feeding and the highest mRNA levels were detected in the second half of day 9 and then maintained at moderate levels till 24 h after pupation (Figure 9b). In the stem cells of group 1, low E75B mRNA levels began to increase and peaked on day 13 of the fifth instar. Relatively moderate transcript levels were maintained until 24 h after pupation (Figure 9b). Day 3 fenoxycarb treatment resulted in very low E75B mRNA levels until day 12; however, a small increase occurred in the second half of day 12 and day 13 of the fifth instar (Figure 9b).

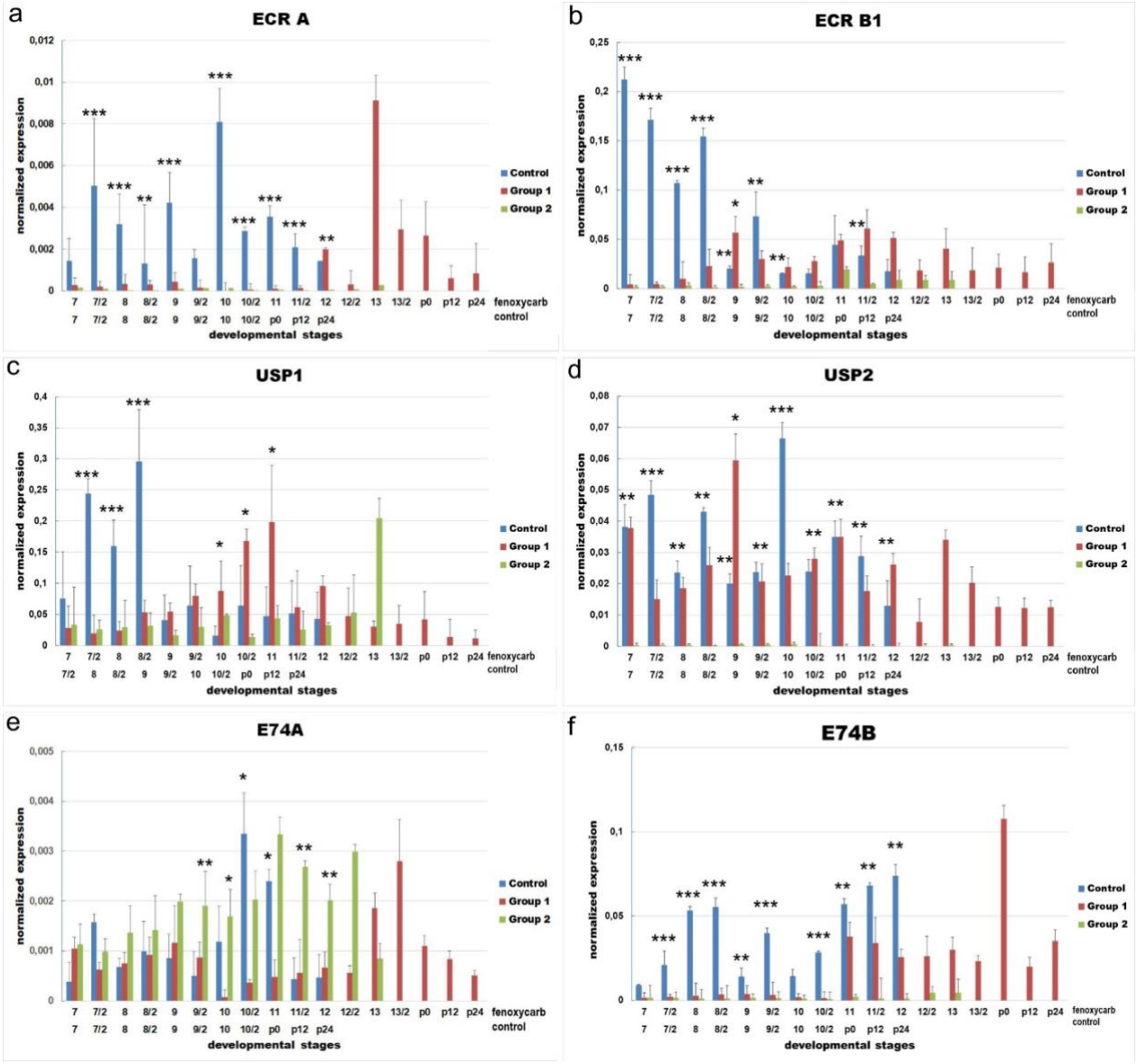

Figure 8. Expression profiles of ecdysone receptors and ecdysone-related transcription factors in the stem cell fractions of untreated and fenoxycarb treated insects (Group 1, day 0 treated insects, and group 2, day 3 treated insects): a) EcR A; b) EcR B1; c) USP1; d) USP2; e) E74A; f) E74B. Expression levels of the genes were normalized using *Bombyx mori* Actin 3 RNA as a housekeeping gene. Asterisks show significantly different expression ($P \leq 0.05$) by one-way ANOVA. Stars above bars into graphs indicate a significant difference between control and treatment groups ($P \leq 0.05$) by ANOVA. Significant differences between * control and day 0 treatment group; ** control and day 3 treatment group; *** control and all treatment groups.

In the stem cells of the control group, BRC Z1 levels showed a fluctuating expression pattern, and the highest level was detected just before pupation (Figure 9c). Relatively high BRC-Z1 levels were detected in the stem cells of group 1 until day 11. After a sudden drop that occurred in the second half of day 11, transcript levels rose sharply on day 13 and then dropped to quite low levels after pupation (Figure 9c). In the stem cells of group 2, the fluctuating expression pattern of BRC Z1 was detected until day 11 but its amount was considerably reduced on days 12 and 13 (Figure 9c).

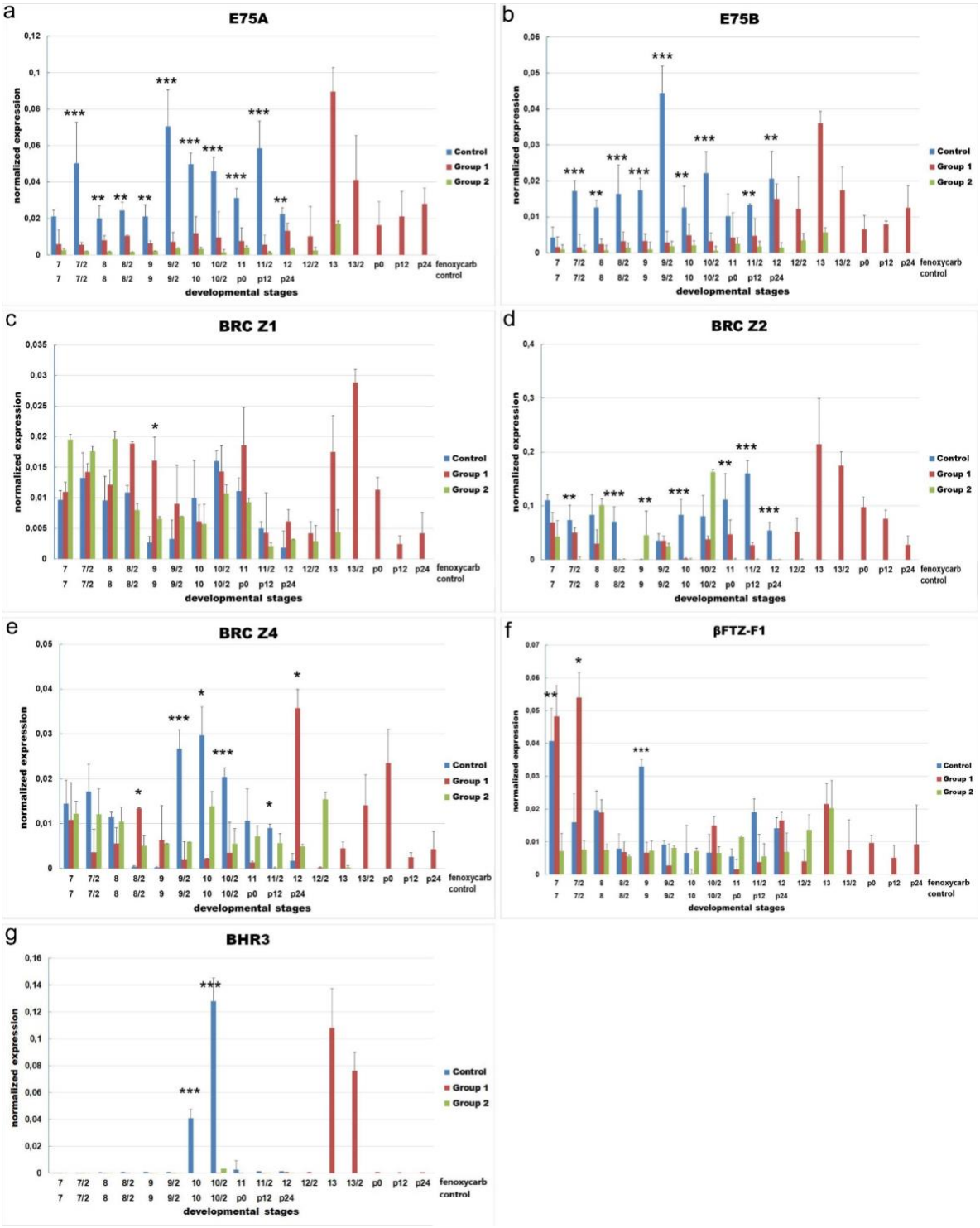

Figure 9. Expression profiles of ecdysone receptors and related transcription factors in the stem cell fractions of untreated and fenoxycarb treated insects (Group 1, day 0 treated insects, and group 2, day 3 treated insects): a) E75A; b) E75B; c) BR-C Z1; d) BR-C Z2; e) BR-C Z4; f) βFTZ-F1; g) BHR3. Expression levels of the genes were normalized using *Bombyx mori* Actin 3 RNA as a standard. Asterisks show significantly different expression ($P \leq 0.05$) by one-way ANOVA. Stars above bars into graphs indicate a significant difference between control and treatment groups ($P \leq 0.05$) by ANOVA. Significant differences between * control and day 0 treatment group; ** control and day 3 treatment group; *** control and all treatment groups.

In the stem cells of the control group, moderate BRC-Z2 mRNA levels decreased suddenly to an undetectable level on day 9. Following this decline, it started to rise again and peaked 12 h after pupation (Figure 9d). In stem cells of group 1, the fluctuating expression pattern of BRC-Z2 was detected until day 13. mRNA levels dropped to an undetectable level on some days. The highest transcript level was detected on day 13, followed by a gradual decline until 24 h after pupation (Figure 9d). In the stem cells of group 2, BRC Z2 expression was detected in only 5 of 13 developmental days, and the highest mRNA level was determined in the second half of day 10 (Figure 9d). In the stem cells of the control group, moderate BRC-Z4 levels on days 7 and 8 decreased to undetectable levels in the second half of day 8 and the first half of day 9. However, transcripts sharply rose in the second half of day 9 and peaked on day 10. Following the peak, its levels decreased gradually until 24 h after pupation (Figure 9e). In the stem cells of group 1, low levels of BRC- Z4 until day 12 peaked suddenly on this day but followed by a sharp decrease. mRNA levels started to rise again from day 13 and produced a second peak at pupation (Figure 9e). In the stem cells of group 2, a fluctuating expression pattern of BRC-Z4 was detected during the experiment (Figure 9e).

Two βFTZ-F1 peaks were detected in the stem cells of the control group on days 7 and 9, respectively. Transcript levels were low on other days during the experiment (Figure 9f). In the stem cells of group 1, the highest βFTZ-F1 levels were detected on day 7 of the fifth instar. After this day, low βFTZ-F1 mRNA levels were maintained during the experiment (Figure 9f). Low βFTZ-F1 levels in the stem cells of group 2 were maintained during the experiment and the highest level was determined on day 13 of the fifth instar (Figure 9f). In the untreated insects, BHR3 transcripts were only detected on day 10 of the fifth instar and peaked in the second half of the day (Figure 9g). A similar expression pattern was detected in the stem cells of group 1. BHR3 transcripts were detected only on day 13 (Figure 9g). Expression of BHR3 was completely suppressed in the stem cells of group 2 during the experiment (Figure 9g).

Discussion

The formation of the pupal midgut is strictly controlled by 20-hydroxyecdysone (20E), juvenile hormone (JH), paracrine factors (Smagghe et al., 2005) and various components from hemolymph (Hakim et al., 2007) and fat body (Smagghe et al., 2003; Hakim et al., 2010). The molecular mechanism of ecdysone during the formation of pupal midgut from stem cells is still unclear. Therefore, this study investigated the critical expression timing of ecdysone-related genes involved in the developmental processes of midgut stem cells by manipulating developmental events. Kamimura & Kiuchi (1998) reported that 1 ng of fenoxycarb administration on day 0 of the fifth instar temporarily suppressed 20E release from the prothoracic gland resulting in a prolonged feeding phase. However, the midgut purge and wandering behavior that emerged in group 1 at the beginning of day 11 were signs of ecdysone release (Sakurai et al., 1998). Due to the prolonged feeding stage, the development of pupal midgut in group 1 was delayed but stem cell proliferation and differentiation occurred almost in the same physiological timing as in the control when considered the spinning activity and gut purge. Based on the morphologic observations and BrdU assay results, proliferation of stem cells occurred in both the control group and group 1 during the late prepupal stage, and following a decrease in cell proliferation these cells began to differentiate into columnar pupal midgut epithelial cells during the early pupal stage. In contrast, fenoxycarb treatment on day 3 of the fifth instar completely inhibited ecdysone release (Kamimura & Kiuchi, 1998). Therefore, pupal midgut formation did not occur in group 2 but proliferated stem cells produced new mature larval epithelial cells.

The type of ecdysone receptors and the expression patterns of ecdysone-related genes are key elements in determining cell fate in insects (Talbot et al., 1993). Our previous study showed the co-expression patterns of EcR A and USP-2 in the midgut stem cells and their high mRNA levels especially during the late prepupal stage (Goncu et al., 2016). The delayed ecdysone secretion due to fenoxycarb treatment on day 0 completely suppressed the first increase of the EcR A; however, achieving critical hemolymph ecdysone levels allowed the EcR A expression to be similar to that of the control group.

Parthasarathy & Palli (2007) reported that EcR A and USP B regulate the differentiation of imaginal diploid cells in *Aedes aegypti* midgut. In *Manduca sexta* L., 1763 (Lepidoptera: Sphingidae) epidermis, EcR A and EcR B1 have been found during the cell divisions but only EcR A isoform has been detected during wing cuticle deposition in wing cells (Jindra et al., 1996). The similarity in the EcR A/USP2 expression patterns in the stem cells of group 1 with the control group and their complete inhibition in group 2 supported the involvement of this heterodimer in the late metamorphic process of stem cells.

Similar expression profiles of EcR B1 and USP 1 showed their involvement as a heterodimeric receptor complex in stem cells. According to the expression timing in the control group and group 1, EcR B1 and USP1 may be responsible for cellular processes such as the reprogramming of stem cells for the formation of the pupal midgut epithelium that occurs just before the initiation of metamorphic events in stem cells. The inhibition of EcR B1 and USP1 in stem cells of group 2 also supports this hypothesis. Riddiford et al. (2000) proposed that the loss of EcR B1 in the general epidermis of *M. sexta* might be critical to switch from the predifferentiation to the differentiation phase. When we evaluate our results and these previous reports, the marked decrease in EcR B1 expression and increase in EcR A expression in the control group and group 1 in the late prepupal stage may be a signal for the onset of differentiation events in midgut stem cells.

Stilwell et al. (2003) found that MsE74A mRNA expression occurs when ecdysteroid titers decrease. In this study, the presence of E74A in the long-term feeding phase of both treated groups both supported this previous report and pointed to a possible relationship between JH and transcriptional regulation of E74A. However, Fletcher et al. (1995) reported that mutated E74A and E74B cause death during the prepupal stage of *D. melanogaster,* and this information indicated their critical role in larval-pupal metamorphosis. In the stem cells of group 1, complete inhibition of E74A just before the termination of feeding suggested that its downregulation might important for the onset of metamorphic events. Maintaining the high expression rate of E74A in stem cells of group 2 supported this possibility. A previous study reported that E74A is produced in most larval and imaginal tissues of *Drosophila* (Boyd et al., 1991) but it can also bind late puffs and activate late genes. Ali et al. (2013) demonstrated that the highest E74A expression in the wing disc of *B. mori* is detected during the late prepupal stage and regulates transcription of late genes like the cuticular protein gene. Therefore, when expression profiles of E74A in stem cells of the control and group 1 insect are considered, E74A may mediate ecdysone for the activation of late genes allowing the stem cell differentiation. In contrast to E74A, almost complete inhibition of E74B expression in the extended feeding periods of treated insects indicated that the expression of this isoform is inhibited by juvenile hormone. Similarly, Stilwell et al. (2003) suggested that MsE74B expression occurs in direct response to ecdysone in the absence of JH in the epidermis of *M. sexta* associating with pupal commitment. In addition to this, its critical expression timing in the control group and group 1 after cessation of feeding and its complete inhibition in group 2 suggested its pivotal role in the preparation of stem cells for remodeling events.

Previous studies have suggested that the E75A gene can be induced directly or indirectly by the juvenile hormone in different insect tissues such as mature midgut cell (Goncu et al., 2016), *Drosophila* S2 cells (Dubrovsky et al., 2004), *Manduca* CH1 cultured cells (Dubrovskaya et al., 2004), but in this study, fenoxycarb treatment inhibited both E75A and E75B expression in the midgut stem cells during prolonged feeding phase of both treated groups. Similar to our results, different publications reported that E75A expression levels decreased in the midgut of *Tribolium castaneum*, Herbst, 1797 (Coleoptera: Tenebrionidae) after methoprene treatment, (Parthasarathy & Palli, 2008), and JH did not increase E75A levels in cultured *M. sexta* wing discs (Keshan et al., 2006). These results suggest that the interaction between JH and E75A is cell-specific. The upregulation of isoforms in control and group 1 insect stem cells during the formation of pupal midgut and their inhibition in stem cells of group 2 strongly suggested the role of E75 isoforms in this process.

Early BR-C gene is known to have an important role in *D. melanogaster* during metamorphosis because a mutation in this gene prevents larva-pupal transformation (Restifo & White, 1992; Fletcher & Thummel, 1995; Kiss et. al., 1998). The presence of all BRC transcripts during the extended feeding period of treated insects showed that JH did not inhibit BR-C gene expression. Similarly, it has been reported that JH does not inhibit the expression of BRC transcripts in *M. sexta* wing discs, moreover, metamorphic competence and production of BRC transcripts occur in the late larval stage when JH is still present in hemolymph (Zhou et al., 1998). Zhou & Riddiford (2002) reported that BR-C Z1 inhibits the activation of pupal cuticle genes in *Drosophila*. In this study, the presence of BR-C mRNAs in midgut stem cells of all groups during the feeding phase suggests that it may have a role in the inhibition of pupa-specific genes. Thus, downregulation of their expression in the middle prepupal stage may be a signal for the activation of genes responsible for pupal midgut formation. However, previous reports along with upregulation in BRC mRNA levels occurring in the stem cells of the control and group 1 and the absence of similar increases in group 2 indicate that they may have a role in the formation of the pupal midgut. BRC isoforms have been reported to be involved in the growth of wing buds by increasing cell division in *Blatta germanica* L., 1767 (Blattodea: Ectobiidae) (Huang et al., 2013) and also imaginal disc differentiation and histoblast proliferation in *Drosophila* (Zhou & Riddiford, 2002).

A previous study on BFTZ-F1 have suggested its role as a cellular competence factor associated with both 20E and JH in many tissues (Bernardo & Dubrovsky, 2012). Bernardo & Dubrovsky (2012) reported that the FTZ-F1 nuclear receptor is involved in JH signaling via JH receptor candidates methoprene-tolerant and germ cell-expressed. In this study, fenoxycarb treatment did not inhibit FTZ-F1 expression, suggesting that βFTZ-F1 is likely associated with JH signaling in stem cells. Our previous study demonstrated that BHR3 is a late prepupal stage-specific gene in the midgut stem cells of *B. mori* (Goncu et al., 2016). Timing of BHR3 expression in the stem cells of control and group 1 indicated that hormonal conditions suitable for BHR3 expression are provided only during the late prepupal stage under high 20E levels (Kamimura & Kiuchi, 1998; Sakurai et al., 1998). The absence of BHR3 transcripts in the stem cells of group 2 also supported this finding. The critical expression timing of BHR3 and its complete inhibition in stem cells of group 2 strongly indicated its possible role (s) for midgut stem cell differentiation. Siaussat et al. (2005) reported that PHR3 and PIE75 are detected as components of a 20E-induced genetic cascade associated with proliferative arrest and chitin precursor synthesis of IAL-PID2 cells.

In conclusion, we classified the genes firstly according to their susceptibility to fenoxycarb and secondly to their specific expression timings at each developmental stage (Figure 5a, b). The obvious inhibitor effect of fenoxycarb was detected in the expression of EcR A, EcR B1, USP 2, E74B, E75A, E75B and BHR3. Expression of USP1, BRC Z2 and BRC Z4 was not completely inhibited but their mRNA levels are reduced after fenoxycarb treatment. BRC Z1 and βFTZ F1 expression were almost not affected by fenoxycarb treatment and E74A was the only gene whose expression was induced by fenoxycarb (Figure 10a).

The expression of E74A and BRC isoforms in the feeding stage of all groups indicated their roles in the maintaining of larval midgut structure. The high expression status of the EcR B1, USP1, and E75 isoforms during the early prepupal stage suggest that these genes could be important for the involvement of stem cells in metamorphic changes and readiness for proliferation. Expression of EcR A, USP2, BRC Z1 and BHR3 may be important during the late prepupal stage when stem cells highly proliferate and prepare for differentiation. The presence of abundant E74B and BR-C Z2 mRNAs during the early pupal stage revealed their possible role in the pupal midgut. When considering all groups, the results obtained were not sufficient to make any inferences regarding the possible function of BFTZ-F1 in midgut stem cells (Figure 10b).

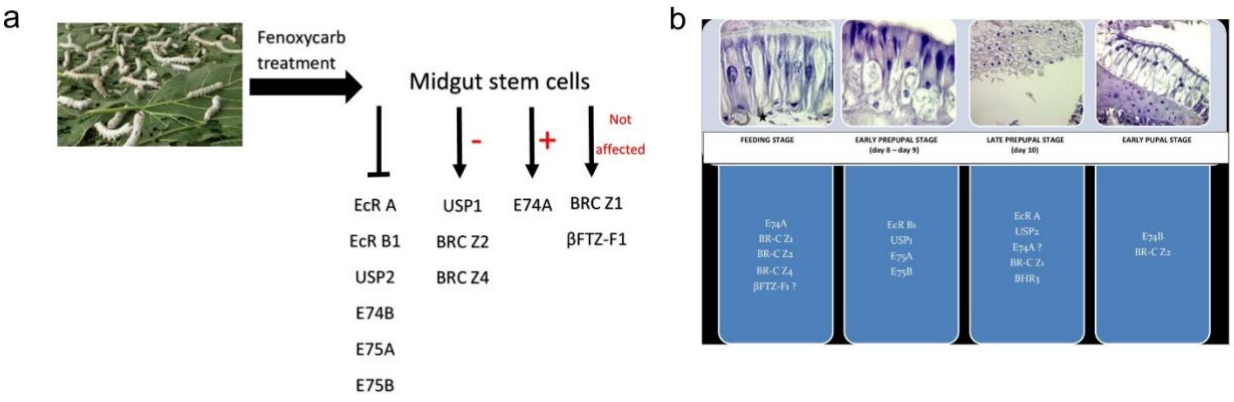

Figure 10. a) Comparison of expression profiles of ecdysone-related genes in the midgut stem cells of untreated and fenoxycarb treated insects showed their sensitivity of fenoxycarb treatment**.** Ecdysone receptors, USP2, E74B, and E75 isoforms were inhibited; however, USP1, BRC Z2 and Z4 were reduced by fenoxycarb. E74A was the only induced gene after fenoxycarb treatment. BRC Z1 and βFTZ-F1 genes seemed non-responsive to the fenoxycarb treatment. b) We grouped ecdysone-related genes in connection with their timing of expression and morphological evaluations of stem cells**.** More evidence is needed for genes with question marks.

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References

Ali, M. S., M. Iwanaga & H. Kawasaki, 2013. Ecdysone-responsive transcriptional regulation determines the temporal expression of cuticular protein genes in wing discs of *Bombyx mori.* Gene, 512 (2): 337-347.

Baldwin, K. M., R. S. Hakim, M. Loeb & S. Sadrud-Din, 1996. "Midgut Development, 31-54". In: Biology of the Insect Midgut (Eds. M. J. Lehane & P. F. Billingsley). Chapman and Hall, London, England, 486 pp.

Bernardo, T. J. & E. B. Dubrovsky, 2012. The *Drosophila* juvenile hormone receptor candidates methoprene-tolerant (MET) and germ cell-expressed (GCE) utilize a conserved LIXXL motif to bind the FTZ-F1 nuclear receptor. The Journal of Biological Chemistry, 287 (10): 7821-7833.

Boyd, L., E. O'Toole & C. S. Thummel, 1991. Patterns of E74A RNA and protein expression at the onset of metamorphosis in *Drosophila*. Development, 112 (4): 981-995.

Cermenati, G., P. Corti, S. Caccia, B. Giordana & M. A. Casartelli, 2007. Morphological and functional characterization of *Bombyx mori* larval midgut cells in culture. Invertebrate Survival Journal, 4 (2): 119-126.

Cranna, N. & L. Quinn, 2009. Impact of steroid hormone signals on *Drosophila* cell cycle during development. Cell Division, 4 (3): 1-13.

Dubrovskaya, V. A., E. M. Berger & E. B. Dubrovsky, 2004. Juvenile hormone regulation of the E75 nuclear receptor is conserved in Diptera and Lepidoptera. Gene, 13 (2): 171-177.

Dubrovsky, E. B., V. A. Dubrovskaya & E. M. Berger, 2004. Hormonal regulation and functional role of *Drosophila* E75A orphan nuclear receptor in the juvenile hormone signaling pathway. Developmental Biology, 268 (2): 258-270.

Fletcher, J. C., K. C. Burtis, D. S. Hogness & C. S. Thummel, 1995. The *Drosophila* E74 gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. Development, 121 (5): 1455-1465.

Fletcher, J. C. & C. S. Thummel, 1995. The ecdysone-inducible Broad-complex and E74 early genes interact to regulate target gene transcription and *Drosophila* metamorphosis. Genetics, 141 (3): 1025-1035.

Franzetti, E., Z. J. Huang, Y. X. Shi, K. Xie, X. J. Deng, J. P. Li, Q. R. Li, W. Y. Yang, W. N. Zeng, M. Casartelli, H. M. Deng, S. Cappellozza, A. Grimaldi, Q. Xia & Q. Feng, 2012. Autophagy precedes apoptosis during the remodeling of silkworm larval midgut. Apoptosis, 17 (3): 305-324.

Gibson, U. E., C. A. Heid & P. M. Williams, 1996. A novel method for real-time quantitative RT-PCR. Genome Research, 6 (10): 995-1001.

Goncu, E. & O. Parlak, 2011. The influence of juvenile hormone analogue, fenoxycarb on the midgut remodeling in *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae) during larval-pupal metamorphosis. Turkish Journal of Entomology, 35 (2): 179-194.

Goncu, E., R. Uranlı, G. Selek & O. Parlak, 2016. Developmental expression of ecdysone-related genes associated with metamorphic changes during midgut remodeling of silkworm *Bombyx mori* (Lepidoptera: Bombycidae). Journal of Insect Science, 164 (1): 1-13.

Hakim, R. S, K. Baldwin & G. Smagghe, 2010. Regulation of midgut growth, development, and metamorphosis. Annual Review of Entomology, 55: 593-608.

Hakim, R. S., M. B. Blackburn, P. Corti, D. B. Gelman, C. Goodman, K. Elsen, M. J. Loeb, D. Lynn, T. Soin & G. Smagghe, 2007. Growth and mitogenic effects of arylphorin in vivo and in vitro. Archives of Insect Biochemistry and Physiology, 64 (2): 63-73.

Hakim, R. S, S. Caccia, M. Loeb & G. Smagghe, 2009. Primary culture of insect midgut cells. In Vitro Cellular & Developmental Biology-Animal, 45 (3-4): 106-110.

Huang, J. H., J. Lozano & X. Belles, 2013. Broad-complex functions in postembryonic development of the cockroach *Blattella germanica* shed new light on the evolution of insect metamorphosis. Biochimica et Biophysica Acta, 1830 (1): 2178-2187.

Jindra, M., F. Malone, K. Hiruma & L. M. Riddiford, 1996. Developmental Profiles and Ecdysteroid Regulation of the mRNAs for Two Ecdysone Receptor Isoforms in the Epidermis and Wings of the Tobacco Hornworm, *Manduca sexta*. Developmental Biology, 180 (1): 258-272.

Kamimura, M. & M. Kiuchi, 1998. Effects of a juvenile hormone analog, fenoxycarb, on 5th stadium larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). Applied Entomology and Zoology, 33 (2): 333-338.

Keshan, B., K. Hiruma & L. M. Riddiford, 2006. Developmental expression and hormonal regulation of different isoforms of the transcription factor E75 in the tobacco hornworm *Manduca sexta*. Developmental Biology, 295 (2): 623-632.

Lee, C. Y., C. R. Simon, C. T. Woodard & E. H. Baehrecke, 2002. Genetic Mechanism for the Stage-and Tissue-Specific Regulation of Steroid Triggered Programmed Cell Death in *Drosophila*. Developmental Biology, 252 (1): 138-148.

Nishiura, J. T., K. Ray & J. Murray, 2005. Expression of nuclear receptor-transcription factor genes during *Aedes aegypti* midgut metamorphosis and the effect of methoprene on expression. Insect Biochemistry and Molecular Biology, 35 (6): 561-573.

Parthasarathy, R. & S. R. Palli, 2007. Stage-and cell-specific expression of ecdysone receptors and ecdysone-induced transcription factors during midgut remodeling in the yellow fever mosquito *Aedes aegypti.* Journal of Insect Physiology, 53 (3): 216-229.

Parthasarathy, R. & S. R. Palli, 2008. Proliferation and differentiation of intestinal stem cells during metamorphosis of the red flour beetle, *Tribolium castaneum*. Developmental Dynamics, 223 (4): 893-903.

Quinn, L., J. Lin, N. Cranna, J. Lee, N. Mitchell & R. Hannan, 2012. “Steroid Hormones in *Drosophila*: How Ecdysone Coordinates Developmental Signaling with Cell Growth and Division, 141-168”. In: Steroids - Basic Science (Ed. H. Abduljabbar). InTech, Rijeka, Croatia, 234pp.

Restifo, L. L. & K. White, 1992. Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in *Drosophila*: salivary glands, muscle, and gut. Roux's Archives of Developmental Biology, 201 (4): 221-234.

Riddiford, L. M., P. Cherbas, & J. W. Truman, 2000. Ecdysone receptors and their biological actions. Vitamins and Hormones, 60: 1-73.

Sakurai, S., M. Kayav & S. I. Satake, 1998. Hemolymph ecdysteroid titer and Ecdysteroid-dependent developmental events in the last larval stadium of the silkworm, *Bombyx mori*: the role of low ecdysteroid titer in larval-pupal metamorphosis and a reappraisal of the head critical period. Journal of Insect Physiology, 44 (10): 867-881.

Siaussat, D., F. Bozzolan, I. Queguiner, P. Porcheron & S. Debernard, 2005. Cell cycle profiles of EcR, USP, HR3 and B cyclin mRNAs associated to 20E-induced G2 arrest of *Plodia interpunctella* imaginal wing cells. Insect Molecular Biology, 14 (2): 151-161.

Smagghe, G., K. Elsen, M. J. Loeb, D. B. Gelman & M. Blackbur, 2003. Effects of a fat body extract on larval midgut cells and growth of Lepidoptera. In Vitro Cellular & Developmental Biology-Animal, 39 (1): 8-12.

Smagghe, G., W. Vanhassel, C. Moeremans, D. Wilde, S. Goto, M. J. Loeb, M. B. Blacburn & R. S. Hakim, 2005. Stimulation of midgut stem cell proliferation and differentiation by insect hormones and peptides. Annals of the New York Academy of Sciences, 1040 (1): 472-475.

Stilwell, G. E., C. A. Nelson, J. Weller, H. Cui, K. Hiruma, J. W. Truman & L. M. Riddiford, 2003. E74 exhibits stage-specific hormonal regulation in the epidermis of the tobacco hornworm, *Manduca sexta*. Developmental Biology, 258 (1): 76-90.

Talbot, W. S., E. A. Swyryd & D. S. Hogness, 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell, 73 (7): 1323-1337.

Tettamanti, G., A. Grimaldi, M. Casartelli, E. Ambrosetti, B. Ponti, T. Congiu, R. Ferrarese, M. R. Rivas-Pena, F. Pennacchio & M. De Eguileor, 2007. Programmed cell death and stem cell differentiation are responsible for midgut replacement in *Heliothis virescens* during prepupal instar. Cell and Tissue Research, 330 (2): 345-359.

Zhou, B., K. Hiruma, T. Shinoda & L. M. Riddiford, 1998. Juvenile hormone prevents ecdysteroid-induced expression of broad complex RNAs in the epidermis of the tobacco hornworm, *Manduca sexta*. Developmental Biology, 203 (2): 233-244.

Zhou, X. & L. M. Riddiford, 2002. Broad specifies pupal development and mediates the ‘status quo’ action of juvenile hormone on the pupal-adult transformation in *Drosophila* and *Manduca*. Development, 129 (9): 2259-2269.

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Original article (Orijinal araştırma)

Effects of pyriproxyfen and *Bacillus thuringiensis* Berliner, 1915 on enzymatic antioxidant defense system and hemocytes of *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae)[1]

Pyriproxyfen ve *Bacillus thuringiensis*'in *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae)'nın enzimatik antioksidan savunma sistemi ve hemosit sayılarına etkileri

Benay TUNÇSOY[2*] **Pınar ÖZALP[3]**

Abstract

With the increasing uses of biological control methods, knowing the physiological and immunological effects of these insecticides on insects is essential for them to be used safely in agricultural areas. The aim of the study is to determine the effects of pyriproxyfen and *Bacillus thuringiensis* subsp. *kurstaki* individually and as a mixture on malondialdehyde levels (MDA), glutathione-s-transferase, acetylcholinesterase, cytochrome P450 enzyme activities in hemolymph, midgut, and fat body and total (THC) and differential hemocyte counts (DHC) of fifth instar larvae of *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) 24, 48 and 72 h after exposure under laboratory conditions (30 ± 1ºC, 65 ± 5% RH). The study was conducted in the Animal Physiology Research Laboratory of the Department of Biology, Faculty of Science and Letter, University of Çukurova between 2016-2018. Effects of these insecticides on antioxidant, detoxification enzyme activities and MDA levels were changed depends on exposure time and the differences of tissues. THC decreased after 24 h, whereas it had increased after 48 and 72 h. DHC induced depends on exposure time and applied insecticide. This study revealed that pyriproxyfen and *B. thuringiensis* applications caused biochemical, physiological reactions and effected the immune defense system of larvae by the alterations in hemocyte counts.

Keywords: Antioxidant defense, detoxification, *Galleria mellonella,* immune defense, pyriproxyfen

Öz

Biyolojik mücadele yöntemlerinin artan kullanımları nedeniyle, bu insektisitlerin böcekler üzerindeki fizyolojik ve immünolojik etkilerinin bilinmesi, tarımsal alanlarda güvenle kullanılabilmesi için büyük önem taşımaktadır. Çalışmanın amacı, pyriproxyfen ve *Bacillus thuringiensis* subsp. *kurstaki*' nin tek başına ve karışım halinde 24,48 ve 72 saatlik etkileri sonucunda, *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae)'nın 5. dönem larvaların hemolenf, orta barsak ve yağ dokusunda malondialdehid (MDA) miktarı, glutatyon-s-transferaz, asetilkolinesteraz, sitokrom P450 aktiviteleri ile total ve diferansiyel hemosit sayıları üzerine etkilerini laboratuvar koşulları altında (30 ± 1ºC, %65 ± 5 RH) belirlemektir. Çalışma, Çukurova Üniversitesi, Fen Edebiyat Fakültesi, Biyoloji Bölümü Hayvan Fizyolojisi araştırma laboratuvarında 2016-2018 yılları arasında gerçekleştirilmiştir. Pyriproxyfen ve *B. thuringiensis*'in etkisinde, larvaların hemolenf, orta barsak ve yağ dokusunda antioksidan ve detoksifikasyon enzim aktiviteleri ile MDA seviyesinde uygulama süresine ve doku farklılıklarına bağlı olarak değişimler belirlenmiştir. Total hemosit sayısında uygulamadan 24 saat sonra azalma, 48 ve 72 saat sonra ise artış meydana gelmiştir. Diferansiyel hemosit sayısı üzerine etkilerinde ise, pyriproxyfen ve *B. thuringiensis* uygulamasına ve uygulama zamanına bağlı olarak değişiklikler meydana geldiği belirlenmiştir. Bu çalışma ile pyriproxyfen ve *B. thuringiensis* uygulamalarının biyokimyasal ve fizyolojik reaksiyonlara neden olduğu ve hemosit sayılarında değişikliğe yol açarak bağışıklık savunma sistemini etkilediği ortaya konmuştur.

Anahtar sözcükler: Antioksidan savunma, detoksifikasyon, *Galleria mellonella*, immun savunma, pfyriproxyfen

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Introduction

Galleria mellonella (L., 1758) (Lepidoptera: Pyralidae) larvae, which adversely affects beehives, are widely used as model organisms for studies in insect physiology and immunology, due to the similarities with the mammalian organisms in the immune response and gastrointestinal tracts. Moreover, *G. mellonella* larvae, in contrast to many other alternative models, can be reared easily in a wide range of temperature and in a short time. Due to the adverse effects of chemical insecticides used in management with *G. mellonella* on human health, problems such as leaving residues in honey and products grown in the surrounding lands, beekeepers are adopting integrated management. A key purpose of integrated management is to use methods that are not harmful to the environment and have minimal non-target effects. In particular, insecticides can be used in combinations due to the restriction of the use of new products in order to prevent the development of resistance of pests against some insecticides. Insecticides with different effect mechanisms used in the mixture increase the effect of each other and consequently this leads to a decrease in cost (Martinez et al., 2004; Attique et al., 2006). The use of combinations of these insecticides at low doses provides different ecological, biological and economic benefits.

In addition to the use of plant-based insecticides, hormones and analogs, predators and parasitic insects, entomopathogens and some microorganisms are also used in pest management. The most important of these methods of pest management is the use of insect growth regulators and microbial insecticides. Insect growth regulators are insecticides that mimic hormones in insects. Pyriproxyfen, belonging to the group of juvenile hormone analogs that is excreted by the corpus allatum in insects, is an organic and heterocyclic compound. Zhao et al. (2020) demonstrated that growth and development of *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) were significantly affected by pyriproxyfen and the fat body tissues were damaged after treatment. The detoxification enzyme activities were also increased by pyriproxyfen and affect the immune signaling pathway. *Bacillus thuringiensis* Berliner, 1915, it is a gram-positive entomopathogenic bacteria that has been used successfully against agricultural pests. It is known that in some cases, the exposure to insecticides results in the increased production of reactive oxygen species and cause oxidative stress in insects (Livingstone, 2001). To understand the detoxification metabolism of organisms against insecticides, some biomarkers are usually used to evaluate the physiological effects of environmental stressors (Boily et al., 2013; Carvalho et al., 2013; Badawy et al., 2015). Lipid peroxidation can be determined by measuring malondialdehyde (MDA) concentration which has been used as a biomarker for oxidative stress in living organisms (Meng et al., 2009). Glutathione-S-transferase (GST), one of the potential biomarkers, is also produced to assess the environmental impact of insecticides. Acetylcholinesterase (AChE) is an important enzyme in the nervous system that is inhibited by many pesticides. The Cytochrome P450 (Cyt P450) superfamily consists of important monooxygenase enzymes that are among the most useful biomarkers of exposure due to its role in biotransformation of xenobiotic (Goksoyr & Farlin, 1992).

The hematological studies are important in the field of insect physiology due to certain vital activities which are performed by hemocytes. Coagulation, phagocytosis, encapsulation, detoxification, storage and distribution of nutritive materials are the primary functions of hemocytes. There is an inherent variability of hemocytes within a species depending on the developmental and physiological stages (Sanjayan et al., 1996; Beetz et al., 2008; Abd el-Aziz & Awad, 2010). Although many studies conducted on the effects of pyriproxyfen and *B. thuringiensis* in insects, there is little information concerning the mixture effects of these insecticides. Consequently, the aim of this study was to evaluate whether sublethal concentrations of pyriproxyfen and *B. thuringiensis* alter cellular and biochemical parameters in insects. To test this hypothesis, detoxification enzymes (AChE, GST and Cyt P450), the amount of the lipid peroxidation products in different tissues of *G. mellonella* and hemocyte counts (total and differential; THC and DHC) in hemolymph were determined.

Materials and Methods

Experimental designs

The larvae used in the experiment were obtained from *G. mellonella* stock cultures reared in the Animal Physiology Research Laboratory of Çukurova University, Faculty of Science and Letters. *G. mellonella* larvae were reared at 30 ± 1ºC, 65 ± 5% RH on a diet composed of bran, honey, glycerol, honeycomb and distilled water (Bronksill, 1961). The study was conducted in the Animal Physiology Research Laboratory of the Department of Biology between 2016-2018. Before bran was added to the food, it was sterilized at 150ºC to prevent contamination. Juvenile hormone analog (10% EC), Pyriproxyfen (Admiral; Sumitomo Chemical, Kwinana, Australia) and larvicide based on *Bacillus thuringiensis* subsp. *kurstaki* (Delfin WG, consisting of 32,000 IU mg-1 spores; Agrikem, Antalya, Turkey consisting of 32,000 IU mg^{-1} spores) were used for determining the activity of antioxidant enzymes and hemocyte counts. LD_{50} concentrations of *B. thuringiensis* and pyriproxyfen were defined in our previous study (Tuncsoy Sezer & Ozalp, 2016). Fifth instar larvae were reared on honeycomb with LD_{50} concentrations of *B. thuringiensis* and pyriproxyfen (*B. thuringiensis* LD_{50} 359 µg mL^{-1} and pyriproxyfen LD_{50} 2.39 µg mL^{-1}) individually and a 1:1 mixture at these concentrations. For enzymatic analysis, hemolymph, midgut, and fat body tissues were dissected after 24, 48 and 72 h from larvae into a chilled eppendorf tubes with cold homogenization buffer (20 mM Tris buffer, pH 7.6). A few crystals of phenylthiourea were added to each sample to prevent melanization (Li et al., 2012). Resulting homogenates were centrifuged at 500 g for 15 min (4ºC) and supernatants recentrifuged at 12,000 g for 45 min (4ºC). Fat body tissues were homogenized at 50 W, 40-50 s. with ultrasonic homogenizer (Bandelin Sonoplus HD 2070, Berlin, Germany). Experiments were run in triplicate being 20 fifth instar larvae in each replicate. The samples for biochemical assays were frozen at -80ºC until use.

Enzyme activities

The TBA assay was used to assess the MDA concentration and the absorbance of the chromophore was measured at 535 nm. The MDA concentration is presented as nmoles of MDA produced per mg protein using a molar extinction coefficient of 1.56 10^5 M^{-1} cm^{-1} (Dubovskiy et al., 2008). For AChE activity, the midgut and fat body tissues were homogenized on ice in five volumes of a Tris-HCl buffer (100 mM, pH 8.0) containing 10% Triton and centrifuged at 12,000 g for 30 min (4ºC). This calorimetric method is based on the coupled enzyme reaction of acetylthiocholine as the specific substrate for AChE and 5,5' dithiobis-2-nitrobenzoate as an indicator for the enzyme reaction at 450 nm (Ellman et al. 1961). GST activity was measured spectrophotometrically using 1-chloro-2,4 dinitrobenzene and reduced glutathione as co-substrate, according to the method of Habig et al. (1974) by using an extinction coefficient of 9.6 mM^{-1} cm^{-1}. For the determination of Cyt P450 enzyme activity, p-nitroanisole was used as the substrate (Rose et al., 1995). The tests were conducted with three replicates. Protein amount was measured according to the Bradford (1976) method using bovine serum albumin as a substrate.

Total and differential hemocyte counts

For determining the THC and DHC, hemolymph was obtained by piercing the cuticle for blood smear slide preparation. Air dried smear was then fixed in neat alcohol for 10 min, stained with Giemsa stain and mounted in Entellan (Sigma Aldrich, Darmstadt, Germany). Phase contrast microscope (Nikon Eclipse E200; Tokyo, Japan) was used to determine the hemocyte types and morphology (Sezer & Ozalp, 2015). Four µL of hemolymph was obtained from larvae by using microcapillary tube (Sigma Aldrich, Darmstadt, Germany) for total hemocyte count. Hemolymph was then transferred to eppendorf tubes that contained 36 µL of anticoagulant buffer (9.8 mM NaOH, 186 mM NaCl, 17 mM Na_2EDTA, and 41 mM citric acid, pH 4.5). The number of circulating hemocytes per mm^3 was calculated using the formula of Jones (1962).

Data analysis

The statistical analyses of the data were performed at $P < 0.05$ by a series of analysis of variance and Student-Newman Keul's test using SPSS 21.00.

Results

MDA level in hemolymph had significantly decreased 24 and 48 h after application of all treatments, however it increased after 72 h when the larvae exposed to pyriproxyfen, *B. thuringiensis* and mixed relative to the control (Figure 1a). In midgut, MDA level had significantly increased after 24 h in all treatments and 72 h after exposure to *B. thuringiensis* and mixed treatments relative to the control (Figure 1b). MDA level in fat body had significantly increased after 24 h in all treatments, nevertheless it significantly decreased after 48 and 72 h relative to the control (Figure 1c).

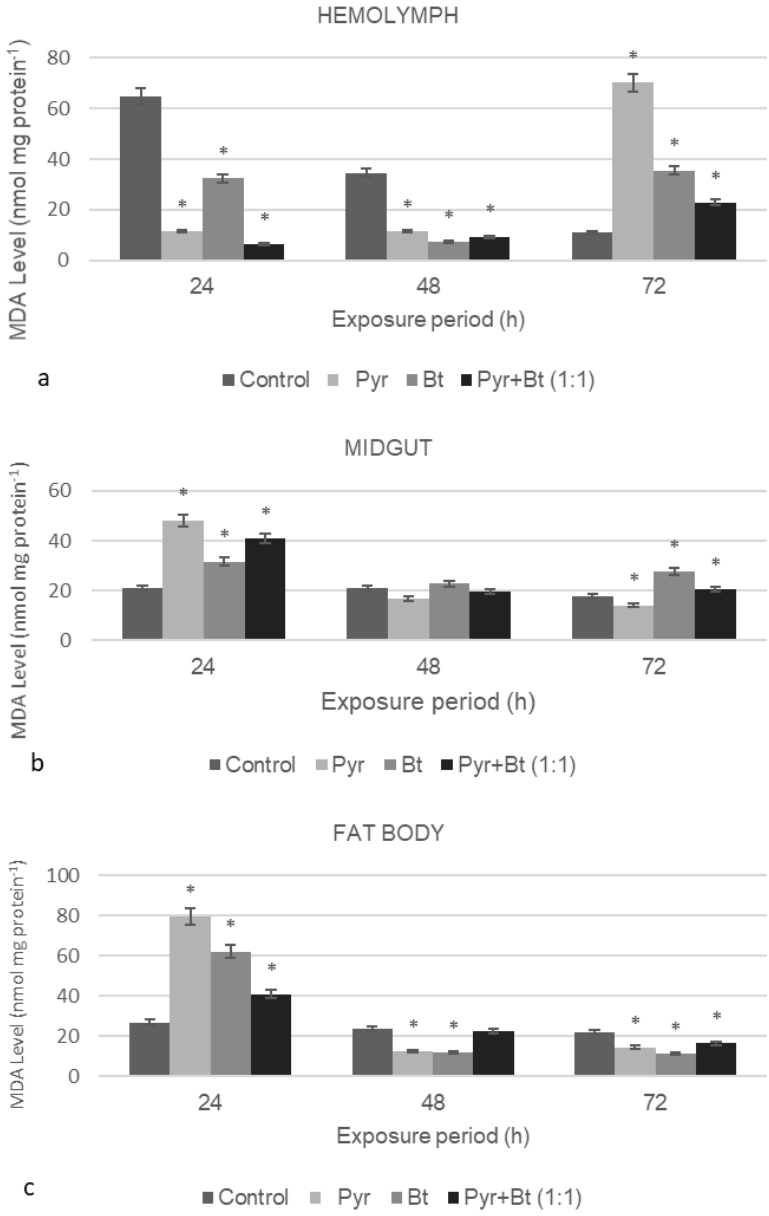

Figure 1. MDA levels of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) MDA level in hemolymph of *Galleria mellonella,* b) in midgut of *Galleria mellonella*, c) in fat body of *Galleria mellonella* were determined. Data shown are means ± SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

AChE activity in hemolymph had significantly increased after 24 and 72 h after exposure to pyriproxyfen, however at the same time point it had significantly decreased in *B. thuringiensis* treatment relative to the control. In the mixed treatment, the enzyme activity had significantly decreased after 24 h, nevertheless it had increased after 72 h relative to the control (Figure 2a). AChE activity in midgut had significantly 24 h after exposure to pyriproxyfen, however it had decreased after 48 h relative to the control. In *B. thuringiensis* treatments, AChE activity had significantly increased after 48 and 72 h relative to the control (Figure 2b). In fat body, AChE activity had significantly increased 24 h after exposure to pyriproxyfen, however it decreased after 48 h relative to the control (Figure 2c).

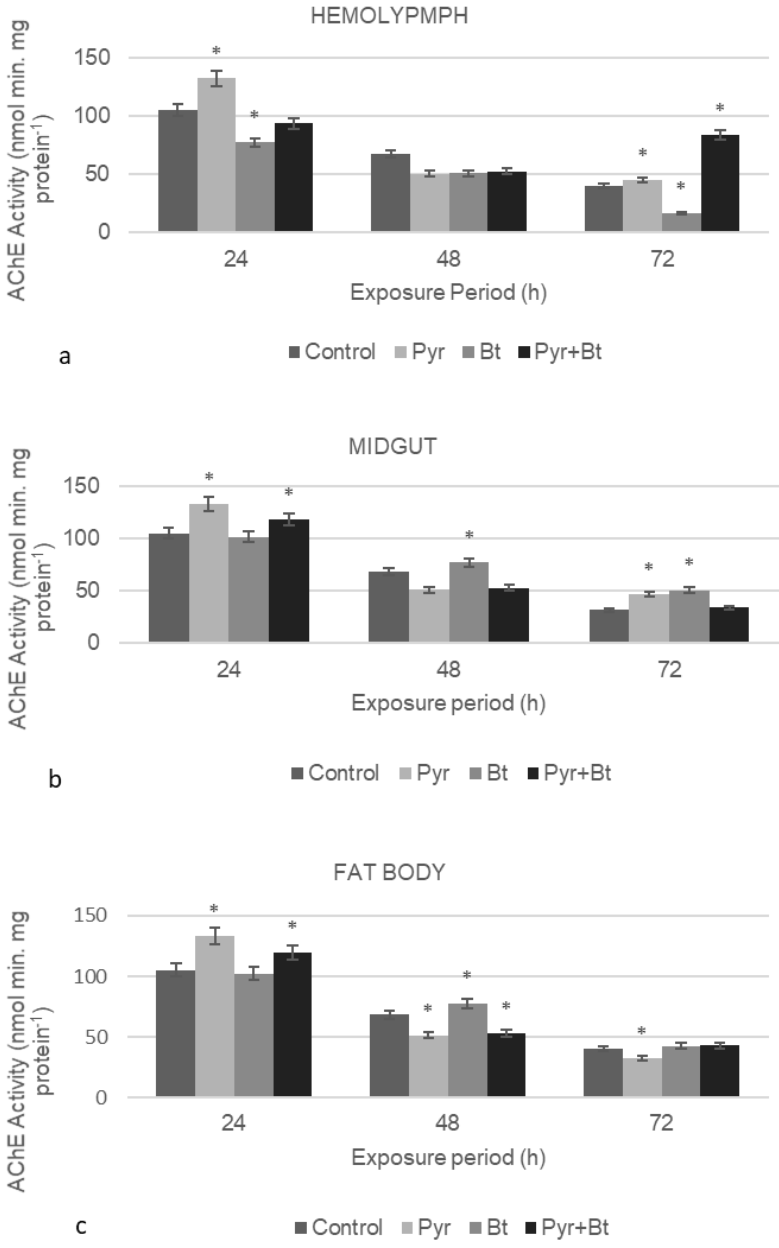

Figure 2. AChE activity of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) AChE activity in hemolymph of *Galleria mellonella,* b) in midgut of *Galleria mellonella*, c) in fat body of *Galleria mellonella* were determined. Data shown are means ± SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

GST activity in hemolymph and midgut had significantly decreased 24 h after application of all treatments. In fat body, GST activity had increased after 24 h in *B. thuringiensis* treatment, however it had decreased in mixed treatment relative to the control. Forty-eight h after exposure to pyriproxyfen and mixture the GST activity had significantly decreased in hemolymph, nevertheless it had increased in *B. thuringiensis* group relative to the control. In midgut, GST had significantly increased in all treatments. After 72 h, the enzyme activity had also significantly increased after exposure to pyriproxyfen and *B. thuringiensis*, however it had decreased in mixed treatment relative to the control. GST activity in fat body had significantly decreased after 72 h after application of all treatments (Figure 3).

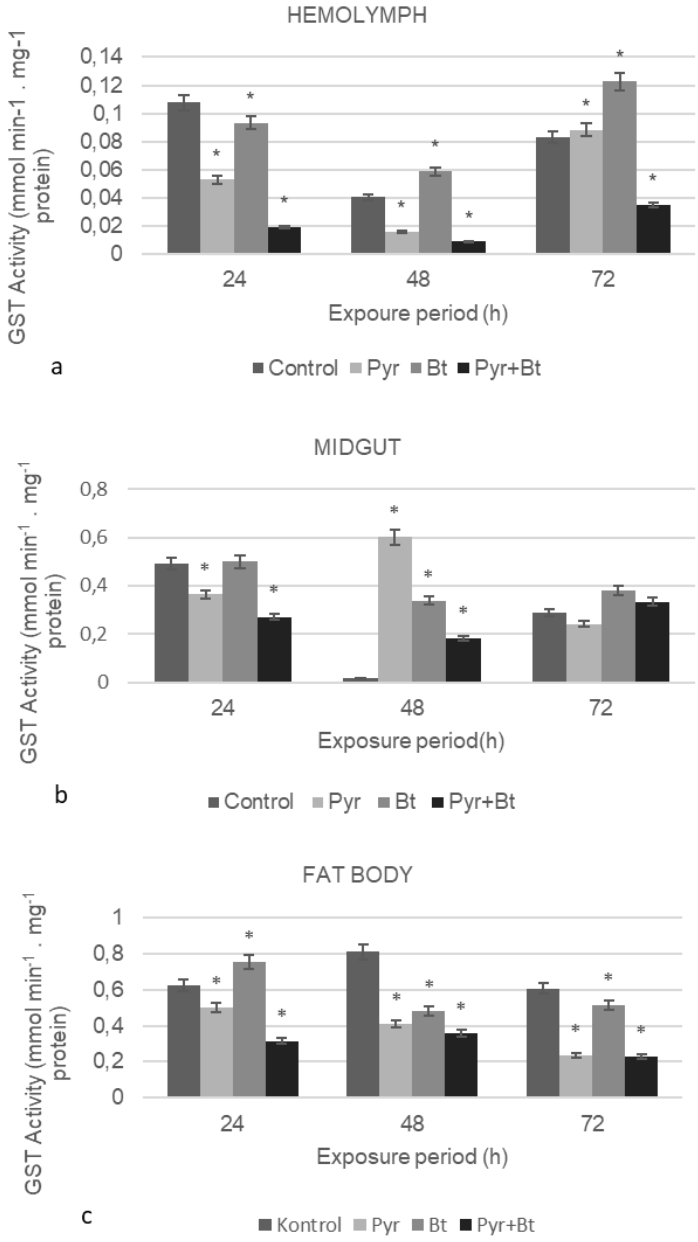

Figure 3. GST activity of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) GST activity in hemolymph of *Galleria mellonella,* b) in midgut of *Galleria mellonella*, c) in fat body of *Galleria mellonella* were determined. Data shown are means ± SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

The Cyt P450 activity in hemolymph had significantly decreased after 24, 48 and 72 h in all treatments, expect pyriproxyfen. After 72, Cyt P450 activity in midgut had significantly increased after exposure to *B. thuringiensis* relative to the control (Figure 4).

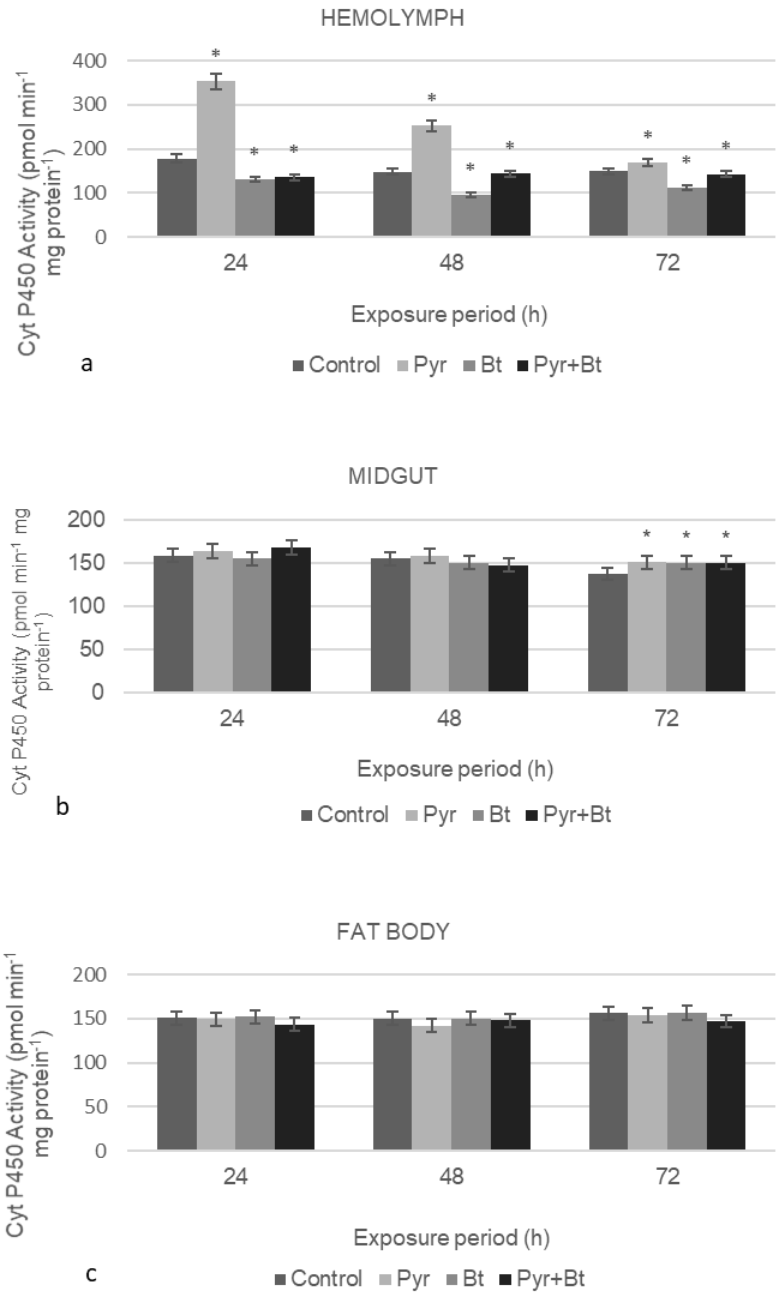

Figure 4. Cyt P450 activity of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) Cyt P450 activity in hemolymph of *Galleria mellonella,* b) in midgut of *Galleria mellonella*, c) in fat body of *Galleria mellonella* were determined. Data shown are means ± SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

The THC in pyriproxyfen, *B. thuringiensis* and mixed treatments had decreased significantly after 24 h, however it increased after 48 and 72 h in all treatments. After 72 h, THC decreased in mixed treatment relative to the pyriproxyfen and *B. thuringiensis* treatments (Figure 5). The highest increase after 48 h was observed with the mixture of pyriproxyfen and *B. thuringiensis*.

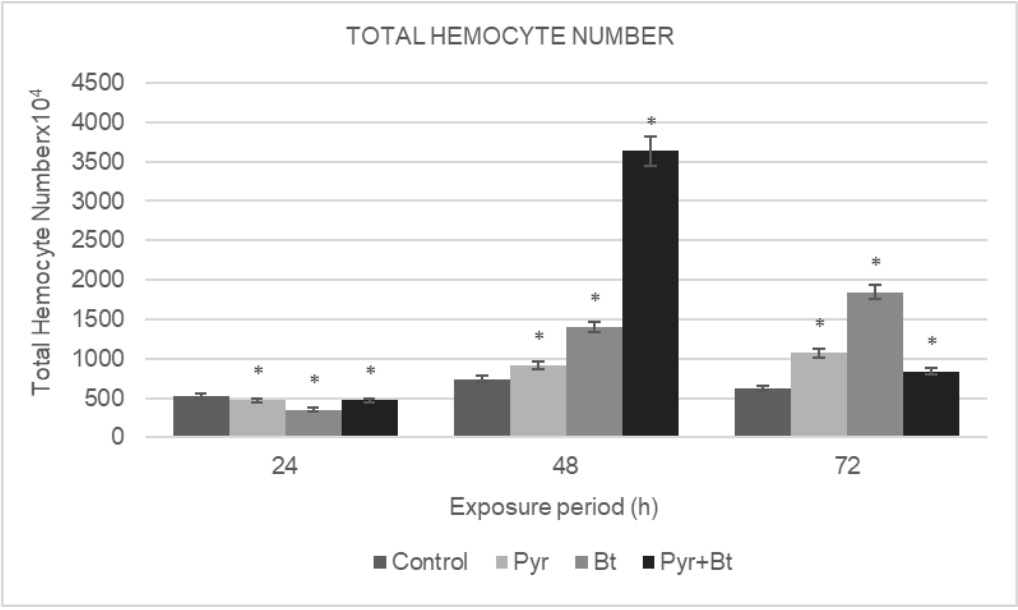

Figure 5. Total hemocyte count of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. Data shown are means ± SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

DHC in larvae revealed the highest number of plasmatocyte and granulocyte in all treatments. The plasmatocyte count in pyriproxyfen and *B. thuringiensis* treatments decreased significantly after 24 h, however it increased in mixed treatment at the same time interval relative to the control. After 48 h, it also increased significantly relative to the control. The highest increase after 72 h was observed with *B. thuringiensis* ($p < 0.05$; Figure 6a). The highest granulocyte count was observed in pyriproxyfen treatment after 24 and 48 h. After 72 h, it decreased in pyriproxyfen and *B. thuringiensis* treatments relative to the control ($p < 0.05$; Figure 6b). The prohemocyte count decreased in all treatments after 24 and 48 h. After 72 h, an increase was observed in only mixed treatment relative to the control ($p < 0.05$; Figure 6c).

The spherulocyte count increased in *B. thuringiensis* and mixed treatments after 24 h, however it decreased after 72 h ($p < 0.05$; Figure 7a). The oenocytoid count decreased in *B. thuringiensis* treatment after 48 h, however it increased in mixed treatment relative to the control. After 72 h, it increased in all treatments relative to the control ($p < 0.05$; Figure 7b).

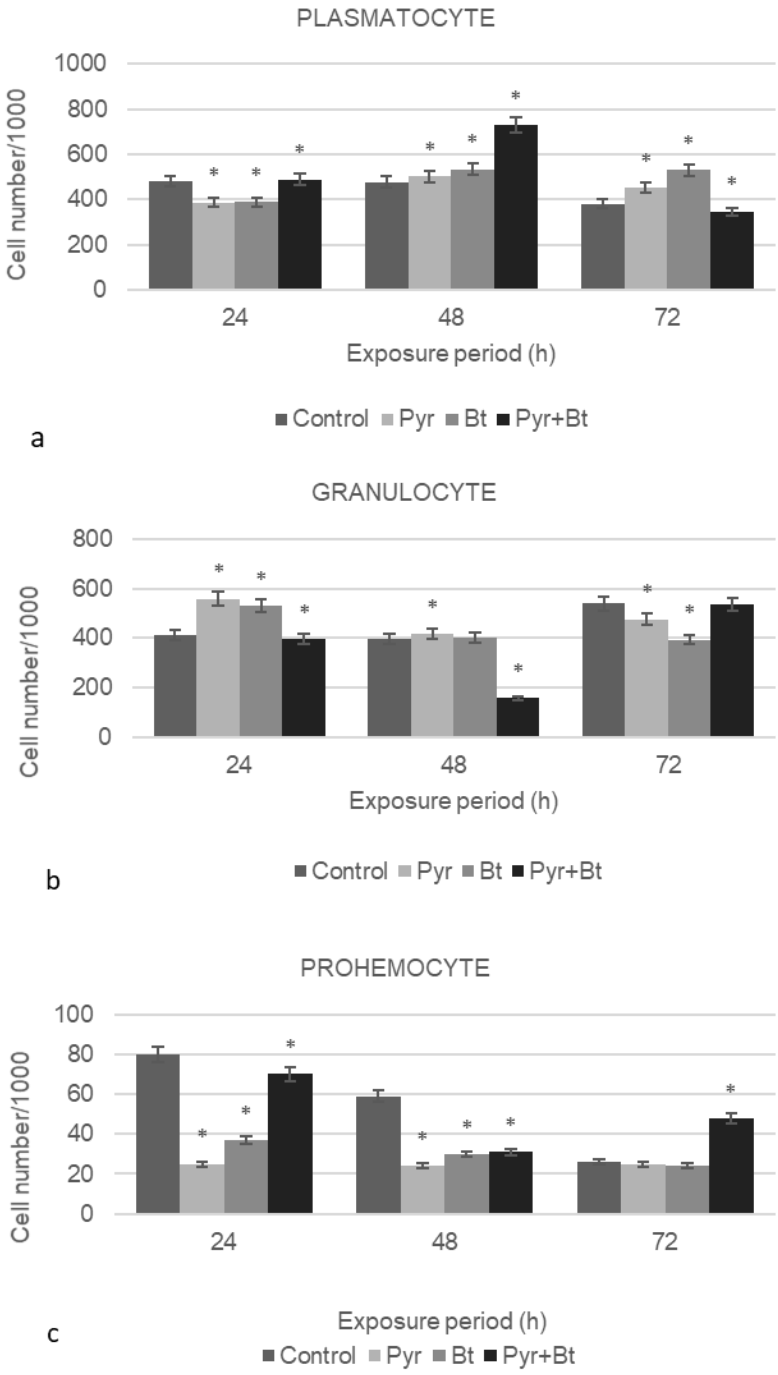

Figure 6. Differential hemocyte counts of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) plasmatocyte, b) granulocyte, c) prohemocyte counts were determined. Data shown are means ± SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

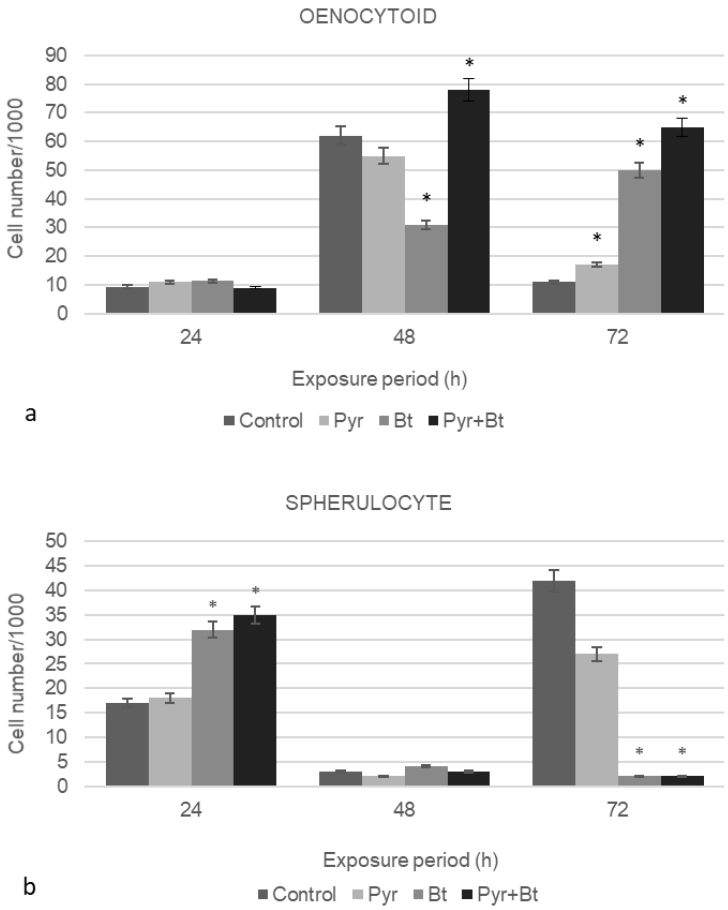

Figure 7. Differential hemocyte counts of fifth instar larvae exposed to pyriproxyfen, *Bacillus thuringiensis* and mixture after 24, 48 and 72 h. Control larvae were not treated with insecticides. a) spherulocyte, b) oenocytoid counts were determined. Data shown are means ± SE (Student-Newman Keul's test; asterisks indicate significant differences at $P < 0.05$).

Discussion

SOD and CAT are major enzymes in the first line of defense against oxidative damage converting the superoxide anion radical to hydrogen and the hydrogen peroxide to water and molecular oxygen, respectively (Hong et al., 2018). In our previous study, the activity of SOD, CAT and GPx in hemolymph and fat body had significantly increased after 72 h for larvae exposed to pyriproxyfen, *B. thuringiensis* and mixture, however in midgut the enzyme activities had significantly decreased relative to the control (Tuncsoy & Ozalp, 2016). Increased activities of antioxidant enzymes in the fat body and hemolymph are consistent with increased rates of adaptive metabolic responses to elevated lipid peroxidation (Hyrsl et al., 2007). In this study, we found a significant increase after 24 h in all treatments in the MDA level of midgut and fat body relative to the control. Similarly, Boctor & Salama (1983) found that *B. thuringiensis* bacteriosis in *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) increased lipid peroxidation. The increase of MDA level in midgut 24 h after exposure of *B. thuringiensis* might be also due to the destruction via endotoxins. Also, an increase after 72 h was observed in hemolymph of *G. mellonella* larvae when exposed to all treatments. It is considered that hemolymph has a vital role in destruction of free radicals in comparison with midgut and fat body. Fahmy (2012) also found that pyriproxyfen increased the CAT activity and MDA level in larvae of *S. littoralis*. Similarly, Dubovskiy et al. (2008) determined that *B. thuringiensis* infection resulted in increased SOD and MDA activity in the *G. mellonella* midgut, however CAT activity decreased on the first and following days after infection with *B. thuringiensis*. The increased MDA level 24

h after application of all treatments in the midgut and fat body of larvae is evidence of oxidative stress. However, the MDA level significantly decreased after 48 and 72 h in midgut and fat body after exposure to pyriproxyfen, *B. thuringiensis* and mixture. These contradictions may be due to the tissue specific differences in antioxidant enzyme activity (Hong et al., 2018).

AChE is an enzyme that found in many central and peripheral tissues, particularly in the nervous and muscular tissues (Massoulié et al., 1993). AChE inactivates the neurotransmitter acetylcholine in the synapses of the insect's central nervous (Casida & Durkin, 2013; Johnson, 2015; Zhu et al., 2017). In this study, it was determined that pyriproxyfen, *B. thuringiensis,* and mixed treatments caused significant changes in AChE activity in hemolymph, midgut, and fat body of the larvae after 24, 48, 72 h. In another study with *G. mellonella*, it was found that AChE activity decreased in hemolymph after exposure to sodium tetraborate, although an increase was observed in fat body (Durmus, 2007). Similarly, Nathan et al. (2008) determined that lethal concentration of azadirachtin, which is juvenile hormone analog, significantly inhibited the activity of AChE only at the high dose in *Nilaparvata lugens* (Stål, 1854) (Hemiptera: Delphacidae) compared with control. It was concluded that the reason of the reduction of AChE activity owing to increased free radical formation when pyriproxyfen and *B. thuringiensis* was applied may be due to the inactivation of this enzyme by free radicals.

Chemical insecticides, which are frequently utilized in fields against pests, enable to destroying of pest in a short time period, although overuse of these insecticides leads to progressing of resistance. Detoxification enzymes, including esterases, GST, and Cyt P450 has a vital role in resistance development. GST catalyze the secondary metabolism of a vast array of compounds which is oxidized by the Cyt P450 family (Wilce & Parker, 1994). The catalytic reactions transform a wide range of endogenous and xenobiotic compounds, such as insecticides (Armstrong, 1997; Zhu et al., 2017). Weirich et al. (2002) reported that the GST activity in the ventricles of honey bees was five times higher than the hemolymph. It was also determined that GST activity in midgut of *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) larvae increased when nickel and diazinon applied individually and in combination; however, when diazinon was applied individually, a decrease was observed in body wall and when nickel was applied individually. GST activity also decreased in fat body (Zawisza-Raszka & Dolezych, 2008). We found similar results with other studies and it was thought that various results in GST activities in the tissues may be due to the differences between tissue metabolism and defenses against reactive oxygen species. The Cyt P450 is also related to the synthesis of insect hormones, such as 20-hydroxyecdysone and juvenile hormone (Scott, 1999; Warren et al., 2002; Pondeville et al., 2013). In this study, P450 activity increased in hemolymph after 24, 48 and 72 h and in midgut 72 h after exposure to pyriproxyfen, *B. thuringiensis* and mixture. The increase in Cyt P450 activity when exposed to pyriproxyfen may be due to involving of monooxygenases in the synthesis of insect hormones. We also determined that Cyt P450 activity increased in midgut 72 h after application of all treatments, and significant increases in hemolymph of pyriproxyfen treatments after 24, 48 and 72 h. It is considered that this effect in hemolymph might be due to the accumulation of enzymes or proteins in non-target regions such as hemolymph, where these molecules bind to the toxin substances entering the body of the insect. Similar results to our findings have been found in insects responding to other chemicals, for example P450 monooxygenase increased in Malpighian tubules and in midgut of lepidopteran insect during the defense response to allelochemical toxicity (Yorulmaz & Ay, 2010). Qiu et al. (2003) also determined that Cyt P450 enzyme increased in midgut of *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae) larvae in proportion to fat body when exposed to pentamethyl benzene and naphthalene.

Hemocytes, which are important in the immune system of insects, decline with the starvation, parasitism, diseases and insecticides as well as changes in the growth of the insects. Thus, it is known that THC may change in stress factors. In this study, we determined that THC had decreased 24 h after exposure to pyriproxyfen, *B. thuringiensis,* and mixture, whereas it increased after 48 and 72 h. Significant

changes in the DHC of five hemocyte types were observed after application of all treatments. Degradation of cellular immune response may be due to the decreased hemocyte count in response to bacterial intoxication. Furthermore, a decrease in the THC may have occurred in starvation, which is an effect of bacteriosis of *B. thuringiensis* (De Block & Stoks, 2008; Lee et al., 2006). Broderick et al. (2010) showed that THC decreased in *Lymantria dispar* (L., 1758) (Lepidoptera: Erebidae) larvae exposed to *B. thuringiensis*. Manachini et al. (2011) also determined that THC in *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera: Dryophthoridae) hemolymph decreased after exposure to a sublethal concentration of *B. thuringiensis*. Insect hormones, such as 20-hydroxyecdysone and juvenile hormone, act in two ways in the immune system. 20-Hydroxyecdysone causes proliferation of hemocytes, while juvenile hormone and analogs have an adverse effect (James & Xu, 2012). Juvenile hormone analogs such as pyriproxyfen have an inhibitory effect on the ecdysone biosynthesis and suppress the release of hemocytes from hematopoietic organs. Kim et al. (2008) determined that juvenile hormones have an antagonistic effect on hemocyte counts and spreading of hemocytes. Zibaee et al. (2012) also showed that THC, plasmatocyte and granulocyte counts decreased in *Eurygaster integriceps* Puton, 1881 (Hemiptera: Scutelleridae) adults after exposure to pyriproxyfen. Ghasemi et al. (2014) also reported that THC, plasmatocyte, prohemocyte and spherulocyte counts decreased in *Ephestia kuehniella* Froggat, 1912 (Lepidoptera: Pyralidae) larvae, while granulocyte and oenocytoid counts increased when exposed to pyriproxyfen.

In conclusion, taking into consideration studies with different insect species, it is thought that juvenile hormone analogs such as pyriproxyfen and entomopathogen *B. thuringiensis* may have an adverse effect on hemocyte counts, inhibit larval hematopoietic functions or cytotoxic effects such as cell proliferation. Moreover, pyriproxyfen and *B. thuringiensis* also negative effects on oxidative stress and detoxification mechanism of *G. mellonella*. Investigation of the toxic effects of *B. thuringiensis* and pyriproxyfen on insects individually and in combination are of useful effects on pest management and improving of new methods which have less adverse effects on environment could overcome potential toxicological effects on non-target organisms.

Acknowledgments

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References

Abd el-Aziz, N. M. & H. H. Awad, 2010. Changes in the hemocytes of *Agrotis ipsilon* larvae (Lepidoptera: Noctuidae) in relation to dimilin and *Bacillus thuringiensis* infections. Micron, 41 (3): 203-209.

Armstrong, R. N., 1997. Structure, catalytic mechanism, and evolution of the glutathione transferases. Chemical Research in Toxicology, 10 (1): 2-18.

Attique, M. N. R., Khaliq, A. & A. H. Sayyed, 2006. Could resistance to insecticides in *Plutella xylostella* (Lep., Plutellidae) be overcome by insecticide mixtures. Journal of Applied Entomology, 130 (2): 122-127.

Badawy, M. E. I., H. M. Nasr & E. I. Rabea, 2015. Toxicity and biochemical changes in the honey bee *Apis mellifera* exposed to four insecticides under laboratory conditions. Apidologie, 46 (2): 177-193.

Beetz, S., T. K. Holthusen, J. Koolman & T. Trenczek, 2008. Correlation of hemocyte counts with different developmental parameters during the last larval instar of the tobacco hornworm, *Manduca sexta*. Archives of Insect Biochemistry, 67 (2): 63-75.

Boctor, I. Z. & H. S. Salama, 1983. Effect of *Bacillus thuringiensis* on the lipid content and compositions of *Spodoptera littoralis* larva. Journal of Invertebrate Pathology, 41 (3): 381-384.

Boily, M., B. Sarrasin, C. Deblois, P. Aras & M. Chagnon, 2013. Acetylcholinesterase in honey bees (*Apis mellifera*) exposed to neonicotinoids, atrazine and glyphosate: laboratory and field experiments. Environmental Science and Pollution Research, 20 (8): 5603-5614.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72 (1-2): 248-254.

Broderick, N. A., K. F. Raffa & J. Handelsman, 2010. Chemical Modulators of the Innate Immune Response Alter Gypsy Moth Larval Susceptibility to *Bacillus thuringiensis.* BMC Microbiology, 10 (129): 1-13.

Bronksill, J. F., 1961. A cage to simplify the rearing of the greater wax moth, *Galleria mellonella* (Pyralidae). Journal Lepidopterist's Society, 15 (2): 102-104.

Carvalho, S. M., L. P. Belzunces, G. A. Carvalho, J. L. Brunet & A. Badiou Beneteau, 2013. Enzymatic biomarkers as tools to assess environmental quality: a case study of exposure of the honeybee *Apis mellifera* to insecticides. Environmental Toxicology and Chemistry, 32 (9): 2117-2124.

Casida, J. E. & K. A. Durkin, 2013. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. Annual Review of Entomology, 58 (1): 99-117.

De Block, M. & R. Stoks, 2008. Compensatory growth and oxidative stress in a damselfly. Proceedings of The Royal Society of London Series B-Biological Sciences, 275 (1636): 781-785.

Dubovskiy, I. M., V. V. Martemyanov, Y. L. Vorontsova, M. J. Rantala, E. V. Gryzanova & V. V. Glupov, 2008. Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of *Galleria mellonella* L. larvae (Lepidoptera: Pyralidae). Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology, 148 (1): 1-5.

Durmus, Y., 2007. Effects of Sodium Tetraborate on Survival, Development, and Activities of Some Enzymes of Greater Wax Moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). University of Bulent Ecevit, Zonguldak, (Unpublished) Master Thesis, 75 pp (in Turkish with abstract in English).

Ellman, G. L., K. O. Courtney, V. Anders & R. M. Featherstone, 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7 (2): 88-95.

Fahmy, N. M., 2012. Impact of two insect growth regulators on the enhancement of oxidative stress and antioxidant efficiency of the cotton leaf worm, *Spodoptera littoralis* (Biosd.). Egyptian Academical Journal of Biological Science, 5 (1):137-149.

Ghasemi, V., S. Moharramipor & J. J. Sendi, 2014. Impact of pyriproxyfen and methoxyfenozide on hemocytes of the mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae). Journal of Crop Protection, 3 (4): 449-458.

Goksoyr, A. & L. Farlin, 1992. The cytochrome P450 system in fish, aquatic toxicology and environmental monitoring. Aquatic Toxicology, 24 (1-2): 1-19.

Habig, W. H., M. J. Pabst & W. B. Jakoby, 1974. Glutathione-s-transferases, the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry, 249 (22): 7130-7139.

Hong, Y., X. Yanga, Y. Huang, G. Yan & Y. Cheng, 2018. Assessment of the oxidative and genotoxic effects of the glyphosate-based herbicide roundup on the freshwater shrimp, *Macrobrachium nipponensis*. Chemosphere**,** 210: 896-906.

Hyrsl, P., E. Büyükgüzel & K. Büyükgüzel, 2007. The Effects of boric acid- induced oxidative stress on antioxidant enzymes and survivorship in *Galleria mellonella*. Archives of Insect Biochemistry and Physiology, 66 (1): 23-31.

James, R. R. & J. Xu, 2012. Mechanisms by which pesticides affect insect immunity. Journal of Invertebrate Pathology. 10 (2): 175-182.

Johnson, R. M., 2015. Honey bee toxicology. Annual Review of Entomology, 60: 415-434.

Jones, J. C., 1962. Current concepts concerning insect hemocytes. Americal Zoology, 2 (1): 209-246.

Kim, Y., S. Jung & N. Madanagopal, 2008. Antagonistic effect of juvenile hormone on hemocyte-spreading behavior of *Spodoptera exigua* in response to an insect cytokine and its putative membrane action. Journal of Insect Physiology, 54 (6): 909-915.

Lee, K. P., J. S. Cory, K. Wilson, D. Raubenheimer & S. J. Simpson, 2006. Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. Proceeding of Royal Society Biological Science, 273 (1588): 823-829.

Li, Z., D. Ptak, L. Zhang, E. K. Walls, W. Zhong & Y. F. Leung, 2012. Phenylthiourea specifically reduces zebrafish eye size. PLoS ONE, 7 (6): e40132, 1-14.

Livingstone, D. R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin, 42 (8): 656-666.

Manachini, B., V. Arizza, D. Parrinello & N. Parrinello, 2011. Hemocytes of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) and their response to *Saccharomyces cerevisiae* and *Bacillus thuringiensis*. Journal of Invertebrate Pathology, 106 (3): 360-365.

Massoulié, J., L. Pezzementi, S. Bon, E. Krejci & F. M. Vallette, 1993. Molecular and cellular biology of cholinesterases. Progress in Neurobiology, 41 (1): 31-91.

Meng, J. Y., C. Y. Zhang, F. Zhu, X. P. Wang & C. L. Lei, 2009. Ultraviolet light-induced oxidative stress: Effects on antioxidant response of *Helicoverpa armigera* adults. Journal of Insect Physiology, 55 (6): 588-592.

Nathan, S. S., M. Y. Choi, H. Y. Seo, C. H. Paik, K. Kalaivani & J. D. Kim, 2008. Effect of azadirachtin on acetylcholinesterase (AChE) activity and histology of the brown planthopper *Nilaparvata lugens* (Stal). Ecotoxicological and Environmental Safety, 70 (2): 244-250.

Pondeville, E., J. P. David, E. Guittard, A. Maria, J. C. Jacques, H. Ranson, C. Bourgouin & C. DauphinVillemant, 2013. Microarray and RNAi analysis of P450s in *Anopheles gambiae* male and female steroidogenic tissues: CYP307A1 is required for ecdysteroid synthesis. PLoS ONE, 8 (12): e79861, 1-9.

Qiu, X., W. Y. Tian & X. Leng, 2003. Cytochrome P450 monooxygenases in the cotton Bollworm (Lepidoptera: Noctuidae): tissue differences and induction. Journal of Economical Entomology, 96 (4): 1283-1289.

Rose, R., L. Barbhaiya, R. Roe, G. Rock & E. Hodgson, 1995. Cytochrome P-450- Associated insecticide resistance and the development of biochemical diagnostic assays in *Heliothis virescens*. Pesticide Biochemistry and Physiology, 51 (3): 178-191.

Sanjayan, K. P., T. Ravikumar & S. Albert, 1996. Changes in the haemocyte profile of *Spilostethus hospes* (Fab.) (Heteroptera: Lygaeidae) in relation to eclosion, sex and mating. Journal of Bioscience, 21 (6): 781-788.

Scott, J. G., 1999. Cythochromes P450 and insecticide resistance. Insect Biochemistry and Molecular Biology, 29 (9): 757-777.

Sezer, B. & P. Ozalp, 2015. Effects of pyriproxyfen on hemocyte count and morphology of *Galleria mellonella*. Fresenius Environmental Bulletin, 24 (2a): 621-625.

Tuncsoy Sezer, B. & P. Ozalp, 2016. Combined effects of pyriproxyfen and *Bacillus thuringiensis* on antioxidant activity of hemolymph, midgut and fat body of *Galleria mellonella* larvae. Fresenius Environmental Bulletin, 25 (5): 1660-1665.

Warren, J. T., A. Petryk, G. Marqués, M. Jarcho, J. P. Parvy, C. Dauphin-Villemant, M. B. O'Connor & L. I. Gilbert, 2002. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. Proceedings of the National Academy of Sciences of the United States of America, 99 (17): 11043-11048.

Weirich, G., A. Collins & V. Williams, 2002. Antioxidant enzymes in the honey bee, *Apis mellifera*. Apidologie, 33 (1): 3-14.

Wilce, M. C. & M. W. Parker, 1994. Structure and function of glutathione S-transferases. Biochimica et Biophysica Acta, 1205 (1): 1-18.

Yorulmaz, S. & R. Ay, 2010. The Enzymes playing role in detoxification of the pesticides in mites and insects. Journal of Agricultural Faculty of Uludag University, 24 (2): 137-148 (in Turkish with abstract in English).

Zawisza-Raszka, A. & B. Dolezych, 2008. Acetylcholinesterase, catalase and glutathione s-transferase activity in beet armyworm (*Spodoptera exigua*) exposed to nickel and/or diazinon. Acta Biologica Hungarica, 59 (1): 31-45.

Zhao, G., H. Guo, H. Zhang, X. Zhang, H. Qian, G. Li & A. Xu, 2020. Effects of pyriproxyfen exposure on immune signaling pathway and transcription of detoxification enzyme genes in fat body of silkworm, *Bombyx mori*. Pesticide Biochemistry and Physiology, 168 (104621): 1-7.

Zhu, Y.C., J. Yao, J. Adamczyk & R. Luttrell, 2017. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). PLoS ONE, 12 (5): e0176837, 1-16.

Zibaee, A., A. R. Bandani & D. Malagoli, 2012. Methoxyfenozide and pyriproxyfen alter the cellular immune reactions of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) against *Beauveria bassiana*. Pesticide Biochemistry and Physiology, 102 (1): 30-37.

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Original article (Orijinal araştırma)

Combined and individual effects of diatomaceous earth and methyl eugenol against stored products insect pests

Metil öjenol ve diyatom toprağının depolanmış ürün zararlısı böceklere karşı tek başına ve birlikte etkisi

Sait ERTÜRK[1*]

Abstract

The insecticidal activity of a proprietary diatomaceous earth, Detech, and methyl eugenol was tested with *Sitophilus granarius* (L., 1758), *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera: Curculionidae), *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrychidae), *Tribolium confusum* Jaquelin Du Val, 1868 (Coleoptera: Tenebrionidae) adults at Stored Products Pest Laboratory of Plant Protection Central Research Institute (Ankara, Turkey) in 2020. A range of doses of Detech (100, 250 and 500 mg kg^{-1}) and methyl eugenol (5, 10 and 15 mg kg^{-1}) were used. To determine insect mortality, at a range of times after treatment, and half-doses of both products were also studied in order to examine simultaneous use for synergistic effect. The greatest effect of Detech on *R. dominica*, *S. zeamais, S. granarius* and *T. confusum* was observed with 500 mg kg^{-1} giving 81, 29, 26 and 18% mortality 28 d after treatment (DAT), respectively. Methyl eugenol at 15 mg kg^{-1} gave complete mortality with *R. dominica* adults 3 DAT. Simultaneous application of both products did not give complete mortality in test insects. The data show that Detech and methyl eugenol are promising treatments for the control of insect pests of stored products.

Keywords: Essential oils, mortality, physical control, stored products insects, synergism

Öz

Tescilli bir diyatom toprağı Detech ve metil öjenolün *Sitophilus granarius* (L., 1758), *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera: Curculionidae), *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrychidae), *Tribolium confusum* Jaquelin Du Val, 1868 (Coleoptera: Tenebrionidae) erginlerine karşı Zirai Mücadele Merkez Araştırma Enstitüsü, Depolanmış Ürün Zararlıları Laboratuvarı (Ankara, Türkiye)'nda 2020 yılında test edilmiştir. Bu amaçla farklı dozlarda Detech (100, 250 ve 500 mg kg^{-1}) ve metil öjenol (5, 10 ve 15 mg kg^{-1}) kullanılmıştır. Böceklerin ölüm oranını belirlemek için farklı maruz kalma süreleri kullanılmıştır. Sinerjistik etkiyi belirlemek amacıyla, her iki ürünün de yarı dozları böcekler üzerinde çalışılmıştır. Diyatom toprağı Detech, *R. dominica, S. zeamais, S. granarius* ve *T. confusum* için en yüksek etkisi 500 mg kg-1 dozda, 28 günlük maruziyet süresinin sonunda sırasıyla %81, %29, %26 ve %18 ölüm oranı şeklinde gerçekleşmiştir. 15 mg kg^{-1}'lık metil öjenol dozu, *R. dominica* için günlük maruziyetten sonra %100 ölüm oranı sağlamıştır. Her iki ürünün aynı anda kullanılması, üzerinde çalışılan böceklerde tam bir ölüm oranına ulaşmamıştır. Elde edilen veriler, Detech ve metil öjenol kullanımının depolanmış ürün zararlısı böcekler ile mücadelede ümit var sonuçlar verdiğini ortaya koymuştur.

Anahtar sözcükler: Uçucu yağlar, ölüm oranı, fiziksel kontrol, depolanmış ürün zararlısı böcekler, sinerjizm

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Introduction

The confused flour beetle, *Tribolium confusum* Jaquelin Du Val, 1868 (Coleoptera: Tenebrionidae), the maize weevil, *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera: Curculionidae), the lesser grain borer, *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrychidae), and the granary weevil, *Sitophilus granarius* (L., 1758) (Coleoptera: Curculionidae), are some of the most destructive insect pests of the stored grain. The damage of these insects is important in terms of both quality and quantity loss of the product. *Sitophilus granarius*, *S. zeamais* and *R. dominica* are known as primary pests because they can easily break the seeds with their mandibles*. Tribolium confusum* is known as a secondary pest; it can easily infest damaged grain kernels and feed on different commodities (Aitken, 1975; Mewis & Ulrichs, 2001; Gałęcki et al., 2019). They not only consume the grain, but also contaminate the commodity with their body parts, feces, and the excretions, the latter of which may cause a bad smell in the commodity (Houssou et al., 2009). Additionally, by altering the microflora, they may cause mycotoxin formation on the products, which are dangerous especially for warm-blooded living organisms (Dawson et al., 2004; Richard et al., 2007). The inability to consume the harvested grain immediately, the demands of the food industry, mills, government and nongovernment organizations are important in determining storage period. Loss of grain and related commodities seriously threat global food security and availability (Oliveira, et al., 2014). It is predicted that the human population will reach 9.1 billion by the year 2050, with the concomitant increased needs for food production (FAO, 2009).

Currently, synthetic residual insecticides and fumigants are most commonly used to control stored products pests worldwide. However, several negative effects, such as the environmental contamination and toxicity to warm-blooded organisms, pesticides residues and pest resistance have arisen from their continuous use. Therefore, research has focused on developing alternative control measures (Halliday et al., 1988; Pacheco et al., 1990; Arthur, 1996; Ribeiro et al., 2003; Boulogne et al., 2012; Boyer et al., 2012; Barres et al., 2016; Hubert et al., 2018).

Inert dusts and botanical derivatives are among of the most promising alternatives to replace synthetic insecticides (Ferizli et al., 2012; Islam & Rahman, 2016; Ertürk et al., 2017; Alkan et al., 2019a, b, c, 2020a, b; Ertürk et al., 2020; Korunic & Fields, 2020; Zhanda et al., 2020). Diatomaceous earth (DE) is a kind of inert dust that is composed mostly of amorphous silica and several oxides of Al, Ca, Mg, Fe, K, Na and Ti. DE originated from single-cell phytoplankton that lived in salty or freshwater sources (Vayias & Athanassiou, 2004; Ashraf et al., 2016; Adarkwah et al., 2017; Baliota & Athanassiou, 2020). The fossils of these tiny creatures turned into diatom rocks and DE is obtained from these rocks by fine grinding. Unlike synthetic chemicals, the mode of action of DE is non-chemical. The main cause of insect mortality occurs by desiccation due to water loss from the entire body. DE particles absorb the epicuticular lipids from insect cuticles, which is a waterproof layer of the integument, consequently, insect mortality is caused by desiccation (Zacher & Kunike, 1931; Ebeling, 1971; Korunic, 1998; Prasantha et al., 2015). However, the use of DE has been limited due to the decrease in the hectoliter weight of the product, non-target effects on workers, and damage to machinery and equipment (Merget et al., 2002).

As an alternative to synthetic pesticides, plant derivatives are recognized as safer and it has been known for a long time that plant metabolites have biological activity against insects (Obeng-Ofori & Reichmuth, 1997; Chang et al., 2009; Regnault-Roger et al., 2012; Dwivedy et al., 2015; Jankowska et al., 2017). Pesticides obtained from essential oils of botanical origin are a promising control option for stored product pests (Upadhyay et al., 2018). To ensure the successful sustainability of pest control, bioactive products from plants can be used in integrated pest management programs (Regnault-Roger et al., 2012; Mossa, 2016). Plants have many secondary metabolites that are synthesized for defense against harmful organisms. Methyl eugenol (ME) is a phenylpropanoid derived from eugenol and is found in more than 450 species across 80 plant families (Herrmann & Weaver, 1999; Tan & Nishida, 2012). ME causes death by inhibiting acetylcholine esterase, an essential enzyme in insect nervous systems (Lee et al., 2001).

DE and ME have mostly been studied separately, so there is a gap in the literature regarding their combined effect on stored product pests. Therefore, this study was conducted to investigate the synergistic effect of these agents against *T. confusum, S. granarius*, *R. dominica* and *S. zeamais,* as well as to reduce the potential risks arising from their individual use.

Materials and Methods

Insects

Adults of *S. granarius* and *S. zeamais* were reared on whole wheat and maize, respectively. Rearing of *T. confusum* and *R. dominica* was performed on cracked wheat with 5% brewer's yeast (by weight) (Athanassiou et al., 2016). In the experiments mixed-sex insects between 7 and 28 d old were used. All insects were incubated in climate cabinet at 25 ± 1ºC and 60 ± 5% RH in the dark (Nüve ID 501, Ankara, Turkey). The laboratory experiments were conducted in the stored products pest laboratory at Plant Protection Central Research Institute, Ankara, Turkey in 2020.

Diatomaceous earth and methyl eugenol

A proprietary DE product, Detech, was used in this study. This product comes from the Central Anatolia Region in Turkey from light-gray soil and consists of Fe_2O_3 1.50%, Al_2O_3 4.70%, CaO 4.75%, SiO_2 80.6%, MgO 0.85%, K_2O 0.50%, Na_2O 0.40% and TiO_2 <0.01% (Bayram et al., 2020) with an average granule size of 14 mm (Sağlam et al., 2020). ME (≥98% purity, CAS Number: 93-15-2) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Contact activity bioassays

The insecticidal activity of Detech and ME against adults of four stored product insects was evaluated by direct application to the grain. For this purpose, white soft winter wheat, *Triticum aestivum* L. (Poaceae), and maize, *Zea mays* L. (Poaceae), were weighed into 2-L plastic bags. Detech was applied as a dust at 100, 250 and 500 mg kg^{-1} of grain and ME was used in liquid form at 5, 10 and 15 ml kg^{-1} grain. The Detech and ME were added to the plastic bags separately. The plastic bags were closed and shaken to achieve equal distribution (Ertürk et al., 2017). To determine the synergistic effect of half doses of both agents, Detech and ME were applied to wheat and maize according to the method described by Khorrami et al., (2018). Also, the label rate of K-Obiol EC25 (25 g/L deltamethrin plus 250 g/L piperonyl butoxide, Bayer AG, Leverkusen, Germany) and untreated grain were prepared (Table 1). For each observation, separate PVC vials were used. Twenty g of treated grain was added to into each PVC vials (7 x 5 cm) with 20 adult insects and then the vials were incubated at 25ºC and 60 ± 5% RH. Mortality of insects was assessed 1, 2, 3, 7, 14, 21 and 28 d after of treatment (DAT). The experiments were included three replicates per treatment, and the experiments were repeated three times.

Table 1. Treatments used in the bioassays

Treatment	Dose (mg kg^{-1})
Detech	100
	250
	500
Methyl Eugenol	5
	10
	15
Detech+Methyl Eugenol	DE 50+ ME 2.5
	DE 125 + ME 5
	DE 250 + ME 7.5
K-Obiol EC25	Label Rate
Control (untreated grain)	-

Statistical analysis

Data from the dose screening tests were first converted to percentage mortality and then arcsine transformation (Zar, 1999; Warton & Hui, 2011). Tukey's multiple comparison test was used to reveal the differences between the treatments at 5% significance level. To determine the relationship between treatments, MINITAB Release 18 was used with a general linear model (Mckenzie & Goldman, 2004).

Results

The ANOVA (treatment × dosage × observation time × insect) revealed that interaction between the experimental factors tested including different rates of Detech and ME against the insect species were significant (Table 2).

For Detech, the greatest effect on *S. granarius* was obtained at 500 mg kg^{-1} with a 25.9% mortality 28 DAT, whereas the lowest rate of 100 mg kg^{-1} did not cause any mortality 1 DAT ($F_{10,88} = 34.5$, $P < 0.05$). The doses of 250 and 500 mg kg^{-1} Detech gave similar mortality results (Table 3). The highest insecticidal efficacy of Detech applied at 100, 250 and 500 mg kg^{-1} on *S. zeamais* were found at 28 DAT, with the highest mortalities being 12, 19 and 29%, respectively ($F_{10,88} = 101$, $P < 0.05$). Mortality caused by 100 mg kg^{-1} 1 DAT was negligible ($F_{10,88} = 26.4$, $P < 0.05$) (Table 4). For *T. confusum,* the highest adult mortality with 100 mg kg^{-1} Detech 28 DAT was <1% ($F_{10,88} = 146$, $P < 0.05$). Mortality did not exceed 18% for the three doses tested (Table 5). Unlike other insect species, the highest mortality for *R. dominica* was at 28 DAT for all doses. The highest mortalities for 100, 250 and 500 mg kg^{-1} Detech 28 DAT were 58, 74 and 81%, respectively. Two DATl, there was no mortality, but by 3 DAT, the lowest mortality <1% at 500 mg kg^{-1} was recorded (Table 6).

Table 2. ANOVA for different applications of Detech and methyl eugenol against *Sitophilus oryzae, Sitophilus granarius, Tribolium confusum* and *Rhyzopertha dominica*

Source	DF	F- Value	P-Value
Treatment	2	8746.1	<0.05
Dosage	2	175.5	<0.05
Insect	3	532.8	<0.05
Observation time	7	631.9	<0.05
Treatment * Dosage	4	44.6	<0.05
Treatment * Insect	6	131.1	<0.05
Treatment * Observation time	14	114.7	<0.05
Dosage * Insect	6	46.0	<0.05
Dosage * Observation time	14	2.1	<0.05
Insect * Observation time	21	24.8	<0.05
Treatment * Dosage * Insect	12	16.9	<0.05
Treatment * Dosage * Observation time	28	2.7	<0.05
Treatment * Insect * Observation time	42	30.3	<0.05
Dosage * Insect * Observation time	42	2.4	<0.05
Treatment * Dosage * Insect * Observation time	84	1.5	<0.05
Error	2304		
Total	2591		

Table 3. Mortality (%, mean ± SE) of *Sitophilus granarius* exposed to wheat grain treated with Detech and methyl eugenol 1 to 28 d after treatment

Treatment		1 DAT	2 DAT	3 DAT	5 DAT	7 DAT	14 DAT	21 DAT	28 DAT
DE (mg kg^{-1})	100	0.0±0.0 c*B♦*	1.1±0.4 deAB	3.7±1.2 deAB	5.9±1.2 cdA	6.7±1.4 cdeA	6.7±1.4 cdA	9.5±1.2 cdeA	11.4±1.2 cdA
	250	0.0±0.0 cC	1.1±0.9 eBC	6.1±0.7 deABC	6.7±1.4 cdABC	7.7±1.0 cdABC	8.6±1.1 cdAB	15.3±1.3 bcdeA	21.1±3.7 cdA
	500	0.1±0.0 cD	3.2±1.0 deCD	6.6±1.5 deBC	17.1±0.4 bcAB	22.5±0.6 bcA	24.9±0.5 bcA	24.9±0.5 bcdA	25.9±0.6 bcA
ME (mg kg^{-1})	5	0.1±0.0 bcC	13.1±1.6 dB	31.5±0.6 cAB	36.9±1.1 bA	42.4±1.2 bA	44.6±1.4 bA	44.6±1.4 bA	53.6±1.3 bA
	10	0.5±0.3 cD	37.4±1.5 cC	67.4±1.6 bB	93.4±1.5 aA	93.4±1.5 aA	93.4±1.5 aA	94.2±1.3 aA	96.3±1.2 aA
	15	5.9±1.2 bC	82.8±2.6 bB	98.1±1.5 aA	98.5±1.2 aA	98.9±0.9 aA	99.9±0.4 aA	99.9±0.4 aA	99.9±0.4 aA
DE+ME (mg kg^{-1})	50+2.5	0.0±0.0 cA	0.1±0.0 eA	0.8±0.4 eA	1.5±0.8 dA	3.5±1.8 deA	4.8±3.1 dA	4.8±3.1 deA	7.6±3.1 dA
	125+5	0.1±0.0 cC	0.8±.0.3 eBC	5.8±1.3 deABC	7.4±1.6 cdAB	10.3±1.4 cdA	10.3±1.4 cdA	10.3±1.4 cdeA	15.2±1.4 cdA
	250+7.5	0.8±0.1 bcC	4.9±1.7 deBC	16.1±1.5 cdAB	19.0±1.9 bcAB	23.9±1.4 bcAB	26.4±1.2 bcA	27.4±1.3 bcA	27.4±1.3 bcA
Control		0.0±0.0 cB	0.0±0.0 eB	0.0±0.0 eB	0.0±0.0 dB	0.0±0.0 eB	1.1±0.9 dAB	2.2±1.4 eA	2.2±1.4 dA
Insecticide		47.9±0.8 aB	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA

* Means followed by the same lowercase letter within columns are not significantly different at P < 0.05.
♦ Means followed by the same uppercase letter within rows are not significantly different at P < 0.05.
DAT, d after treatment; DE, Detech, ME: methyl eugenol, Insecticide, K-Obiol EC 25.

Table 4. Mortality (%, mean ± SE) of *Sitophilus zeamais* exposed to maize grain treated with Detech and methyl eugenol 1 to 28 d after treatment

Treatment		1 DAT	2 DAT	3 DAT	5 DAT	7 DAT	14 DAT	21 DAT	28 DAT
DE (mg kg^{-1})	100	0.1±0.0 c*B♦*	0.5±0.2 cAB	1.1±0.9 bAB	1.1±0.9 bAB	2.0±1.0 bAB	4.4±1.4 bAB	8.0±1.9 bA	12.4±2.5bA bA
	250	0.5±0.2 cB	1.1±0.9 cB	1.1±0.9 bB	2.0±1.0 bB	3.0±1.5 bAB	9.7±1.8 b bAB	16.0±1.6 bA	19.4±3.8 bA
	500	1.1±0.8 cB	3.3±1.9 cB	3.3±1.9 bAB	3.3±1.9 bAB	3.7±2.4 bAB	10.3±1.4 bAB	16.7±0.8 bAB	28.7±1.6 bA
ME (mg kg^{-1})	5	21.5±1.7 bC	79.6±1.6 bB	96.5±1.8 aA	97.1±1.6 aA	99.5±0.7 aA	99.5±0.7 aA	99.5±0.7 aA	100.0±0.0 aA
	10	22.1±3.6 bB	96.9±2.6 aA	99.5±0.7 aA	99.9±0.4 aA	99.9±0.4 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA
	15	30.0±1.2 bB	98.0±1.0 aA	99.9±0.4 aA	99.9±0.4 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA
DE+ME (mg kg^{-1})	50+2.5	0.0±0.0 cB	0.1±0.0 cB	0.1±0.0 bB	0.1±0.0 bB	0.1±0.0 bB	0.5±0.2 bB	2.2±2.0 bA	16.7±1.4 bA
	125+5	0.0±0.0 cA	0.8±0.1 cA	0.8±0.1 bA	0.8±0.1 bA	0.8±0.1 bA	1.0±0.4 bA	7.4±1.6 bA	18.7±1.3 bA
	250+7.5	0.5±0.2 cB	1.5±1.2 cB	1.5±1.2 bB	1.5±1.2 bB	1.5±1.2 bB	4.9±1.7 bAB	8.2±1.8 bAB	20.5±0.4 bA
Control		0.3±0.1 cB	0.4±0.1 cB	0.4±0.1 bB	0.4±0.1 bB	0.4±0.1 bB	1.8±1.0 bAB	2.4±1.2 bAB	2.4±1.2 bA
Insecticide		65.9±2.7 aB	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA

* Means followed by the same lowercase letter within columns are not significantly different at P < 0.05.
♦ Means followed by the same uppercase letter within rows are not significantly different at P < 0.05.
DAT, d after treatment; DE, Detech, ME: methyl eugenol, Insecticide, K-Obiol EC 25.

Table 5. Mortality (%, mean ±SE) of *Tribolium confusum* exposed to wheat grain treated with Detech and methyl eugenol 1 to 28 d after treatment

Treatment		1 DAT	2 DAT	3 DAT	5 DAT	7 DAT	14 DAT	21 DAT	28 DAT
DE (mg kg^{-1})	100	0.0±0.0 b*A♦	0.0±0.0 cA	0.0±0.0 eA	0.0±0.0eA eA	0.0±0.0 dA	0.0±0.0dA dA	0.0±0.0dA dA	0.5±0.7 eA
	250	0.0±0.0 bB	0.0±0.0 bB	0.0±0.0 eB	0.0±0.0 eB	0.0±0.0 dB	0.1±0.4 dB	0.1±0.4 dB	3.2±1.0 dA
	500	0.0±0.0 bB	0.1±0.0 cB	0.1±0.0 eB	0.5±0.1 eB	0.5±0.1 cdB	0.5±0.1 cdB	0.5±0.1 cdB	18.3±1.5 bA
ME (mg kg^{-1})	5	0.0±0.0 bC	1.6±1.1 bcC	9.1±3.0 cdB	29.2±0.7 cB	46.5±0.4 bB	87.6±2.4 bA	95.3±2.2 bA	98.9±1.4 aA
	10	0.0±0.0 bD	1.9±1.5 bcD	18.7±2.5 bcD	31.6±1.8 cC	63.1±1.1 bB	98.9±1.4 bA	100.0±0.0 aA	100.0±0.0 aA
	15	0.5±0.3 bE	9.6±2.2 bD	39.3±1.2 bC	63.6±1.8 bB	97.7±1.8 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA
DE+ME (mg kg^{-1})	50+2.5	0.0±0.0 bA	0.0±0.0 cA	0.0±0.0 eA	0.1±0.0 eA	0.1±0.0 dA	0.1±0.1 dA	0.5±0.2 cdA	0.5±0.2 deA
	125+5	0.0±0.0 bA	0.5±0.1 cA	0.5±0.1 dA	0.5±0.1 deA	0.5±0.1 cdA	0.5±0.1 cdA	0.8±0.2 cdA	1.8±0.2 dA
	250+7.5	0.1±0.0 bA	0.5±0.2 cA	0.5±0.2 deA	1.1±0.9 dA	3.2±1.0 cA	3.2±1.0 cA	3.2±1.0 cA	7.2±1.0 cA
Control		0.0±0.0 bA	0.0±0.0 cA	0.0±0.0 eA	0.0±0.0 eA	0.0±0.0 eA	0.1±0.0 dA	0.1±0.0 dA	1.1±0.9 eA
Insecticide		45.3±0.6 aB	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA

* Means followed by the same lowercase letter within columns are not significantly different at P < 0.05.
♦ Means followed by the same uppercase letter within rows are not significantly different at P < 0.05.
DAT, d after treatment; DE, Detech, ME: methyl eugenol, Insecticide, K-Obiol EC 25.

Table 6. Mortality (%, mean ± SE) of *Rhyzopertha dominica* exposed to wheat grain treated with Detech and methyl eugenol 1 to 28 d after treatment

Treatment		1 DAT	2 DAT	3 DAT	5 DAT	7 DAT	14 DAT	21 DAT	28 DAT
DE (mg kg^{-1})	100	0.0±0.0 c*C♦	0.0±0.0 cC	0.0±0.0 bC	0.5±0.2 cC	3.5±1.8 cdC	28.1±2.0 cB	54.0±1.2 bA	57.6±1.6 bcA
	250	0.0±0.0 cC	0.0±0.0cC cC	0.0±0.0 bC	0.8±0.3 bcC	12.7±0.8 bcB	23.7±1.7 cdB	56.8±0.5 bA	73.6±0.2 bcA
	500	0.0±0.0 cE	0.0±0.0 cE	0.5±0.2 bDE	9.5±1.2 bD	31.6±0.5 bC	53.4±0.6 bBC	67.0±1.5 bAB	80.8±2.9 bA
ME (mg kg^{-1})	5	4.4±2.5 bcC	82.7±2.8 bB	98.7±1.8 aA	99.7±0.8 aA	99.7±0.8 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA
	10	10.7±1.9 bC	88.9±1.5 aB	99.5±0.7 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA
	15	16.5±2.4 bB	99.7±0.8 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA
DE+ME (mg kg^{-1})	50+2.5	0.0±0.0 cD	0.0±0.0 cD	0.0±0.0 bD	1.1±0.9 bcCD	3.0±1.5 bcC	17.1±0.4 bcB	29.2±0.7 bcAB	50.0±0.6 bcA
	125+5	0.0±0.0 cD	0.0±0.0 cD	0.1±0.0 bD	3.0±1.5bcCD bcCD	15.3±1.3 cdBC	20.0±1.9 cdB	30.2±0.9 cdAB	66.4±1.8 bcA
	250+7.5	0.0±0.0 cC	0.0±0.0 cC	0.5±0.1 bC	4.4±1.4 bcC	16.5±2.4 bcC	38.5±0.6 cdB	48.8±0.6 cdB	79.6±1.7 bA
Control		0.0±0.0 cB	0.0±0.0 cB	0.1±0.0 bB	0.5±0.7 cAB	0.5±0.7 dAB	0.5±0.7 dAB	0.5±0.7 dAB	1.0±0.3 dA
Insecticide		64.8±2.8 aB	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA	100.0±0.0 aA

* Means followed by the same lowercase letter within columns are not significantly different at P < 0.05.
♦ Means followed by the same uppercase letter within rows are not significantly different at P < 0.05.
DAT, d after treatment; DE, Detech, ME: methyl eugenol, Insecticide, K-Obiol EC 25.

The efficacy of ME on test species was revealed by differences in mortality. The greatest effect on *S. granarius* was nearly complete mortality with 15 ml kg^{-1} 28y DAT ($F_{10,88}$ = 69.7, P < 0.05). One DAT, complete mortality was not seen in *S. granarius,* the minimum mortality was <14% at 5 ml kg^{-1} ($F_{10,88}$ = 34.5, P < 0.05)

(Table 3). For *S. zeamais,* complete mortality was recorded 7 DAT at 15 ml kg^{-1} ($F_{10,88}$ = 175, P < 0.05). The minimum insecticidal effect was with 5 ml kg^{-1} gave 227% mortality 1 DAT ($F_{10,88}$ = 26.4, P < 0.05) (Table 4). For *T. confusum,* complete mortality was observed in 14 DAT with 15 ml kg^{-1} ($F_{10,88}$ = 227, P < 0.05). At the same dose, the mortality was <1% 1 DAT ($F_{10,88}$ = 99.5, P < 0.05) (Table 5). With *R. dominica,* the lowest mortality of 4% was observed 1 DAT at 5 ml kg^{-1} ($F_{10,88}$ = 29.6, P < 0.05). Complete mortality with all the three doses was observed from 14 DAT (Table 6).

No mortality was observed at the lowest combined dose of the agents (50 mg kg^{-1} Detech plus 2.5 ml kg^{-1} ME) 1 DAT ($F_{10,88}$ = 34.5, P < 0.05) with *S. granarius*. The greatest effect of the combined products (250 mg kg^{-1} Detech plus 7.5 ml kg^{-1} ME) on *S. granarius* was 27% mortality 28 DAT ($F_{10,88}$ = 69.7, P < 0.05) (Table 3). The combined reduced doses had a different effect on *S. zeamais*. The greatest mortality (250 mg kg^{-1} Detech plus 7.5 ml kg^{-1} ME) of 29% was observed 28 DAT ($F_{10,88}$ = 101, P < 0.05). Increasing dose and time resulted in increased insect mortality (Table 4). With *T. confusum*, mortality with combined products ranged from 0 to 7% (Table 5). There was no mortality observed for *R. dominica* 2 DAT. The lowest mortality 3 DAT was <1% with 125 mg kg^{-1} Detech plus 5 ml kg^{-1} ME. The highest morality of 80% was observed 28 DAT with 250 mg kg^{-1} Detech plus 7.5 ml kg^{-1} ME (Table 6). The mortality with K-Obiol EC 25 varied from 45 to 66% 1 DAT. However, complete mortality was observed from 2 DAT onwards. The mortality in the control did not exceed ~2%.

Discussion

The present study showed that different doses of Detech and ME had different efficacy against *T. confusum, R. dominica, S. granarius* and *S. zeamais*. The highest mortality of 81% for *R. dominica* was with Detech at 500 mg kg^{-1} 28 DAT. In a study conducted with diatomaceous earth products, Insecto and SilicoSec, mortality of *R. dominica* adults in different types of grain treated with 750 mg kg^{-1} varied between 63 and 97% 14 DAT (Kavallieratos et al., 2005). Similarly, Protector, another commercial DE product, did not give complete mortality in *R. dominica* adults in wheat treated with 0.5 g kg^{-1} 14 DAT (Baldassari et al., 2008). However, in the present study not all of the tested doses of Detech gave complete mortality of *S. zeamais, R. dominica, T. confusum* and *S. granarius*. Doumbia et al. (2014) shown that a complete mortality was achieved with *S. zeamais* adults 2 DAT in maize treated with DE at 3 g kg^{-1}. The mode of action of diatomaceous earth is known as abrasion of the insect cuticle and death occurs due to dehydration (Carlson & Ball, 1962; Ebeling, 1971; Krzyzowski et al., 2019). So, this effect may be due to the use of relatively low dose in the present study. Unlike other insects treated with Detech, *T. confusum* had the minimum mortality under similar conditions. Some studies showed that in order to achieve the desired level of mortality of *T. confusum*, DE doses equal to 500 mg kg^{-1} or above need to be used (Athanassiou et al., 2004; Athanassiou & Kavallieratos, 2005). The maximum Detech dose used in the present study was 500 mg kg^{-1}. The results obtained for *T. confusum* are similar to those in the literature. In the experiments with Detech, the desired mortality rates could not be obtained even after the longest time and the highest dose applications. Ashraf et al. (2016) stated that although DE, Inert-PMS, was effective against test insects, shorter periods gave lower mortality and mortality increased with time. The factors include the structure of the epicuticule, insect behavior or mobility, body shape and size of the insects and tolerance for water loss (Ebeling, 1971; Rigaux et al., 2001; Losic & Korunić, 2017, Korunić et al., 2020). Therefore, the tolerance of different insect species to DE products varies. A recent study revealed that *Cryptolestes pusillus* (Schönherr, 1817) (Coleoptera: Laemophloeidae) was by far more susceptible to DE compared to *R. dominica, T. confusum, Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae), *Oryzaephilus surinamensis* (L., 1758) (Coleoptera: Silvanidae), *S. oryzae* and *S. granarius* (Baliota & Athanassiou, 2020). In the present study, as in the previous studies with different DEs, *T. confusum* was as most tolerant species to Detech. It is also known that increased RH reduces the efficacy of the DEs (Mewis & Ulrichs, 2001; Ertürk, 2020). The present study was conducted at a constant temperature and RH, therefore, no comparison could be made in the efficacy at different temperature and RH.

The present results indicate that ME is a potential and effective plant-derived synthetic chemical insecticide to minimize pests damage in stored products, such as wheat and maize. In present study, *S. zeamais* was the most susceptible insect to ME 1 DAT. However, from 2 DAT onwards, the highest mortality was obtained with *R. dominica*, the most susceptible species to ME. ME is a benzene derivative and has a high insecticidal activity. The fact that ME has more methoxy groups is a feature that increases its knockdown effect (Smith et al., 2002). The presence of a double bond in the side chain of the aromatic ring in ME, an analog of eugenol, and the substitution of the methoxy group is positively correlated with toxicity (Mossa, 2016; Imai & Masuda, 2017). Norambuena et al. (2016) reported that the main essential oil components of *Laureliopsis philippiana* (Looser) Schodde (Atherospermataceae) were ME (61%) and safrole (17%) and the *a*essential oil toxicity ranged from low to high as *S. granarius* < *S. zeamais* < *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). In the present study, similar to the previous study, *S. zeamais* was found to be more sensitive to ME than *S. granarius*. The insecticidal activity of ME essential oil increased with increasing dose and time. Previous studies showed that mortality of insect species even in the same genus could be different (Ertürk et al., 2014; Liang et al., 2017; Guo et al., 2017; Alkan 2020a, b). In the present study, *T. confusum* was the most tolerant species to ME and this was similar to other studies (Shojaei et al., 2017). However, the differences are thought to be due to the physiological properties of insects. With the expectation of an increased efficacy of DE and ME together, both agents were also investigated in combination at half doses. Based on the results, it is considered that a Detech and ME combination is feasible against *R. dominica* but it was not effective against *T. confusum, S granarius* and *S. zeamais*. In a previous study, Form N, a mixture of SilicoSec and other ingredients was reported to provide higher mortality of *S. oryzae, R. dominica* and *T. castaneum* than DE alone, especially in barley (Paponja et al., 2020). Korunic & Fields (2020) revealed that the simultaneous usage of diatomaceous earth with dill essential oil, silica gel, pyrethrin, disodium octaborate tetrahydrate and yeast were more effective than DE alone. The combined application of DE and ME in maize and wheat, in the present study did not affect the test insects at the desired level. It is thought that the low effect obtained by using Detech and ME together may be due to the low concentrations used. Therefore, it was assumed that insects were not sufficiently in contact with the DE and ME. One of the most important factors limiting the use of DE in the protection of stored grain is the necessity for high concentrations for successful insect control. Therefore, reducing the amount of DE while maintaining its effectiveness is an important challenge.

The result of the present study indicates a possible synergistic effect of the combination of DE and ME on *R. dominica, T. confusum, S granarius* and *S. zeamais.* This is particularly important, as such a combination is expected to enhance a DE treatment so it can cause rapid mortality without adverse effects on the physicochemical characteristics of the grain. At the same time, such a formulation can be registered more easily than combinations with synthetic insecticides, as ME is a natural substance. Additional work is needed to clarify the data that would be needed for regulatory approval.

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References

Adarkwah, C., D. Obeng-Ofori, C. Ulrichs & M. Schöller, 2017. Insecticidal efficacy of botanical food by-products against selected stored-grain beetles by the combined action with modified diatomaceous earth. Journal of Plant Diseases and Protection, 124 (3): 255-267.

Aitken A. D., 1975. Insect Travelers. Volume I. Coleoptera. Technical Bulletin, Ministry of Agriculture, Fisheries and Food, No. 31, 191 pp.

Alkan, M., 2020a. Chemical composition and insecticidal potential of different *Origanum* spp. (Lamiaceae) essential oils against four stored product pests. Turkish Journal of Entomology, 44 (2): 149-163.

Alkan, M., 2020b. Chemical composition of *Achillea millefolium* L. (Asteraceae) essential oil and insecticidal effect against *Sitophilus granarius* (Coleoptera: Curculionidae) and *Rhyzopertha dominica* (Coleoptera: Bostrichidae). Plant Protection Bulletin, 60 (1): 85-93.

Alkan, M., T. Atay, S. Ertürk & İ. Kepenekci, 2019a. Comparison of bioactivities of native diatomaceous earth against Turkestan cockroach [*Blatta lateralis* Walker (Blattodea: Blattidae)] nymphs. Applied Ecology and Environmental Research, 17 (3): 5987-5994.

Alkan, M., S. Erturk, T. A. Firat & E. Ciftci, 2019b. Study of insecticidal and behavioral effects and some characteristic of native diatomaceous earth against the bean weevil, *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae). Fresenius Environmental Bulletin, 28 (4): 2916-2922.

Alkan, M., S. Ertürk, T. Atay & A. Çağlayan, 2019c. Insecticidal efficacy of local diatomaceous earths against adult and larvae of *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae). Turkish Journal of Entomology, 43 (3): 347-354.

Arthur, F. H., 1996. Grain protectants: current status and prospects for the future. Journal of Stored Products Research, 32 (4): 293-302.

Ashraf, M., W. Wakil, F. Hafeez & M. Farooq, 2016. Persistence and insecticidal efficacy of a diatomaceous earth formulation, inert-PMS, in stored wheat grain against *Cryptolestes ferrugineus* (Stephens), *Liposcelis paeta* Pearman, *Rhyzopertha dominica* (F.) and *Tribolium castaneum* (Herbst). Turkish Journal of Entomology, 40 (2): 107-115.

Athanassiou, C. G. & N. Kavallieratos, 2005. Insecticidal effect and adherence of PyriSec® in different grain commodities. Crop Protection, 24 (8): 703-710.

Athanassiou, C. G., N. G. Kavallieratos & N. S. Andris, 2004. Insecticidal effect of three diatomaceous earth formulations against adults of *Sitophilus oryzae* (Coleoptera: Curculionidae) and *Tribolium confusum* (Coleoptera: Tenebrionidae) on oat, rye and triticale. Journal of Economic Entomology, 97 (6): 2160-2167.

Athanassiou, C. G., N. G. Kavallieratos, A. Chiriloaie, T. N. Vassilakos, V. Fatu, S. Drosu & R. Dudoiu, 2016. Insecticidal efficacy of natural diatomaceous earth deposits from Greece and Romania against four stored grain beetles: the effect of temperature and relative humidity. Bulletin of Insectology, 69 (1): 25-34.

Baldassari, N., C. Prioli, A. Martini, V. Trotta & P. Baronio, 2008. Insecticidal efficacy of a diatomaceous earth formulation against a mixed age population of adults of *Rhyzopertha dominica* and *Tribolium castaneum* as function of different temperature and exposure time. Bulletin of Insectology, 61 (2): 355-360.

Baliota, G. V. & C. G. Athanassiou, 2020. Evaluation of a Greek diatomaceous earth for stored product insect control and techniques that maximize its insecticidal efficacy. Applied Sciences, 10 (18): 6441.

Barres, B., A. Micoud, M. F. Corio-Costet, D. Debieu, S. Fillinger, A. S. Walker, C. Délye, J. Grosman & M. Siegwart, 2016. Trends and challenges in pesticide resistance detection. Trends in Plant Science, 21 (10): 834-853.

Bayram, A., A. A. Isikber, Ö. Sağlam & R. Şen, 2020. "Evaluation of repellency effect of diatomaceous earth formulation (Detech®) on three coleopteran stored grain insects, 56-64". 12th Conference of the Working Group Integrated Protection of Stored Product (September 3-6, 2019, Pisa, Italy), 429 pp.

Boulogne, I., P. Petit, H. Ozier-Lafontaine, L. Desfontaines & G. Loranger-Merciris, 2012. Insecticidal and antifungal chemicals produced by plants: a review. Environmental Chemistry Letters, 10 (4): 325-347.

Boyer, S., H. Zhang & G. Lempérière, 2012. A review of control methods and resistance mechanisms in stored-product insects. Bull Entomol Res 102 (2): 213-229.

Carlson, S. D. & H. J. Ball, 1962. Mode of action and insecticidal value of a diatomaceous earth as a grain protectant. Journal of Economic Entomology, 55 (6): 964-970.

Chang, C. L., I. K. Cho & Q. X. Li, 2009. Insecticidal activity of basil oil, trans-anethole, estragole, and linalool to adult fruit flies of *Ceratitis capitata*, *Bactrocera dorsalis*, and *Bactrocera cucurbitae*. Journal of Economic Entomology, 102 (1): 203-209.

Dawson, W. A. J. M., M. Jestoi, A. Rizzo, P. Nicholson & G. L. Bateman, 2004. Field evaluation of fungal competitors of *Fusarium culmorum* and *F. graminearum*, causal agents of ear blight of winter wheat, for the control of mycotoxin production in grain. Biocontrol Science and Technology, 14 (8): 783-799.

Doumbia, M., B. G. Douan, K. E. Kwadjo, D. K. Kra, V. Martel & M. Dagnogo, 2014. Effectiveness of diatomaceous earth for control of *Sitophilus zeamais* (Coleoptera: Curculionidae), *Tribolium castaneum* and *Palorus subdepressus* (Coleoptera: Tenebrionidae). Journal of Stored Products Research, 57: 1-5.

Dwivedy, A. K., M. Kumar, N. Upadhyay & N. K. Dubey, 2015. Green chemistry in agricultural pest management programmes. Medicinal Chemistry, (Special Issue): 1-2.

Ebeling, W., 1971. Sorptive dusts for pest control. Annual Review of Entomology, 16 (1): 123-158.

Ertürk, S., T. Atay, U. Toprak & M. Alkan, 2020. The efficacy of different surface applications of wettable powder formulation of Detech® diatomaceous earth against the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). Journal of Stored Products Research, 89: 101725.

Ertürk, S., A. G. Ferizli & M. Emekci, 2017. Evaluation of diatomaceous earth formulations for the control of rice weevil, *Sitophilus oryzae* (L.), 1763 (Coleoptera: Curculionidae) in stored rice. Turkish Journal of Entomology, 41 (3): 347-354.

Ertürk, S., A. Yılmaz, T. Fırat Akdeniz & M. Alkan, 2014. Fumigant effect of trans-anethole and carbon dioxide mixture against to *Rhyzopertha dominica*, *Tribolium castaneum* and *Sitophilus oryzae*. Plant Protection Bulletin, 57 (3): 391-400.

FAO, 2009. How to feed the world in 2050. (Web page: http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf) (Date accessed: October 2020).

Ferizli, A. G., G. Beriş & M. Emekçi, 2012. Effects of diatomaceous earth on the mortality and progeny production of *Rhyzopertha dominica* (Coleoptera: Bostrychidae). Journal of Agricultural Sciences, 17 (2): 85-94.

Gałęcki, R., T. Bakuła, M. Wojtacki & K. Żuk-Gołaszewska, 2019. Susceptibility of ancient wheat species to storage pests *Sitophilus granarius* and *Tribolium confusum*. Journal of Stored Products Research, 83: 117-122.

Guo, S. S., W. J. Zhang, C. X. You, J. Y. Liang, K. Yang, Z. F. Geng & C. F. Wang, 2017. Chemical composition of essential oil extracted from *Laggera pterodonta* and its bioactivities against two stored product insects. Journal of Food Processing and Preservatio, 41 (2): 1-9.

Halliday, W. R., F. H. Arthur & J. L. Zettler, 1988. Resistance status of red flour beetle (Coleoptera: Tenebrionidae) infesting stored peanuts in the southeastern United States. Journal of Economic Entomology, 81 (1): 74-77.

Herrmann, K. M. & L. M. Weaver, 1999. The shikimate pathway. Annual Review of Plant Physiology and Plant Molecular Biology, 50 (1): 473-503.

Houssou, P. A., B. C. Ahohuendo, P. Fandohan, K. Kpodo, D. J. Hounhouigan & M. Jakobsen, 2009. Natural infection of cowpea (*Vigna unguiculata* (L.) Walp.) by toxigenic fungi and mycotoxin contamination in Benin, West Africa. Journal of Stored Products Research, 45 (1): 40-44.

Hubert, J., V. Stejskal, C. G. Athanassiou & J. E. Throne, 2018. Health hazards associated with arthropod infestation of stored products. Annual Review of Entomology, 63 (1): 553-573.

Imai, T. & R. Masuda, 2017. Insecticidal activities of methyleugenol and β-asarone, from the herbal medicines Saishin and Sekishōkon, and other alkoxy-propenyl-benzene derivatives against the cigarette beetle *Lasioderma serricorne* (Coleoptera: Anobiidae). Applied Entomology and Zoology, 52 (2): 183-188.

Islam, M. S. & M. M. Rahman, 2016. Diatomaceous earth induced alterations in the reproductive attributes in the housefly *Musca domestica* L. (Diptera: Culicidae). Elixir Applied Zoology, 96 (2016): 41241-41244.

Jankowska, M., J. Rogalska, J. Wyszkowska & M. Stankiewicz, 2017. Molecular targets for components of essential oils in the insect nervous system-a review. Molecules, 23 (1): 34 (1-20).

Kavallieratos, N. G., C. G. Athanassiou, F. G. Pashalidou, N. S. Andris & Z˘. Tomanovic´, 2005. Influence of grain type on the insecticidal efficacy of two diatomaceous earth formulations against *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae). Pest Management Science, 61 (7): 660-666.

Khorrami, F., O Valizadegan, M. Forouzan & A. Soleymanzade, 2018. The antagonistic/synergistic effects of some medicinal plant essential oils, extracts and powders combined with diatomaceous earth on red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae). Archives of Phytopathology and Plant Protection, 51 (13-14): 685-695.

Korunić, Z., 1998. Diatomaceous earths, a group of natural insecticides. Journal of Stored Products Research, 34 (2-3): 87-97.

Korunić, Z. & P. G. Field, 2020. Evaluation of three new insecticide formulations based on inert dusts and botanicals against four stored-grain beetles. Journal of Stored Products Research, 88: 101633.

Korunić, Z., A. Liška, P. Lucić, D. Hamel & V. Rozman, 2020. Evaluation of diatomaceous earth formulations enhanced with natural products against stored product insects. Journal of Stored Products Research, 86: 101565.

Krzyzowski, M., J. Francikowski, B. Baran & A. Babczynska, 2019. Physiological and behavioral effects of different concentrations of diatomaceous earth on common stored product pest *Callosobruchus maculatus*. Journal of Stored Products Research, 82: 110-115.

Lee, S. E., B. H. Lee, W. S. Choi, B. S. Park, J. G. Kim & B. C. Campbell, 2001. Fumigant toxicity of volatile natural products from Korean spices and medicinal plants towards the rice weevil, *Sitophilus oryzae* (L). Pest Management Science, 57 (6): 548-553.

Liang, J. Y., W. T. Wang, Y. F. Zheng, D. Zhang, J. L. Wang, S. S. Guo & J. Zhang, 2017. Bioactivities and chemical constituents of essential oil extracted from *Artemisia anethoides* against two stored product insects. Journal of Oleo Science, 66 (1): 71-76.

Losic, D. & Z. Korunić, 2017. "Diatomaceous Earth, a Natural Insecticide for Stored Grain Protection: Recent Progress and Perspectives, 219-247". In: Diatom Nanotechnology: Progress and Emerging Applications (Ed. D. Losic). Royal Society of Chemistry Publishing, Cambridge, UK, 270 pp.

Mckenzie, Jr. J. D. & J. M. R. Goldman, 2004. The Student Guide to MINITAB Release 14 + MINITAB Student Release 14 Statistical Software (Book + CD). Pearson Education, Boston, MA. 560 pp.

Merget, R., T. Bauer, H. Küpper, S. Philippou, H. Bauer, R. Breitstadt & T. Bruening, 2002. Health hazards due to the inhalation of amorphous silica. Archives of Toxicology, 75 (11-12): 625-634.

Mewis, I. & C. Ulrichs, 2001. Action of amorphous diatomaceous earth against different stages of the stored product pests *Tribolium confusum, Tenebrio molitor, Sitophilus granarius and Plodia interpunctella*. Journal of Stored Products Research, 37 (2): 153-164.

Mossa, A. T. H., 2016. Green pesticides: essential oils as biopesticides in insect-pest management. Journal of Environment Science and Technology, 9 (5): 354-378.

Norambuena, C., G. Silva, A. Urbina, I. Figueroa & J. C. Rodríguez-Maciel, 2016. Insecticidal activity of *Laureliopsis philippiana* (Looser) Schodde (Atherospermataceae) essential oil against *Sitophilus* spp. (Coleoptera: Curculionidae). Chilean Journal of Agricultural Research, 76 (3): 330-336.

Obeng-Ofori, D. & C. Reichmuth, 1997. Bioactivity of eugenol, a major component of essential oil of *Ocimum suave* (Wild.) against four species of stored-product Coleoptera. International Journal of Pest Management, 43 (1): 89-94.

Oliveira, C. M., A. M. Auad, S. M. Mendes & M. R. Frizzas, 2014. Crop losses and the economic impact of insect pests on Brazilian agriculture. Crop Protection, 56: 50-54.

Pacheco, I. A., M. R. Sartori & S. Bolonhezi, 1990. "Resistance to malathion, pirimiphos-methyl and fenitrothion in Coleoptera from stored grains, 1029-1038". Proceedings of the 5th International Working Conference on Stored Product Protection (9-14 September 1990, Bordeaux, France), 2064 pp.

Paponja, I., V. Rozman & A. Liška, 2020. Natural formulation based on diatomaceous earth and botanicals against stored product insects. Insects, 11 (9): 613.

Prasantha, B. D. R., C. Reichmuth, C. Adler & D. Felgentreu, 2015. Lipid adsorption of diatomaceous earths and increased water permeability in the epicuticle layer of the cowpea weevil *Callosobruchus maculatus* (F.) and the bean weevil *Acanthoscelides obtectus* (Say) (Chrysomelidae). Journal of Stored Products Research, 64 (Part A): 36-41.

Regnault-Roger, C., C. Vincent & J. T. Arnason, 2012. Essential oils in insect control: low-risk products in a high-stakes world. Annual Review of Entomology, 57: 405-424.

Ribeiro, B. M., R. N. C. Guedes, E. E. Oliveira & J. P. Santos, 2003. Insecticide resistance and synergism in Brazilian populations of *Sitophilus zeamais* (Coleoptera: Curculionidae). Journal of Stored Products Research, 39 (1): 21-31.

Richard, E., N. Heutte, L. Sage, D. Pottier, V. Bouchart, P. Lebailly & D. Garon, 2007. Toxigenic fungi and mycotoxins in mature corn silage. Food and Chemical Toxicology, 45 (12): 2420-2425.

Rigaux, M., E. Haubruge & P. G. Fields, 2001. Mechanisms for tolerance to diatomaceous earth between strains of *Tribolium castaneum* (Coleoptera: Tenebrionidae). Entomologia Experimentalis et Applicata, 101 (1): 33-39.

Sağlam, Ö., R. Şen, H. Bozkurt & A. A. Işıkber, 2020. “Insecticidal efficacy of three commercial diatomaceous earths, Detech®, Demite® and Silicosec® against cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae: Bruchinae), 47-53”. 12th Conference of the Working Group Integrated Protection of Stored Product (September 2019, Pisa, Italy), 429 pp.

Shojaei, A., K. Talebi, I. Sharifian & S. M. Ahsaei, 2017. Evaluation of detoxifying enzymes of *Tribolium castaneum* and *Tribolium confusum* (Col.: Tenebrionidae) exposed to essential oil of *Artemisia dracunculus* L. Biharean Biologist, 11 (1): 5-9.

Smith, R. L., T. B. Adams, J. Doull, V. J. Feron, J. I. Goodman, L. J. Marnett & I. G. Sipes, 2002. Safety assessment of allylalkoxybenzene derivatives used as flavouring substances-methyl eugenol and estragole. Food and chemical Toxicology, 40 (7): 851-870.

Tan, K. H. & R. Nishida, 2012. Methyl eugenol: its occurrence, distribution, and role in nature, especially in relation to insect behavior and pollination. Journal of Insect Science, 12 (1): 1-74.

Upadhyay, N., A. K. Dwivedy, M. Kumar, B Prakash & N. K. Dubey, 2018. Essential oils as eco-friendly alternatives to synthetic pesticides for the control of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Journal of Essential Oil Bearing Plants, 21 (2): 282-297.

Vayias, B. J. & C. G. Athanassiou, 2004. Factors affecting the insecticidal efficacy of the diatomaceous earth formulation SilicoSec against adults and larvae of the confused flour beetle, *Tribolium confusum* DuVal (Coleoptera: Tenebrionidae). Crop Protection, 23 (7): 565-573.

Warton, D. I. & F. K. C. Hui, 2011. The arcsine is asinine: the analysis of proportions in ecology. Ecology, 92 (1): 3-10.

Zacker, F. & G. Kunike, 1931. Untersuchungen uber die insektizide Wirkung von Oxyden und Karbonaten. Arbeiten aus der Biologischen Reichsanstalt für Land- und Forstwirtschaft, 18 (2): 201-231.

Zar, J. H., 1999. Biostatistical Analysis, Fourth Edition. Published by PrenticeHall, Upper Saddle River, New Jersey, USA, 929 pp.

Zhanda, J., B. M. Mvumi & H. Machekano, 2020. Potential of three enhanced diatomaceous earths against *Sitophilus zeamais* Motschulsky and *Prostephanus truncantus* (Horn) on stored maize grain. Journal of Stored Products Research, 87: 101608.

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Original article (Orijinal araştırma)

Comparison of hermaphrodites of hybrid *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH strain and its parents on reproduction capacity[1]

Hibrit *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH ırkı ve ebeveynlerinin hermafroditlerinin üreme kapasitelerinin karşılaştırılması

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Abstract

Entomopathogenic nematodes (EPNs) are microscopic roundworms used in biocontrol. EPNs are obligate insect parasites, they live in soil, and they are especially effective against soilborne insects. They are a good alternative to chemical pesticides thanks to their advantages, such as prolonged longevity, broad host range and mass production suitability. However, EPNs cannot compete with chemical pesticides due to high production costs and short shelf life. The aim of this study was to determine the reproduction capacity of the Turkish hybrid *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH strain and then compare it with its parents to improve its liquid culture yield. In this way, it is aimed to reveal the effects behind the high reproduction potential of the hybrid HBH strain. All experiments were performed at Bursa Uludağ University, Faculty of Agriculture, Department of Plant Protection, in 2020. All cadavers were periodically dissected, hermaphrodites were counted and their body lengths were measured. Compared to its parents, the hybrid HBH strain had greater hermaphrodite counts, with mean 66 individuals within 12 days, and hermaphrodite body length, with mean 3.88 mm. The results obtained from this study should provide information for commercial EPN production development.

Keywords: Body length, hermaphrodite, *Heterorhabditis bacteriophora*, in vitro, reproduction capacity

Öz

Entomopatojen nematodlar (EPN) biyolojik mücadelede kullanılan mikroskobik ölçülerdeki yuvarlak solucanlardır. EPN'ler zorunlu böcek parazitleri olup, toprak altında yaşamakta ve toprak kökenli böceklere karşı etkili olmaktadırlar. Uygulamalarının kolay olması, uzun sürekli etkinliği, geniş konukçu aralığı ve kitle üretimi yapılabilmesi gibi avantajları sayesinde kimyasal ilaçlara iyi bir alternatif olmaktadırlar. Bununla beraber, yüksek üretim maliyetleri ve kısa raf ömrü nedeniyle EPN'ler kimyasal ilaçlar ile rekabet edememektedir. Bu çalışma, sıvı kültürdeki üretim verimini artırmak amacıyla Türk hibriti *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH ırkının üreme kapasitesini belirlemek ve ebeveynleri ile karşılaştırmak amacıyla yapılmıştır. Bu sayede hibrit HBH ırkının yüksek üreme potansiyelinin arkasında yatan etkilerin ortaya çıkarılması hedeflenmiştir. Tüm denemeler Bursa Uludağ Üniversitesi Ziraat Fakültesi Bitki Koruma Bölümü'nde 2020 yılında gerçekleştirilmiştir. Bütün kadavralar periyodik olarak disekte edilmiş, hermafroditler sayılmış ve vücut uzunlukları ölçülmüştür. Ebeveynleri ile karşılaştırıldığında, hibrit HBH ırkı, 12 günde ortalama 66 hermafrodit birey ile hermafrodit sayısında ve ortalama 3.88 mm ile hermafrodit vücut uzunluğu açısından daha yüksek değerlere sahip olmuştur. Bu çalışma ile elde edilen sonuçlar, ticari EPN üretim çalışmalarına bilgi sağlayacaktır.

Anahtar sözcükler: Vücut uzunluğu, hermafrodit, *Heterorhabditis bacteriophora*, in vitro, üreme kapasitesi

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Introduction

Many insect pathogens occur in the phylum Nematoda, but species within the families Steinernematidae and Heterorhabditidae have gained significant importance as biocontrol agents in plant protection. Entomopathogenic nematodes (EPN) are important biocontrol agents, and they can be especially used to control soil-dwelling insect pests (Lacey & Georgis, 2012). They have a high potential on a broad host range of insects thanks to their host-seeking ability (Divya & Sankar, 2009; Lacey & Georgis, 2012). These nematodes are associated with bacteria in the genus *Photorhabdus* Boemare et al., 1993 (Enterobacteriales: Enterobacteriaceae) for *Heterorhabditis* Poinar, 1976 (Rhabditida: Heterorhabditidae) and *Xenorhabdus* Thomas & Poinar, 1979 (Enterobacteriales: Enterobacteriaceae) for *Steinernema* Travassos, 1927 (Rhabditida: Steinernematidae) (Boemare et al., 1996). The life cycles of EPNs are quite similar. The only stage living outside the host is the infective juveniles (IJs). The IJs carry cells of the bacterial symbiont in their intestine. When the IJs find a susceptible host, they invade and penetrate the host's hemocoel through natural openings (i.e., anus, mouth or spiracles). The IJs then release the symbiotic bacterium that kills the host within 48 h by septicemia (Kaya & Gaugler, 1993). The bacterium produces antibiotics to prevent other microorganisms from colonizing the cadaver (Bode, 2009; Sajnaga & Kazimierczak, 2020). Besides serving as a food source for the nematode, the bacterium also provides proper nutrients for nematode development. After 2-3 weeks, the food resource depletes in the cadaver, and IJs disperse through the soil to find new hosts.

EPNs can be applied with standard spray equipment used for pesticides, they can resist shear stress, they can be applied simultaneously with many pesticides, and they can be mass-produced to manage with a broad host range (Wright et al., 2005; Garcia-del-Pino et al., 2013; van Niekerk & Malan, 2014; Sabino et al., 2019). Although they have many advantages, high production costs and limited shelf life are still significant obstacles to the large-scale application (Grewal, 2000; Perry et al., 2012). Therefore, there are many studies focused on reducing production costs. Previous research has established that, many local strains of EPNs have better infectivity and reproduction capacity than commercial ones (Malan & Moore, 2016; De Waal et al., 2018; James et al., 2018; Mokrini et al., 2020), and can be used to increase mass production yield and lower expenses. Another way to improve beneficial traits of EPNs is genetic selection and hybridization. Over the past two decades, considerable research has focused on improving some beneficial traits by selection (Johnigk et al., 2002; Ehlers et al., 2005; Mukuka et al., 2010; Salame et al., 2010; Anbesse et al., 2013).

Between 2010 and 2013, several hybrid *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) strains were obtained from hybridization of Turkish isolates of *H. bacteriophora*. Among these strains, HBH had superior genetic traits, such as high reproduction capacity, high virulence, high resistance to stress conditions, and prolonged longevity (Susurluk et al., 2013). EPNs have been registered in Turkey, and they have an increasing market share. Since Turkey does not have any commercial EPN strain and liquid production technology, imported commercial EPN products are used. The HBH hybrid strain has superior features, and it has the potential to be a commercial strain. Consequently, it is important to understand the reason of the hybrid strain's characteristics to optimize and improve mass production, longevity and stress tolerance.

This study aimed to compare the reproduction capacity and hermaphrodite length of HBH hybrid strain with its parents. There may be a positive correlation between body length and ovary size, which can also mean longer individuals have higher reproductive potential. The study also collected data to determine the reason for its higher reproduction capacity. It is hoped that the results of this study will contribute information for future in vitro liquid culture experiments.

Materials and Methods

This study was conducted at Bursa Uludağ University, Department of Plant Protection, Nematology Laboratory, in 2020.

EPN strains and *Galleria mellonella* larvae

Two parent strains, *H. bacteriophora* HB4 and HB1138, and one hybrid HBH strain patented by Susurluk (TPMK Patent No: TR 2013 06141 B) were used. Parent strains were isolated from two different regions of Turkey. The hybrid strain was obtained from hybridization of those parent strains. *H. bacteriophora* is one of Turkey's most abundant EPN species, and our hybrid strain has superior genetic traits (Susurluk et al., 2001, 2013; Kongu & Susurluk, 2014). The last instar of *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae) larvae are used for the production of EPNs. Larvae are reared with a modified diet consisting of 200 g bran, 150 g maize flour, 100 g soy flour, 100 g milk powder, 50 g yeast, 200 g glycerin and 200 g honey. All larvae were kept in glass jars and incubated around 30-32ºC.

Experimental design and inoculation of the strains

For determining reproduction capacity, 30 last instar larvae were inoculated with EPNs and divided into five replicates, consisting six larvae per Petri dish. Prior to inoculation, 10 µl samples were taken from EPN cultures and counted under a microscope. Inoculation dose were adjusted to 100 IJs/larva, and a total of 600 IJs were applied per Petri dish. After inoculation, Petri dishes were sealed with Parafilm and incubated at 24ºC. Cadavers were dissected on 4, 6, 8, 10 and 12 days after inoculation (DAI). For dissection, dead larvae were transferred to a bigger Petri dish containing a thin layer of Ringer's solution. All cadavers were dissected with a needle and all extracted nematodes transferred to a fresh Ringer's solution.

Reproduction capacity criteria

Hermaphrodites of *Heterorhabditis* spp. are positively correlated with the offspring and reproduction capacity. Namely, higher numbers of hermaphrodites are the sign of higher yield. Moreover, the longer body length of the hermaphrodites leads to longer ovaries, which also results in more reproduction yield. Thus, after each dissection process, hermaphrodites of each strain were counted under stereomicroscope, and body lengths of the individuals were measured using Leica Application Suite 3.2 software (Leica, Weztlar, Germany). Body lengths of 15 hermaphrodites were measured for each strain.

Statistical analyses

Statistical analysis for both the reproduction capacity and hermaphrodite body measurements was performed using JMP 7 software (SAS Institute, Cary, NC, USA). One-way ANOVA analysis was performed on data obtained from experiments.

Results and Discussion

Reproduction capacity

Reproduction capacity was determined based on periodic counts of hermaphrodites. For all strains, the first hermaphrodites were observed 6 DAI. Among the three strains, the hybrid HBH strain had the best results for overall hermaphrodite number over the 12 days ($F_{2,15} = 23.8$, $p < 0.001$). The hybrid strain had almost two times more hermaphrodites than its parent HB1138. As evident in Figure 1, reproduction dynamics of the same species can differ between strains. HB4 strain reached its peak total hermaphrodite number 8 DAI, while it was 10 DAI for HBH and 12 DAI for HB1138 (Figure 1). There was clear decrease of the hermaphrodites in all strains following their peak day, which was related to their second or third generation in the cadaver.

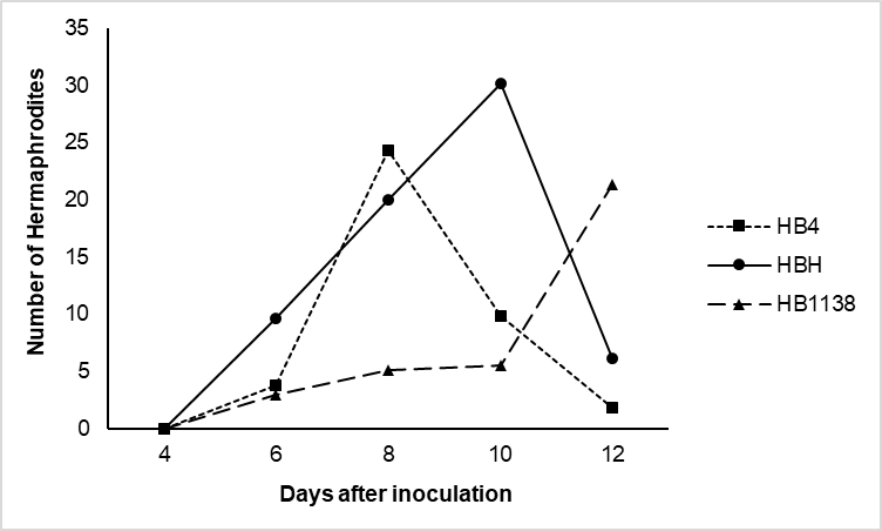

Figure 1. Daily changes in the number of hermaphrodites.

Body length measurement

The reproduction capacity of the strains is also related to hermaphrodite body length. The longer body length leads to a longer ovary, which increases the number of eggs. The HB1138 had the shortest hermaphrodite body length (2.07 ± 0.21 mm, mean ± SD, n = 15), while HB4 and HBH lengths were closer (3.61 ± 0.26 and 3.88 ± 0.29 mm, respectively), with HBH being the longest ($F_{2,15} = 204$, $p < 0.001$).

Entomopathogenic nematodes are useful for biological control of insect pests. Management of soil-dwelling insects still a significant problem due to the low activity of pesticides into soil. Therefore, EPNs are a good alternative to chemicals. In addition, their application is relatively easy, they have a broad host range and they are a safe solution compared to chemicals. However, even though they have many advantages, they still need more development to become a commercial substitute for chemical pesticides. EPNs have high mass production costs, expensive commercial products and short shelf life. For this reason, many studies have been conducted over several decades to reduce production costs, directly or indirectly. Optimization of in vivo and in vivo culture is one of the most studied topics (van Zyl & Malan, 2014; Ferreira et al., 2016; Ulu & Susurluk, 2018; Dunn et al., 2020).

Mass production of EPNs in liquid culture is the most common and feasible way for commercial production (Dunn et al., 2021). There are many commercial EPN strains with optimized liquid culture parameters. However, new endemic species or strains, which may have positive genetic traits, are important for mass production yield, effectiveness, host range, resistance to stress factors and other factors. Thus, considerable research has attempted to optimize in vitro liquid culture production of local species or strains (Upadhyay, 2015; Ramakuwela et al., 2016; Dunn et al., 2020). High liquid culture yield, high effectiveness or longer shelf life lowers commercial product expenses indirectly and contributes EPNs to compete with chemicals (Grewal, 2000; Shapiro-Ilan et al., 2012; Devi & George, 2018).

Another way to improve EPNs is genetic selection or hybridization. With these tools, it is possible to obtain superior strains, which have the potential to improve on current strains and to become a commercial product. There are many studies focused on the genetic selection of EPNs. The main idea of these studies is to improve the resistance of EPNs to several stress factors, longevity or effectiveness (Shapiro-Ilan et al., 1997; Ehlers et al., 2005; Mukuka et al., 2010; Nimkingrat et al., 2013a, b; Santhi et al., 2016). Likewise, in a project between 2010 and 2013, many hybrid strains of *H. bacteriophora* were obtained from hybridization local Turkish isolates from different regions. Among these hybrid strains, HBH was the superior strain compared with its parents and other hybrid strains (Susurluk et al., 2013; Kongu & Susurluk, 2014). To

understand the reason for the high reproduction capacity of HBH, we counted hermaphrodites of all strains periodically and measured their body length. Although there are very few similar studies, Ferreira et al. (2014) aimed to determine in vitro liquid culture production yield of a South African EPN isolate of *Heterorhabditis zealandica* (Wouts, 1979) (Rhabditida: Heterorhabditidae). As they mentioned, it is important to monitor the body length of different life stages to improve mass production. Another study by Hirao et al. (2010) aimed to characterize the population development of *Steinernema carpocapsae* (Weiser, 1955) and *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae). They measured the body length of adults and recorded significant differences. They found that even in the same species, the body length of parental adults was doubled in F1 adults.

EPNs have a considerable market share around the world (Glare et al., 2012). However, they still need to overcome some obstacles to increase their market and become preferable. The main problems of EPNs are high mass production expenses, high commercial product cost, and short shelf life (Ali & Wharton, 2013; Ramakuwela et al., 2016; Kagimu & Malan, 2019). The present study was designed to determine the reproduction capacity of HBH hybrid strain and compare it with its parent strains. Reproduction capacity is an important genetic trait for in vitro mass production yield, and it is closely related with hermaphrodites in *Heterorhabditis* species. The results of this research support the idea that Turkey can have its own hybrid strain with better reproduction capacity. It also provides the first comprehensive assessment of the reproduction capacity of local Turkish strains and a hybrid strain. EPNs are registered in Turkey, and their market share is increasing gradually. Local strains, which are adapted to local conditions, have been shown to have better success. In the future, we think that Turkey should use a local EPN species or strains instead of foreign commercial ones for more effective insect management. We hope that determining the reproduction capacity of this hybrid strains will contribute to future liquid culture optimization studies in Turkey. However, it is recommended that further research should be undertaken on liquid culture methods.

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References

Ali, F. & D. A. Wharton, 2013. Cold tolerance abilities of two entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. Cryobiology, 66 (1): 24-29.

Anbesse, S., N. H. Sumaya, A. V. Dörfler, O. Strauch & R. Ehlers, 2013. Stabilization of heat tolerance traits in *Heterorhabditis bacteriophora* through selective breeding and creation of inbred lines in liquid culture. BioControl, 58 (1): 85-93.

Bode, H. B., 2009. Entomopathogenic bacteria as a source of secondary metabolites. Current Opinion in Chemical Biology, 13 (2): 224-230.

Boemare, N., C. Laumond & H. Mauleon, 1996. The entomopathogenic nematode-bacterium complex: biology, life cycle and vertebrate safety. Biocontrol Science and Technology, 6 (3): 333-346.

De Waal, J. Y., M. Addison & A. Malan, 2018. Potential of *Heterorhabditis zealandica* (Rhabditida: Heterorhabditidae) for the control of codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae) in semi-field trials under South African conditions. International Journal of Pest Management, 64 (2): 102-109.

Devi, G. & J. George, 2018. Formulation of Insecticidal Nematode. Annual Research & Review in Biology, 24 (5): 1-10.

Divya, K. & M. Sankar, 2009. Entomopathogenic nematodes in pest management. Indian Journal of Science and Technology, 2 (7): 53-60.

Dunn, M. D., P. D. Belur & A. P. Malan, 2020. In vitro liquid culture and optimization of *Steinernema jeffreyense* using shake flasks. BioControl, 65 (2): 223-233.

Dunn, M. D., P. D. Belur & A. P. Malan, 2021. A review of the in vitro liquid mass culture of entomopathogenic nematodes. Biocontrol Science and Technology, 31(1): 1-21.

Ehlers, R., J. Oestergaard, S. Hollmer, M. Wingen & O. Strauch, 2005. Genetic selection for heat tolerance and low temperature activity of the entomopathogenic nematode-bacterium complex *Heterorhabditis bacteriophora-Photorhabdus luminescens*. BioControl, 50 (5): 699-716.

Ferreira, T., M. Addison & A. Malan, 2014. In vitro liquid culture of a South African isolate of *Heterorhabditis zealandica* for the control of insect pests. African Entomology, 22 (1): 80-92.

Ferreira, T., M. Addison & A. Malan, 2016. Development and population dynamics of *Steinernema yirgalemense* (Rhabditida: Steinernematidae) and growth characteristics of its associated *Xenorhabdus indica* symbiont in liquid culture. Journal of Helminthology, 90 (3): 364-371.

Garcia-del-Pino, F., X. Alabern & A. Morton, 2013. Efficacy of soil treatments of entomopathogenic nematodes against the larvae, pupae and adults of *Tuta absoluta* and their interaction with the insecticides used against this insect. BioControl, 58 (6): 723-731.

Glare, T., J. Caradus, W. Gelernter, T. Jackson, N. Keyhani, J. Köhl, P. Marrone, L. Morin & A. Stewart, 2012. Have biopesticides come of age? Trends in Biotechnology, 30 (5): 250-258.

Grewal, P. S., 2000. Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. Pest Management Science, 56 (5): 401-406.

Hirao, A., R. Ehlers & O. Strauch, 2010. Life cycle and population development of the entomopathogenic nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda, Rhabditida) in monoxenic liquid culture. Nematology, 12 (2): 201-210.

James, M., A. P. Malan & P. Addison, 2018. Surveying and screening South African entomopathogenic nematodes for the control of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Crop Protection, 105: 41-48.

Johnigk, S., S. Hollmer, O. Strauch, U. Wyss & R. Ehlers, 2002. Heritability of the liquid culture mass production potential of the entomopathogenic nematode *Heterorhabditis bacteriophora*. Biocontrol Science and Technology, 12 (2): 267-276.

Kagimu, N. & A. P. Malan, 2019. Formulation of South African entomopathogenic nematodes using alginate beads and diatomaceous earth. BioControl, 64 (4): 413-422.

Kaya, H. K. & R. Gaugler 1993. Entomopathogenic nematodes. Annual Review of Entomology, 38 (125): 181-206.

Kongu, Y. & I. A. Susurluk, 2014. Comparison of virulence of hybridized entomopathogenic nematode *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) strains and their parents. Turkish Journal of Entomology, 38 (2): 125-134.

Lacey, L. A. & R. Georgis, 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. Journal of Nematology, 44 (2): 218-225.

Malan, A. P. & S. D. Moore, 2016. Evaluation of local entomopathogenic nematodes for the control of false codling moth, *Thaumatotibia leucotreta* (Meyrick, 1913), in a citrus orchard in South Africa. African Entomology, 24 (2): 489-501.

Mokrini, F., S. E. Laasli, Y. Benseddik, A.B. Joutei, A. Blenzar, H. Lakhal, M. Sbaghi, M. Imren, G. Özer, T. Paulitz, R. Lahlali, & A. A. Dababat 2020. Potential of Moroccan entomopathogenic nematodes for the control of the Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae). Scientific Reports, 10 (1): 19204.

Mukuka, J., O. Strauch, C. Hoppe & R. Ehlers, 2010. Improvement of heat and desiccation tolerance in *Heterorhabditis bacteriophora* through cross-breeding of tolerant strains and successive genetic selection. BioControl, 55 (4): 511-521.

Nimkingrat, P., S. Khanam, O. Strauch & R. Ehlers, 2013a. Hybridisation and selective breeding for improvement of low temperature activity of the entomopathogenic nematode *Steinernema feltiae*. BioControl, 58 (3): 417-426.

Nimkingrat, P., O. Strauch & R. Ehlers, 2013b. Hybridisation and genetic selection for improving desiccation tolerance of the entomopathogenic nematode *Steinernema feltiae*. Biocontrol Science and Technology, 23 (3): 348-361.

Perry, R. N., R. Ehlers & I. Glazer, 2012. A realistic appraisal of methods to enhance desiccation tolerance of entomopathogenic nematodes. Journal of Nematology, 44 (2): 185-190.

Ramakuwela, T., J. Hatting, M. D. Laing, S. Hazir & N. Thiebaut, 2016. In vitro solid-state production of *Steinernema innovationi* with cost analysis. Biocontrol Science and Technology, 26 (6): 792-808.

Sabino, P. H. S., A. S. Negrisoli, V. Andaló, C. C. Filgueiras, A. Moino & F. S. Sales, 2019. Combined application of entomopathogenic nematodes and insecticides in the control of leaf-miner *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) on Tomato. Neotropical Entomology, 48 (2): 314-322.

Sajnaga, E. & W. Kazimierczak, 2020. Evolution and taxonomy of nematode-associated entomopathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus*: an overview. Symbiosis, 80 (1): 1-13.

Salame, L., I. Glazer, M. T. Chubinishvilli & T. Chkhubianishvili, 2010. Genetic improvement of the desiccation tolerance and host-seeking ability of the entomopathogenic nematode *Steinernema feltiae*. Phytoparasitica, 38 (4): 359-368.

Santhi, V. S., D. Ment, L. Salame, V. Soroker & I. Glazer, 2016. Genetic improvement of host-seeking ability in the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* toward the Red Palm Weevil *Rhynchophorus ferrugineus*. Biological Control, 100 (1): 29-36.

Shapiro-Ilan, D., I. Glazer & D. Segal, 1997. Genetic improvement of heat tolerance in *Heterorhabditis bacteriophora* through hybridization. Biological Control, 8 (2): 153-159.

Shapiro-Ilan, D. I., R. Han & C. Dolinksi, 2012. Entomopathogenic nematode production and application technology. Journal of Nematology, 44 (2): 206-217.

Susurluk, A., I. Dix, E. Stackebrandt, O. Strauch, U. Wyss & R. U. Ehlers, 2001. Identification and ecological characterisation of three entomopathogenic nematode-bacterium complexes from Turkey. Nematology, 3 (8): 833-841.

Susurluk, I. A., T. C. Ulu & Y. Kongu, 2013. Tolerances of hybridized entomopathogenic nematode *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) strains to heat and desiccation. Turkish Journal of Entomology, 37 (2): 221-228.

Ulu, T. C. & A. Susurluk, 2018. Effects of several ingredients on in vitro mass production of *Heterorhabditis bacteriophora* HBH strain. Mediterranean Agricultural Sciences, 31 (3): 209-212.

Upadhyay, D. 2015. Lab-scale in vitro mass production of the entomopathogenic nematode *Heterorhabditis bacteriophora* using liquid culture fermentation technology. American Journal of Bioscience and Bioengineering, 3 (6): 203-207.

van Niekerk, S. & A. P. Malan, 2014. Compatibility of biological control agents and agrochemicals to entomopathogenic nematodes, *Steinernema yirgalemense* and *Heterorhabditis zealandica*. African Entomology, 22 (1): 49-56.

van Zyl, C. & A. Malan, 2014. Optimization of inoculation techniques for in vivo mass culture of entomopathogenic nematodes through nematode and insect host manipulation. African Entomology, 22 (2): 405-416.

Wright, D. J., A. Peters, S. Schroer & J. Fife, 2005. "Application Technology, 91-106". In: Nematodes as Biocontrol Agents (Eds. P. S. Grewal, R. U. Ehlers & D. I. Shapiro-Ilan), CABI, Wallingford, UK, 528 pp.

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Original article (Orijinal araştırma)

Effects of some *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) isolates on root-knot nematodes under laboratory conditions[1]

Bazı *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) izolatlarının laboratuvar koşullarında kök-ur nematodları üzerine etkileri

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Abstract

The aim of this study to isolate and diagnose entomopathogenic fungi obtained from soil samples taken from the plateaus and pastures of Sakarya Province in 2019, and to investigate the use of five isolates (S43/1, S43/2, S43/3, S42/1, S42/2) of the diagnosed as *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) for control *Meloidogyne incognita* (Kofoid & White, 1919) and *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Meloidogynidae). The study was conducted at the Laboratory of Nematology in Plant Protection Central Research Institute (Republic of Turkey Ministry of Agriculture and Forestry) in 2020. The experiments were performed under laboratory conditions. Counts of dead larvae were made 24, 48 and 72 h post application and mortality rates calculated. Isolate S43/1 was the most effective isolate for control *M. javanica* at 10^8 cfu/ml causing 98.5% mortality after 24 h and 100% after 48 h. Isolate S42/2 was the most effective against *M. incognita* at 10^8 cfu/ml and with 97.1% mortality after 24 h and 100% after 72 h.

Keywords: Biocontrol, *Meloidogyne incognita, Meloidogyne javanica*, *Metarhizium anisopliae*

Öz

Bu çalışmanın amacı, 2019 yılında Sakarya İli yayla ve meralarından alınan toprak örneklerinden elde edilen entomopatojen fungusların izolasyonunun, teşhisinin yapılması ve *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) olarak teşhisi yapılan beş adet izolatın (S43/1, S43/2, S43/3, S42/1, S42/2), *Meloidogyne incognita* (Kofoid & White, 1919) ve *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Meloidogynidae) ile mücadelede kullanım olanaklarının araştırılmasıdır. Denemeler 2020 yılında Türkiye Cumhuriyeti Tarım ve Orman Bakanlığı, Zirai Mücadele Merkez Araştırma Enstitüsü Müdürlüğü Nematoloji Laboratuvarı'nda laboratuvar koşullarında yürütülmüştür. Uygulamadan 24, 48 ve 72 saat sonra ölü larva sayımları yapılmış ve ölüm oranları hesaplanmıştır. S43/1 izolatı *M. javanica'*nın kontrolünde en etkili izolat olmuş, 10^8 cfu/ml dozunda 24 saat sonunda %98,5 ve 48 saat sonunda ise %100 ölüme neden olmuştur. S42/2 izolatı *M. incognita*'ya karşı en etkili izolat olmuş 24 saat sonunda %97.1 ve 72 saat sonunda %100 ölüme neden olmuştur.

Anahtar sözcükler: Biyolojik savaş, *Meloidogyne incognita, Meloidogyne javanica*, *Metarhizium anisopliae*

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Introduction

Root-knot nematodes, *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Heteroderidae) cause economic damage on many crops all over the world. Their characteristic symptoms occur on underground parts of plants. Infected roots swell at the point of infection and galls form. Several control methods are available for reducing root knot nematode populations. The most common and effective method used in their management is chemical control. However, due to the negative effects of the chemicals used on human, animal and environmental health, it has become necessary to develop new control strategies. One of the alternative control strategies is the use of biological control agents (Devi, 2018). Biological control of nematodes is one of the most important strategies in nematode control for sustainable agriculture (Mokhtari et al., 2009).

Certain soil-dwelling fungi are pathogenic on plant pests, including nematodes (Dijksterhuis et al., 1994). Some fungi are biological control agents and are important in the control of nematodes (Butt et al., 2001). Given that many fungi live within the rhizosphere along with nematodes and are in contact with them, they can be effective in reducing nematode populations in many soil and geographic contexts (Siddiqui & Mahmood, 1996). About 80 species of fungus belonging to more than 30 genera are effective against nematodes (Viaene & Abawi, 2000; Sun et al., 2006; Bakr et al., 2014). *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) is an entomopathogenic fungus that occurs naturally in soil all over the world and causes disease in various insects and is widely used in biological control programs (Richards & Rogers, 1990; Driver et al., 2000; Liu et al., 2007; Hoe et al., 2009; Abdollahi, 2018). *Metarhizium anisopliae*, the agent of green muscardine disease of insects, is the most important entomopathogenic fungus (Driver et al., 2000). This is a facultative parasite which can infect a range of insects and is a well-studied for microbial control of insect pests (Liu et al., 2007; Hoe et al., 2009).

The exact mode of action of *M. anisopliae* on nematodes is still unknown but it is likely to be similar to other fungi with sticky spores or conidia. The conidia germinate, parasitize and kill the nematode, by direct penetration and producing the infective hyphae inside the nematode body. This fungus produces some cyclic peptides, destruxins which may be important in its pathogenicity (Kershaw et al., 1999; Hsiao & Ko, 2001). Prior to infection of the host, the fungus produces destruxins A and B that can kill the host (Roberts, 1966).

There are only a few reports of the impact of *M. anisopliae* on nematodes. The effectiveness of *M. anisopliae* on nematodes has been demonstrated in studies conducted with *Rotylenchulus reniformis* Linford & Oliveira, 1940 (Tylenchida: Hoplolaimidae) (Tribhuvaneshwar et al., 2008), *Heterodera avenae* Wollenweber, 1924 (Tylenchida: Heteroderidae) (Ghayedi & Abdollahi, 2013) and several root-knot nematode species (Jahanbazian et al., 2014).

This study aimed to isolate and identify entomopathogenic fungi from 50 soil samples taken from the meadows and pasture areas of Sakarya Province, Turkey. The activity of five isolates of *M. anisopliae* against *Meloidogyne incognita* (Kofoid & White, 1919) and *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Meloidogynidae) was investigated under laboratory conditions.

Materials and Methods

Soil samples were collected from the plateaus and pastures of Sakarya Province in 2019. From these five isolates (S43-1, S43-2, S43-3, S42-1, S42-2) of *M. anisopliae* were tested against *M. incognita* and *M. javanica*. The study was conducted at the Plant Protection Central Research Institute of the Laboratory of Nematology (Republic of Turkey Ministry of Agriculture and Forestry) in 2020.

Collection of soil samples

In July 2019, 50 soil samples were collected from meadows and pastures of Sakarya Province. Samples were taken from various locations to depths of 15-20 cm with the locations were chosen arbitrarily (Mracek & Becvar, 2000). Five to six subsamples were taken 5-6 m apart at each location and mixed thoroughly. About 1 kg of soil was collected at each location. The coordinates of the samples were recorded, each soil sample was labeled with a code number and brought to the laboratory in plastic bags.

Production of *Galleria mellonella* larvae

The final instar of the larvae of *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae), also known as the large wax moth, were used in the isolation of entomopathogenic fungi from the soil samples. *Galleria mellonella* larvae were grown in artificial medium consisting of 45 g beeswax, 90 g granulated yeast, 307 g maize flour, 225 g honey mixture in glass jars at 27 ± 1ºC (Kaya & Stock, 1997). A proportion of the reared larvae were retained for pupal and adult development to ensure the continuation of the culture.

Entomopathogenic fungus isolation from soil

Entomopathogenic fungus isolation from the soil was performed using *G. mellonella* as bait (Zimmermann, 1986; Griffin et al., 2000). Prior to the isolation, the final instar of *G. mellonella* were submerged in 50 ± 3ºC water for 5 s and then transferred to a cold-water bath for 3 s to prevent spinning of web when they were released into the soil samples (Woodring & Kaya, 1988). Soil samples (300 g) were dispensed into 500 ml plastic containers and moistened with sterile water; 10 larvae of the same size were added to these containers and kept in an incubator at 22-25ºC in the dark. Samples were checked every 3 d for 10-15 d and dead larvae were removed. This was repeated three times for each sample.

Dead larvae collected from the soil were placed in 3% sodium hypochlorite for 3 min, then rinsed twice with sterile distilled water and placed in 9-cm Petri dishes of potato dextrose agar (PDA; Merck, Darmstadt, Germany). Agar plates were incubated at 24 ± 1ºC, with a 12:12 h L:D photoperiod for one week. Fungal colonies on the PDA medium were subcultured on PDA into 6-cm Petri dished, and a single spore isolation was made from the fungus colony that developed and stored in 20% glycerol at -20ºC in inclined tubes with PDA at 4ºC.

Molecular identification of entomopathogenic fungi

DNA extraction and PCR conditions

Total DNA was extracted according to DArT DNA isolation method (http://www.diversityarrays.com). Fungal mycelia were scraped from the surface of 15-d cultures grown on PDA and placed into 1.5 ml Eppendorf tubes. The samples were homogenized in 650 µl buffer solution [125 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 0.8 M NaCl, 1% CTAB, 1% sarcosyl and 2% PVP-40 (K29-32), 0.5% sodium disulfite] and then incubated at 65ºC for 1 h. The suspension was mixed with one volume of chloroform: isoamylalcohol (24:1) and centrifuged for 20 min at 13,000 g. DNA was precipitated with the same volume of ice-cold isopropanol. The pellet was washed with 70% cold ethanol, air-dried and suspended in 50 µl of sterile water. DNA concentrations were determined spectrophotometrically and finally diluted to 20 ng/µl.

The nuclear internal transcribed-spacer region (ITS1-5.8S-ITS2) of the isolates obtained was amplified using primer pairs ITS1 (5'- TCC GTA GGT GAA CCT GCGG -'3) and ITS4 (5'- TCC TCC GCT TAT TGA TATGC -'3) described by White et al. (1990). PCR reactions were performed in 50 µl containing 5 µl 10× reaction buffer, 0.4 µM of each primer, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 1.5 U *Taq* DNA polymerase (Thermo Fischer Scientific, Waltham, MA, USA) and 20 ng DNA. PCR cycling conditions were as follows: 4 min at 94ºC; 45 s at 94ºC, 45 s at 56ºC, 1 min at 72ºC for 35 cycles; and 10 min at 72°C. Amplification products were checked on 1.2% agarose gel stained with ethidium bromide. PCR products were sequenced in both directions using the same primers by Macrogen (Seoul, Korea).

Sequence data was optimized with the software package Seqman (DNAStar Inc., Madison, WI, USA). The alignments of the resulting sequences and the reference isolates from Genbank were performed by MEGA7 (Kumar et al., 2016). The tree showing genetic relationships among the isolates was constructed by the neighbor-joining method, Kimura 2-parameter distances and 1000 bootstrap values.

Generation of second stage juvenile of root-knot nematode species

Pure cultures of *M. incognita* and *M. javanica*, previously identified and stored in the Nematology Laboratory of the Directorate of Plant Protection Central Research Institute (Ankara), were used in this study. Serial cultures of susceptible tomato plants (Tueza F1) were used to culture these nematodes. The infected roots were washed carefully under tap water and egg masses were collected from these roots under the microscope and left to hatch at room temperature. The juveniles that hatched in the first 24 h were discarded and subsequent hatching were monitored and juveniles collected daily. The suspension with nematodes obtained was adjusted to 100 juveniles/ml.

Preparation of entomopathogenic fungi

Metarhizium anisopliae isolates were inoculated on Sabouraud dextrose agar in 9-cm Petri dishes and incubated at 25 ± 1ºC in the dark for 15 d. After the incubation, 2 ml of sterile distilled water with 0.03% Tween 80 was added to each Petri dish to suspend the spores by scraping with a glass rod. The spore suspensions were filtered through a four-layer cheesecloth and separated from mycelium for 3 min. Spore suspensions were homogenized with a vortex mixer and diluted to 1×10^6, 10^7 and 10^8 spores/ml with a Neubauer hemocytometer (Saruhan et al., 2015). Spore germination rates were determined before using the isolates. One hundred µl of the suspension diluted 1×10^4 was spread on PDA in three Petri dishes and incubated in the dark at 25 ± 1ºC for 24 h. In each Petri dish, 100 spores were examined and the germination rate determined as ≥90% (Güven et al., 2015).

Application of entomopathogenic fungi to root knot nematode species

The experiments were conducted in the laboratory in 24-well culture plates. Second stage juveniles of each nematode species were transferred to separate wells in the culture plates with a dropper at 100 juveniles/ml. Then, 1×10^6, 10^7 and 10^8 spores/ml suspensions of *M. anisopliae* isolates were added to the wells. Each test was done with three replicates and two repeats. Viability rates of nematodes were determined after 24, 48 and 72 h, and nematode mortality rates calculated (Abbasi et al., 2018). Dead juveniles were examined under light microscope to determine if they had been killed by fungal infection. Only pure water was used in the control.

Statistical analysis

The data were evaluated using Duncan multiple comparison test in SPSS program. Square root transformation was applied to non-normally distributed data, followed by ANOVA Duncan test.

Results and Discussion

Metarhizium-like fungi were isolated from 50 soil samples collected from different meadow and pastures in Sakarya Province. Five isolates from samples S42 and S43 were identified as *M. anisopliae* based on morphological characteristics (Humber, 1997) (Figure 1) and confirmed by DNA sequence analysis of ribosomal DNA-ITS region. PCR amplification with the primers ITS1 and ITS4 yielded a single DNA fragment of about 580 bp for all isolates. BLASTn search of their sequences showed high level of genetic similarity with DNA sequences of *M. anisopliae* available in GenBank. The resulting sequences were deposited in the GenBank database (Accessions MW073447 to MW073451). Neighbor-joining tree derived from DNA sequences of *M. anisopliae* and closely related fungi corresponded to the morphological classification (Figure 2). The sequence alignments revealed high level of identity between the isolates of the same species. *Metarhizium anisopliae* isolates were placed in a unique group different from other *Metarhizium* spp.

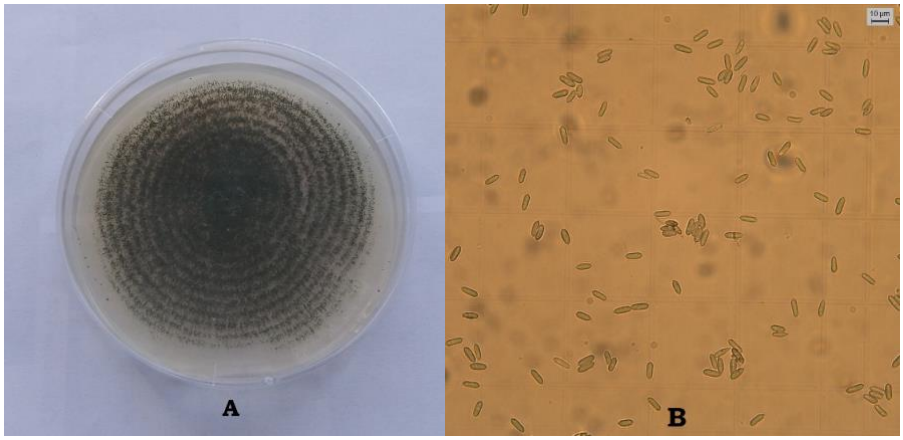

Figure 1. a) 15-day colony of *Metarhizium anisopliae* on PDA, and b) conidia at 40x magnification.

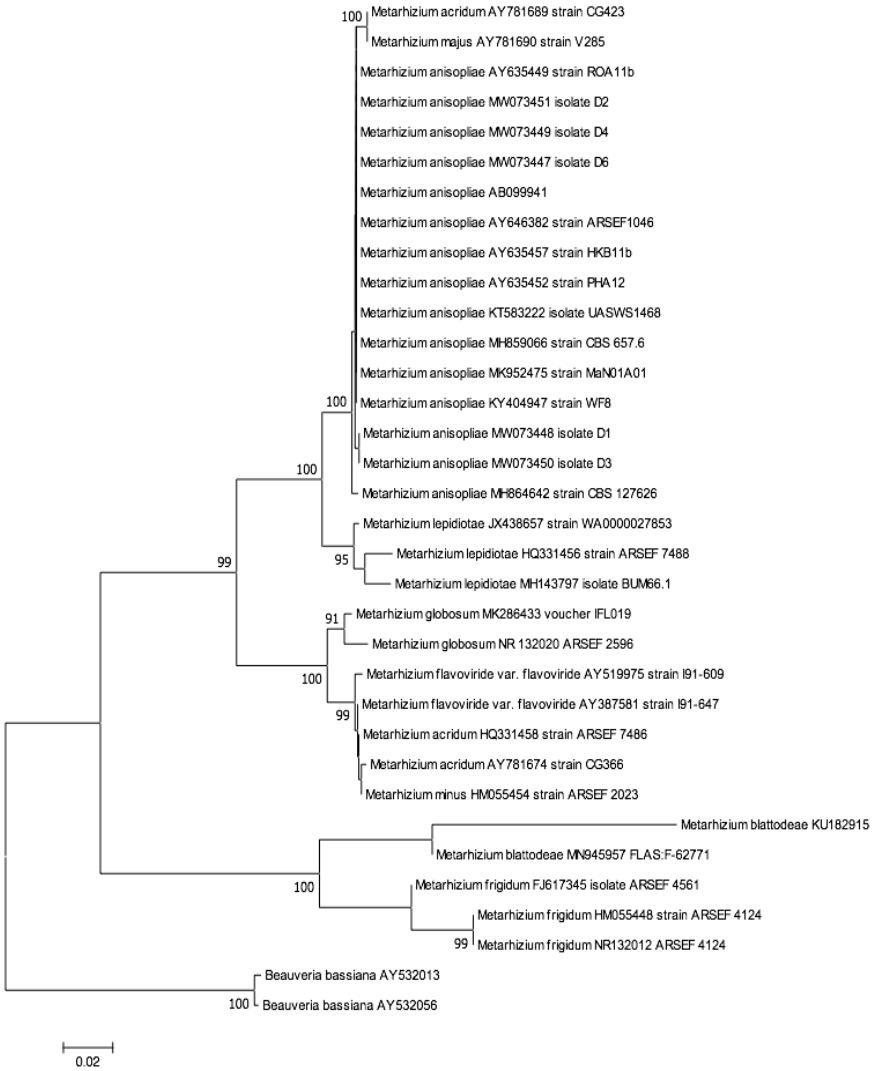

Figure 2. Phylogenetic analysis of *Metarhizium* spp. isolates based on ITS rDNA sequences. The bootstrap values were obtained from 1000 replications and the values greater than 90% are shown in the dendrogram. The ITS sequences of *Beauveria bassiana* were used as an outgroup.

The efficacy of five *M. anisopliae* isolates against both *M. incognita* and *M. javanica* increased as the dose increased (Tables 1 & 2).

Table 1. Mortality (%) of *Meloidogyne javanica* larvae caused by different *Metarhizium anisopliae* isolates after 24, 48 and 72 h

Doses	*Metarhizium anisopliae* isolate									
	S43/1		S43/2		S43/3		S42/1		S42/2	
24 h										
10^{-6}	69.9 ± 4.74	d	17.9 ± 1.93	f	17.9 ± 1.93	f	16.5 ± 1.46	f	15.7 ± 1.73	f
10^{-7}	91.9 ± 0.50	ab	31.0 ± 3.23	e	31.0 ± 3.23	e	77.7 ± 4.40	c	78.5 ± 1.93	c
10^{-8}	98.5 ± 0.44	a	88.5 ± 2.48	b	88.5 ± 2.48	b	95.2 ± 0.74	ab	91.9 ± 1.03	ab
48 h										
10^{-6}	85.3 ± 1.28	ef	27.7 ± 2.99	h	58.9 ± 7.26	g	17.3 ± 1.43	i	19.4 ± 2.24	i
10^{-7}	93.9 ± 0.64	abcd	91,4 ± 1,43	bcde	86.7 ± 2.09	def	89.3 ± 2.23	cde	81.1 ± 2.01	f
10^{-8}	100.0 ± 0.00	a	95.8 ± 1.31	abc	98.3 ± 0.95	ab	97.7 ± 0.50	ab	94.6 ± 1.01	abc
72 h										
10^{-6}	86.8 ± 1.45	c	70.4 ± 1.12	e	75.2 ± 2.17	d	24.6 ± 2.54	f	26,1 ± 3,67	f
10^{-7}	95.3 ± 0.44	ab	95.1 ± 1.39	ab	91.8 ± 1.98	b	96.8 ± 0.61	a	86.8 ± 1.44	c
10^{-8}	100.0 ± 0.00	a	99.8 ± 0.18	a	99.7 ± 0.22	a	99.1 ± 0.33	a	100.0 ± 0.00	a

$F_{1,47}$h = 224, 212 and 446 for 24, 48 and 72 h, respectively.

For *M. javanica,* isolate S43/1 was 98.5% effective at 10^8 cfu/ml after 24 h and 100% after 48 h. After 72 h, isolates S43/1 and S42/2 were also 100% effective at 10^8 cfu/ml. isolates S43/2, S43/3 and S42/1 were 99.8, 99.7, 99.1% effective, respectively.

Table 2. Mortality (%) of *Meloidogyne incognita* larvae caused by different *Metarhizium anisopliae* isolates after 24, 48, 72 h

Doses	*Metarhizium anisopliae* isolate									
	S43/1		S43/2		S43/3		S42/1		S42/2	
24 h										
10^{-6}	5.1 ± 0.53	d	3.8 ± 0.83	e	1.54 ± 0.44	f	-0.4 ± 0.34	g	-0.5 ± 0.35	g
10^{-7}	5.8 ± 0.50	d	5.5 ± 0.79	d	85.5 ± 2.12	ab	71.9 ± 2.77	c	74.7 ± 1.40	bc
10^{-8}	88.9 ± 1.97	a	95.7 ± 0.48	a	95.4 ± 0.79	a	94.7 ± 0.62	a	97.1 ± 0.41	a
48 h										
10^{-6}	5.3 ± 0.62	d	3.3 ± 0.78	e	1.2 ± 0.49	f	-0.7 ± 0.58	h	-0.5 ± 0.44	gh
10^{-7}	78.9 ± 2.29	c	5.0 ± 0.91	d	89.7 ± 1.49	abc	82.3 ± 1.58	abc	80.9 ± 1.55	bc
10^{-8}	96.4 ± 0.57	ab	98.6 ± 0.51	ab	98.5 ± 0.44	ab	96.7 ± 0.32	ab	99.7 ± 0.22	a
72 h										
10^{-6}	11.6 ± 2.28	c	75.3 ± 1.38	b	1.2 ± 0.49	d	-0.9 ± 0.68	f	0.7 ± 0.58	de
10^{-7}	93.1 ± 1.05	a	86.5 ± 0.56	ab	94.6 ± 1.46	a	86.7 ± 1.04	ab	96.0 ± 0.48	a
10^{-8}	97.6 ± 0.63	a	99.3 ± 0.34	a	99.0 ± 0.38	a	98.6 ± 0.35	a	100.0 ± 0.00	a

$F_{1,47}$ = 788, 558, and 528 for 24, 48 and 72 h, respectively.

For *M. incognita*, isolate S42/2 was 97.1% effective at 10^8 cfu/ml after 24 h, whereas isolates S43/2 and S43/3 were 95.7 and 95.4% effective, respectively. Isolate S42/2 was also effective in counts made after 3 d and it was 100% effective after 72 h. This was followed by S42/1, S43/3, S42/1 and S4 /1 (99.31%, 98.97, 98.61, 97.58). Some species of *Metarhizium* have the ability to colonize roots (Bruck, 2005). Some isolates of *M. anisopliae* have endophytic behavior (Leger, 2008). The fungus produces sticky conidia that attach to nematode cuticles (Ghayedi & Abdollahi, 2013). The conidia germinate, parasitize and kill the nematode, by direct penetration producing infective hyphae inside the nematode body. The fungus produces some cyclopeptides and destruxins which are important in its pathogenicity (Kershaw et al., 1999).

In previous studies in Turkey, *M. anisopliae* isolates were obtained from grass, *Lolium* spp. Lam. (Poales: Poaceae) by Sevim et al. (2012) in the Eastern Black Sea Region, Er (2013) in pistachio, *Pistacia vera* L. (Sapindales: Anacardiaceae) orchards Gaziantep, Gürlek et al. (2018) in the walnut, *Juglans* spp.

(L.) (Fagales: Juglandaceae) orchards in Kırşehir; Tuncer et al. (2018) in nut weevil, *Curculio nucum* (L., 1758) (Coleoptera: Curculionidae), and Keskin et al. (2019) in Düzce and their effectiveness against various pests investigated. However, no study has been conducted in Turkey on the efficacy of *M. anisopliae* on root-knot nematodes.

In the present study, it was observed that fungal efficacy increased with increased concentrations and the lowest effect was recorded at 10^6 cfu/ml for all isolates. These results are consistent with other studies. A study assessing the efficacy of *M. anisopliae* and *Trichoderma harzianum* Rifai on *M. javanica* under laboratory and greenhouse conditions, found that these fungi had no antagonistic effect on each other and that both fungi were effective against root knot nematodes, but there was no significant benefit from combined application (Abdollahi, 2015).

Abdollahi (2018) determined the effectiveness of *M. anisopliae* IMI 330189 and different oak, *Quercus* spp. (Fagales: Fagaceae) tree debris on *M. javanica* in tomato and demonstrated that the combination of *M. anisopliae* and oak tree debris caused a reduction of over 90% in nematode development with 100 and 150 g/kg soil. Although, *M. anisopliae* caused a 76% decrease in the number of galls formed in the roots compared to the control, its combination with oak debris caused a decrease of 86% and did not reduce the effect of entomopathogenic fungi.

In another study (Youssef et al., 2020), the effect of different concentrations of *Beauveria bassiana* (Bals.-Criv.) Vuill*., M. anisopliae* and *Paecilomyces lilacinum* (Thom) Samson spores and filtrates on *M. incognita* egg hatch and juvenile mortality in cowpea was investigated in climatic chamber. The highest mortality with the standard dilution occurred with *P. lilacinus* at 84.5%, followed by *B. bassiana* and *M. anisopliae* at 81.1% and 78.5%, respectively. With 10^8 cfu/ml^{1}n, *P. lilacinus* at 85.3% mortality was followed by *M. anisopliae* at 83.6%. All three fungi were effective against *M. incognita* and could be used in biological control of the agent.

In a study of the nematicidal activity of *B. bassiana* against *Meloidogyne hapla* Chitwood, 1949 was evaluated under greenhouse conditions (Liu et al., 2008), it was observed that hatching and mortality rates were directly proportional to the density of *B. bassiana*. It has been observed that *B. bassiana* significantly reduces larval density, egg sac and gall formation in the soil and root.

In the present study, five *M. anisopliae* isolates from Turkey gave over 95% mortality after 72 h at 10^8 cfu/ml. Therefore, based on this study conducted under laboratory conditions, it is considered that detailed semi-field and field trials would be justified. If efficacy is demonstrated in the semi-field and field trials these isolates could be used to control root knot nematodes (*Meloidogyne* spp.) in integrated pest control programs.

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References

Abbasi, M. W., N. Ahmed, M. J. Zaki & S. S. Shuakat, 2018. Nematicidal effects of *Bacillus subtilis* and *Bacillus pumilus* against *Meloidogyne incognita* infecting pea. Advances in Agricultural Science, 6 (4): 52-59.

Abdollahi, M., 2015. Effect of *Metarhizium anisopliae* and *Trichoderma harzianum* on root knot nematode *Meloidogyne javanica*. Biological Control Pest and Diseases, 3 (1): 67-76.

Abdollahi, M., 2018. Application of *Metarhizium anisopliae* against *Meloidogyne javanica* in soil amended with oak debris. World Academy of Science, Engineering and Technology International Journal of Agricultural and Biosystems Engineering,12 (2): 40-46.

Bakr, R. A., M. E. Mahdy & E. M. Mousa, 2014. Biological control of root-knot nematode *Meloidogyne incognita* by *Arthrobotrys oligospora*. Egyptian Journal Plant Protection, 9 (1): 1-11.

Bruck, D. J., 2005. Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management. Biological Control, 32 (1): 155-163.

Butt, T. M., C. Jackson & N. Magan, 2001. "Fungal Biological Control Agents: Progress, Problem and Potential, 1-9". In: Fungi as Biocontrol Agents (Eds. T. M. Butt, C. Jackson & N. Magan). CABI International, Wallingford, Oxon, U.K., 390 pp.

Devi, G., 2018. Nematophagous fungi:*Metarhizium anisopliae*. International Journal of Environment, Agriculture and Biotechnology (IJEAB), 3 (6): 2110-2113.

Dijksterhuis, J., M. Veenhuis, W. Harder & B. Nordbring-Hertz, 1994. Nematophagous fungi: physiological aspects and structure-function relationships. Advances in Microbial Physiology, 36: 111-143.

Driver, F., R. J. Milner & J. W. H. Trueman, 2000. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycological Research, 104 (2): 143-150.

Er, M. K., 2013. Gaziantep, Adıyaman ve Kahramanmaraş Antepfıstığı bahçelerinde bulunan entomopatojen fungusların tespiti. Türkiye Biyolojik Mücadele Dergisi, 4 (2): 155-163 (in Turkish with abstract in English).

Ghayedi, S. & M. Abdollahi, 2013. Biocontrol potential of *Metarhizium anisopliae* (Hypocreales:Clavicipitaceae), isolated from suppressive soils of Boyer-Ahmad region, Iran, against J2s of *Heterodera avenae*. Journal of Plant Protection Research, 53 (2): 165-171.

Griffin, C. T., R. Chaerani, D. Fallon, A. P. Reid & M. J. Downes, 2000. Occurrence and distribution of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis indica.* Journal of Helminthology, 74 (2): 143-150.

Gürlek, S. A., F. S. Milletli Sezgin & E. Sevim, 2018. Isolation and characterization of *Beauveria* and *Metarhizium* spp. from walnut fields and their pathogenicity against the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). Egyptian Journal of Biological Pest Control, 28 (50): 1-6.

Güven Ö., D. Çayır, R. Baydar & İ. Karaca, 2015. Entomopatojen fungus *Beauveria bassiana* (Bals.) Vull. izolatlarının patates böceği [*Leptinotarsa decemlineata* Say. (Coleoptera: Chrysomelidae)] üzerindeki etkisi. Türkiye Biyolojik Mücadele Dergisi, 6 (2): 105-114 (in Turkish with abstract in English).

Hoe, P. K., C. F. J. Bong, K. Jugah & A. Rajan, 2009. Evaluation of *Metarhizium anisopliae* var. *anisopliae* (Deuteromycotina: Hyphomycete) isolates and their effects on subterranean termite *Coptotermes curvignathus* (Isoptera: Rhinotermitidae). American Journal of Agricultural and Biological Sciences, 4 (4): 289-297.

Hsiao, Y. M. & J. L. Ko, 2001. Determination of destruxins, cyclic peptide toxins produced by different strains of *Metarhizium anisopliae* and their mutants induced by ethyl methane sul-fonate and ultraviolet using HPLC method. Toxicon, 39 (6): 837-841.

Humber, R. A., 1997. "Fungi: Identification, 153-185". In: Manual of Techniques in Insect Pathology (Ed. L. A. Lacey). Academic Press, San Diego, Chapter V-3, 409 pp.

Jahanbazian, L., M. Abdollahi & M. Hussienvand, 2014. Inhibitory effect of *Metarhizium anisopliae* against *Meloidogyne incognita*, the causal agent of root knot of tomato, under laboratory condition. National Conference of Modern Topic in Agriculture,12 (2): 40-46.

Kaya, H. K. & S. P. Stock, 1997. "Techniques in Insect Pathology, 281-324". In: Manual of Techniques in Insect Pathology (Ed. L. A. Lacey). Academic Press, San Diego, Chapter V-3, 409 pp.

Kershaw, M. J., E. R. Moorhouse, R. Bateman, S. E. Reynolds & A. K. Charnley, 1999. The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. Journal of Invertebrate Pathology, 74 (3): 213-223.

Keskin Y., S. Karabörklü & N. Altın, 2019. Bazı yerel entomopatojen fungusların toprak koşullarındaki etkinliklerinin *Tenebrio molitor* L. (Col.: Tenebrionidae) larvaları kullanılarak araştırılması. Türkiye Teknoloji ve Uygulamalı Bilimler Dergisi, 2 (1): 26-31 (in Turkish with abstract in English).

Kumar, S., G. Stecher & K. Tamura, 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 33 (7): 1870-1874.

Leger, St. R., 2008. Studies on adaptations of *Metarhizium anisopliae* to life in the soil. Journal of Invertebrate Pathology, 98 (3): 271-276.

Liu, B. L., T. M. Rou, Y. K. Rao & Y. M. Tzeng, 2007. Effect of pH and aeration rate on the production of Destruxins A and B from *Metarhizium anisopliae*. International Journal of Applied Science and Engineering, 5 (1): 17-26.

Mokhtari, S., N. Sahebani & H. R. Etebarian, 2009. Study on biological control and systemic induction of peroxidase enzyme activity in tomato plant infected with root-knot nematode (*Meloidogyne javanica*) by *Pseudomonas fluorescens* CHA0 antagonist. Journal of Agriculture, 11 (1): 151-161.

Mracek, Z. & S. Becvar, 2000. Insect aggregations and entomopathogenic nematode occurrence. Nematology, 2 (3): 297-301.

Richards, M. G. & P. B. Rodgers, 1990. Commercial development of insect biocontrol agents. The exploitation of micro -organisms in applied biology. Aspects of Applied Biology, 318 (1189): 357-373.

Roberts, D. W., 1966. Toxins from the entomogenous fungus *Metarhizium anisopliae*. 1. Production in submerged and surface cultures, and in inorganic and organic nitrogen media. Journal of Invertebrate Pathology, 8 (2): 212-221.

Saruhan, I., I. C. Erper Tuncer & I. Akca, 2015. Efficiency of some entomopathogenic fungi as biocontrol agents against *Aphis fabae* Scopoli (Hemiptera:Aphididae). Pakistan Journal of Agricultural Sciences, 52 (2): 273-278.

Sevim, A., M. Höfte & Z. Demirbağ, 2012. Genetic variability of *Beauveria bassiana* and *Metarhizium anisopliae* var. *anisopliae* isolates obtained from the Eastern Black Sea Region of Turkey. Turkish Journal of Biology, 36 (3): 255-265.

Siddiqui, Z. A. & I. Mahmood, 1996. Biological control of plant parasitic nematodes by fungi: a review. Bioresource Technology, 58 (3): 229-239.

Sun, M. H., L. Gao, Y. X. Shi, B. J. Li & X. Z. Liu, 2006. Fungi and actinomycetes associated with *Meloidogyne* spp. eggs and females in China and their biocontrol potential. Journal of Invertebrate Pathology, 93 (1): 22-28.

Tribhuvaneshwar, S. M. K. & S. Bhargava, 2008. Efficacy of green muscardine fungi, *Metarhizium anisopliae* against reniform nematode, *Rotylenchulus reniformis* on tomato. Indian Journal of Nematology, 38 (2): 242-244.

Tuncer, C., R. Kushiyev, J. Liu & İ. Akça, 2018. *Metarhizium anisopliae* ve *Beauveria bassiana* izolat ve preparatlarının *Curculio nucum*'a karşı etkinlikleri. Anadolu Tarım Bilimleri Dergisi, 33 (2):116-123 (in Turkish with abstract in English).

Viaene, N. M. & G.S. Abawi, 2000. *Hirsutella rhossiliensis* and *Verticillium hlamydosporium* as biocontrol agents of the root-knot nematode *Meloidogyne hapla* on Lettuce. Journal of Nematology, 32 (1): 85-100.

White, T. J., T. Bruns, S. Lee & J. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, 18 (1): 315-322.

Woodring, J. L. & H. K. Kaya, 1988. Steinernematid and Heterorhabditid Nematodes: A Handbook of Biology and Techniques. Arkansas Agricultural Experiment Station, Fayetteville, 30 pp.

Youssef, M. M. A., W. M. A. Nagdi & D. E. M. Lotfy, 2020. Evaluation of the fungal activity of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces lilacinus* as biocontrol agents against root-knot nematode, *Meloidogyne incognita* on cowpea. Bulletin of the National Research Centre, 44 (112): 1-11.

Zimmermann, G., 1986. The "Galleria bait method" for detection of entomopathogenic fungi in soil. Journal Applied Entomology, 102 (1-5): 213-215.

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Original article (Orijinal araştırma)

Resistance of local okra cultivars against *Meloidogyne incognita* (Kofoid & White, 1919) (Nematoda: Meloidogynidae), effects of nematode infestation on growth parameters and leaf macro- micronutrients

Yerel bamya çeşitlerinin *Meloidogyne incognita* (Kofoid & White, 1919) (Nematoda: Meloidogynidae)'ya karşı dayanıklılığı, nematod enfeksiyonunun bamyanın morfolojik özelliklerine ve yapraklarda makro-mikro element içeriğine etkisi

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Abstract

Okra is an important species of vegetable grown in Turkey and around the world. Root-knot nematodes cause serious yield losses in vegetables. Twenty-six cultivars okra were obtained from the Aegean Agricultural Research Institute in 2002. Resistance of this cultivars to nematode was determined and changes induced with nematode infestation on plant morphology and macro- and micronutrients in leaves measured. The experiments were conducted in completely randomized design with four replicates in climate-controlled glasshouse at Alata Horticultural Research Institute in 2019. The cultivars were tested against *Meloidogyne incognita* (Kofoid & White, 1919) (Nematoda: Meloidogynidae) at 25 ± 2ºC. Sixty days after inoculation, root gall indexes varied with an average of 4.56. Compared to uninoculated controls, nematode inoculated reduced plant height by 46.0%, stem thickness by 9.06%, root lengths by 39.7%, leaf widths by 15.1%, leaf lengths by 20.5% and increase root weight by 14.5%. Leaf macro- and micronutrients were determined. Compared to the control, nematode inoculation reduced leaf N, P, K and Mg concentrations by 2.31, 5.85, 5.24 and 10.3%, respectively, and increased Ca, Fe, Zn, Mn and Cu concentrations by 7.91, 17.0, 13.4, 118 and 15.6%, respectively.

Keywords: *Meloidogyne incognita*, morphological traits, nutrient, okra, resistance

Öz

Bamya dünyada ve ülkemizde yetiştiriciliği yapılan önemli sebze türlerinden birisidir. Kök-ur nematodları sebzelerde önemli ürün kayıplarına neden olmaktadır. Ege Tarımsal Araştırma Enstitüsü'nden 2002 yılında getirilen 26 yerel bamya çeşidinin nematodlara karşı dayanıklılık durumları, bitki morfolojisinde meydana gelen değişikliklere ve bitki yapraklarındaki makro-mikro besin elementleri içeriğine bakılmıştır. Deneme tesadüf parselleri deneme desenine göre 4 tekrarlamalı olarak Alata Bahçe Kültürleri Araştırma Enstitüsü Müdürlüğü'ne ait kontrollü cam seralarda 2019 yılında yürütülmüştür. Yerel bamya çeşitleri *Meloidogyne incognita* (Kofoid & White, 1919) (Nematoda: Meloidogynidae), popülasyonuna karşı 25 ± 2ºC'de testlenmiştir. İnokulasyondan sonra bitkiler 60. günde bitki sökümleri yapılarak kök gal indeksi değerlendirmesi yapılmış ve ortalama 4.56 ur indeksi oluşturduğu belirlenmiştir. Bitkisel özelliklerde kök-ur nematod uygulamasının kontrol bitkilerine göre bitki boyunu ortalama %46.0, gövde kalınlığını %9.06, kök uzunluğunu %39.7, yaprak eni uzunluğunu %15.1, yaprak boyu uzunluğunu %20.50 oranında azalttığı ve bamya kök ağırlığını ise ortalama %14.5 oranında arttırdığı belirlenmiştir. Bitki yapraklarında makro-mikro besin içeriklerini ise kök-ur nematod uygulamasının kontrol bitkilere göre N, P, K ve Mg değerlerini ortalama olarak sırasıyla %2.31, 5.85, 5.24 ve 10.3 oranında azalttığı belirlenmiştir. Buna karşılık kök-ur nematod uygulamasının kontrol bitkilere göre Ca, Fe, Zn, Mn ve Cu değerlerini ortalama olarak sırasıyla %7.91, 17.0, 13.9, 118 ve 15.6 oranında arttırdığı belirlenmiştir.

Anahtar sözcükler: *Meloidogyne incognita*, morfolojik özellikler, besin elementi, bamya, dayanıklılık

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Introduction

Okra [*Abelmoschus esculentus* (L.) Moench], a member of the Malvaceae, is commonly grown in tropical and subtropical regions of the world (Marin et al., 2017). Geographical origin of okra is thought to be Ethiopia and was initially distributed from there to southern Africa, the Mediterranean Basin, the Arabian Peninsula and South Asia, then the entire world (Sathish & Eswar, 2013).

Okra is largely consumed as human food and fruits are rich in vitamins, protein and crude fiber. With its carbohydrate, protein, oil, mineral and vitamin supply, okra is an important food for human nutrition (Abd El-Kader et al., 2010). Okra seeds are used as an oil source and fruits are also used as a fiber source. Carbohydrate and pectin-containing mucilage is obtained from the fruits and used as a thickener by the food industry (Alegbejo et al., 2008). Worldwide, almost 10 Mt of okra is produced on about 2.7 Mha (FAO, 2019). Okra is cultivated in all regions of Turkey. Commercial cultivation is common in Aegean, Marmara, Mediterranean and Central Anatolia Regions. In Turkey, annually 31 kt of okra is produced on 6 kha (FAO, 2019).

Stress is defined as conditions that influences or hampers plant growth, development and metabolism. Stress factors can be biotic or abiotic. Root-knot nematodes cause plant biotic stress resulting in serious economic losses in various plant species worldwide. Among the pests and diseases affecting okra production and limiting production area, root-knot nematode (*Meloidogyne* spp.*)* is the most important (Hussain et al., 2011). The symptoms of nematode infestation are slow growth rate, limited root development, leaf yellowing and wilting. Root-knot nematode causes wilting, chlorosis, stunted growth, and root galling, and when the nematode population exceeds the economic threshold level, it generally results in destruction of the roots, weak growth and reduced yield (Daramola et al., 2015; Mukhtar et al., 2017). Root-knot nematode-induced yield losses of 29 to 91% have been reported (Pandey & Kalra, 2003). Mukhtar et al. (2013) indicated *Meloidogyne incognita* (Kofoid & White, 1919) (Nematoda: Meloidogynidae) as the dominant species largely influencing plant growth and yield. Sikora and Fernandez (2005) reported that *Meloidogyne* spp. caused yield loss of up to 27% in okra. Subsequent cultivation of okra in the same field allows the nematodes to become widespread and colonizing the whole field (Hussain et al., 2011). In addition to these direct impacts, nematodes also increase *Fusarium* spp. infection, resulting greater damage and loss. In Turkey, *M. incognita*, *Meloidogyne arenaria* (Neal, 1889) and *Meloidogyne javanica* (Treub, 1885) (Nematoda: Meloidogynidae) have been reported as the most common and economically-damaging species in vegetable production (Kaşkavalcı & Öncüer, 1999; Özarslandan & Elekcioğlu, 2010; Çetintaş & Çakmak, 2016). Also, *Meloidogyne luci* can be a serious problem in field crops and greenhouse vegetables in northern areas of Turkey (Aydınlı, 2018).

For integrated management of nematodes, non-host plants are incorporated into the crop rotations, resistant cultivars are used, biological control and solarization are practiced, and nematicide and fumigants are applied. In large fields, due to the high cost, nematicides are not applied to okra. Generally non-host plants are incorporated into crop rotations in such contexts. Cultivars with nematode resistance usually give, greater yield than susceptible cultivars.

This study was conducted to determine the resistance of local okra cultivars against root-knot nematodes. Use of resistant cultivars in okra farming is the most effective, economic and practical method for control of nematodes. In this study, resistance of 26 okra cultivars against *M. incognita* was determined and nematode-induced morphological changes and effects on leaf macro- and micronutrients were determined.

Materials and Methods

Twenty-six okra from various geographic origins for Turkey were obtained from the large collection maintained at the Aegean Agricultural Research Institute, Menemen, Izmir, Turkey (Karagül, 2003). Twenty-four local okra cultivars, commonly cultivated in various regions of Turkey, and two commercially available and common local cultivars cv. Sultani (Marmara and Aegean Regions) and cv. Bornova (Aegean Region) were used in this study (Table 1). Testing of okra cultivars against nematodes were conducted in climate-controlled glasshouse at Alata Horticultural Research Institute in September-November 2019.

Resistance of the okra cultivars against root-knot nematode of *M. incognita* was investigated at 25 ± 1ºC and 60 ± 10% RH under controlled conditions. The root-knot nematode (*M. incognita*) used was produced on susceptible sivri pepper cv Alata F_1 (Alata Horticultural Research Institute, Mersin). At the end of nematode production period, egg masses on the roots were collected under a binocular microscope, and 2nd stage juveniles (J2s) were obtained with the aid of modified Baermann-funnel technique. J2s were counted under light microscope to prepare inoculum. Okra seeds were directly sown into pots. Experiments were conducted in a completely randomized design with four replicates. Treatments included a uninoculated control and nematode inoculation. The potting mix consisted of 80% sand, 5% silt and 15% soil disinfested by autoclaving. Each pot was inoculated with ~3,000 J2s at about 2 cm deep near plants that had grown to ~15 cm at the 2-4 leaf stage. Sixty days after inoculation (DAI), the plants were removed from the pots and root galls scored and morphological measurements made. Leaf samples were taken from the control and inoculated plants in each replicate and leaf macro- and micronutrients were determined. Nitrogen concentrations were determined in ground samples by a modified Kjeldahl method (Kacar, 1972). Phosphorus concentration were determined from the extracts obtained through wet digestion in nitric-perchloric acid mixture with the use of vanadomolybdophosphoric yellow method (Kacar & Kovancı, 1982). Potassium, calcium and magnesium, iron, zinc, manganese and copper concentrations were determined from the samen extracts using inductively coupled plasma (ICP) (Kacar, 1972).

Root gall index

Following the inoculation, plants were removed from the pots on 60 DAI and root galls were scored according to Hartman & Sasser (1985) based on 0-5 scale for number of eggs and galls. According to this scale, 0-2 indicates resistant and 3-5 indicates susceptible plants.

Plant measurements

Plant samples were arbitrary selected from each control (uninoculated) and nematode-inoculated pots 60 DAI and plant height (cm), root length (cm), leaf length (cm), leaf width (cm), fresh root weight (g) and stem thickness (mm) were measured.

Leaf macro- and micronutrients

Sixty DAI, leaf samples were taken from the control (nematode non-inoculated) and inoculated plants of each replicate and leaf macro- and micronutrients were determined. For this purpose, N, P, K, Ca and Mg as %, and Fe, Zn, Mn and Cu as ppm concentrations were determined.

Statistical Analysis

The relative effect of nematode inoculation on plant measurements and leaf macro- and micronutrients was determined with the use of Abbott formula [change = (1 − nematode-inoculated / control) × 100]. Experimental data were subjected to analysis of variance using of statistical analysis software (JMP v8.0.2.) and significant means were compared by Tukey HSD test at P = 0.05 significance level.

Results

Root gall index

Differences in root gall indexes were significant. Twenty-six okra cultivars were found to be nematode susceptible. Root gall indexes varied between 3-5 and an average value of 4.6 (Table 1). Differences between gall indexes of the cultivars were mainly attributed genetic characteristics of the plants. The greatest gall index (5) was obtained for cultivars 17, 19, 32, 40, 45, 57, 60, 62, 64, 68, 87, 88, 100 and 109 and the lowest (3.25) for cultivar 104 (Table 1). All local cultivars tested in present study were found to be susceptible to root-knot nematode (Figure 1).

Table 1. Local okra cultivars used in present study and gall indexes of 26 local okra cultivars

Cultivar No	Cultivar name	Origin	Index
3	TR 37126	Kastamonu	4.5
5	TR 39467	Kırklareli	4.8
11	TR 40293	Kırşehir	4.0
17	TR 42994	Konya	5.0
19	TR 43092	Kütahya	5.0
30	TR 57340	Sinop	4.0
32	TR 57344	Sinop	5.0
40	TR 61765	Trabzon	5.0
45	TR 69231	Karaman	5.0
56	TR 66081	Yozgat	4.5
57	TR 66070	Yozgat	5.0
60	TR 66087	Yozgat	5.0
62	TR 66076	Yozgat	5.0
64	TR 43185	Kütahya	5.0
67	TR 66581	Yozgat	4.0
68	TR 66594	Yozgat	5.0
84	Kılıçlı IIII	Mersin	3.8
87	Karşıyaka III	İzmir	5.0
88	Sultani Bamya	Marmara-Aegean Regions	5.0
89	Bornova Bamyası	Aegean Region	3.5
97	Tarsus Yalamık 1	Mersin	4.3
100	Şanlıurfa 1 Karaali	Şanlıurfa	5.0
104	Şanlıurfa 2	Şanlıurfa	3.2
108	Hilvan 3 Arpalı Köyü	Şanlıurfa	4.0
109	Siverek	Şanlıurfa	5.0
112	Denizli	Denizli	4.0
Mean			4.6

Figure 1. Nematode infestation response of three okra cultivars (17, 60 and 68).

Plant height, stem thickness and root length

In root-knot nematode-inoculated plants, the tallest plant height (35.8 cm) was in cultivar 45 and the shortest (16.8 cm) in cultivar 5. In control plants, the tallest plant height (67.5 cm) was in cultivar 87 and the shortest (34.3 cm) in cultivar 100. The change in plant height ranged from 17.3 to 71.3% with a mean of 46.0% (Table 2).

Table 2. Effects of root-knot nematode inoculation on plant growth parameters of 26 local okra cultivars

Cultivar No	Plant height (cm)			Stem thickness (mm)			Root length (cm)		
	Control*	Nematode	Change (%)	Control	Nematode	Change (%)	Control	Nematode	Change (%)
3	42.2 eh	21.7 hk	48.7	2.45 ij	3.29 dh	-34.3	19.7 ad	9.1 cf	53.7
5	54.6 be	16.8 k	69.2	5.39 a	3.24 di	39.9	17.7 bg	7.4 df	58.3
11	37.7 fh	28.4 be	24.6	3.05 gj	2.47 hi	19.0	18.6 af	8.9 cf	51.9
17	47.9 dg	23.7 ej	50.5	4.70 aj	3.67 cf	21.9	11.4 j	6.8 ef	40.4
19	49.7 df	23.2 fk	53.5	3.54 eh	3.22 di	9.0	15.8 ei	8.3 df	47.5
30	36.9 gh	29.9 bc	19.2	3.50 fi	5.49 a	-56.9	17.6 bg	8.4 df	52.6
32	54.9 ae	23.2 fk	57.8	5.04 ab	3.22 di	36.1	16.1 di	7.0 df	56.7
40	57.2 ad	31.6 ab	44.7	5.09 ab	3.04 ei	40.3	12.9 hj	10.2 be	20.8
45	64.5 ac	35.8 a	44.4	4.55 ae	3.82 ce	16.0	20.4 ab	13.0 ab	36.4
56	40.7 fh	20.3 ik	50.1	4.41 af	2.67 gi	39.5	15.3 fi	8.8 cf	42.6
57	48.0 dg	22.1 gk	54.0	4.62 aj	3.82 ce	17.3	22.2 a	15.5 a	30.3
60	48.9 dg	19.5 jk	60.3	2.89 gj	4.99 ab	-72.7	20.1 ac	15.1 a	25.1
62	32.8 h	20.6 ik	37.2	3.16 gj	3.64 cg	-15.2	14.7 gj	7.5 df	49.1
64	36.9 gh	26.8 bg	27.6	3.56 dh	3.85 ce	-8.2	14.5 gj	14.9 a	-3.3
67	36.7 gh	26.6 ch	27.6	4.25 bf	3.83 ce	9.9	20.0 ac	7.9 df	60.8
68	67.3 ab	19.3 jk	71.3	4.99 ab	3.07 ei	38.5	16.0 ei	7.8 df	51.0
84	36.5 gh	26.3 ch	27.8	2.72 gj	2.92 ei	-7.4	19.9 ac	9.2 cf	53.8
87	67.5 a	23.9 ej	64.6	4.61 ad	3.52 cg	23.6	15.1 fj	9.1 cf	39.3
88	33.1 h	27.4 bf	17.3	3.73 cg	3.65 cf	2.1	17.3 bg	14.4 a	17.0
89	37.7 fh	24.8 ci	34.1	5.09 ab	3.50 dg	56.7	15.3 fi	7.0 df	54.5
97	44.9 dh	20.7 ik	53.9	4.70 ac	2.75 fi	41.5	17.6 bg	5.7 f	67.7
100	34.3 h	26.0 ch	24.2	2.25 j	4.14 bd	-84.0	12.5 ij	10.5 bd	16.4
104	66.0 ac	29.2 bd	55.8	4.41 af	2.71 fi	38.6	19.3 ae	9.0 cf	53.6
108	66.3 ac	21.6 hk	67.5	2.63 hj	2.32 i	11.8	18.7 af	12.2 ac	35.0
109	54.1 ce	23.7 ej	56.1	5.43 a	3.45 dg	36.5	13.5 hj	13.4 ab	0.7
112	54.0 ce	24.6 di	54.4	5.32 a	4.48 bc	15.8	16.5 ch	13.2 ab	20.5
Mean	48.1	24.5	46.0	4.20	3.49	9.1	16.9	10.0	39.7
LSD (5%)	12.76	5.04		1.05	0.97		3.71	3.59	
F prob.	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001	

* Control, uninoculated; nematode, nematode-inoculated.

In nematode-inoculated plants, the greatest stem thickness (5.49 mm) was in cultivar 30 and the lowest (2.32 mm) in cultivar 108. In control plants, the greatest stem thickness (5.43 mm) was in cultivar 109 and the lowest (2.25 mm) in cultivar 100. The change stem thicknesses ranged from -84.0% to 71.3% with mean of 9.06% (Table 2).

In nematode-inoculated plants, the greatest root length (15.1 cm) was in cultivar 57 and the lowest (5.70 cm) in cultivar 97. In control plants, the longest root length (22.2 cm) was in cultivar 57 and the shortest (11.4 cm) in cultivar 17. The change in root lengths ranged -3.3 to 67.7% with a of 39.7% (Table 2).

Root weight, leaf width and leaf length

In nematode-inoculated plants, the greatest root weight (5.77 g) was in cultivar 60 and the lowest (0.68 g) in cultivar 108. In control plants, the greatest root weight (2.56 g) was in cultivar 109 and the lowest (1.27 g) in cultivar 17. Root-knot nematode changed root weight by -258 to 60.2% with a mean of -14.5% (Table 3).

Table 3. Effects of root-knot nematode inoculation on plant growth parameters of 26 local okra cultivars

Cultivar No	Root weight (g)			Leaf width (cm)			Leaf length (cm)		
	Control*	Nematode	Change (%)	Control	Nematode	Change (%)	Control	Nematode	Change (%)
3	1.84 dg	1.68 df	8.7	8.4 gi	10.6 bc	-26.9	7.8 hi	9.0 cd	-15.5
5	2.44 ab	0.98 gk	59.8	14.3 ce	6.6 h	54.0	11.4 ad	6.3 h	44.5
11	1.68 eh	1.26 fk	25.0	7.6 i	12.0 ab	-57.2	9.0 fi	7.4 dh	17.8
17	1.27 eh	4.01 b	-215.8	8.2 hi	8.7 cg	-6.4	8.1 hi	7.9 dh	3.1
19	1.47 eh	1.52 ei	-3.4	11.9 ae	9.3 ce	21.5	11.0 ae	8.5 cf	22.7
30	2.40 ac	1.47 ei	38.8	11.5 be	9.2 cf	20.6	12.0 ac	8.6 ce	28.2
32	1.34 fh	1.27 fk	5.2	11.1 cf	7.5 eh	32.4	11.0 ae	6.6 gh	39.5
40	1.29 gh	2.40 c	-86.1	13.3 ac	9.5 ce	29.1	12.1 ac	9.1 cd	25.0
45	1.86 cf	2.15 ce	-15.6	11.6 be	9.1 cg	21.7	11.0 ae	8.6 ce	22.5
56	1.77 dh	1.31 fk	26.0	12.0 ae	7.7 dh	36.2	11.6 ad	7.5 dh	35.7
57	1.43 eh	1.36 fk	4.9	10.6 dg	8.1 dh	23.6	10.7 bf	7.6 dh	28.4
60	1.61 eh	5.77 a	-258.4	13.0 ad	12.7 a	2.3	11.2 ad	10.9 ab	2.9
62	1.50 eh	0.70 jk	53.3	9.0 fi	7.2 gh	20.1	9.3 eh	7.5 dh	20.2
64	1.41 eh	1.86 cf	-31.9	10.4 eh	7.3 fh	30.5	10.1 dg	6.9 eh	32.1
67	1.68 eh	1.57 dh	6.6	12.1 ae	8.3 dh	31.5	10.8 bf	7.7 dh	28.6
68	1.61 eh	0.85 ik	47.2	11.1 cf	11.5 ab	-3.6	11.6 ad	11.1 ab	4.1
84	1.55 eh	0.88 hk	43.2	12.1 ae	9.1 cf	24.4	10.9 be	8.4 cf	23.0
87	1.31 fh	2.45 c	-87.0	10.6 dg	9.5 cd	10.1	10.4 cg	8.9 cd	14.4
88	1.42 eh	1.80 cf	-26.8	11.9 ae	9.3 ce	22.1	10.8 bf	8.2 cg	24.2
89	1.52 eh	1.66 dg	-9.2	13.1 ac	9.5 cd	27.1	11.2 ad	8.2 cg	27.1
97	2.25 ad	1.34 fk	40.4	8.1 hi	7.5 eh	7.4	7.8 i	6.9 eh	11.5
100	1.68 eh	2.24 c	-33.3	8.7 gi	10.4 bc	-19.2	8.7 gi	9.7 bc	-11.5
104	1.49 eh	1.39 fj	6.7	13.1 ac	8.1 dh	38.3	11.2 ad	6.8 fh	39.3
108	1.71 dh	0.68 k	60.2	13.9 ab	9.0 cg	35.8	12.7 a	7.9 dh	38.2
109	2.56 a	3.66 b	-43.0	14.4 a	9.2 cf	35.9	12.4 ab	8.3 cg	33.2
112	1.94 be	1.81 cf	6.7	11.3 cf	13.4 a	-18.9	11.6 ad	12.2 a	-5.9
Mean	1.69	1.85	-14.5	11.3	9.23	15.1	10.6	8.32	20.5
LSD (5%)	0.56	0.70		2.44	1.97		1.82	1.74	
F prob.	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001	

* Control, uninoculated; nematode, nematode-inoculated.

In nematode-inoculated plants, the greatest leaf width (13.4 cm) was in cultivar 112 and the lowest (6.58 cm) in cultivar 5. In control plants, the greatest leaf width (14.4 cm) was in cultivar 109 and the lowest (7.60 cm) in cultivar 11. The change in leaf width ranged from -57.2 to 54.0% with a mean of 15.1% (Table 3).

In nematode-inoculated plants, the greatest leaf length (12.2 cm) was in cultivar 112 and the lowest (6.30 cm) in cultivar 5. In control plants, the greatest leaf length (12.7 cm) was in cultivar 108 and the lowest (7.50 cm) in cultivar 97. The change in leaf length ranged from -15.5 to 44.5% with a mean of 20.5% (Table 3).

Nitrogen, phosphorus and potassium

In nematode-inoculated plants, the greatest N concentration (2.39%) was in cultivar 100 and the lowest (1.19%) in cultivar 17. In control plants, the greatest N concentration (2.76%) was in cultivar 109 and the lowest (1.28%) in cultivar 19. The change ranged from 47.1 to 73.4% with a mean of 2.31% (Table 4).

In nematode-inoculated plants, the greatest P concentration (0.35%) was in cultivar 11 and the lowest (0.13%) in cultivar 62. In control plants, the greatest P concentration (0.55%) was in cultivar 109 and the lowest (0.17%) in cultivar 19. The change ranged from -50% to 67% with a mean of 5.85% (Table 4).

In nematode-inoculated plants, the greatest K concentration (1.80%) was in cultivar 100 and the lowest (0.55%) in cultivar 62. In control plants, the greatest K concentration (2.43%) was in cultivar 57 and the lowest (0.60%) in cultivar 62. The change ranged from -86.1% to 63.8% with a mean of 5.24% (Table 4).

Table 4. Nematode-induced changes in leaf N, P and K concentrations of 26 local okra cultivars

Cultivar No	N (%)			P (%)			K (%)		
	Control*	Nematode	Change (%)	Control	Nematode	Change (%)	Control	Nematode	Change (%)
3	2.03 gi	1.37 j	32.5	0.18 lm	0.14 jk	22.2	1.24 kj	0.74 m	40.3
5	1.95 ij	2.24 bc	-14.9	0.19 km	0.21 df	-10.5	0.86 n	1.60 b	-86.1
11	2.11 fh	2.36 ab	-11.9	0.28 bd	0.36 a	-28.6	0.92 mn	1.17 fj	-27.2
17	2.17 eg	1.19 k	45.2	0.27 be	0.16 jk	40.7	1.39 hi	0.69 m	50.4
19	1.28 m	2.22 bd	-73.4	0.17 m	0.23 ce	-35.3	0.99 m	1.62 b	-63.6
30	1.75 k	2.33 ab	-33.1	0.20 im	0.30 b	-50.0	0.88 n	1.40 cd	-59.1
32	2.01 hi	1.56 i	22.4	0.26 dg	0.21 df	19.2	2.28 b	1.14 gj	50.0
40	2.40 b	1.39 j	42.1	0.28 bd	0.16 hk	42.9	1.52 fg	1.09 ik	28.3
45	2.29 be	2.04 e	10.9	0.24 ei	0.19 fh	20.8	1.31 ij	1.02 k	22.1
56	1.82 jk	2.27 ac	-24.7	0.20 im	0.24 cd	-20.0	1.23 jl	1.40 cd	-13.8
57	2.36 bc	1.35 j	42.8	0.30 bc	0.17 hj	43.3	2.43 a	0.88 l	63.8
60	2.23 cf	1.72 gh	22.9	0.22 hl	0.17 gj	22.7	1.14 kl	1.32 de	-15.8
62	2.38 b	1.26 jk	47.1	0.27 cf	0.13 k	51.9	1.48 gh	0.55 n	62.8
64	1.53 i	2.22 bd	-45.1	0.19 km	0.23 ce	-21.1	0.87 e	1.20 fh	-37.9
67	2.29 be	1.39 j	39.3	0.22 gk	0.20 eg	9.1	1.41 gi	1.08 jk	23.4
68	2.26 be	1.25 jk	44.7	0.21 im	0.17 hj	19.1	1.62 ef	0.66 m	59.3
84	1.906 ik	2.27 ac	-19.5	0.22 gk	0.25 c	-13.6	1.13 l	1.27 ef	-12.4
87	2.03 gi	2.34 ab	-15.3	0.21 im	0.25 c	-19.1	1.49 gh	1.25 eg	16.1
88	1.32 m	2.15 ce	-62.9	0.31 b	0.23 ce	25.8	1.87 c	1.39 cd	25.7
89	2.19 df	2.36 ab	-7.8	0.25 dh	0.24 cd	4.0	1.34 ij	1.20 fi	10.5
97	2.32 bd	2.07 de	10.8	0.23 fj	0.23 ce	0.0	1.18 kl	1.16 gj	1.7
100	2.22 cf	2.39 a	-7.7	0.22 gk	0.26 c	-18.2	1.50 g	1.80 a	-20.0
104	1.94 ij	1.87 f	3.6	0.21 im	0.21 df	0.0	1.72 de	1.11 hk	35.5
108	1.75 k	1.83 fg	-4.6	0.20 jm	0.21 df	-5.0	0.60 o	1.02 k	-70.0
109	2.76 a	1.63 hi	40.9	0.56 a	0.19 fi	66.1	1.80 cd	0.87 l	51.7
112	1.89 ik	2.35 ab	-24.3	0.21 im	0.24 cd	-14.3	1.69 e	1.68 b	0.6
Mean	2.05	1.90	2.3	0.24	0.21	5.9	1.38	1.17	5.2
LSD (5%)	0.15	0.15		0.04	0.03		0.11	1.11	
F prob.	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001	

* Control, uninoculated; nematode, nematode-inoculated.

Calcium, magnesium and iron

In nematode-inoculated plants, the greatest Ca concentration (4.70%) was in cultivar 100 and the lowest (2.91%) in cultivar 56. In control plants, the greatest Ca concentration (4.84%) was in cultivar 87 and the lowest (2.73%) in cultivar 64. The change ranged from -59.0% and 24.4% with a mean of -7.9%. However, nematode inoculation generally increased Ca concentrations (Table 5).

In nematode-inoculated plants, the greatest Mg concentration (0.89%) was observed in cultivar 68 and the lowest (0.53%) in cultivar 100. In control plants, the greatest Mg concentration (0.98%) was obtained from cultivar 17 and the lowest (0.52%) from cultivar 64. The change ranged from 17.7 to -38.8% with a mean of 10.3%. However, nematode inoculation generally reduced Mg concentrations (Table 5).

In nematode-inoculated plants, the greatest Fe concentration (289 ppm) was in cultivar 100 and the lowest (76 ppm) in cultivar 19. In control plants, the greatest Fe concentration (331 ppm) was in cultivar 57 and the lowest (85 ppm) in cultivar 30. The change ranged from -155% and 56.0% and with a mean of -17.0%. However, nematode inoculation increased Fe concentrations (Table 5).

Table 5. Nematode-induced changes in leaf Ca, Mg and Fe concentrations of 26 local okra cultivars

Cultivar No	Ca (%)			Mg (%)			Fe (ppm)		
	Control*	Nematode	Change (%)	Control	Nematode	Change (%)	Control	Nematode	Change (%)
3	3.39 il	3.98 ce	-17.4	0.68 j	0.66 eh	2.9	130 f	82 mn	37.5
5	3.69 ei	3.44 i	6.8	0.85 d	0.70 dg	17.7	105 im	111 1jk	-5.4
11	3.41 4ik	3.87 df	-13.5	0.84 de	0.71 df	15.5	91 no	110 jk	-21.0
17	4.15 2bc	4.37 b	-5.3	0.98 a	0.60 hj	38.8	165 d	110 jk	33.2
19	3.22 km	3.49 hi	-8.4	0.80 eg	0.87 ab	-8.8	92 mo	76 n	17.2
30	3.49 hk	3.53 hi	-1.2	0.90 bc	0.61 gj	32.2	85 o	105 kl	-23.4
32	3.90 cf	3.79 fg	2.8	0.74 hi	0.65 eh	12.2	291 b	128 hi	56.0
40	3.84 dg	4.03 cd	-5.0	0.73 i	0.63 fi	13.7	111 gk	228 b	-105.1
45	3.60 gj	3.84 ef	-6.7	0.76 gi	0.64 fi	15.8	104 in	137 gh	-31.9
56	3.58 gj	2.91 j	18.7	0.78 fh	0.70 dg	10.3	107 hl	186 cd	-73.0
57	4.02 bd	4.09 c	-1.7	0.84 de	0.71 df	15.5	331 a	180 de	45.6
60	3.97 ce	3.42 i	13.9	0.86 cd	0.63 fi	26.7	91 no	83 mn	8.6
62	4.20 bc	4.35 b	-3.6	0.86 cd	0.71 df	17.4	112 gj	105 kl	6.1
64	4.84 a	3.66 gh	24.4	0.52 l	0.55 ij	-5.8	121 fg	114 jk	6.1
67	3.58 gj	4.44 b	-24.0	0.82 df	0.77 cd	6.1	94 lo	172 ef	-81.8
68	3.31 jm	4.37 b	-32.0	0.82 df	0.89 a	-8.5	113 gi	127 hi	-13.1
84	3.42 ik	4.04 cd	-18.1	0.75 hi	0.67 eh	10.7	97 ko	198 c	-103.1
87	2.73 n	4.34 b	-59.0	0.62 k	0.73 de	-17.7	98 jo	138 gh	-41.2
88	3.77 dh	3.54 hi	6.1	0.77 gi	0.65 eh	15.6	173 cd	120 ij	30.3
89	3.01 mn	2.94 j	2.3	0.84 de	0.67 eh	20.2	107 il	108 jl	-0.8
97	3.62 fi	4.02 ce	-11.1	0.76 hi	0.83 ac	-9.2	96 lo	144 g	-50.9
100	4.28 b	4.70 a	-9.8	0.63 k	0.53 j	15.9	114 gi	289 a	-154.7
104	3.97 be	4.01 ce	-1.0	0.73 i	0.78 bd	-6.9	186 c	129 hi	31.0
108	3.69 ei	4.28 b	-16.0	0.91 b	0.78 bd	14.3	121 fh	121 ij	-0.2
109	3.29 jm	4.27 b	-29.8	0.76 gi	0.64 ei	15.8	151 e	162 f	-7.1
112	3.09 lm	3.62 gh	-17.2	0.68 j	0.63 fi	7.4	94 lo	95 lm	-0.6
Mean	3.66	3.90	-7.9	0.78	0.69	10.3	130	137	-17.0
LSD (5%)	0.30	0.18		0.05	0.09		13.8	13.6	
F prob.	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001	

* Control, uninoculated; nematode, nematode-inoculated.

Zinc, manganese and copper

In nematode-inoculated plants, the greatest Zn concentration (113 ppm) was in cultivar 109 and the lowest (12.3 ppm) in cultivar 68. In control plants, the greatest Zn concentration (392 ppm) was in cultivar 57 and the lowest (12.5 ppm) in cultivar 19. The change ranged from -212% to 94.4% and mean increase in Zn concentrations was calculated as -13.9% (Table 6). However, nematode inoculation generally increased Zn concentrations

In nematode-inoculated plants, the greatest Mn concentration (336 ppm) was in cultivar 30 and the lowest (41.2 ppm) in cultivar 3. In control plants, the greatest Mn concentration (98.0 ppm) was in cultivar 89 and the lowest (32.4 ppm) in cultivar 108. The change ranged from -744% to 24.8% with a mean of -118% (Table 6). However, nematode inoculation generally increased Mn concentrations.

In nematode-inoculated plants, the greatest Cu concentration (6.59 ppm) was in cultivar 100 and the lowest (1.79 ppm) in cultivar 57. In control plants, the greatest Cu concentration (8.89 ppm) was in cultivar 57 and the lowest (1.34 ppm) in cultivar 60. The change ranged from -129% to 79.9% and mean increase in Cu concentrations was calculated as -15.6% (Table 6). However, nematode inoculation generally increased Cu concentrations.

Table 6. Nematode-induced changes in leaf Zn, Mn and Cu concentrations of 26 local okra cultivars

Cultivar No	Zn (ppm)			Mn (ppm)			Cu (ppm)		
	Control*	Nematode	Change (%)	Control	Nematode	Change (%)	Control	Nematode	Change (%)
3	132.3 c	15.0 ln	88.7	43.7 jl	41.2 o	5.8	2.15 p	3.90 f	-81.4
5	23.5 fg	29.1 h	-23.4	36.0 mn	75.5 lm	-110.0	2.09 p	4.01 f	-91.9
11	17.9 gi	23.8 ij	-32.7	38.0 ln	54.9 no	-44.6	3.38 ik	2.95 ij	12.7
17	128.5 c	13.4 mn	89.6	71.2 de	141.4 ef	-98.6	5.04 e	2.78 j	44.8
19	12.5 i	14.9 ln	-19.2	60.7 gh	45.7 o	24.8	3.26 jl	3.51 gh	-7.7
30	13.2 hi	41.2 e	-212.1	39.9 lm	336.4 a	-743.9	1.96 p	3.29 h	-67.9
32	329.4 b	18.5 kl	94.4	79.9 c	64.6 mn	19.2	6.98 b	5.22 c	25.2
40	30.5 f	39.1 ef	-28.2	64.3 fg	116.8 gh	-81.6	4.28 g	4.39 e	-2.6
45	25.8 fg	23.6 ij	8.6	68.4 ef	205.1 c	-199.9	4.70 f	3.97 f	15.5
56	23.8 fi	33.8 g	-42.4	54.5 i	48.8 no	10.5	2.96 mn	4.09 ef	-38.2
57	329.4 a	51.6 d	84.3	92.6 ab	96.4 ik	-4.0	8.89 a	1.79 m	79.9
60	27.1 fg	35.3 fg	-30.2	41.2 km	119.0 g	-189.1	1.34 q	2.34 k	-74.6
62	20.9 fi	14.0 mn	32.9	80.4 c	80.1 km	0.4	2.66 o	3.28 hi	-23.3
64	20.7 fi	60.2 c	-190.9	89.1 b	220.4 c	-147.3	2.54 o	5.81 b	-128.7
67	17.4 gi	12.9 mn	26.1	54.8 hi	109.4 gi	-99.7	2.78 no	2.18 kl	21.6
68	24.2 fh	12.3 n	49.3	56.6 hi	55.9 no	1.2	2.96 mn	3.92 f	-32.4
84	16.1 gi	26.6 hi	-65.3	57.7 hi	174.1 d	-201.7	3.50 ij	5.28 c	-50.9
87	20.6 fi	19.9 jk	3.5	54.9 hi	100.0 hj	-82.2	2.71 no	3.80 fg	-40.2
88	104.9 d	47.2 d	55.0	48.1 j	262.0 b	-444.7	6.52 c	4.88 d	25.2
89	21.4 fi	19.9 jk	7.1	98.0 a	150.4 e	-53.5	3.61 i	3.35 h	7.2
97	24.3 fh	65.5 b	-169.1	47.0 jk	98.2 ik	-109.1	2.64 o	4.00 f	-51.5
100	15.5 gi	35.3 fg	-127.9	66.3 eg	176.7 d	-166.6	3.20 km	6.59 a	-105.9
104	78.9 e	16.8 kn	78.8	65.0 fg	88.4 jl	-36.1	4.76 f	2.27 k	52.3
108	21.7 fi	17.4 km	20.2	32.4 n	83.8 jl	-158.5	3.99 h	2.25 kl	43.6
109	70.1 e	112.8 a	-60.8	76.8 cd	127.4 fg	-66.0	3.12 lm	3.29 h	-5.5
112	19.5 fi	18.8 kl	3.4	81.8 c	158.3 de	-93.6	6.20 d	1.94 lm	68.7
Mean	60.4	31.5	-13.9	61.5	124.3	-118.0	3.78	3.66	-15.6
LSD (5%)	11.66	4.51		5.94	18.46		0.25	0.32	
F prob.	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001	

* Control, uninoculated; nematode, nematode-inoculated.

Discussion

In Turkey, resistance of local okra cultivars against *M. incognita* has not been studied previously. Okra is severely damaged by root-knot nematodes and most cultivars are susceptible to *M. incognita*. Twenty-six cultivars were not resistant to *M. incognita.* Root gall indexes for the local okra cultivars varied between 3.3 and 5 with a mean of 4.6 indicating that these cultivars as susceptible to the nematode. Similar studies have been conducted elsewhere, especially in Asian and African countries. Hussain et al. (2014) conducted a study under field conditions in three regions of Pakistan with 12 okra cultivars and reported root gall indexes as between 4 and 5. Basil et al. (2019) inoculated 10 okra cultivars and reported root gall indexes as between 3.7 and 4.2. Karajeh & Salameh (2015) conducted a nematode inoculation study in Jordan with 36 okra cultivars and reported moderate resistance against the nematode. Kedarnath et al. (2017) conducted a study in India on seven okra cultivars and reported gall indexes of 3 to 4 for plants 60 DAI and at harvest. These results are similar to the present study, but not all their results. Sheela et al. (2006) investigated root gall indexes of 123 okra cultivars and based on gall index of 3 reported that theses okra cultivars were moderately resistant. Similarly, Muhammad et al. (2017) conducted a study under field conditions in Pakistan with 12 okra cultivars and reported root gall indexes of 2-4. As seen in previous studies, okra is generally not resistant to root-knot nematodes. Although a range of research has evaluated the reaction of okra cultivars to root-knot nematodes, there are no reports of fully resistant cultivars, only those that are moderately resistant and susceptible (Mohanta & Mohanty, 2012; Hussain et al., 2014; Marin et al., 2017).

No studies similar to the present study have been reported, but a few studies examined plant growth parameters in okra, tomatoes and soybeans. In present study, effects of *M. incognita* inoculation on plant growth parameters were investigated. Compared to uninoculated control plants, root-knot nematode inoculation reduced plant height, stem thickness, root length, leaf width and leaf length but root weight was increased depending on gall formation. Kumar et al. (2012) investigated the effects of nematode inoculation on root length, shoot length, fresh and dry root weights and reported significantly effects. Root and shoot lengths were greater in control plants. Mean root length of the control and inoculated plants was reported as 40 and 32 cm, shoot length as 49 and 32 cm, root dry weights as 4.8 and 7.0 g and root fresh weights as 13.4 and 11.5 g, respectively. Odeyemi et al. (2016) reported *M. incognita*-induced reductions of between 13.7 and 75.6% in plant height, between 13.7 and 67.2% plant fresh weight and between 13.0 and 53.3% in fruit weight. In another study conducted on okra, Claudius-Cole (2018) reported mean number of fruits as 18.4 for control plants and 13.8 for *M. incognita*-inoculated plants; mean fruit weight as 167 g in control plants and 81.3 g for inoculated plants. Yield loss was reported as 51.4%. Pandey et al. (2019) reported (60 DAI) significant effects of nematode inoculation on plant length, plant diameter, root length and root diameter and reported. Plant length, plant diameter, root length and root diameter were greater in control plants in current study. Azam et al. (2011) examined *M. luci* infestation in tomatoes and reported decreased plant height and weights with increasing Pi density. Root-knot nematode symptoms in aboveground plant parts are reported to be stunted growth, chlorosis in leaves and reductions in plant weight. Gall formation-induced browning in leaves and recessed plant growth are generally encountered in nematode-infested okras and yield reductions vary between 29 and 91% (Pandey & Kalra, 2003). Studies on soybean and cucumber found that root-knot nematodes infestation increased root weights (Kayani et al., 2017). There was a general reduction in the growth parameters measured for the three okra cultivars for infested versus uninfested plants. The present findings on plant growth parameters are consistent with the findings of earlier studies. As a result, the galls formed in response to the feeding the root-knot nematodes disrupt the structure of the root and prevent uptake of water and nutrients from the soil, changing the distribution of photosynthesis products in the plant, increasing the movement of these products towards the root region, especially during the development and reproduction of the nematode. As a result, the inhibition of plant growth causes a decrease in plant height and stem diameter, and shrinkage of leaves, shortening of plant root, increase in plant root diameter and increase in plant root weight (Fortnum et al., 1991; Carneiro et al., 1999; Maleita et al., 2012).

Plant-pathogen-nutrient interactions are complex and the mechanisms have not been fully elucidated. Nutrients are essential elements for plant growth and development, and are essential for various physiological processes including protein synthesis, photosynthetic electron transfer, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormonal structure (Dordas, 2008; Santana-Gomes et al., 2013). In the present study, the concentrations of macro- and micronutrients were investigated in the leaves of okra cultivars. Compared to uninoculated control plants, nematode inoculation reduced leaf N, P, K and Mg concentrations and increased Ca, Fe, Zn, Mn and Cu concentrations. While macronutrients are generally reduced by nematode infestation, micronutrient accumulation was higher in leaf tissues.

There has been no reported study on the nematode effect of the concentrations of macro- and micronutrients in plant leaves. Similar studies have been done with plants such as tomatoes, soybeans, ridge gourd and coffee. Therefore, comparisons are made here with these plants. Miamoto et al. (2017) conducted a study on soybean and reported greater leaf Ca, Fe, Zn, Mn and Cu concentrations in nematode, *Pratylenchus brachyurus* (Godfrey, 1929) (Nematoda: Pratylenchidae), inoculated plants than in control plants. S, Cu and Zn, cofactors of plant enzymes and influencing cell wall formation and composition, produce some substances and improve plant resistance against nematodes through generating a physical barrier. Dietrich et al. (2004) reported lower N, Ca, Mg, Fe and Mn concentrations in

nematode-infested plants [*Arabidopsis thaliana* (L.) (Tracheophyta: Brassicaceae)] than in control plants. Activation of plant defense mechanism increased energy consumption and thus reduced the concentrations of these nutrients. In contrast, Carneiro et al. (2002) reported greater Mg, Fe, Mn and Zn concentrations in nematode-inoculated soybean plants. N and Ca concentrations were lower in shoots than in root-knot nematode-infested roots. Pathogens alters plant nutrient concentrations. Goncalves et al. (1995) indicated that *M. incognita* reduced P, Mg, Fe, Mn and B concentrations of coffee plants. Blevins et al. (1995) reported high Ca and low K and Mn concentrations in *Heterodera glycines* Ichinohe, 1952 (Nematoda: Heteroderidae) nematode-infested roots of soybean. Hajji et al. (2016) reported that nematodes significantly increased Cu, Zn, Fe, Mn, Mg and K concentrations and decreased Ca concentration in tomato roots. Carneiro et al. (2002) reported decreased N and P and increased Ca concentration in nematode-infested soybean roots. Decreased leaf size of nematode-infested plants resulted in increased Ca concentrations. Melakeberhan et al. (1985) reported that *M. incognita* reduced plant dry weight and increased Ca concentration of bean plants. Pandey et al. (2019) reported that as compared to the control plants, *M. incognita* reduced plant protein (20.4 vs 40.3%), N (20.0 vs 40.4%) and P (11.4 vs 92.0%) concentrations and increased K (27.3 vs 208%) concentration of ridge guard. Pandey et al. (2017) compared the control and *M. incognita*-infested mung bean plants and compared to the control plants, N concentrations of nematode-inoculated plants decreased by 4.1 to 88.6%, protein concentrations by 4.1 to 88.5%, P concentrations by 4.6 to 52.3% and K concentrations by 7.1 to 37.9%. Hurchanik et al. (2004) reported that nematode infestations significantly reduced Ca, Mg, P and B concentrations and increased Mn, Cu, Zn concentrations and Ca/B ratio in roots of coffee plants. Macro- and micronutrients present in okra are responsible for plant growth and development. According to the authors, nematode infestation causes abiotic and biotic stress in plants. As a result, nutrient uptake and accumulation of plants are affected. Root-knot nematode infestations cause physiological and biochemical changes. Such changes cause cell growth and tissue thickening in the roots, affecting the water nutrient uptake of the roots. As a result, it negatively affects plant growth and reduce yield. (Hussain et al., 2016; Débia et al., 2019).

Conclusion

In present study, root-knot nematode induced large and small galls on roots of local okra cultivars and all cultivars were identified as susceptible to the nematode. Similar findings were also reported for previous studies conducted in different parts of the world. Nematode damage impaired plant water and nutrient uptake from the soil, thus resulted in stunted growth. Compared to uninoculated control plants, nematode inoculation reduced plant height, stem thickness, root length, leaf width and leaf length, and increased root weight. Leaf samples were taken from the control and inoculated plants and leaf macro- and micronutrients were determined. It was observed that nematode inoculation reduced leaf N, P, K and Mg concentrations, but increased Ca, Fe, Zn, Mn and Cu concentrations. Since plant-pathogen-nutrient interactions are complex and the mechanisms involved have not been fully elucidated, further research is recommended on these mechanisms. Given environmental hazards and potential residues, nematicides are not recommended in okra production. Fumigation is hard to implement over large fields. Therefore, in control of root-knot nematode in okra fields, incorporation of non-host plants into crop rotations is recommended. Further breeding studies are recommended to identify the resistance gene of okras against root-knot nematode and to develop resistant cultivars.

References

Abd El-Kader, A. A., S. M. Saaban & M. S. Abd El-Fattah, 2010. Effect of irrigation levels and organic compost on okra plants (*Abelmoschus esculentus* L.) grown in sandy calcareous soil. Agriculture and Biology Journal of North America, 1 (3): 225-231.

Alegbejo, M., M. Ogunlana & O. Banwo, 2008. Survey for incidence of okra mosaic virus in northern Nigeria and evidence for its transmission by beetles. Spanish Journal of Agricultural Research, 6 (3): 408-411.

Aydınlı, G., 2018. Detection of the root-knot nematode *Meloidogyne luci* Carneiro et al., 2014 (*Tylenchida: Meloidogynidae*) in vegetable fields of Samsun Province, Turkey. Turkish Journal of Entomology, 42 (3): 229-237.

Azam, T., S. Hisamuddin, S. Singh & M. I. Robab, 2011. Effect of different inoculum levels of *Meloidogyne incognita* on growth and yield of *Lycopersicon esculentum*, and internal structure of infected root. Archives of Phytopathology and Plant Protection, 44 (18): 1829-1839.

Basil, H. K., E. H. Alaa, M. A. R. Asmaa & S. S. Brent, 2019. Screening for susceptibility and tolerance to *Meloidogyne incognita* and *M. javanica* in okra cultivars in Iraq. Arab Journal of Plant Protection, 37 (3): 279-283.

Blevins, D. G., V. H. Dropkin & V. D. Luedders, 1995. Macronutrient uptake, translocation, and tissue concentration of soybeans infested with the soybean cyst nematode and elemental composition of cysts isolated from roots. Journal of Plant Nutrition, 18 (3): 579-591.

Carneiro, R. G., P. Mazzafera & L. C. C. B. Ferraz, 1999. Carbon partitioning in soybean infected with *Meloidogyne incognita* and *M. javanica*. The Journal of Nematology, 31 (3): 348-355.

Carneiro, R. G., P. Mazzafera, L. C. C. B. Ferraz, T. Muraoka & P. C. O. Trevelin, 2002. Uptake and translocation of nitrogen, phosphorus and calcium in soybean infected with *Meloidogyne incognita* and *M. javanica*. Fitopatologia Brasileira, 27 (2): 141-50.

Çetintaş, R. & B. Çakmak, 2016. *Meloidogyne* species infesting tomatoes, cucumbers and eggplants grown in Kahramanmaraş Province, Turkey. Turkish Journal of Entomology, 40 (4): 355-364.

Claudius-Cole, A. O., 2018. Comparative effect of *Rotylenchulus reniformis* and *Meloidogyne incognita* on the productivity of okra in Nigeria. Australian Journal of Basic and Applied Sciences, 12 (9): 20-25.

Daramola, F. Y., J. O. Popoola, A. O. Eni & O. Sulaiman, 2015. Characterization of root-knot nematodes (*Meloidogyne* spp.) associated with *Abelmoschus esculentus*, *Celosia argentea* and *Corchorus olitorius*. Asian Journal of Biological Sciences, 8 (1): 42-50.

Débia, P. J. G., B. C. Bolanho, H. H. Puerari & C. R. Dias-Arieira, 2019. *Meloidogyne javanica* parasitism and its impacts on the vegetative parameters, physicochemical composition, and antioxidant potential of beet. Pesquisa Agropecuária Brasileira, 54: e00695, 1-8.

Dietrich, R., K. Ploss & M. Heil, 2004. Constitutive and induced resistance to pathogens in *Arabidopsis thaliana* depends on nitrogen supply. Plant Cell and Environment, 27 (7): 896-906.

Dordas, C., 2008. Role of nutrients in controlling plant diseases in sustainable agriculture a review. Agronomy for Sustainable Development, 28 (1): 33-46.

FAO, 2019. FAOSTAT database collections. Food and Agriculture Organization of the United Nations. (Web page: http://www.fao.org/faostat/en/#data/QC) (Date accessed: 13 January 2021).

Fortnum, B. A., M. J. Kasperbauer, P. G. Hunt & W. C. Bridges, 1991. Biomass partitioning in tomato plants infected with *Meloidogyne incognita*. The Journal of Nematology, 23 (3): 291-297.

Godfrey G. H., 1929. A destructive root disease of pineapples and other plants due to *Tylenchus brachyurus* n.sp. Phytopathology, 19 (7): 611-629.

Goncalves, W., P. Mazzafera, L. C. C. B. Ferraz, M. B. Silvarolla & M. M. A. De Lima, 1995. Biochemical basis of coffee tree resistance to *Meloidogyne incognita*. Plantations Recherche Development, 2 (1): 54-58.

Hajji, L., M. A. Elouaer, H. Regaieg, B. N. M'Hamdi & R. N. Horrigue, 2016. Biochemical and plant nutrient alterations induced by *Meloidogyne javanica* and *Fusarium oxysporum* f.sp.*radicis lycopersici* co-infection on tomato cultivars with differing level of resistance to *Meloidogyne javanica*. Springer European Journal of Plant Pathology, 148 (2): 463-472.

Hartman, K. M. & J. N. Sasser, 1985. "Identification of *Meloidogyne* Species on the Basis of Differential Host Test and Perennial Pattern Morphology, 69-77". In: An Advanced Treatise on Meloidogyne. Vol II. Methodology (Eds. K. R. Barker, C. C. Carter& J. N. Sasser). Raleigh, NC: North Carolina States Graphics, 223 pp.

Hurchanik, D., D. P. Schmitt, N. V. Hue & B. S. Sipes, 2004. Plant nutrient partitioning in coffee infected with *Meloidogyne konaensis*. The Journal of Nematology, 36 (1): 76-84.

Hussain, M., M. Kamran, K. Singh, M. Zouhar, P. Rysánek & S. A. Anwar, 2016 Response of selected okra cultivars to *Meloidogyne incognita*. Crop Protection, 82: 1-6.

Hussain, M. A., T. Mukhtar & M. Z. Kayani, 2011. Assessment of the damage caused by *Meloidogyne incognita* on okra (*Abelmoschus esculentus*). The Journal of Animal and Plant Sciences, 21 (4): 857-861.

Hussain, M. A., T. Mukhtar & M. Z. Kayani, 2014. Characterization of susceptibility and resistance responses to root-knot nematode (*Meloidogyne incognita*) infection in okra germplasm. Pakistan Journal of Agricultural Sciences, 51 (2): 319-324.

Kacar, B., 1972. Bitki ve Toprağın Kimyasal Analizleri II: Bitki Analizleri, Ankara Üniversitesi Ziraat Fakültesi Yayınları, No: 453, Ankara, Türkiye, VI1+121 s (in Turkish).

Kacar, B. & İ. Kovancı, 1982. Bitki, toprak ve gübrelerde kimyasal fosfor analizleri ve sonuçlarının değerlendirilmesi. Ege Üniversitesi Ziraat Fakültesi Yayınları, No: 354, İzmir, Türkiye, 646 s (in Turkish).

Karagül, S., 2003. Yerel Bamya (*Abelmoschus esculentus* (L) Moench) Çeşit ve Tiplerinin Karekterizasyonu. Çukurova Üniversitesi Fen Bilimleri Enstitüsü, (Unpublished) Yüksek Lisans Tezi, Tez No:135698, Adana, Türkiye, 115 s (in Turkish with English abstract).

Karajeh, M. R. & N. M. Salameh, 2015. Evaluation of okra landraces and accessions response to the root-knot nematode, *Meloidogyne javanica*. Jordan Journal of Agricultural Sciences, 11 (3): 735-745.

Kaşkavalcı, G. & C. Öncüer, 1999. Investigations on distribution and economic importance of *Meloidogyne* Goeldi, 1887 (Tylenchida: Meloidogynidae) species found in the major areas of hot climate vegetables in Aydın province. Turkish Journal of Entomology, 23 (2): 149-160 (in Turkish with abstract in English).

Kayani, M. Z., T. Mukhtar & M. A. Hussain, 2017. Effects of southern root-knot nematode population densities and plant age on growth and yield parameters of cucumber. Elsevier Crop Protection, 92 (1): 207-212.

Kedarnath, N. G., D. M. Ravichandra, B. M. R. Preethi, B. S. Reddy & R. S. Pavithra, 2017. Screening of okra (*Abelmoschus esculentus*) cultivars for resistance against root knot nematode (*Meloidogyne incognita*) under field condition in Karnataka, India. International Journal of Current Microbiology and Applied Sciences, 6 (11): 3420-3426.

Kumar, V., A. U. Singh & R. K. Jain, 2012. Comparative efficacy of bioagents as seed treatment for management of *Meloidogyne incognita* infecting okra. Nematologia Mediterranea, 40 (2): 209-211.

Maleita, C. M., M. J. Simões, C. Egas, R. H. C. Curtis & I. M. O. Abrantes, 2012. Biometrical, biochemical, and molecular diagnosis of Portuguese *Meloidogyne hispanica* isolates. Plant Disease, 96 (6): 865-874.

Marin, M. V., L. S. Santos, L. A. Gaion, H. O. Rabelo, C. A. Franco, G. M. M. Diniz, E. H. C. Silva & L. T. Braz, 2017. Selection of resistant rootstocks to *Meloidogyne enterolobii* and *Meloidogyne incognita* for okra (*Abelmoschus esculentus* L. Moench). Chilean Journal of Agricultural Research, 77 (1): 58-67.

Melakeberhan, H., J. M. Webster & R. C. Brooke, 1985. Response of *Phaseolus vulgaris* to a single generation of *Meloidogyne incognita.* Nematologica, 31 (2): 190-202.

Miamoto, A., M. T. R. Silva, C. R. Dias-Arieira & H. H. Puerari, 2017. Alternative products for *Pratylenchus brachyurus* and *Meloidogyne javanica* management in soya bean plants. Journal of Phytopathology, 165 (10): 635-640.

Mohanta, S. & K. C. Mohanty, 2012. Screening of okra germplasms/varieties for resistance against *Meloidogyne incognita*. Journal of Plant Protection and Environment, 9 (1): 66-68.

Muhammad, A. U. H., M. Kamra, S. Muhammad, Z. Muhammad, Y. Faiqa & S. Hania, 2017. Reaction of two summer vegetables (okra and chillies) germplasm against root knot nematode (*Meloidogyne incognita*). Plant Protection, 1 (1): 23-27.

Mukhtar, T., I. Arshad, M. Z. Kayani, M. A. Hussain, S. B. Kayani, A. M. Rahoo & M. Ashfaq, 2013. Estimation of damage to okra (*Abelmoschus esculentus*) by root-knot disease incited by *Meloidogyne incognita.* Pakistan Journal of Botany, 45 (3): 1023-1027.

Odeyemi I. S., S. O. Afolami & F. T. Oguejiofor, 2016. Susceptibility of okra accessions to root knot nematode. International Journal of Vegetable Science, 22 (3): 289-294.

Özarslandan, A. & İ. H. Elekcioğlu, 2010. Investigation on virulence of *Meloidogyne incognita* (Kofoid & White, 1919), *Meloidogyne arenaria* (Neal, 1889) and *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Meloidogynidae) populations on resistant and susceptible tomato cultivars. Turkish Journal of Entomology, 34 (4): 495-502 (in Turkish with English abstract).

Pandey, R. K., S. Bhandari, K. Giri, P. Wangle & H. K. Manandhar, 2019. Influence of different fertilizers and nematicides on number of nematode galls and yield of okra in summer season in Chitwan, Nepal. Azarian Journal of Agriculture, 6 (2): 23-36.

Pandey, R. & A. Kalra, 2003. Root knot disease of ashwagandha *Withania somnifera* and its ecofriendly cost-effective management. Journal of Mycology and Plant Pathology, 33 (2): 240-245.

Pandey, R. K., D. K. Nayak & K. K. Rajesh, 2017. Effects of macronutrient contents of resistant and susceptible greengram cultivars as influenced by root-knot nematode, *Meloidogyne incognita*. International Journal of Current Research, 9 (4): 49166-49170.

Santana-Gomes, S. M., C. R. Dias-A, M. Roldi, T. S. Dadazio, P. M. Marini & D. A. O. Barizão, 2013. Mineral nutrition in the control of nematodes. African Journal of Agricultural Research, 8 (21): 2413-2420.

Sathish, D. & A. Eswar, 2013. A Review on: *Abelmoschus esculentus* (Okra). International Research Journal of Pharmaceutical and Applied Sciences, 3 (4): 129-132.

Sheela, M. S., Jiji, R. Malu & S. Shaiju, 2006. Screening of okra varieties for resistance against *Meloidogyne incognita*. Indian Journal Nematolojia, 36 (2): 292-293.

Sikora, R. A. & E. Fernandez, 2005. “Nematode Parasites of Vegetables, 319-392”. In: Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. CABI Publishing, 871 pp.

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Original article (Orijinal araştırma)

Current occurrence and prevalence of root-knot nematodes species, *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Meloidogynidae) in ware potato fields of Turkey

Yemeklik patates alanlarında kök-ur nematodu türlerinin, *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Meloidogynidae) mevcut durumu ve yaygınlığı

Emre EVLİCE[1*]

Abstract

The study was conducted to determine the occurrence, frequency and density of species of *Meloidogyne* Goeldi, 1892 (Tylenchida: Meloidogynidae) in ware potato cultivation areas of Turkey in Plant Protection Central Research Institute. Soil samples were taken from Afyonkarahisar (149), Aksaray (69), Bolu (94), Kayseri (94), Konya (127), Nevşehir (91), Niğde (226) and Sivas (77) Provinces in 2018-2019. *Meloidogyne* juveniles were extracted by modified Baarmenn funnel and counted under inverted microscope. *Meloidogyne* populations were identified by species-specific primers. *Meloidogyne* spp. were detected in 84 of 927 soil samples. *Meloidogyne* spp. was detected in Aksaray, Kayseri, Nevşehir, and Niğde while was not found Afyonkarahisar, Bolu, Konya, and Sivas Provinces. In survey areas, the occurrence of *Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley, 1980 and *Meloidogyne hapla* (Chitwood, 1949) (Tylenchida: Meloidogynidae) was 8.7 and 1.5%, respectively. Also, *M. chitwoodi* and *M. hapla* mixed populations were found in 1.2% of samples. Mean density of *Meloidogyne* spp. J2s was determined as 182, 175, 162 and 90 J2s/100 ml of soil in Niğde, Nevşehir, Aksaray and Kayseri Provinces, respectively. In conclusion, important ware potato cultivation areas of Turkey were found to be infested with *Meloidogyne* spp., but the important seed potato-growing areas were found to be free of *Meloidogyne* spp.

Keywords: *Meloidogyne chitwoodi, Meloidogyne hapla*, seed potato, survey, ware potato

Öz

Bu çalışma Türkiye'deki yemeklik patates ekiliş alanlarında *Meloidogyne* Goeldi, 1892 (Tylenchida: Meloidogynidae) türlerinin bulunuş, yaygınlık ve yoğunluklarının belirlenmesi amacıyla Zirai Mücadele Merkez Araştırma Enstitüsü'nde yapılmıştır. Toprak örnekleri 2018-2019 yıllarında Afyonkarahisar (149), Aksaray (69), Bolu (94), Kayseri (94), Konya (127), Nevşehir (91), Niğde (226) ve Sivas (77) illerinden alınmıştır. *Meloidogyne* ikinci dönem larvaları modifiye Baarmenn huni tekniği ile elde edilmiş ve inverted mikroskopta sayılmıştır. *Meloidogyne* spp. popülasyonları türe spesifik primerler kullanılarak teşhis edilmiştir. Çalışma sonucunda 927 toprak örneğinin 84'ünün *Meloidogyne* spp. ile bulaşık olduğu belirlenmiştir. Aksaray, Kayseri, Nevşehir ve Niğde illerinde *Meloidogyne* spp. tespit edilirken Afyonkarahisar, Bolu, Konya ve Sivas illerinde bulunmamıştır. Sürvey alanında *Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley, 1980 ve *Meloidogyne hapla* (Chitwood, 1949) (Tylenchida: Meloidogynidae) yaygınlığı sırasıyla %8.7 ve %1.5 olarak belirlenmiştir. Ayrıca *M. chitwoodi* ve *M. hapla* karışık popülasyonlarının yaygınlığı ise %1.2 olarak belirlenmiştir. *Meloidogyne* spp. J2 ortalama yoğunluğu Niğde, Nevşehir, Aksaray ve Kayseri illerinde sırasıyla 182, 175, 162 ve 90 J2/100 ml toprak/J2 olarak belirlenmiştir. Çalışma sonucunda, önemli yemeklik patates ekiliş alanlarının yoğun olarak *Meloidogyne* spp. ile bulaşık olduğu ancak Türkiye'deki önemli tohumluk patates ekiliş alanlarının ise *Meloidogyne* spp. açısından temiz olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Meloidogyne chitwoodi*, *Meloidogyne hapla*, tohumluk patates, sürvey, yemeklik patates

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Introduction

Potato, *Solanum tuberosum* L. (Solanaceae) is the most important non-grain crop in the world and the fourth major food crop after maize, rice and wheat with an annual production of 370 Mt (FAOSTAT, 2019). Considered one of the most promising products in the fight against hunger and poverty, potatoes are highly recommended by FAO as a food security crop due to the growing population and food demand (Thomas & Sansonetti, 2009). Potato crops are damaged by numerous pests and pathogens, including nematodes (Niere & Karuri, 2018). Plant-parasitic nematodes are one of the crucial factors restricting potato yield and quality in many potato-growing areas (Lima et al., 2018). Potato cyst nematodes, *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 and *Globodera pallida* (Stone, 1973) Behrens, 1975 (Tylenchida: Heteroderidae) and root-knot nematodes, *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Meloidogynidae) (RKN) are the major parasites of potato (Lima et al., 2018). Estimated annual yield losses in potato due to plant-parasitic nematodes is over 13% worth about US$6 billion worldwide (Ravichandra, 2014). While cyst nematodes have been more troublesome in the past, there have been drastic rises in root-knot nematodes due to the ban of some effective nematicides and soil fumigants such as 1,3-D, cadusafos and ethoprophos and also lacking resistant cultivars (Wesemael et al., 2011).

Root-knot nematodes include the most economically damaging plant-parasitic nematodes to crops worldwide (Karssen & Moens, 2006). Although the genus *Meloidogyne* includes about 100 species, *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949*, Meloidogyne javanica* (Treub, 1885) Chitwood, 1949*, Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949*, Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley, 1980*, Meloidogyne fallax* Karssen, 1996 and *Meloidogyne hapla* Chitwood, 1949 (Tylenchida: Meloidogynidae) are the most common species. These species account for over 95% of the *Meloidogyne* occurrences in the world (Adam et al., 2007; Jones et al., 2013). The impact of these species is increasing due to their wide host ranges and it is estimated that they can affect more than 5,500 plant species (Trudgill & Blok, 2001) with crop losses of about 5% worldwide (Agrios, 2005).

Potato is one of the main hosts of *Meloidogyne* spp., and the prevalence and damage of these species have been increasing in recent decades. The damage of RKN to potatoes has been determined in all continents except Antarctica, including the major potato producing countries including China (Mao et al., 2019), India (Singh & Kumar, 2015), the USA (Golden et al., 1980), Germany (Müller et al., 1996), France (Djian-Caporalino, 2012), Holland (Keidel et al., 2007), South Africa (Fourie et al., 2001), and Australia (Nobbs et al., 2001). In potato production, *M. arenaria*, *Meloidogyne enterolobii* Yang & Eisenback, 1983, *M. incognita* and *M. javanica* and are present in warmer climates, while *M. chitwoodi*, *M. fallax, M. hapla* and *Meloidogyne minor* Karssen et al., 2004 are encountered in relatively colder climates (Nyczepir et al., 1982; Nobbs et al., 2001; Thoden et al., 2012; Onkendi & Moleleki, 2013; Medina et al., 2017). Of these, *M. arenaria, M. chitwoodi*, *M. fallax*, *M. hapla, M. incognita* and *M. javanica* are significant for potato production (Lima et al., 2018). In Turkey, *M. chitwoodi*, *M. hapla, M. incognita*, and *M. javanica* have been determined in potato-growing areas, however *M. chitwoodi* is the most common one among them (Kepenekci et al., 2006; Özarslandan & Elekcioğlu, 2010; Erdoğus et al., 2011; Evlice & Bayram, 2016; Demirbaş Pehlivan et al., 2020).

There are no reports of specific aboveground symptoms in potato associated with root-knot nematode infestation, however varying degrees of stunting, yellowing, and wilting under moisture stress can be seen in some of the infested plants. Root-knot nematodes reproduce on potato roots and tubers, but the size and shape of galls or knots could vary depending on the species and density of nematodes (Niere & Karuri, 2018). The economic threshold on potato cultivation in the USA and the Netherlands are 1 and 10 J2s/100 ml soil for *M. chitwoodi*, respectively (Brodie et al., 1993; Norshie et al., 2011) and 50 eggs/250 ml soil for *M. hapla* (Brodie et al., 1993).

Galls of temperate species, *M. chitwoodi*, *M. fallax*, *M. hapla* and *M. minor*, are usually smaller than those caused by other species and cause extensive lateral root formation. However, these species cause numerous small pimple-like swelling on the tuber surface (Elling, 2013). Even 5% necrotic spotting in the flesh of tubers make them inappropriate for the fresh market, and if 5-15% of the tubers have defects when harvested, the whole field crop can be discarded (King & Taberna, 2013).

For management, in most countries, at least a 2-year rotation is used in potato production. There are no commercially available potato cultivars resistant to root-knot nematodes. Under such conditions, *Meloidogyne* spp. populations can increase rapidly. For this reason, the most important and widely used management strategy is to apply nematicides.

This study has been conducted to determine (1) occurrence of the root-knot nematodes and species, (2) frequency and density of root-knot nematode species in ware potato-growing areas in Turkey.

Materials and Methods

Sampling strategy and sample processing

Surveys were conducted in 8 potato-growing provinces (Afyonkarahisar, Aksaray, Bolu, Kayseri, Konya, Nevşehir, Niğde and Sivas) between 2018 and 2019. A total of 927 soil samples were taken from fields immediately after potato harvest, 0.1% of the annual ware potato production area in each province (Figure 1). Soil sample of 2-3 kg consisting of 50 subsamples according to a grid pattern from 0-30 cm depth was randomly obtained from each field using an auger. The soil samples and symptomatic potato tubers (Figure 2) were placed in a plastic container. For each sampled area, GPS coordinates of sampling sites were recorded.

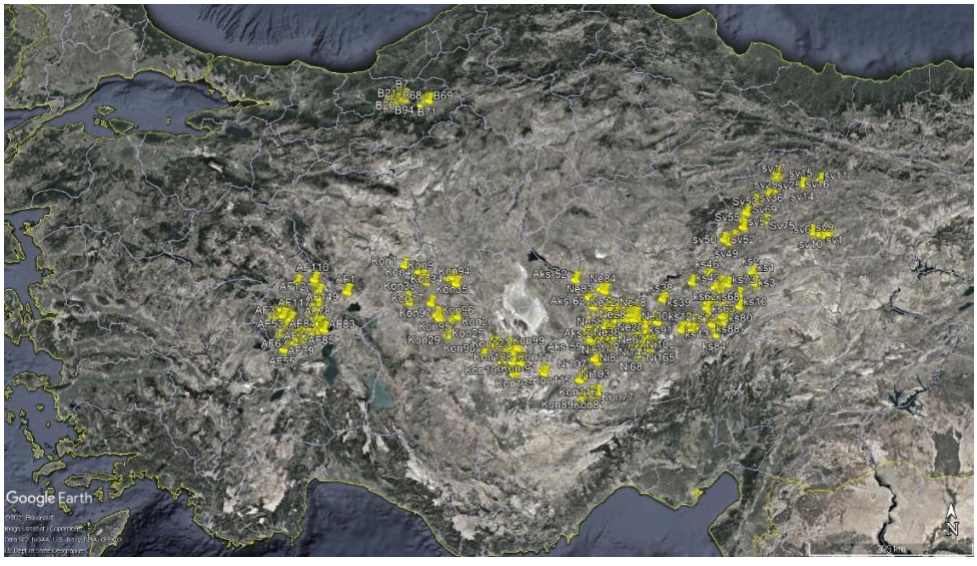

Figure 1. Soil samples taken from potato-growing areas in this study (mapped using www.earth.google.com).

Soil samples were mixed thoroughly and 200 ml subsamples were processed for nematode extraction for each field. Root-knot nematodes were extracted by the modified Baermann funnel technique (Hooper, 1986). After 24 h, nematode suspension was transferred to 100 ml graduated cylinder and collected in a 50 ml sample bottle for examination. The whole suspension was checked for *Meloidogyne* spp. and counted under an invert microscope (Leica DMI 400B).

DNA extraction

DNA were extracted from second-stage juveniles (J2s) for each isolate according to Waeyenberge et al. (2000). For this purpose, 10 *Meloidogyne* spp. J2s was picked from extracted soil samples using a small needle and cut into two pieces with a sterile scalpel under a stereomicroscope (Leica M165C) in 20 µl of molecular grade water on a glass microscope slide, then 10 µl with the nematode pieces was transferred by pipette into a 0.2 ml centrifuge tube containing 8 µl of worm lysis buffer (WLB; 500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM MgCl2, 10 mM DTT, 4.5% Tween 20, 0.1% gelatin). After adding 2 µl of proteinase K (600 g/ml; Thermo Fisher Scientific, Waltham, MS, USA) the suspension was centrifuged at 13,500 rpm for 2 min and frozen to -80ºC for 10 min. After freezing, the contents were thawed rapidly. The lysis mix was incubated at 65ºC for 1 h followed by 95ºC for 10 min for a proteinase K inactivation. After incubation, the tubes were centrifuged for 1 min at 14,000 rpm and stored at -20ºC until used.

Figure 2. Potato tuber infested with *Meloidogyne chitwoodi*, pimple-like galls and necrotic spots on their surface.

DNA Amplification protocols

The primers used in PCR amplification for the identification of root-knot nematodes are given in Table 1. All multiplex PCR amplifications were conducted in a total volume of 25 µl consisting of 10x PCR Buffer, 200 µM dNTPs 0.2 µM of each primer, 1 unit Taq DNA polymerase (Thermo Fisher Scientific) and 2 µl template DNA. All DNA amplifications were conducted with a thermocycler (Techne, TC-5000).

Table 1. Primers used in the multiplex PCR to identify the species of *Meloidogyne* spp.

Species	Fragments	Primer	Primer sequences (5'-3')	Reference
Meloidogyne chitwoodi	540 bp	JMV1	GGATGGCGTGCTTTCAAC	Wishart et al., 2002
Meloidogyne fallax	670 bp	JMV2	TTTCCCCTTATGATGTTTACCC	
Meloidogyne hapla	440 bp	JMVhapla	AAAAATCCCCTCGAAAAATCCACC	
Meloidogyne incognita	150 bp	MincF1	GCACCTCTTTCATAGCCACG	Devran et al., 2018
		MincR1	GGTGCGCGATTGAACTGAGC	
Meloidogyne javanica	670 bp	Fjav	GGTGCGCGATTGAACTGAGC	Zijlstra et al., 2000
		Rjav	CAGGCCCTTCAGTGGAACTATAC	
Meloidogyne arenaria	420 bp	Far	TCGGCGATAGAGGTAAATGAC	Zijlstra et al., 2000
		Rar	TCGGCGATAGACACTACAACT	

The cycling parameters were as follows for *M. chitwoodi*, *M. fallax*, and *M. hapla*: an initial denaturation step at 94ºC for 3 min followed by 35 cycles at 94ºC for 30 s, 55ºC for 30 s, then 72ºC for 90 s and final extension step at 72ºC for 10 min; and for *M. incognita*, *M, javanica*, *M. arenaria*: initial denaturing step for 3 min at 95ºC followed by 35 cycles at 95ºC for 30 s, 56ºC for 30 s, then 72ºC for 60 s and a final extension step at 72ºC for 7 min. PCR products were separated by electrophoresis in Tris-EDTA buffer with 1.7% agarose gel stained with Pronosafe (Condalab, Spain) at 80 V for 1 h and then visualized with an image acquisition system (Vilber QUANTUM ST4 1000, Collégien, France).

***Meloidogyne* spp. community analyses**

The incidence, occurrence, and frequency of root-knot nematodes determined in the study at the genus and species level in potato-growing areas were calculated according to the formulas below (Norton, 1978).

$$Occurence\ of genus\ or\ species = \frac{Number\ of\ sample\ with\ root-knot\ nematodes\ infection}{Total\ number\ of\ sample\ surveyed} X100$$

$$Absolute\ frequency = \frac{Number\ of\ sample\ containing\ species}{Number\ of\ sample\ collected} X100$$

$$Relative\ frequency = \frac{Frequency\ of\ occurence\ of\ species}{Sum\ of\ frequency\ of\ all\ Meloidogyne\ spp.} X100$$

Results

Species identification

Multiplex PCR analysis by using JMV1/JMV2/JMVhapla primers produced a DNA fragment size of 540 bp specific to *M. chitwoodi* for 70 populations, 440 bp specific to *M. hapla* for three populations, and also both 540 bp and 440 bp for 11 populations. However, there was no 670-bp amplification product which was characteristic for the *M. fallax*. In addition, primer sets Far/Rar, MincF1/MincR1 and Fjav/Rjav, specific for *M. arenaria, M. incognita* and *M. javanica*, respectively, did not yield any amplification products. These results showed that 70 populations were *M. chitwoodi*, three populations were *M. hapla*, and 11 populations were mixed *M. chitwoodi* and *M. hapla.* There was no evidence of the existence of *M. arenaria*, *M. fallax, M. incognita* or *M. javanica* as previously reported in potato areas in Central Anatolia (Table 2).

***Meloidogyne* spp. community analyses**

J2s of *Meloidogyne* spp. were detected in 84 of 927 soil samples in all surveyed areas. *Meloidogyne* spp. was detected in Aksaray, Kayseri, Nevşehir and Niğde Provinces, but not in Afyonkarahisar, Bolu, Konya and Sivas Provinces (Tables 2 & 3). The occurrence, frequency and population density of *Meloidogyne* genus and species differed according to the location sampled. The occurrence of RKNs in all surveyed areas was 9.1% with a mean density of 176 J2s/100 ml of soil. *Meloidogyne chitwoodi* was the most abundant species in all provinces infested with *Meloidogyne* spp. The occurrence of *M. chitwoodi* and *M. hapla* in the field surveys were 8.7 and 1.5%, respectively, and *M. chitwoodi* and *M. hapla* mixed populations were 1.2%. *Meloidogyne chitwoodi* was the most prevalent species in all surveyed areas, with 96.4 and 85.3% absolute and relative frequency, respectively. This was followed by *M. hapla* with 16.7 and 14.7% absolute and relative frequency, respectively.

The highest occurrence of RKNs was found in Nevşehir with 25.3%, followed by Niğde, Aksaray, and Kayseri with 23.5, 8.7, and 2.1%, respectively. The occurrence and absolute frequencies were 25.3, 22.1%, 8.7 and 2.1%, and 100, 94.3, 100 and 100% in Nevşehir, Niğde, Aksaray, and Kayseri Provinces, respectively. *Meloidogyne hapla* was only found as the sole species in Niğde, but it was found in mixed populations with *M. chitwoodi* in Aksaray and Nevşehir Provinces. Additionally, *M. hapla* was not detected in any samples from Kayseri Province. Its occurrence and absolute frequencies were found to be 4.4, 3.5

and 2.9%, and 17.4, 15.1 and 33.3% in Nevşehir, Niğde and Aksaray Provinces, respectively. Mean density of *Meloidogyne* spp. J2s were similar in Nevşehir (175 J2s/100 ml of soil), Niğde (182 J2s/100 ml of soil) and Aksaray (162 J2s/100 ml of soil) Provinces, but lower in Kayseri (90 J2s/100 ml of soil).

Table 2. Occurrence of *Meloidogyne* spp. on potatoes collected from ware potato fields in Turkey

Province	District	Village	Altitude (m)	Coordinate	Species	J2s/100 ml soil
Aksaray	Gülağaç	-	1192	38º21'8.15"N 34º20'49.16"E	*M. chitwoodi*	44
		Demirci	1190	38º20'42.49"N 34º18'40.94"E	*M. chitwoodi*	108
	Güzelyurt	Alanyurt	1204	38º20'1.76"N 34º19'7.08"E	*M. chitwoodi, M. hapla*	237
			1212	38º20'40.34"N 34º20'16.93"E	*M. chitwoodi, M. hapla*	194
		Bozcayurt	1200	38º21'30.02"N 34º22'23.10"E	*M. chitwoodi*	83
	Merkez	Yenipınar	1412	38º12'37.95"N 34º16'50.61"E	*M. chitwoodi*	305
Kayseri	İncesu	Örenşehir	1046	38º41'42.26"N 35º14'46.92"E	*M. chitwoodi*	83
	Melikgazi	Yeşilyurt	1086	38º48'43.61"N 35º36'49.44"E	*M. chitwoodi*	97
Nevşehir	Acıgöl	Kurugöl	1288	38º22'32.75"N 34º32'27.47"E	*M. chitwoodi*	124
	Derinkuyu	Çakıllı	1342	38º24'28.98"N 34º39'13.37"E	*M. chitwoodi*	149
		Doğala	1387	38º25'37.96"N 34º36'26.69"E	*M. chitwoodi*	53
		Kuyulutatlar	1305	38º22'2.92"N 34º32'4.62"E	*M. chitwoodi*	196
			1292	38º23'20.81"N 34º31'48.68"E	*M. chitwoodi*	79
		Til	1500	38º25'29.67"N 34º47'23.46"E	*M. chitwoodi*	148
		Yazıhüyük	1287	38º21'22.85"N 34º36'42.45"E	*M. chitwoodi*	207
		-	1389	38º20'50.37"N 34º46'51.10"E	*M. chitwoodi, M. hapla*	417
		-	1320	38º20'37.51"N 34º44'35.28"E	*M. chitwoodi*	202
		-	1336	38º21'23.18"N 34º44'23.79"E	*M. chitwoodi, M. hapla*	581
		-	1363	38º23'6.14"N 34º44'52.66"E	*M. chitwoodi*	299
		-	1323	38º20'34.63"N 34º44'36.57"E	*M. chitwoodi, M. hapla*	45
		-	1336	38º21'23.28"N 34º44'23.78"E	*M. chitwoodi*	217
		-	1361	38º22'51.59"N 34º43'22.27"E	*M. chitwoodi*	102
		-	1449	38º27'26.94"N 34º42'11.31"E	*M. chitwoodi, M. hapla*	52
		-	1392	38º21'26.63"K 34º45'39.54"D	*M. chitwoodi*	74
	Merkez	Çardak	1373	38º32'43.71"N 34º46'8.02"E	*M. chitwoodi*	48
			1409	38º32'0.91"N 34º45'33.11"E	*M. chitwoodi*	294
		İcik	1384	38º29'56.68"N 34º35'42.48"E	*M. chitwoodi*	61
			1363	38º30'51.49"N 34º35'55.92"E	*M. chitwoodi*	121
		Kaymaklı	1423	38º28'56.74"N 34º45'10.41"E	*M. chitwoodi*	56
			1406	38º27'13.72"N 34º44'38.68"E	*M. chitwoodi*	81
	Ürgüp	Bahçeli	1474	38º31'0.78"N 34º47'23.01"E	*M. chitwoodi*	427
Niğde	Altunhisar	-	1212	38º0'30.00"N 34º19'39.00"E	*M. chitwoodi*	95
	Çiftlik	Azatlı	1572	38º12'39.63"N 34º31'2.85"E	*M. hapla*	61
			1582	38º13'0.93"N 34º31'22.52"E	*M. chitwoodi*	349
			1541	38º12'9.00"N 34º28'35.00"E	*M. chitwoodi*	57
			1580	38º11'51.02"K 34º29'9.67"D	*M. chitwoodi*	134
		Bozköy	1575	38º13'10.00"N 34º29'10.00"E	*M. chitwoodi, M. hapla*	167
			1591	38º13'43.00"N 34º29'10.00"E	*M. chitwoodi*	69
			1569	38º13'4.00"N 34º29'18.00"E	*M. chitwoodi*	60
		Divarlı	1546	38º12'15.00"N 34º28'51.00"E	*M. chitwoodi*	45
			1542	38º12'49.93"N 34º27'55.76"E	*M. hapla*	301
			1643	38º13'45.00"N 34º28'16.00"E	*M. chitwoodi*	59
		Kitreli	1422	38º11'48.28"N 34º21'31.36"E	*M. chitwoodi*	74

Table 2 Continued

Province	District	Village	Altitude (m)	Coordinate	Species	J2s/100 ml soil
		Ağcaşar	1320	38º18'35.03"K 34º45'15.70"D	*M. chitwoodi, M. hapla*	1159
		Alay	1302	38º17'5.84"N 34º41'32.33"E	*M. chitwoodi*	34
			1311	38º16'14.72"N 34º40'44.60"E	*M. chitwoodi*	56
		Bağlama	1308	38º16'2.60"N 34º40'35.93"E	*M. chitwoodi*	67
			1330	38º16'29.16"N 34º39'25.84"E	*M. chitwoodi*	295
			1325	38º16'16.63"N 34º39'32.55"E	*M. chitwoodi*	86
			1318	38º15'23.97"N 34º39'40.18"E	*M. chitwoodi*	45
			1315	38º16'8.33"N 34º40'25.80"E	*M. chitwoodi*	413
		Edikli	1368	38º14'7.20"N 34º55'10.86"E	*M. chitwoodi*	102
			1363	38º14'2.05"N 34º55'10.63"E	*M. chitwoodi*	147
			1428	38º17'0.66"N 34º53'35.87"E	*M. chitwoodi*	309
			1429	38º17'43.11"N 34º53'18.59"E	*M. chitwoodi*	416
		Kiledere	1306	38º16'36.40"N 34º40'45.45"E	*M. chitwoodi*	74
			1297	38º17'11.49"N 34º41'13.01"E	*M. chitwoodi*	56
			1300	38º17'34.37"N 34º41'11.16"E	*M. chitwoodi*	85
		Konaklı	1343	38º7'31.52"N 34º49'52.42"E	*M. chitwoodi*	167
			1339	38º8'40.37"N 34º49'41.42"E	*M. chitwoodi*	191
			1335	38º10'48.69"N 34º50'5.69"E	*M. chitwoodi*	368
			1343	38º11'37.52"N 34º49'23.86"E	*M. chitwoodi*	152
			1366	38º13'33.99"N 34º51'5.63"E	*M. chitwoodi*	202
Niğde	Merkez		1426	38º16'39.74"N 34º52'28.59"E	*M. chitwoodi*	64
		Orhanlı	1452	38º19'29.20"N 34º50'21.20"E	*M. chitwoodi*	445
			1426	38º17'16.40"N 34º52'58.64"E	*M. chitwoodi*	83
			1425	38º16'16.69"N 34º52'27.42"E	*M. chitwoodi*	367
			1452	38º18'27.21"N 34º52'48.99"E	*M. chitwoodi, M. hapla*	281
			1459	38º18'56.21"N 34º51'54.59"E	*M. chitwoodi, M. hapla*	384
			1474	38º19'38.19"N 34º56'26.21"E	*M. chitwoodi*	194
			1465	38º19'1.57"N 34º55'27.45"E	*M. chitwoodi*	226
			1529	38º20'43.58"K 34º48'48.41"D	*M. chitwoodi*	416
		Ovacık	1332	38º4'35.66"N 34º48'1.81"E	*M. chitwoodi*	281
			1383	38º8'33.71"K 34º46'39.37"D	*M. chitwoodi*	67
		Tırhan	1304	38º14'35.99"N 34º42'25.47"E	*M. chitwoodi*	81
			1296	38º15'0.00"N 34º43'50.54"E	*M. hapla*	194
			1293	38º15'9.37"N 34º43'28.00"E	*M. chitwoodi*	84
			1296	38º14'41.51"N 34º42'54.20"E	*M. chitwoodi, M. hapla*	106
			1297	38º14'17.73"N 34º43'4.81"E	*M. chitwoodi*	73
		Yeşilgölcük	1307	38º16'27.08"N 34º45'37.98"E	*M. chitwoodi*	124
			1308	38º15'56.28"N 34º45'41.84"E	*M. chitwoodi*	64
			1308	38º15'17.14"N 34º46'0.56"E	*M. chitwoodi*	81
			1304	38º14'57.90"N 34º45'51.97"E	*M. chitwoodi*	50
			1305	38º13'59.33"N 34º46'13.14"E	*M. chitwoodi*	63

Table 3. Community analyses of *Meloidogyne* spp. on potato in the survey areas

	Survey area	Afyonkarahisar	Aksaray	Bolu	Kayseri	Konya	Nevşehir	Niğde	Sivas
Number of samples	928	149	69	94	94	127	91	226	77
Meloidogyne spp. infestation	84	0	6	0	2	0	23	53	0
M. chitwoodi infestation	81	0	6	0	2	0	23	50	0
M. hapla infestation	14	0	2	0	0	0	4	8	0
M. chitwoodi+*M. hapla* infestation	11	0	2	0	0	0	4	5	0
Occurence of *Meloidogyne* spp.	9.1%	-	8.7%	-	2.1%	-	25.3%	23.5%	-
Occurence of *M. cihtwoodi*	8.7%	-	8.7%	-	2.1%	-	25.3%	22.1%	-
Occurence of *M. hapla*	1.5%	-	2.9%	-	-	-	4.4%	3.5%	-
Occurence of *M. chitwoodi*+*M. hapla*	1.2%	-	-	-	-	-	4.4%	2.2%	-
Absolute frequency of *M. chitwoodi*	96.4%	-	100%	-	100%	-	100%	94.3%	-
Absolute frequency of *M. hapla*	16.7%	-	33.3%	-	-	-	17.4%	15.1%	-
Relative frequency of *M. chitwoodi*	85.3%	-	75%	-	100%	-	85.2%	86.2%	-
Relative frequency of *M. hapla*	14.7%	-	25%	-	-	-	14.8%	14.8%	-

Discussion

The survey area covered about 58% of the potato production and harvested area of Turkey (TUIK, 2019). The findings showed that a total of 927 soil samples were obtained from potato-growing areas in eight provinces of Turkey, of which 9.1% were infested with *Meloidogyne* spp. *Meloidogyne chitwoodi* and *M. hapla* were detected at 8.7 and 1.5%, respectively, and *M. chitwoodi* and *M. hapla* were found in mixed populations in 1.2% of the samples. Nine root-knot species, *M. arenaria*, *Meloidogyne artiellia* Franklin, 1961, *M. chitwoodi*, *Meloidogyne ethiopica* Whitehead, 1968, *Meloidogyne exigua* Goeldi, 1887, *M. hapla*, *M. incognita*, *M. javanica* and *Meloidogyne thamesi* Chitwood, 1952 have been identified in different regions and different crops in Turkey (Yüksel, 1966; 1967; Elekçioğlu, 1992; Özarslandan et al., 2009; Aydınlı et al., 2013; Kepenekci et al., 2014; İmren et al., 2014). However, four root-knot species, *M. chitwoodi*, *M. hapla*, *M. incognita* and *M. javanica*, have been found in association with potato in Turkey (Kepenekci et al, 2006; Özarslandan et al., 2009; Erdoğus et al., 2011; Demirbaş Pehlivan et al., 2020). The first report of root-knot nematode in potatoes appears to have been made by Kepenekci et al (2006) as *M. hapla* from potato tubers collected from Aksaray, Nevşehir and Niğde Provinces. Following that, *M. chitwoodi* was found in Niğde potato-growing areas by Özarslandan et al (2009). This finding has been supported by other studies in which only *M. chitwoodi* was detected and *M. chitwoodi* has since be found in Aksaray, Balıkesir, Bitlis, Isparta, İzmir, Kayseri, Konya, Kütahya, Manisa and Nevşehir Provinces (Özarslandan & Elekcioğlu, 2010; Ulutaş et al., 2012; Özarslandan et al., 2013; Evlice & Bayram, 2016). The present findings are similar to previous studies, and the relative frequency of *M. hapla* (14.7%) was quite low compared to *M. chitwoodi* (85.3%). *Meloidogyne incognita* was first identified in the potato-growing areas of Edirne, then in İzmir (Erdoğus et al., 2011; Demirbaş Pehlivan et al., 2020). Additionally, *M. javanica*, *M. hapla* and *M. chitwoodi* was determined in potato fields in İzmir (Yıldız et al., 2009; Demirbaş Pehlivan et al., 2020). The occurrence of *Meloidogyne* spp. in potato cultivation areas was higher in İzmir (18.4%) than found overall in the present survey (9.1%) but lower than Nevşehir (25.3%) and Niğde (23.5%). Differing from the present findings, the dominant species in the potato cultivation areas of İzmir Province was determined as *M. incognita* and the relative frequency of *M. incognita*, *M. javanica*, *M. hapla* and *M. chitwoodi* was found 61, 24.4, 12.2 and 2.4%, respectively (Demirbaş Pehlivan et al., 2020). This was an expected result due to the widespread cultivation of good hosts of root-knot nematodes in the Aegean Region and the prevalence of tropical root-

knot nematodes species in these areas (Kaşkavalcı & Öncüer, 1999; Yağcı & Kaşkavalcı, 2018). Similar results were obtained in other surveys conducted in warmer regions worldwide (Okendi & Moleleki, 2013; Medina et al., 2017). Temperate species such as *M. chitwoodi* and *M. hapla* are cold temperature nematodes whereas tropical species such as *M. incognita* and *M. javanica* prefer at higher temperatures and cannot tolerate cold temperatures (Evans & Perry, 2009). The base threshold temperature is 4ºC and 8ºC for *M. chitwoodi* and *M. hapla* while 10ºC and 13ºC for *M. incognita* and *M. javanica*, respectively (Inserra et al., 1983; Lahtinen et al., 1988; Madulu & Trudgill, 1994; Ploeg & Maris, 1999).

The results presented here show that the mean density of *Meloidogyne* spp. ranged from 34 to 1159 J2s/100 ml of soil. *Meloidogyne chitwoodi* and *M. hapla* are the most important species within *Meloidogyne* for potatoes (Brodie et al., 1993). The economic threshold of *M. chitwoodi* in potatoes is reported to be 1 and 10 J2s/100 ml soil in the USA and Holland, respectively, whereas it is as 50 eggs/250 ml soil for *M. hapla* (Brodie et al., 1993; Norshie et al., 2011). Therefore, in the present study, the population density of RKN was above the economic damage threshold in all areas where it was determined. Crop loss from RKN infestation can be about 25%, depending on cultivars, environmental factors and population density, but may reach 100% in potato fields (Mai et al., 1981; Lima et al., 2018). The economic loss of cultivated potatoes caused by *M. chitwoodi* can reach US$ 9 900/ha (Ingham et al., 2007). Potato roots and tubers can be invaded by root-knot nematodes. However, the first generation resides mostly on the root system, with subsequent generations entering the tubers (Pinkerton et al., 1991). Depending on the nematode density and species, infested roots and tubers may have galls of varying sizes and shapes. *Meloidogyne chitwoodi* and *M. hapla* produced galls that are normally smaller than those induced by other species (Niere & Karuri, 2018). Even 5% necrotic spotting in the flesh of tubers makes them commercially unacceptable for the fresh market. The entire crop can be rejected if 5 to 15% of the field tubers have visual defects when processed (King & Taberna, 2013). While potato genotypes resistant to *M. chitwoodi* have been identified, there is currently no commercially available resistant cultivars (Brown et al., 2006; Brown et al., 2009; Norshie et al., 2011). For this reason, the application of nematicides is the most commonly used control measure (Jones et al., 2017). The approved rates in the USA for some nematicides for control of *M. chitwoodi* are higher than for other *Meloidogyne* spp. (Lima et al., 2018). Crop rotation or succession with non- or poor hosts, shortening the growing period, planting certified seed and destruction of volunteer potato plants are some of the other control methods (Jones et al., 2017).

This study demonstrated that important ware potato cultivation areas, such as in Nevşehir and Niğde, where seed potato cultivation has been prohibited, are heavily infested with *M. chitwoodi*. However, the most important seed potato-growing areas in Turkey including Afyonkarahisar, Konya and Sivas were free from *Meloidogyne* spp. Therefore, quarantine measures should be applied meticulously to prevent infestations in these areas. Turkey's annual requirement is about 510 kt of seed potatoes and the use of seed potatoes is about 340 kt (BÜGEM, 2019; TUİK, 2019). The difference of about 45% between production and use is due to the use of non-seed potatoes for planting. Although *Meloidogyne* spp. causes pimple-like swellings on the surface of potato tubers, these symptoms depend on the population density and the duration of vegetative growth. For this reason, asymptomatically infested tubers are observed in most cases, and the use of these tubers as seeds must be avoided to prevent the spread of *Meloidogyne* spp. to uninfested areas.

References

Adam, M. A. M., M. S. Phillips & V. C. Blok, 2007. Molecular diagnostic key for identification of single juveniles of seven common and economically important species of root-knot nematode (*Meloidogyne* spp.). Plant Pathology, 56 (1): 190-197.

Agrios, G. N., 2005. Plant Pathology (Fifth Edition). Elsevier Academic Press, California, USA, 922 pp.

Aydınlı, G., S. Mennan, Z. Devran, S. Sirca & G. Urek, 2013. First report of the root-knot nematode *Meloidogyne ethiopica* on tomato and cucumber in Turkey. Plant Disease, 97 (9): 1262.

Brodie, B. B., K. Evans & J. Franco, 1993. "Nematode Parasites of Potatoes, 87-132". In: Plant Parasitic Nematodes in Temperate Agriculture (Eds. K. Evans, D. L. Trudgill & J. M. Webster). CAB International, Wallingford, England, 656 pp.

Brown, C. R., H. Mojtahedi, S. James, R. G. Novy & S. Love, 2006. Development and evaluation of potato breeding lines with introgressed resistance to Columbia root-knot nematode (*Meloidogyne chitwoodi*). American Journal of Potato Research, 83 (1): 1-8.

Brown, C. R., H. Mojtahedi, L. H. Zhang & E. Riga, 2009. Independent resistant reactions expressed in root and tuber of potato breeding lines with introgressed resistance to *Meloidogyne chitwoodi*. Phytopathology, 99 (9): 1085-1089.

BÜGEM, 2019. Patates bülteni Aralık 2019 (Web page: https://www.tarimorman.gov.tr/BUGEM/Belgeler/M%C4%B0LL%C4%B0%20TARIM/PATATES%20ARALIK%20B%C3%9CLTEN%C4%B0.pdf) (Date accessed: February 2021) (in Turkish).

Demirbaş Pehlivan, H., G. Kaşkavalcı, E. B. K. Uludamar, H. Toktay & H. Elekcioğlu, 2020. Identification and prevalence of potato cyst nematodes and root-knot nematodes in the potato production areas of İzmir Province, Turkey. Turkish Journal of Entomology, 44 (2): 259-272.

Devran, Z., İ. Polat, İ. Mıstanoğlu & Ö. Baysal, 2018. A novel multiplex PCR tool for simultaneous detection of three root-knot nematodes. Australasian Plant Pathology, 47 (4): 389-392.

Djian-Caporalino, C., 2012. Root-knot nematodes (*Meloidogyne* spp.), a growing problem in French vegetable crops. EPPO Bulletin, 42 (1): 127-137.

Elekçioğlu, İ. H., 1992. Untersuchungen zum Auftreten und zur Verbreitung phytoparasitarer Nematogen in den land wirtschafflichen Hauptkulturen des astmediterranen Gebretes der Turkei. Plits, 10 (5): 120 pp.

Elling, A. A., 2013. Major emerging problems with minor *Meloidogyne* species. Phytopathology, 103 (11): 1092-1102.

Erdoğus, F. D., H. C. Akgül & S. Bayram, 2011. A new host for *Meloidogyne incognita* (Kofoid & White 1919) Chitwood, 1949 (Nemata: Tylenchida) in Turkey: Potato. Journal of Agricultural Science and Technology A, 1: 100-105.

Evans, A. A. & R. N. Perry, 2009. "Survival mechanisms, 201-222". In: Root-knot nematodes (Eds. R. N. Perry, M. Moens & J. L. Starr). CAB International, Landon, UK, 488 pp.

Evlice, E. & Ş. Bayram, 2016. Identification of root-knot nematode species (*Meloidogyne* spp.) (Nemata: Meloidogynidae) in the potato fields of Central Anatolia (Turkey) using molecular and morphological methods. Türkiye Entomoloji Bülteni, 6 (4): 339-347 (in Turkish with abstract in English).

FAOSTAT, 2019. FAOSTAT-Agriculture. (Web page: http://www.fao.org/faostat/en/#data/QC) (Date accessed: February 2021).

Fourie, H., C. Zijlstra & A. H. Mcdonald, 2001. Identification of root-knot nematode species occurring in South Africa using the SCAR-PCR technique. Nematology, 3 (7): 675-698.

Golden, A. M., J. H. O'Bannon, G. S. Santo & A. M. Finley, 1980. Description and SEM observations of *Meloidogyne chitwoodi* n. sp. (Meloidogynidae), a root-knot nematode on potato in the Pacific Northwest. Journal of Nematology, 12 (4): 319-327.

Hooper, D. J., 1986. "Handling, Fixing, Staining and Mounting Nematodes, 59-80". In: Laboratory Methods for Work with Plant and Soil Nematodes (Eds. J. F. Southey). Her Majesty's Stationery Office, London, 202 pp.

İmren, M., A. Özarslandan, E. C. Kasapoğlu, H. Toktay & İ. H. Elekcioğlu, 2014. Türkiye buğday faunası için yeni bir tür, *Meloidogyne artiellia* (Franklin)'nın morfolojik ve moleküler yöntemlerle tanımlanması. Turkish Journal of Entomology, 38 (2): 189-196 (in Turkish with abstract in English).

Ingham, R. E., P. B. Hamm, M. Baune, N. L. David & N. M. Wade, 2007. Control of *Meloidogyne chitwoodi* in potato with shank-injected metam sodium and other nematicides. Journal of Nematology, 39 (2): 161-168.

Inserra, R. N., G. D. Griffin & D. V. Sisson, 1983. Effects of temperature and root leachates on embryogenic development and hatching of *Meloidogyne chitwoodi* and *M. hapla*. Journal of Nematology, 15 (1): 123-127.

Jones, J. T., A. Haegeman, E. G. J. Danchin, H. S. Gaur, J. Helder, M. G. K. Jones, T. Kikuchi, R. Manzanilla-López, J. E. Palomares-Rius, W. M. L. Wesemael & R. N. Perry, 2013. Top 10 plant-parasitic nematodes in molecular plant pathology: Top 10 plant-parasitic nematodes. Molecular Plant Pathology, 14 (9): 946-961.

Jones, R. K., S. G. Storey, R. Knoetze & H. Fourie, 2017. "Nematode Pests of Potato and Other Vegetable Crops, 231-260". In: Nematology in South Africa: A View from the 21st Century (Eds. H. Fourie, V. W. Spaull, R. Jones, M. S. Daneel & D. de Waele). Springer International Publishing, Switzerland, 569 pp.

Karssen, G. & M. Moens, 2006. "Root-Knot Nematodes, 59-90". In: Plant Nematology (Eds. R. N. Perry & M. Moens). CABI Publishing, Wallingford, 568 pp.

Kaşkavalcı, G. & C. Öncüer, 1999. Investigations on distribution and economic importance of *Meloidogyne* Goeldi, 1887 (Tylenchida: Meloidogynidae) species found in the major areas of hot climate vegetables in Aydın province. Turkish Journal of Entomology, 23 (2): 149-160 (in Turkish with abstract in English).

Keidel, H., T. G. Van Beers, J. Doornbos & L. P.G. Molendijk, 2007. Monitoring nulsituatie: rapport resultaten meetronde 2005-2006. (Web page: https://www.researchgate.net/publication/254844952_Samenvatting_Monitoring_Nulsituatie-Rapport_Resultaten_meetronde_2005-2006) (Date accessed: February 2021).

Kepenekci, İ., E. Evlice & G. Öztürk, 2014. Taxonomic characteristics of *Meloidogyne exigua* Goeldi which is a new root-knot nematode for Turkey and other root-knot nematode species. Bitki Koruma Bülteni, 54 (1): 1-9 (in Turkish with abstract in English).

Kepenekci, İ., H. Toktay, E. Evlice & A. Özarslandan, 2006. Potato (*Solanum tuberosum* L.) fig (*Ficus* spp.) and mulberry (*Morus* spp.) new host records of root-knot nematodes in Turkey. Pakistan Journal of Nematology, 24 (2): 217-219.

King, B. A. & J. P. Taberna Jr, 2013. Site-specific management of *Meloidogyne chitwoodi* in Idaho potatoes using 1, 3-dichloropropene; approach, experiences, and economics. Journal of Nematology, 45 (3): 202-213.

Lahtinen, A. E., D. L. Trudgill & K. Tlikkala, 1988. Threshold temperature and minimum thermal time requirements for the complete life cycle of *Meloidogyne hapla* from northern Europe. Nematologica, 34 (4): 443-451.

Lima, F. S. O., V. S. Mattos, E. S. Silva, M. A. S. Carvalho, R. A. Teixeira, J. C. Silva & V. R. Correa, 2018. "Nematodes Affecting Potato and Sustainable Practices for Their Management, 107-124". In: Potato - From Incas to All Over the World (Ed. M. Yildiz). Intech Open, London, 148 pp.

Madulu, J. D. & D. L. Trudgill, 1994. Influence of temperature on the development and survival of *Meloidogyne javanica*. Nematologica, 40 (1-4): 230-243.

Mai, W. F., B. B. Brodie, M. B. Harrison & P. Jatala, 1981. "Nematodes, 93-101". In: Compendium of Potato Diseases (Ed. W. J. Hooker). American Phytopathological Society, St Paul, Minnesota, 125 pp.

Mao, Y., Y. Hu, C. Li, H. Zhang, D. Yu, X. Liu, G. Yang & C. Wang, 2019. Molecular identification of *Meloidogyne* species isolated from potato in China and evaluation of the response of potato genotypes to these isolates. Nematology, 21 (8): 847-856.

Medina, I. L., C. B. Gomes, V. R. Correa, V. S. Mattos, P. Castagnone-Sereno, R. M. D. G. Carneiro, 2017. Genetic diversity of *Meloidogyne* spp. parasitising potato in Brazil and aggressiveness of *M. javanica* populations on susceptible cultivars. Nematology, 19 (1): 69-80.

Müller, J., D. Sturhan, H. J. Rumpenhorst, H. J. Braasch & J. G. Unger, 1996. On the occurrence of a root-knot nematode (*Meloidogyne chitwoodi*) new to Germany. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes, 48 (6): 126-131.

Niere, B. & H. Karuri, 2018. "Nematode Parasites of Potato and Sweet Potato, 222-251". In: Plant Parasitic Nematodes in Subtropical and Tropical Agriculture (Eds. R. A. Sikora, D. Coyne, J. Hallmann & P. Timper). CABI Publishing, Wallingford, 876 pp.

Nobbs, J. M., Q. Liu, D. Hartley, Z. Handoo, V. M. Williamson, S. Taylor, G. Walker & J. Curran, 2001. First record of *Meloidogyne fallax* in Australia. Australasian Plant Pathology, 30 (4): 373.

Norshie, P. M., T. H. Been & C. H. Schomaker, 2011. Estimation of partial resistance in potato genotypes against *Meloidogyne chitwoodi*. Nematology, 13 (4): 477-489.

Norton, D. C., 1978. Ecology of Plant-Parasitic Nematodes. Wiley-Interscience, New York, 268 pp.

Nyczepir, A. P., J. H. O'Bannon, G. S. Santo & A. M. Finley, 1982. Incidence and distinguishing characteristics of *Meloidogyne chitwoodi* and *M. hapla* in potato from the Northwestern United States. Journal of Nematology, 14 (3): 347-353.

Onkendi, E. M. & L. N. Moleleki, 2013. Distribution and genetic diversity of root-knot nematodes (*Meloidogyne* spp.) in potatoes from South Africa. Plant Pathology, 62 (5): 1184-1192.

Özarslandan, A., Z. Devran, N. Mutlu & İ. H. Elekçioğlu, 2009. First report of Columbia root-knot nematode (*Meloidogyne chitwoodi*) in potato in Turkey. Plant Disease, 93 (3): 316.

Özarslandan, A. & İ. H. Elekcioğlu, 2010. Identification of the Root-knot nematode species (*Meloidogyne* spp.) (Nemata: Meloidogynidae) collected from different parts of Turkey by molecular and morphological methods. Turkish Journal of Entomology, 34 (3): 323-335.

Özarslandan, A., M. İmren, A. Öcal & İ. H. Elekçioğlu, 2013. Bitlis ili patates üretim alanlarında kök-ur nematodu (*Meloidogyne chitwoodi* Golden, O'Bannon, Santo et Finley, 1980)'nun saptanması. Turkish Journal of Entomology, 37 (3): 389-395 (in Turkish with abstract in English).

Pinkerton, J. N., G. S. Santo & H. Mojtahedi, 1991. Population dynamics of *Meloidogyne chitwoodi* on Russet Burbank potatoes in relation to Degree-Day accumulation. Journal of Nematology, 23 (3): 283-290.

Ploeg, A. & P. Maris, 1999. Effects of temperature on the duration of the life cycle of a *Meloidogyne incognita* population. Nematology, 1 (4): 389-393.

Ravichandra, N. G., 2014. Horticultural Nematology. New Delhi Springer, India, 412 pp.

Singh, R. & U. Kumar, 2015. Assessment of nematode distribution and yield losses in vegetable crops of Western Uttar Pradesh in India. International Journal of Science and Research, 4 (5): 2812-2816.

Thoden, T. C., G. W. Korthals, J. Visser & W. van Gastel-Topper, 2012. A field study on the host status of different crops for *Meloidogyne minor* and its damage potential on potatoes. Nematology, 14 (3): 277-284.

Thomas, G. & G. Sansonetti, 2009. New light on a hidden treasure: international year of the potato 2008, an end-of-year review. (Web page: http://www.fao.org/3/i0500e/i0500e.pdf) (Date accessed: February 2021).

Trudgill, D. L. & V. C. Blok, 2001. Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. Annual Review of Phytopathology, 39 (1): 53-77.

TÜİK, 2019. Türkiye İstatistik Kurumu. (Web page: http://www.biruni.tuik.gov.tr/medas/?kn=92&locale=tr) (Date accessed: February 2021) (in Turkish).

Ulutaş, E., G. Kaskavalci & E. Pehlivan, 2012. "Determination of the effects of plant-parasitic nematodes on potato plant development in İzmir, Turkey, 200". 31th International Symposium of the European Society of Nematologists Proceedings (23-27 September 2012, Adana, Turkey), 270 pp.

Waeyenberge, L., A. Ryss, M. Moens, J. Pinochet & T. C. Vrain, 2000. Molecular characterization of 18 *Pratylenchus* species using rDNA restriction fragment length polymorphism. Nematology, 2 (2): 135-142.

Wesemael, W. M. L., N. Viaene & M. Moens, 2011. Root-knot nematodes (*Meloidogyne* spp.) in Europe. Nematology, 13 (1): 3-16.

Wishart, J., M. S. Phillips & V. C. Blok, 2002. Ribosomal intergenic spacer: a polymerase chain reaction diagnostic for *Meloidogyne chitwoodi, M. fallax*, and *M. hapla*. Phytopathology, 92 (8): 884-892.

Yağcı, M., & G. Kaşkavalcı, 2018. Ege bölgesi şeftali alanlarında görülen kök ur nematodu türleri (*Meloidogyne* spp.)'nin belirlenmesi ve yayılışının saptanması. Ege Üniversitesi Ziraat Fakültesi Dergisi, 55 (3): 305-310 (in Turkish with abstract in English).

Yıldız, V., Ç. Güneş, N. Bulun & U. Gözel, 2009. "Ödemiş (İzmir) İlçesi patates üretim alanlarında tespit edilen kök-ur nematodu: *Meloidogyne chitwoodi* (Goeldi, 1892, Nemata: Heteroderidae), 35" Türkiye III. Bitki Koruma Kongresi Bildirileri (15-18 Temmuz 2009, Van), 380 pp (in Turkish with abstract in English).

Yüksel, H. Ş., 1966. Karadeniz Bölgesi'nde tesadüf edilen *Meloidogyne incognita* varyasyonu hakkında. Bitki Koruma Bülteni, 6 (1): 35-38 (in Turkish with abstract in English).

Yüksel, H. Ş., 1967. Iğdır Ovasında ilk defa bulunan *Meloidogyne hapla* ve bunun *Meloidogyne incognita*'nın kanatlı varyasyonundan ayırt edici özellikleri. Atatürk Üniversitesi Ziraat Fakültesi Zirai Araştırma Enstitüsü Teknik Bülteni, No: 17, 20 pp (in Turkish).

Zijlstra, C., D. T. Donkers-Venne & M. Fargette, 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. Nematology, 2 (8): 847-853.

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Original article (Orijinal araştırma)

Arthropoda fauna of Zindan Cave (Isparta, Turkey) with notes on new records and some ecological characteristics[1]

Zindan Mağarası (Isparta, Türkiye) Arthropoda faunası, yeni kayıtlar ve bazı ekolojik özellikler üzerine notlar

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Abstract

The aims of the study were to investigate the Arthropoda fauna of Zindan Cave (Aksu, Isparta Province, Turkey) and to consider some ecological characteristics of the collected species such as feeding habits, cave-dwelling categories, and zone distributions in caves. Twenty-seven species of Arthropoda were determined between May 2015 and December 2016, from Zindan Cave. The species found belong to the following orders: nine to Collembola, six to Coleoptera, five to Araneae, two to Diplopoda and one to Orthoptera, Trichoptera, Diptera, Trombidiformes and Isopoda. Nine species are endemic of Turkey. *Traegaardhia distosolenidia* Zacharda, 2010 (Acari: Rhagidiidae) and *Folsomia asiatica* Martynova, 1971 (Collembola: Isotomidae) are newly recorded for the fauna of Turkey. The presence of *Heteromurus sexoculatus* Brown, 1926 (Collembola: Entomobryidae) in Turkey is confirmed. Twenty-four of the 27 species were from the dark zone and half of the collected species (6 troglobites and 8 troglophiles) are ecologically adapted to cave ecosystems. Species can be divided into three groups according to trophic preferences: 15 scavengers, 10 predators and two omnivores. Food habits, cave-dwelling categories, and zone distributions of collected species are discussed.

Keywords: Arthropoda, biospeleology, cave-dwelling categories, habitat, Zindan Cave

Öz

Çalışmanın amacı, Zindan Mağarası (Aksu, Isparta, Türkiye) Arthropoda faunasını belirlemek ve toplanan türlerin beslenme alışkanlıkları, ekolojik sınıflandırılması ve zon dağılımları gibi bazı ekolojik özelliklerini ortaya koymaktır. Zindan Mağarası'ndan Mayıs 2015 ile Aralık 2016 arasında 27 Arthropoda türü belirlenmiştir. Bu türlerden dokuzu Collembola, altısı Coleoptera, beşi Araneae, ikisi Diplopoda takımlarına ait iken, Orthoptera, Trichoptera, Diptera, Trombidiformes ve Isopoda takımlarına aittir. Bu türlerden dokuzu Türkiye için endemiktir. *Traegaardhia distosolenidia* Zacharda, 2010 (Acari: Rhagidiidae) ve *Folsomia asiatica* Martynova, 1971 (Collembola: Isotomidae) türleri, Türkiye faunası için yeni kayıtlardır. Aynı zamanda Türkiye için *Heteromurus sexoculatus* Brown, 1926 (Collembola: Entomobryidae) varlığı doğrulanmıştır. Toplanan 27 türden yirmi dördü karanlık zondan ve türlerin yarısının (6 troglobit ve 8 troglofil) ekolojik olarak mağara ekosistemine adapte olduğunu ortaya konmuştur. Ayrıca türlerin trofik tercihlerine göre: 15 çöpcül, 10 predatör ve iki omnivor olmak üzere üç gruba ayrıldığı belirlenmiştir. Sonuç olarak, toplanan türlerin beslenme alışkanlıkları, ekolojik sınıflandırılması ve zon dağılımları tartışılmıştır.

Anahtar sözcükler: Arthropoda, biyospeleoloji, ekolojik sınıflandırılma, habitat, Zindan Mağarası

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Introduction

Cave ecosystems, with the relative simplicity of their communities, and the temporal and spatial isolation of their biota, are considered as natural ecological and evolutionary laboratories (Poulson & White, 1969). Cave-dwelling species help us to understand the differentiation of species in the biogeography and evolutionary process, as island biota (Cooper et al., 2007; Page et al., 2008; Juan et al., 2010).

Subterranean environments that are characterized by relatively stable abiotic conditions such as moisture, temperature, water chemistry and continuous darkness are unique ecosystems separated from the surface. These ecosystems are poor in food because of the mentioned features, and dependent on the food resources of the epigean ecosystems (Hüppop, 2012). Bacteria, archaea and fungi are primary consumers of organic material (e.g., rotten wood and guano deposits) deposited in caves. Also, most Collembola and some Acari are attracted by colonies of decomposers. Invertebrates such as Isopoda, Diplopoda and some Coleoptera are consuming decayed organic material. These invertebrates also transfer of nutrients through fragmentation and foster microbial activity. Furthermore, these species in turn increases the rate of organic matter decomposition. Predators such as Chilopoda, Araneae, Pseudoscorpiones, carnivorous Acarina and some Coleoptera are hunting invertebrates which feed on dead organic material and microbes. Subterranean food webs mostly consist of more generalist species, as in soil food webs (Parimuchová et al., 2021).

Caves, which are one of the subterranean environments, are one of the most interesting earth formations on the planet. Thus, they have been studied for several aspects from past to present (Palmer, 1991). One of these study areas is biospeleology which studies living organisms in caves. Although the first study on cave organisms dates back to the seventeenth century in Europe (Romero, 2009) and cave-dwelling fauna of Europe and North America are particularly well known, in many parts of the world including Turkey, they are still poorly known (Juberthie & Decu, 1994; Peck et al., 1998; Kunt et al., 2010).

Turkey is defined as a "cave heaven" with about 40,000 caves (Nazik & Bayarı, 2018). However, only 1,250 of these are registered to the cave inventory of Turkey (Kopar, 2009; Nazik et al., 2005). In Turkey, the first biospeleological study was performed by Colonel Dr. Abdullah Bey at the Yarımburgaz Cave (Istanbul) in 1865 (Erguvanlı, 1975; Yamaç et al., 2021). Despite this early start, most of the biospeleological studies in Turkey has mostly continued through non-systematic sampling by foreign researchers until Kunt et al. (2010). Detailed studies of Turkish cave ecosystems have been available only recently (Aydın & Şen, 2020).

The number of terrestrial cave taxa of worldwide is estimated at over 21,000 species including more than 7,000 aquatic species (Juan et al., 2010). In addition, caves and other subterranean environments are poorly studied. In many regions of the world, cave-dwelling species still remain to be investigated (Gibert & Deharveng, 2002; Juan et al., 2010).

In studies conducted in Turkish caves, more than 200 invertebrates have been identified, 105 of which are endemic to Anatolia. More than 80% of the identified species were Arthropoda (Kunt et al., 2010; Antić et al., 2016). The reason for high endemism of the species living in the caves is that these species adapt to separated subterranean environments (Armas & Alayon Garcia, 1984; Juberthie & Decu, 1994). Although cave ecosystems have high endemism and rich fauna, there is still no detailed study on any cave fauna in Turkey (Kunt et al., 2010). Thus, the aims of the present study were (1) to determine the Arthropoda fauna of Zindan Cave, (2) to assign some ecological attributes of the collected species such as food habits, cave-dwelling categories and zone distributions, (3) to give some remarks on morphological characters linked to cave adaptation, and (4) to give notes on new records for Turkey.

Materials and Methods

Zindan Cave

Zindan Cave is located within the borders of the Aksu District of Isparta Province (37º48'42" N, 31º05'03" E) in the northern section of the Western Taurus Mountains. It was formed during the Resiyen (Upper Triassic period) and its length is 765 m (Bozcu, 2007). Although the cave is 765 m long; the last 265 m is dangerous for less-experienced cavers. Thus, only the first 500 m of the cave were investigated. The average temperature in the cave is 12-15ºC, and the RH ranges from 80 to 85% between June and December.

Zindan Cave has been used by humans for sanctuary, dungeon, bat manure collection and tourism from A.D. 169-180 until the present. Today, the cave is used only for tourism. This cave, which has been exposed to humans in different time periods from the past to the present, is thought to have received the most damage when it was dedicated to tourism. With early contributions of Durmuş Kaya and Stephen Mitchell, the study of its biology has only recently recommenced. Zindan Cave, popular with tourists since 2003 and with sanctuary at the entrance, can be reached by a single arch bridge, from the Roman period, over Zindan Stream, a main branch of Köprüçay (Eurymedon) River (Kaya & Mitchell, 1985; Alp, 2013).

Zones of Zindan Cave

At the beginning of the study, the cave was divided into three zones based on the light intensity: entrance, twilight and dark zones. In first part of the cave to 30 m where light can easily reach was deemed the entrance zone. The part between 30-45 m where light breaks and creates a dim environment was deemed the twilight zone. The part from the end of the twilight to the end of the cave was deemed the dark zone.

Ecological classification of collected species

The ecological classification of the cave-dwelling species was made according to Barr (1968): accidental, trogloxene, troglophile and troglobite species. The classification of Barr (1968) was made by taking into consideration not only distribution and ecology but also the evolutionary adaptations of the species (Trajano, 2012). Rendoš et al. (2016) used an ecological classification method specific to the cave-dwelling Collembola. This classification is based on the proximity of habitats of the Collembola species to the surface. For the purposed of this study, the ecological classification of Ruffo (1957), which is primarily used by Italian speleobiologists (Sket, 2008), was also used. The troglomorphic properties of the species they identified were changed according to the method applied by Bar (1968).

Sampling

During 2015-2016, arthropod samples in Zindan Cave were determined monthly using three methods of collection: (1) careful searching over all cave surfaces (Di Russo et al., 2007), (2) extraction from organic matters (bat guano and vertebrate carcass), and (3) bolting water with plankton net (Kováč et al., 2005b). Samples were placed directly into tubes containing 70% ethanol. Cave animals are known to have extremely long generation times. Reproductive ability of cave-dependent species are slower than epigean species, therefore, pitfall traps were not used. Also, the species richness in the cave ecosystems is limited because the cave ecosystems are separated from epigean conditions. Therefore, identified and morphologically similar species were not collected in subsequent collection trips. However, the number and ecological information of these species were recorded. Sufficient sampling was made for diagnosis. Samples were lodged in the collection of the Biology Department of Süleyman Demirel University, Isparta, Turkey. Sampling was conducted by the first author who was equipped with both LED headlamp and a handheld lamp.

Results

Twenty-seven Arthropoda species were identified from Zindan Cave (Figure 1). Of these species, nine belong to Collembola, five to Araneae, six to Coleoptera, two to Diplopoda and one to Orthoptera, Trichoptera, Diptera, Trombidiformes and Isopoda. A complete list follows.

***Traegaardhia distosolenidia* Zacharda, 2010 (Acari: Trombidiformes: Rhagidiidae)**

Material examined. 11.10.2015, ♀, 2 nymphs, dark zone. Det. Miloslav Zacharda.

General and Turkey distribution. The species was identified only from the Taquisara cave in Italy (Zacharda et al., 2010). This is a new record for Turkey.

Ecological classification and trophic behavior. Troglobite and predator.

Note. It was observed that they hunted Collembola specimens on the puddles in the cave and also, specimens were actively moving on the water surface.

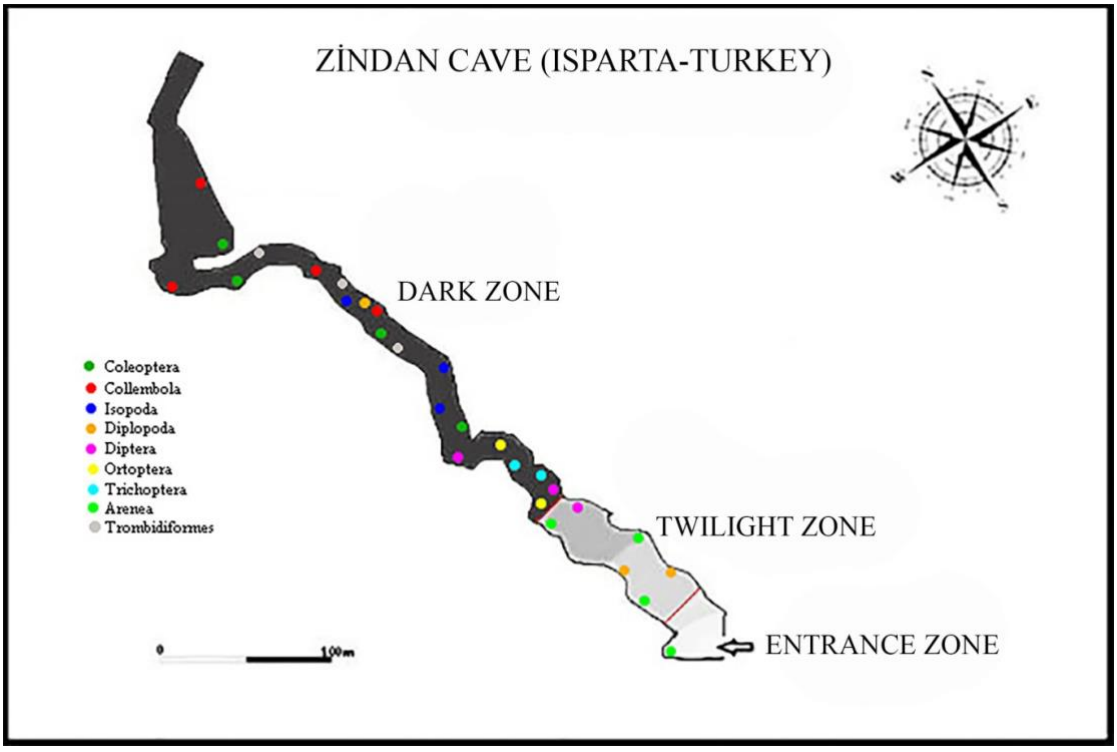

Figure 1. Zindan Cave and distribution of species according to zones (Modified from Karadem, 2011).

***Tegenaria percuriosa* Brignoli, 1972 (Arachnida: Araneae: Agelenidae)**

Material examined. 17.05.2015, 9♀♀, 5♂♂, twilight zone. Det. Kadir Boğaç Kunt.

General and Turkey distribution. Endemic to Turkey (Kunt et al., 2010). The type locality is Zindan Cave. Antalya Province, Dim Cave; Isparta Province, Barla Cave; Konya Province, Hacı Akif Cave; Sivas and Ankara Provinces, unknown localities (Kaya et al., 2010).

Ecological classification and trophic behavior. Troglophile and predator.

Note. It is reported that an adult male of this species has previously been found on the wall surface at the 30-50 m of the cave (Gasparo, 2007). It was observed that this species feed on the *Limonia nubeculosa* Meigen, 1804 (Diptera: Limoniidae) specimens.

***Maimuna vestita* (C.L.Koch, 1841) (Arachnida: Araneae: Agelenidae)**

Material examined. 17.05.2015, 3♂♂, transition zone. Det. Kadir Boğaç Kunt.

General and Turkey distribution. East Mediterranean (Vrenozi & Jäger, 2012). Aydın, Bursa, Istanbul, Izmir, Muğla Provinces (Brignoli, 1978; Kaya & Uğurtaş, 2007).

Ecological classification and trophic behavior. Trogloxene and predator.

Note. This species is also known from epigean ecosystems. It especially lives in rocky habitats (Kaya & Uğurtaş, 2007). It was observed that the specimens built their webs near or under the stones in the cave. The main food sources of the species are small Diptera species.

***Metellina mengei* (Blackwall, 1870) (Arachnida: Araneae: Tetragnathidae)**

Material examined. 16.06.2016, 7♀♀, 2♂♂, dark zone. Det. Kadir Boğaç Kunt.

General and Turkey distribution. It distributes from Europe to Georgia (Marusik et al., 2012). Antalya, Bilecik, Bursa and Çankırı Provinces (Marusik et al., 2012).

Ecological classification and trophic behavior. Troglophile and predator.

Note. This species lives on leaf litter in meadows and woodlands, but has also been recorded in caves (Marusik et al., 2012).

***Diplocephalus turcicus* Brignoli, 1972 (Arachnida: Araneae: Linyphiidae)**

Material examined. 15.09.2016, 2♀♀, 3♂♂, dark zone. Det. Kadir Boğaç Kunt.

General and Turkey distribution. Greece (Deltshev, 2008) and Turkey (Kunt et al., 2010). Burdur Province, Insuyu Cave; Isparta Province, Zindan Cave; Konya Province, Hacı Akif Cave (Brignoli, 1972; Kunt et al., 2010).

Ecological classification and trophic behavior. Troglophile and predator.

Note. This species was observed that they are hunting on Guano.

***Lepthyphantes leprosus* (Ohlert, 1865) (Arachnida: Araneae: Linyphiidae)**

Material examined. 15.09.2016, 3♀♀, ♂, twilight zone. Det. Kadir Boğaç Kunt.

General and Turkey distribution. Bulgaria, Macedonia, Serbia, Croatia, Albania, Greece, Turkey (Deltshev, 2008). Bitlis Province, Sultan Seyit Cave; Diyarbakır Province, Korkha Cave (Kunt et al., 2010).

Ecological classification and trophic behavior. Troglophile and predator.

Note. Although species of *Lepthyphantes* have been reported from various caves (Mammola et al., 2018; Prokopenko, 2019), this species is considered as troglophile in this study because of its dependence on the epigean ecosystems.

***Eurygyrus bilseli* (Verhoeff, 1940) (Diplopoda: Callipodida: Schizopetalidae)**

Material examined. 16.06.2015, 26.09.2015, 11.10.2015, 4♀♀, 3♂♂, twilight and dark zone. Det. Dragan Antic and Henrik Enghof.

General and Turkey distribution. Endemic to Turkey (Enghoff, 2006). Burdur Province, İnsuyu Cave; Isparta Province, unknown locality; Konya Province, Hacı Akif Cave; Tınaztepe Cave; Mersin Province, Cennet Cave (Enghoff, 2006; Kunt et al., 2010).

Ecological classification and trophic behavior. Trogloxene and scavenger.

Note. The species was observed that it was active in darkness. At night, specimens were seen extensively around the stream bed outside the cave.

***Mesoiulus taurus* Antic, 2016 (Diplopoda: Julida: Julidae)**

Material examined. 17.06.2015, 26.09.2015, 11.10.2015, 15.12.2015, 17.05.2016, 4♀♀, 2♂♂, dark zone. Det. Dragan Antic and Henrik Enghof.

General and Turkey distribution. Endemic to Turkey. The type locality is Zindan Cave. There are four endemic *Mesoiulus* species distributed in Turkish caves including this species. The three others are *Mesoiulus ciliciensis* Strasser 1975 from Mersin Province, *Mesoiulus kosswigi* Verhoeff, 1936 from Istanbul Province and *Mesoiulus turcicus* Verhoeff, 1898 from Bilecik Province (Enghoff, 2006).

Ecological classification and trophic behavior. Troglobite and scavenger.

Note. This species is distributed between 200-400 m into the cave. Many of specimens were collected on the moss formed under the projector used for lighting 250 m into the caver.

***Trichonethes kosswigi* (Strouhal, 1953) (Malacostraca: Isopoda: Trichoniscidae)**

Material examined. 16.06.2015, 11.10.2015, 17.05.2016, 4♀♀, 3♂♂, dark zone. Det.Stefano Taiti.

General and Turkey distribution. This species is endemic to Turkey and the type locality of the species is Zindan Cave (Argano & Manicastri, 1988). Antalya Province, Damlataş Cave and Indağı Cave; Burdur Province, Insuyu Cave; Isparta Province, Barla Cave and Inönü Cave; Konya Province, Su Çıktığı Cave, Tınaztepe Cave; Haci Akif Cave, Child Thrown Hole Cave, Körükini Cave and Asarini Cave (Argano & Manicastri, 1988).

Ecological classification and trophic behavior. Troglobite and scavenger.

Note. This species is a troglobite species due to lack of eyes, reduced pigment and a completely whitish structure. This species lives between 150 and 300 m into the cave. This species was observed that they feed on guano, organic residues and carrion.

***Lipothrix lubbocki* (Tullberg, 1872) (Entognatha: Collembola: Sminthuridae)**

Material examined. 11.10.2015, ♀, dark zone. Det. Igor Kaprus.

General and Turkey distribution. The species distributes throughout Europe (Popa & Šustr, 2017). This species is also reported from North Africa (Popa, 2012). The species was only known from Ordu Province in Turkey (Özata, 2015).

Ecological classification and trophic behavior. Accidentally and scavenger.

Note. This species is known that the species occurs in forests, especially pine forests (Kováč et al., 2005a). Thus, the presence in the cave was accidental.

***Lepidocyrtus lignorum* (Fabricius, 1793) (Entognatha: Collembola: Entomobryidae)**

Material examined. 11.10.2015, 2♀♀, dark zone. Det. Igor Kaprus.

General and Turkey distribution. Holarctic Region (Dányi & Traser, 2008). Forest habitats in Antalya Province (Sevgili & Özata, 2014).

Ecological classification and trophic behavior. Trogloxene and scavenger.

Note. This species is known that the species lives in the meadows and pastures (Komonen & Kataja-Aho, 2017). Thus, the presence in the cave was accidental.

***Heteromurus sexoculatus* Brown, 1926 (Entognatha: Collembola: Entomobryidae)**

Material examined. 11.10.2015, 8 specimens, dark zone. Det. Igor Kaprus.

General and Turkey distribution. Romania, Israel, Iran, Iraq and Rhodes Island (Greece) (Ellis, 1974; Shayanmehr et al., 2013). The presence of the species in Turkey was doubtful according to Sevgili & Özata (2014). An exact locality of the species is the first to be provided through this study. Thus, the presence of the species in Turkey is confirmed.

Ecological classification and trophic behavior. Troglophile and scavenger.

Note. Shayanmehr et al. (2013) reported that the species distributed on leaf litter and soil in Iran. Species probably occurs in dark and humid habitats.

***Pseudosinella horaki* Rusek, 1985 (Entognatha: Collembola: Entomobryidae)**

Material examined. 11.10.2015, 4 adults, dark zone. Det. Igor Kaprus.

General and Turkey distribution. Slovakia, Hungary, Moldova, Czech Republic and Turkey (Dániel & Nándor, 2012). In a study conducted in Ordu Province, the species was reported from pine and spruce forest-floor debris and localities over 1,300 m (Özata et al., 2017).

Ecological classification and trophic behavior. Trogloxene and scavenger.

Note. This species is known that the species distributes under-forests such as *Larix decidua* Mill., *Picea abies* (L.) H. Karst. and *Pinus sylvestris* L. (Özata et al., 2017; Čuchta et al., 2019). Thus, the presence in the cave was accidental.

***Folsomia asiatica* (Martynova, 1971) (Entognatha: Collembola: Isotomidae)**

Material examined. 11.10.2015, 12 specimens, dark zone. Det. Igor Kaprus.

General and Turkey distribution. Azerbaijan, Tajikistan, Iran (Rad & Morteza, 2015). This is a new record for Turkey.

Ecological classification and trophic behavior. Troglophile and scavenger.

Note. This species is reported to be linked to hypogenous life due to the lack of pigment and eyes. However, Rad & Morteza (2015) reported the species from soil and leaf litter under oak trees (*Quercus infectoria* G.Olivier) from Iran. So, it is considered to be a troglophile species.

***Folsomia penicula* Bagnall, 1939 (Entognatha: Collembola: Isotomidae)**

Material examined. 11.10.2015, 2 specimens, dark zone. Det. Igor Kaprus.

General and Turkey distribution. Palearctic region (Potapov, 2001). The species has been reported from the Turkey-Syria border (Sevgili & Özata, 2014). Özata et al. (2017) reported it from forest habitat from Ordu Province.

Ecological classification and trophic behavior. Accidental and scavenger.

Note. The general habitat of the species is tree debris in forests (Shayanmehr et al., 2013). The species, which is generally epigenous was collected from algae in 300 m into the cave.

***Folsomia manolachei* Bagnall, 1939 (Entognatha: Collembola: Isotomidae)**

Material examined. 11.10.2015, 8 specimens, dark zone. Det. Igor Kaprus.

General and Turkey distribution. Europe and Asia (Potapov, 2001). This species has been only reported from Ordu Province (Özata et al., 2017).

Ecological classification and trophic behavior. Accidental and scavenger.

Note. The general habitat of the species is forests and meadows (Raschmanová et al. 2017). The species, which is epigene, was collected in a small puddle 350 m into the cave. Also, it was collected on the algae in the cave.

***Isotomiella minor* (Schäffer, 1896) (Entognatha: Collembola: Isotomidae)**

Material examined. 11.10.2015, 11 specimens, dark zone. Det. Igor Kaprus.

General and Turkey distribution. Europe (Folsom, 1932). This species is also a cosmopolitan species (Potapov, 2001; Özata et al., 2017). Antalya (Sevgili & Özata, 2014) and Ordu (Özata et al., 2017) Provinces.

Ecological classification and trophic behavior. Trogloxene and scavenger.

Note. This species is reported that the species lives on leaf litter and soil in Iran (Shayanmehr et al., 2013). The species, which is epigene, was collected in a small puddle 400 m into the cave. Species probably occurs in dark and humid habitats.

***Parisotoma notabilis* (Schäffer, 1896) (Entognatha: Collembola: Isotomidae)**

Material examined. 11.10.2015, 3 adults, dark zone. Det. Igor Kaprus.

General and Turkey distribution. Europe (Porco et al., 2012). Turkey-Syria border (Sevgili & Özata, 2014; Özata et al., 2017). The species is widespread and common in Ordu Province (Özata et al., 2017).

Ecological classification and trophic behavior. Trogloxene and scavenger.

Note. The general habitat of the species is tree debris in forests and meadows, but also it is typically present in arable fields, pastures, urban soils and caves (Shayanmehr et al., 2013; Von Saltzwedel et al., 2017). The species, which is epigene, was collected in a small puddle 350 m into the cave. It is assumed that the species can live in many habitats and is also adapted to cave habitats.

***Agabus dilatatus* (Brullé, 1832) (Insecta: Coleoptera: Dytiscidae)**

Material examined. 16.06.2015, 11.10.2015, 16.05.2016, 4♀♀, 3♂♂, dark zone. Det. Mustafa Darilmaz.

General and Turkey distribution. Europe: Albania, Bulgaria, Georgia, Greece, Italy, Macedonia, Russia (South Territory), Turkey, Ukraine. North Africa: Algeria, Egypt, Morocco, Tunisia. Asia: Cyprus, Iran, Iraq, Israel, Kyrgyzstan, Lebanon, Sinai, Syria, Turkey, Uzbekistan (Vafaei et al., 2009; Scheers & Thant, 2017). Adana, Ankara, Bursa, Gümüşhane, Isparta, İzmir, İzmit, Konya, Rize, Trabzon and Van Provinces (Kıyak et al., 2007).

Ecological classification and trophic behavior. Accidental in caves and predator (Karaman, 2007; Özgenç, 2011).

Note. Specimens were collected from ponds and puddles 280 m into the cave. This species is known to live in groundwater (Karaman, 2007; Özgenç, 2011). The specimens may have been accidentally transported by groundwater to the cave. The species was observed with *Agabus biguttatus* (Olivier, 1795) in the same water sources.

***Agabus biguttatus* (Olivier, 1795) (Insecta: Coleoptera: Dytiscidae)**

Material examined. 16.06.2015, 11.10.2015, 16.05.2016, 3♀♀, 2♂♂, dark zone. Det. Mustafa Darilmaz.

General and Turkey distribution. Species is widely distributed in Europe, North Africa and Asia (Kıyak et al., 2007; Vafaei et al., 2009; Özgenç, 2011). Adana, Aksaray, Ankara, Bilecik, Bursa, Çankırı, Elazığ,

Gaziantep, Gümüşhane, Isparta, İzmir, Kastamonu, Sakarya, Trabzon and Yozgat Provinces (Darılmaz & Kıyak, 2006; Kıyak et al., 2007).

Ecological classification and trophic behavior. Accidental in caves and predator (Karaman, 2007; Özgenç, 2011).

Note. The specimens were collected from the ponds and puddles 280 m into the cave. The species are known to live in groundwater (Karaman, 2007; Özgenç, 2011). The specimens may have been accidentally transported by groundwater to the cave. These species were observed with *A. dilatatus* in the same water sources.

***Choleva (Cholevopsis) bertiae* Giachino & Vailati, 2000 (Insecta: Coleoptera: Leiodidae)**

Material examined. 17.05.2015, 16.07.2015, 11.10.2015, 15.12.2015, 28.02.2016, 16.06.2016, 15.09.2016, 3♀♀, 4♂♂, dark zone. Det. Michel Perreau.

General and Turkey distribution. Endemic to Turkey. Isparta Province, Zindan Cave; Eskişehir Province, Sarıkaya Cave Tütüncüini Cave; Antalya Province, Zeybeyni Cave; Antakya Province, unknown locality (Fidan et al., 2014).

Ecological classification and trophic behavior. Troglobite and scavenger.

Note. This species was observed that Choleva specimens were feeding a variety of foods such as bat guano, dead bats and mice.

***Pisidiella spatulifera* Jeannel, 1930 (Insecta: Coleoptera: Leiodidae)**

Material examined. 16.06.2015, 16.07.2015, 11.10.2015, 15.12.2015, 28.02.2016, 15.09.2016, 5♀♀, 6♂♂, dark zone. Det. Michel Perreau.

General and Turkey distribution. Endemic to Turkey. Isparta Province, Zindan Cave; Konya Province, Hacı Akif Cave (Jeannel, 1955).

Ecological classification and trophic behavior. Troglobite and scavenger.

Note. This species was observed that *Pisidiella* specimens were feeding a variety of foods such as bat guano, dead bats and mice in small groups. Although their eyes are completely blind, they are very sensitive to light and other senses have developed.

***Pisidiella ovoidea* Jeannel, 1955 (Insecta: Coleoptera: Leiodidae)**

Material examined. 16.06.2015, 3♀♀, 4♂♂, dark zone. Det. Michel Perreau.

General and Turkey distribution. Endemic to Turkey. Isparta Province, Zindan Cave (Jeannel, 1955).

Ecological classification and trophic behavior. Troglobite and scavenger.

Note. This species was observed that *Pisidiella* specimens were feeding a variety of foods such as bat guano, dead bats and mice in small groups. Although their eyes are completely blind, they are very sensitive to light and other senses have developed.

***Quedius magarasiensis* Bordoni, 1978 (Insecta: Coleoptera: Staphylinidae)**

Material examined. 15.09.2016, 3♀♀, 4♂♂, dark zone. Det. Sinan Anlaş.

General and Turkey distribution. Endemic to Turkey. The type locality is Zindan Cave (Bordoni, 2010; Kunt et al., 2010).

Ecological classification and trophic behavior. Troglophile and predator.

Note. The presence of the species in the cave depends on guano. If there are no guano, they will absent in the cave. Although the distribution of the species in the cave is the dark zone, it is the possible to reach them in every area where bat roosts exist, as guano provides the food cycle of this species in the cave.

***Limonia nubeculosa* Meigen, 1804 (Insecta: Diptera: Limoniidae)**

Material examined. 16.06.2015, 2♀♀, 3♂♂, dark zone. Det. Hasan Koç.

General and Turkey distribution. This species is distributed throughout the Palearctic Region, and also in the Nearctic Region (Canada, USA) (Driauach & Belqat, 2016). Aydın, Denizli, Eskişehir, Isparta and Muğla Provinces, and European part of Turkey (Özgül et al., 2009; Bilgin et al., 2015).

Ecological classification and trophic behavior. Trogloxene and omnivore.

Note. The species, which can be observed in the various habitats at aboveground environments, is very common in cave environments during the winter and summer seasons (Barnes et al., 2009). The species feed on insects, carrions, guano and plant residues in caves. Also, it is a food source for cave spiders (Manenti et al., 2015).

***Troglophilus adamovici* Us, 1974 (Insecta: Orthoptera: Rhaphidophoridae)**

Material examined. 15.12.2016, ♀, twilight and dark zone. Det. Mehmet Sait Taylan.

General and Turkey distribution. Endemic to Turkey (Taylan, 2011). The type locality is Zindan Cave. Konya Province, Balatini Cave, Körükini Cave, Ferzene Cave, Tınaztepe Cave (Rampini & Di Russo, 2003; Kunt et al., 2010).

Ecological classification and trophic behavior. Troglophile and predator.

Note. This species was collected from 150 m into the cave. Also, this species is dependent on food resources of the epigean ecosystems.

***Stenophylax nycterobius* McLachlan, 1875 (Insecta: Trichoptera: Limnephilidae)**

Material examined. 25.09.2015, 11.10.2015, 5 larvae and 2♀♀, 2♂♂, dark zone. Det. Füsun Sipahiler.

General and Turkey distribution. The species is distributed very widely in Europe (Ibrahimi et al., 2013). Also, this species is known from Monte Cucco Cave from Italy (Corallini & Marchetti, 2016). Although the species is reported in the Central Anatolia, Turkey, there is no exact locality record (Moretti & Gianotti, 1964).

Ecological classification and trophic behavior. Trogloxene and omnivore.

Note. It is reported that this species uses caves for diapause during the summer (Malicky & Winkler, 1974). It was observed that the species completed all developmental stages in the cave. Larvae are omnivorous shredders. They feed on particulate organic matter and by hunting aquatic invertebrates. The larvae living in puddles inside the cave make their structures by using materials in the cave and they are different from the aboveground environment. Also, the larvae were seen under the drops flowing from the stalactites in the parts where there was no water. It was observed that the movements of Trichoptera adults slowed down due to the low temperature of the cave.

Discussion

In this study, 27 arthropod species were collected. Kunt et al. (2010) reported eight species, six of which are endemic (E) to Turkey, from Zindan Cave. These species were *T. percuriosa* (E), *Harpactocrates troglophilus* Brignoli, 1978 (Arachnida: Araneae: Dysderidae) (E), *D. turcicus*, *C. bertiae* (E), *Q. magarasiensis* (E), *T. adamovici* (E), *Cylisticus convexus* (De Geer, 1778) (Malacostraca: Isopoda: Cylisticidae*), T. kosswigi* (E). However, Kunt et al. (2010) overlooked two endemic Leiodidae species *P.*

spatulifera and *P. ovoidea* mentioned by Jeannel (1955) from the cave. Thus, 10 species, eight of which are endemic to Turkey, were previously known from the cave (Jeannel, 1955; Kunt et al., 2010). In the present study, *C. convexus* and *H. troglophilus* species were not collected. Probably *C. convexus* was previously collected accidentally, given that it is known from Europe and Northern Asia (excluding China), North America and South America. *Harpactocrates troglophilus* has been described from Zindan Cave. However, the species were found in mesovoid shallow substratum traps outside the cave (personal observation). Also, Kunt et al. (2019) described a new genus (*Kut* gen. nov.) with the type species *H. troglophilus*. They reported that the species was probably not present in Zindan Cave and they also collected the species from Kurucaova Village (Konya Province) which is outside of the cave ecosystem. Therefore, the presence of the species in the cave is questionable. In addition, new data has showed that nine species are endemic for Turkey and *M. taurus* has recently been given as a new species from the cave. Based on these data, Zindan Cave is the type locality of *M. taurus*, *P. ovoidea* and *Q. magarasiensis*.

It was observed that most of collected species (24 of 27 species) occurred in the dark zone. Of the rest, two of them were in the twilight zone, one in a transition zone and two species were in both the twilight and dark zones. In addition, half of the collected species (6 troglobites and 8 troglophiles) are ecologically adapted to the cave ecosystem while the other half (8 trogloxenes and 5 accidental species) were not ecologically adapted. Also, the results show that the species are divided into three groups according to trophic behavior: 15 scavengers, 10 predators and only two omnivore species (*L. nubeculosa* and *M. nycterobia*) (Manenti et al., 2015).

As a result of the present study, it has been determined that more than 33% of the collected species are endemic to Turkey. Zindan Cave is the type locality of *M. taurus* and the species is only known from this cave. Also, *T. distosolenidia*, *H. sexoculatus* and *F. asiatica* are firstly reported from Turkey. Especially, *T. distosolenidia* has an interesting distribution pattern with distribution in Italy and Turkey.

Zindan Cave continues be used for tourism, which can be hazardous both for the cave and the cave-dwelling organisms. Warm lighting for tourism activities constitutes the biggest problem in the cave. These problems are: (1) algae are growing in the artificially lit areas, (2) various formations as stalactites, stalagmites and columns inside the cave are damaged by algae, (3) owing to tourism and lighting, populations of bats in the cave are decreasing because baby bats are stressed and fall from their roosts, (4) warm lighting is thought to threaten especially troglobite and troglophile species which are adapted to the cave. Lighting also causes accidental species to penetrate deeper into the cave.

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References

Alp, A. O., 2013. Aksu - Zindan Mağarası ΜΗΤΗΡ ΘΕΩΝ ΟΥΕΓΕΙΝΟΣ Kutsal Alanının Restitüsyonu. Adalya, 16: 119-140 (in Turkish with abstract in English).

Antić, D., C. Ç. Çetin, E. Turantepe & M. F. Gürbüz, 2016. A new cave-dwelling species of the genus *Mesoiulus* Berlese, 1886 (Diplopoda, Julida, Julidae) from Turkey. Zootaxa, 4196 (2): 296-300.

Argano, R. & C. Manicastri, 1988. Il genere *Trichonethes* Strouhal, 1953 di Turchia (Isopoda, Oniscidea, Trichoniscidae). Fragmenta Entomologica, 21 (1): 9-16.

Armas, L. F. De & G. Alayón Garcia, 1984. Sinopsis de los aracnidos cavernicolas de Cuba (excepto acaros). Zoologie, 276: 1-25.

Aydın, G. & İ. Şen, 2020. Determination of arthropod biodiversity and some ecological parameters of Erdal Şekeroğlu (Isparta, Turkey) and Kadıini (Antalya, Turkey) cave ecosystems with evaluation of usability of insects in cave mapping. Turkish Journal of Entomology, 44 (4): 539-557.

Barnes, J. K., M. E. Slay & S. J. Taylor, 2009. Adult Diptera from Ozark Caves. Proceedings of the Entomological Society of Washington, 111 (2): 335-353.

Barr, T. C., 1968. "Cave Ecology and the Evolution of Troglobites, 35-102". In: Evolutionary Biology, Vol. 2 (Eds. T. Dobzhansky, M. K. Hecht & W. C. Steere) Springer, Boston, MA, USA, 452 pp.

Bilgin, S., O. Özgül & H. Koç, 2015. Türkiye için beş yeni kayıt ile Sündiken Dağları (Eskişehir) Limoniidae (Diptera) faunasına katkılar. Türkiye Entomoloji Bülteni, 5 (1): 3-9 (in Turkish with abstract in English).

Bordoni, A., 2010. Catalogue of the Staphylinidae of Cyprus and Asia Minor (Coleoptera). Fragmenta Entomologica, 42 (1): 35-348.

Bozcu, A., 2007. Zindan mağarası ve çevresinin jeolojik -arkeolojik özellikleri. Fen Bilimleri Enstitüsü Dergisi, 11 (1): 54-63 (in Turkish with abstract in English).

Brignoli, P. M., 1972. Terzo contributo alla conoscenza dei ragni cavernicola di Turchia (Araneae). Fragmenta Entomologica, 8 (3): 161-190.

Brignoli, P. M., 1978. Ragni di Turchia V. Specie nuove o interessanti, cavernicole ed epigee, di varie famiglie (Araneae). Revue Suisse de Zoologie, 85 (3): 461-541.

Cooper, S., J. Bradbury, K. M. Saint, R. Leys, A. D. Austin & W. F. Humphreys, 2007. Subterranean archipelago in the Australian arid zone: mitochondrial DNA phylogeography of amphipods from central Western Australia. Molecular Ecology, 16 (7): 1533-1544.

Corallini, C. & O. Marchetti, 2016. Parasitic infestation in a female of *Micropterna nycte*robia McLachlan 1875 (Trichoptera, Limnephilidae) found in Monte Cucco cave (Central Italy). Zoosymposia, 10 (1): 165-171.

Čuchta, P., D. Miklisová & Ľ. Kováč, 2019. The succession of soil Collembola communities in spruce forests of the High Tatra Mountains five years after a windthrow and clear-cut logging. Forest Ecology and Management, 433: 504-513.

Dániel, W. & T. G. Nándor, 2012. Eco-faunistic study on the Collembola fauna in the Vasvár-Nagymákfa area (Őrség, Western Hungary). Natura Somogyiensis, 22: 39-52.

Dányi, L. & G. Traser, 2008. An annotated checklist of the springtail fauna of Hungary (Hexapoda: Collembola). Opuscula Zoologica Instituti Zoosystematici et Oecologici Universitatis Budapestinensis, 38: 3-82.

Darılmaz, M. & S. Kıyak, 2006. A contribution to the knowledge of the Turkish water beetles fauna (Coleoptera). Munis Entomology & Zoology, 1 (1): 129-144.

Deltshev, C., 2008. Faunistic diversity and zoogeography of cave-dwelling spiders on the Balkan Peninsula. Advances in Arachnology and Developmental Biology, 12: 327-348.

Di Russo, C., M. Rampini & I. Landeck, 2007. The cave crickets of northeast Turkey and trans Caucasian regions, with descriptions of two new species of the genera Dolichopoda and *Troglophilus* (Orthoptera, Rhaphidophoridae). Journal of Orthoptera Research, 16 (1): 67-76.

Driauach, O. & B. Belqat, 2016. Additions to the Limoniidae and Pediciidae Fauna of Morocco, with an updated checklist (Diptera, Tipuloidea). ZooKeys, 563: 129-146.

Ellis, W. N., 1974. The spring fauna of Collembola (Insecta) from Rhodos, with descriptions of some new taxa. Beaufortia, 292 (22): 105-152.

Enghoff, H., 2006. The millipedes of Turkey (Diplopoda). Steenstrupia, 29 (2): 175-198.

Erguvanlı, K., 1975. Türkiye'de Miralay Dr. Abdullah Bey'in jeoloji öğretiminde ve araştırmalarında öncülüğü. Türk Mikrobiyoloji Cemiyeti Dergisi, 5: 19-28 (in Turkish with abstract in English).

Fidan, E. C., Ş. Ümit & H. Çalışkan, 2014. Second record of rare cave-dwelling beetle, *Choleva (Cholevopsis) bertiae* Giachino & Vailati, 2000 (Coleoptera: Leiodidae), from Tütüncüini and Sarikaya caves in Eskişehir. Hacettepe Journal of Biology and Chemistry, 42 (4): 473-477.

Folsom, J. W., 1932. Hawaiian Collembola. Proceeding of Society of Hawaiian Entomology, 8 (1): 51-92.

Gasparo, F., 2007. Note su *Tegenaria percuriosa* Brignoli, 1972, con descrizione del maschio (Araneae, Agelenidae). Atti e Memorie della Commissione Grotte "E. Boegan", 41: 95-103.

Gibert, J. & L. Deharveng, 2002. Subterranean Ecosystems: A Truncated Functional Biodiversity. BioScience, 52 (6): 473-481.

Hüppop, K., 2012. "Adaptation to Low Food, 1-9" In: Encyclopedia of Caves. 2nd Edition (Eds. W. B. White & D. C. Culver). Elsevier, 966 pp.

Ibrahimi, H., A. Gashi & L. Grapci-Kotori, 2013. First records of the genus *Micropterna* Stein, 1873 (Insecta: Trichoptera) in Kosovo with distributional and ecological notes. Natura Croatica, 22 (1): 147-155.

Juan, C., M. T. Guzik, D. Jaume & S. J. Cooper, 2010. Evolution in caves: Darwin's 'wrecks of ancient life in the molecular era. Molecular Ecology, 19 (18): 3865-3880.

Juberthie, C. & V. Decu, 1994. Encyclopaedia Biospeologica. Société de Biospéologie, France, 834 pp.

Karadem, A., 2011. Cr-39 dedektörleri ile mağaralarda radon konsantrasyonu ölçümleri. Süleyman Demirel Üniversitesi, (Unpublished) Yüksek Lisans Tezi, Isparta, 65 s (in Turkish with abstract in English).

Karaman, B., 2007. Trabzon İli Sucul Coleoptera (Insecta) Faunası. Gazi Üniversitesi, (Unpublished) Yüksek Lisans Tezi, Ankara, 72 s.

Kaya, D. & S. Mitchell, 1985. The Sanctuary of the God Eurymedon at Tymbriada in Pisidia. Anatolian Studies, 35: 39-55.

Kaya, R., K. Kunt, Y. Marusik & İ. Uğurtaş, 2010. A new species of *Tegenaria* Latreille, 1804 (Araneae, Agelenidae) from Turkey. ZooKeys, 51: 1-16.

Kaya, R. S. & İ. H. Uğurtaş, 2007. A faunistic study on spiders (Araneae) of Terzioğlu Island (Uluabat Lake, Bursa). Journal of Biological and Environmental Sciences, 1 (1): 31-36.

Kıyak, S., M. C. Darilmaz, A. Salur & S. Canbulat, 2007. Diving beetles (Coleoptera: Dytiscidae, Noteridae) of the southwestern Anatolian region of Turkey. Munis Entomology & Zoology, 2 (1): 103-114.

Komonen, A. & S. Kataja-Aho, 2017. Springtails (Collembola) in meadows, pastures and road verges in Central Finland. Entomologica Fennica, 28 (3): 157-163.

Kopar, İ., 2009. Aladağlar'da [Orta Toroslar (Yahyalı-Kayseri)] iki fosil mağara: Zindan-i Köşk-1 ve Zindan-i Köşk - 2 mağaraları. Türk Coğrafya Dergisi, 53: 69-83 (in Turkish with abstract in English).

Kováč, Ľ., A. Mock, P. Ľuptáčik, V. Košel, P. Fenďa, J. Svatoň & P. Mašán, 2005b. "Terrestrial Arthropods of the Domica Cave System and the Ardovská Cave (Slovak Karst) - Principal Microhabitats and Diversity, 61-70". In: Contributions to Soil Zoology in Central Europe I (Eds. K. Tajovský, J. Schlaghamerský & V. Pižl). České Budějovice, Czech Republic, 221 pp.

Kováč, L., N. Kostúrová & D. Miklisová, 2005a. Comparison of collembolan assemblages (Hexapoda, Collembola) of thermophilous oak woods and *Pinus nigra* plantations in the Slovak Karst (Slovakia). Pedobiologia, 49 (1): 29-40.

Kunt, K. B., M. Elverici, E. A. Yağmur, & R. S. Özkütük, 2019. Kut gen. nov., a new troglomorphic spider genus from Turkey (Araneae, Dysderidae). Subterranean Biology, *32*: 95-109.

Kunt, B. K., A. E. Yağmur, S. Özkütük & S. Anlaş, 2010. Checklist of the cave dwelling invertebrates (Animalia) of Turkey. Biological Diversity and Conservation, 3 (2): 26-41.

Malicky, H. & G. Winkler, 1974. Untersuchungen über die Höhlenimmigration von *Micropterna nycterobia* (Trichoptera, Limnephilidae). Oecologia, 15 (4): 375-382.

Mammola, S., P. Cardoso, C. Ribera, M. Pavlek & M. Isaia, 2018. A synthesis on cave-dwelling spiders in Europe. Journal of Zoological Systematics and Evolutionary Research, 56 (3): 301-316.

Manenti, R., E. Lunghi & G. F. Ficetola, 2015. The distribution of cave twilight-zone spiders depends on microclimatic features and trophic supply. Invertebrate Biology, 134 (3): 242-251.

Marusik, Y., R. Özkütük & K. B. Kunt, 2012. Spiders (Araneae) new to the fauna of Turkey. 10. Two new species records of Tetragnathidae. Life Sciences and Biotechnology, 2 (2): 69-73.

Moretti, G. & F. Gianotti, 1964. Considerazioni biogeografiche sul popolamento cavernicolo dei Tricotteri. Archivio botanico e biogeografico italiano, (IV serie), 9 (4): 341-348.

Nazik, L. & S. Bayarı, 2018. Mağara zengini ülke: Türkiye. Mavi Gezegen, 24: 7-19 (in Turkish with abstract in English).

Nazik, L., K. Törk, K. Tuncer, E. Özel, H. İnan & F. Savaş, 2005. "Türkiye mağaraları, 31-46". Ulusal Mağara Günleri Sempozyumu Bildiriler Kitabı (24-26 Haziran 2005, Beyşehir, Konya), Türkiye Tabiat Koruma Derneği, 46 s (in Turkish).

Özata, M. A., H. Sevgili & I. J. Kaprus, 2017. New records of springtail fauna (Hexapoda: Collembola: Entomobryomorpha) from Ordu Province in Turkey. Turkish Journal of Zoology, 41 (1): 24-32.

Özgenç, F., 2011. Çorum İli Dytiscidae (Coleoptera) Familyası Türlerinin Erkek Genitalinin Araştırılması. Gazi Üniversitesi, (Unpublished) Yüksek Lisans Tezi, Ankara, 71 s (in Turkish with abstract in English).

Özgül, O., H. Koç, M. Barlas & A. Tonguç, 2009. Güneybatı Anadolu Bölgesi Limoniidae (Diptera, Nematocera) faunası. Selçuk Üniversitesi Fen Fakültesi Fen Dergisi, 2 (33): 61-66 (in Turkish with abstract in English).

Page, T. J., W. F. Humphreys, & J. M. Hughes, 2008. Shrimps down under: evolutionary relationships of subterranean crustaceans from Western Australia (Decapoda: Atyidae: Stygiocaris). Plos One, 3 (2): e1618.

Palmer, M. W., 1991. Estimating species richness: the second-order jackknife reconsidered. Ecology, 72 (4): 1512-1513.

Parimuchová, A., L. P. Dušátková, L. Kováč, T. Macháčková, O. Slabý & S. Pekár, 2021. The food web in a subterranean ecosystem is driven by intraguild predation. Scientific Reports, 11 (1): 1-11.

Peck, S. B., A. E. Ruiz-Baliú & G. F. Garcés González, 1998. The cave-inhabiting beetles of Cuba (Insecta: Coleoptera): Diversity, distribution and ecology. Journal of Cave and Karst Studies, 60 (3): 156-166.

Popa, I., 2012. Two new records of springtails (Hexapoda: Collembola) for the Romanian fauna - Maramureş county. Travaux de l'Institut de Speologie "Emile Racovitza", 51: 73-79.

Popa, I. & V. Šustr, 2017. First record of *Lipothrix italica* (Cassagnau, 1968) (Hexapoda : Collembola) in Romania. Travaux de l'Institut de Speologie "Emile Racovitza", 56: 51-55.

Porco, D., M. Potapov, A. Bedos, G. Busmachiu, W. M. Weiner, S. Hamra-Kroua & L. Deharveng, 2012. Cryptic diversity in the ubiquist species *Parisotoma notabilis* (Collembola, Isotomidae): A Long-Used Chimeric Species? Plos One, 7 (9): e46056.

Potapov, M., 2001. Synopses on Palaearctic Collembola Pt 3: Isotomidae. Staatliches Museum für Naturkunde, Görlitz, 603 pp.

Poulson, T. L. & W. B. White, 1969. The cave environment. Science, 165 (3897): 971-981.

Prokopenko, E. V., 2019. The first record of *Lepthyphantes centromeroides* Kulczyński, 1914 (Aranei: Linyphiidae) from the Ukrainian Carpathians. Kavkazskij Entomologiceskij Bulleten, 15 (1): 23-24.

Rad, A. A. & K. Morteza, 2015. The first study of Isotomidae fauna (Collembola: Apterygota) in Lorestan Province, Western Iran, with new records for Iranian fauna. Journal of Biodiversity and Environmental Sciences (JBES), 7 (1): 128-133.

Rampini, M. & C. Di Russo, 2003. Una nuova specie di Troglophilus di Turchia (Orthoptera, Rhaphidophoridae). Fragmenta Entomologica, 34 (2): 235-247.

Raschmanová, N., M. Žurovcová, Ľ. Kováč, L. Paučulová, V. Šustr, A. Jarošová & D. Chundelová, 2017. The cold-adapted population of *Folsomia manolachei* (Hexapoda, Collembola) from a glaciated karst doline of Central Europe: evidence for a cryptic species? Journal of Zoological Systematics and Evolutionary Research, 55 (1): 19-28.

Rendoš, M., N. Raschmanová, Ľ. Kováč, D. Miklisová, A. Mock & P. Ľuptáčik, 2016. Organic carbon content and temperature as substantial factors affecting diversity and vertical distribution of Collembola on forested scree slopes. European Journal of Soil Biology, 75: 180-187.

Romero, A., 2009. Cave Biology: Life in Darkness Cave Biology. Cambridge University Press, Cambridge. 306 pp.

Ruffo, S., 1957. Le attuali conoscenze sulla fauna cavernicola della Regione Pugliese. MemBiogeogr Adriat, 3:1-43.

Scheers, K. & S. Thant, 2017. *Agabus didymus* (Olivier, 1795) and *A. dilatatus* (Brullé, 1832), two species new to the fauna of Croatia with notes on the ecology and distribution of both species (Coleoptera: Dytiscidae). Bulletin de la Société royale belge d'Entomologie/Bulletin van de Koninklijke Belgische Vereniging voor Entomologie, 153 (1): 54-58.

Sevgili, H. & M. A. Özata, 2014. Checklist of the springtails (Hexapoda: Collembola) of Turkey. Zoology in the Middle East, 60 (2): 162-168.

Shayanmehr, M., E. Yahyapour, M. Kahrarian & E. Y. Lafooraki, 2013. An introduction to Iranian Collembola (Hexapoda): an update to the species list. ZooKeys, 335: 69-83.

Sket, B., 2008. Can we agree on an ecological classification of subterranean animals? Journal of Natural History, 42 (21-22): 1549-1563.

Taylan, M. S., 2011. Türkiye Mağara Çekirgeleri (Orthoptera, Rhaphidophoridae): Morfolojik ve Moleküler Veriler Işığında Taksonomi, Filogeni ve Biyocoğrafyaları. Akdeniz Üniversitesi, (Unpublished) Doktora Tezi, Antalya, 203 s (in Turkish with abstract in English).

Trajano, E., 2012. "Ecological Classification of Subterranean Organisms, 275-277". In: Encyclopedia of Caves (Eds. W. B. White & D. C. Culver). Elsevier, Amsterdam, 945 pp.

Vafaei, R., M.C. Darilmaz, E. Nazari, U. Incekara & N. Piazak, 2009. Contributions to the knowledge of Iranian aquatic coleoptera fauna (Dytiscidae, Gyrinidae, Helophoridae and Hydrophilidae) with new records and notes on the rare species Coleostoma Transcaspicum Reitter, 1906. Acta Entomologica Serbica, 14 (1): 101-107.

Von Saltzwedel, H., S. Scheu & I. Schaefer, 2017. Genetic structure and distribution of *Parisotoma notabilis* (Collembola) in Europe: Cryptic diversity, split of lineages and colonization patterns. Plos One, 12 (2): e0170909.

Vrenozi, B. & P. Jäger, 2012. A faunistic study on ground-dwelling spiders (Araneae) in the Tirana district, Albania. Arachnologische Mitteilungen, 44 (7): 81-87.

Yamaç, A., É. Gilli, E. Tok & K. Törk, 2021. Caves and Karst of Turkey-Vol. 1: History, Archaeology and Caves. Springer, Switzerland AG, 108 pp.

Zacharda, M., D. Fong, H. H. Hobbs III, E. Piva, M. E. Slay & S. J. Taylor, 2010. A review of the genus *Traegaardhia* (Acari, Prostigmata, Rhagidiidae) with descriptions of new species and a key to species. Zootaxa, 2474 (1): 1-64.

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Original article (Orijinal araştırma)

Host suitability of wheat cultivars to *Pratylenchus thornei* Sher & Allen, 1953 and *Pratylenchus neglectus* (Rensch, 1924) (Tylenchida: Pratylenchidae)

Buğday çeşitlerinin *Pratylenchus thornei* Sher & Allen, 1953 ve *Pratylenchus neglectus* (Rensch, 1924) (Tylenchida: Pratylenchidae)'a karşı konukçu uygunluğu

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Abstract

Root lesion nematodes (RLNs), *Pratylenchus thornei* Sher & Allen, 1953 and *Pratylenchus neglectus* (Rensch, 1924) (Tylenchida: Pratylenchidae) are important plant-parasitic nematodes that cause economic yield losses in wheat cropping systems worldwide. The use of resistant and tolerant cultivars is the most effective method to control these nematodes in wheat. There are currently no commercial wheat cultivars identified as completely resistant to the RLN species. The aim of this research was to evaluate 19 Turkish spring wheat cultivars for reaction to *P. thornei* and *P. neglectus* under *in-vitro* conditions over 16 weeks in 2019. In the result of the study, nine wheat cultivars (Adana99, Ata89, Bürküt, Cumhuriyet75, Gönen98, Marmara86, Meta2002, Troya and Uludağ) were found to have moderate resistance against *P. thornei*, whereas five wheat cultivars (Adana99, Alibey, Ata89, Ceyhan99 and Uludağ) were moderately resistant to *P. neglectus*. The study also showed that Adana99, Ata89 and Uludağ are resistant to both nematode species, and these cultivars, thus, are considered to be excellent sources of genes for further development RLN resistant commercial wheat cultivars.

Keywords: *Pratylenchus neglectus, Pratylenchus thornei*, resistance, root lesion nematode, wheat

Öz

Kök yara nematodları (RLN), *Pratylenchus thornei* Sher & Allen, 1953 ve *Pratylenchus neglectus* (Rensch, 1924) (Tylenchida: Pratylenchidae) dünya genelinde buğday yetiştiriciliğinde ekonomik ürün kayıplarına neden olan önemli bitki paraziti nematodlarıdır. Dayanıklı ve tolerant çeşit kullanımı bu nematodlar ile mücadelede en etkin metot olarak bilinmektedir. Günümüzde, RLN türlerine karşı tamamen dayanıklı ticari herhangi bir çeşit yoktur. Bu çalışmada 19 adet yazlık buğday çeşidinin kontrollü koşullarda 16 hafta süreyle *P. thornei* ve *P. neglectus*'a karşı reaksiyonları 2019 yılında değerlendirilmiştir. Çalışma sonucunda Adana99, Ata89, Bürküt, Cumhuriyet75, Gönen98, Marmara86, Meta2002, Troya ve Uludağ'ın içinde olduğu dokuz buğday çeşidi *P. thornei*'ye karşı orta dayanıklı, buna karşın Adana99, Alibey, Ata89, Ceyhan99 ve Uludağ'ın içinde olduğu beş buğday çeşidi *P. neglectus*'a karşı orta dayanıklı bulunmuştur. Ayrıca çalışmada Adana99, Ata89 ve Uludağ'ın her iki nematod türüne dayanıklılık gösterdiği ve bu çeşitlerin RLN'e karşı dayanıklı ticari buğday çeşitleri geliştirmek için oldukça iyi dayanıklılık gen kaynakları olduğu düşünülmektedir.

Anahtar sözcükler: *Pratylenchus neglectus, Pratylenchus thornei*, dayanıklılık, kök yara nematodu, buğday

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Introduction

Wheat is the most important food crop and accounts for almost one-third of the world's edible grain production (FAOSTAT, 2020). In Turkey, wheat is cultivated mostly in arid and semiarid areas with an average size of 9.83 Mha (TUIK, 2019). There is variability caused by abiotic and biotic stress factors in the amount of production; however, total wheat grain production reached 19 Mt in 2019 (FAOSTAT, 2020). Unfortunately, the production is still inadequate to meet nutritional requirements by a growing population (Sirat & Sezer, 2014). Also, wheat production is often subjected to various constraints, such as lack of fertilizer and irrigation water, and soilborne pathogens including several plant-parasitic nematodes (Shroyer et al., 1990; Dababat & Fourie, 2018).

Root lesion nematodes (RLNs), *Pratylenchus*, are migratory plant-parasitic nematodes that are economically important in many crops, including cereals, and have spread to agricultural soils around the world (Nicol & Ortiz-Monasterio, 2004; Thompson, 2008; Moens & Perry, 2009; Mokrini et al., 2016; Thompson et al., 2017). Wheat is severely affected by several RLNs species of which *Pratylenchus thornei* Sher & Allen, 1953 and *Pratylenchus neglectus* (Rensch, 1924) (Tylenchida: Pratylenchidae) are the most destructive (Yu et al., 2012; Dababat et al., 2016). *Pratylenchus thornei* and *P. neglectus* have a broad host ranges including cereals and legumes (O'Brien, 1982; Vanstone & Russ, 2001a, b; Owen et al., 2014). Yield losses caused by *P. neglectus* in Europe, North America and Australia have not been fully investigated; nevertheless, 16-23% yield loss has been recorded in southern Australia (Taylor et al., 1999). Also, the spring wheat yield losses associated with *P. neglectus* populations in Oregon, USA were 36-85% (Smiley et al., 2005; Smiley, 2010). In contrast, 38-85% wheat yield loss due to *P. thornei* is reported in Australia, 50% in Oregon, USA, 12-37% in Mexico and 70% in Israel (Nicol et al., 2004), as well as 19-32% in Turkey (Toktay, 2008).

Management of root lesion nematodes is difficult for several reasons. Adult female RLNs are highly fecund and can survive/feed in numerous places: in the soil, in the root fragments or feed on weeds for extended periods. Growing resistant cultivars, applying chemicals, cultural practices and biological control are the main methods that have been used to control root lesion nematodes (Urwin et al., 1997). The effectiveness of these methods to control plant-parasitic nematodes increases when used in combination. The ideal control approach relies upon the availability of resistant cultivars and the value of the crop (Riggs & Schuster, 1997). Plants, where nematodes cannot multiply are considered completely resistant whereas plants, where nematodes can freely multiply, are defined as non-resistant or susceptible (Cook & Evans, 1987). Tolerant plants are described as plants that are poorly damaged by severe nematode infection and intolerant plants are described as plants that are severely damaged (Cook & Evans, 1987).

Although, plant resistance does not exist in many crops plants and efficiency is frequently limited to a few races of a nematode species. This situation may result in cultivars becoming susceptible to virulent nematode biotypes or related species in field populations (Gheysen et al., 1996; Whitehead, 1998). Using more widely-based resistance would lessen this problem for resistant cultivars. A few reports indicate that *P. thornei* and *P. neglectus* often occur together in the major wheat cultivating areas of Turkey (Sahin et al., 2008; İmren et al., 2017; Dababat et al., 2018). The major objective of this study was to find novel sources of resistance to *P. thornei* and *P. neglectus* among a core set of spring wheat cultivars for further pyramiding into elite cultivars. The current research aimed to (1) examine the host suitability of wheat cultivars to *P. thornei* and *P. neglectus* and (2) provide a common wheat pool with RLN resistance information to producers.

Materials and Methods

Plant material

The 19 spring wheat cultivars obtained from institutes of the General Directorate of Agricultural Research and Policies (Eastern Aegean Agricultural Research Institute, Field Crops Central Research Institute, Mediterranean Agricultural Research Institute and GAP Agricultural Research Institute) were evaluated for their suitability to *P. thornei* and *P. neglectus* (Table 1). Durum wheat cv. Gatcher and GS50a were used as susceptible and resistant check lines, respectively (Thompson et al., 2008). Two independent experiments were performed for phenotyping cultivars against *P. thornei* (Experiment 1) and *P. neglectus* (Experiment 2) under *in-vitro* conditions in a growth room at 21 ± 3°C with a 16:8 h L:D photoperiod for 16 weeks. Seeds of each cultivar were surface-disinfested with 70% ethanol for 1 min, washed in twice sterilized water, air dried, transferred to Petri dishes containing moistened filter paper and maintained for 3-4 days at 23-25°C to promote germination. A germinated seed of each cultivar with three seminal roots were transplanted to plastic tubes (1.5 x 12 cm) filled with a pasteurized mix of field soil and sand (1:3 v/v). Seven replicates of each cultivar were arranged in a randomized block design.

Nematode inoculum

Pathogenicity experiments were conducted with populations of *P. thornei* (PT18) and *P. neglectus* (PN2) obtained from the provincial center and Gerede District, Bolu Province in 2019, respectively (Dababat et al., 2019). The carrot discs were used to culture and maintain nematode populations *in-vitro* according to Moody et al. (1973). Nematode growth on the carrot discs was monitored and when the desired density was reached, the Baermann-funnel method was use to collect nematodes from chopped carrot discs in a moist chamber for 3 days (OEPP/EPPO, 2013). The nematode suspension was used to inoculate seedlings 1 week after planting, at a density of 400 individuals/ml of water (Keil et al., 2009; Toktay et al., 2012). The suspension of each nematode was pipetted into three 2-cm deep holes at a distance of 0.5 cm from the seedling. The plants were kept in the growth chamber and watered daily. The plants were fertilized with liquid fertilizer [NPK(Mg), 15-8-15-(2), Spiess-Urania Chemicals GmbH, Hamburg, Germany] at 3 and 6 weeks after planting.

Assessment of resistance

The experiment was terminated 16 weeks after the inoculation, and a modified Baermann funnel method was used to extract motile nematodes (juveniles and adults) from 80 g of soil and roots from each tube (Hooper, 1986). To the nematode density of the suspension was determined for three subsamples from each tube under a stereomicroscope (Zeiss Stemi 305, Carl Zeiss, Jena, Germany) at 64x magnification. For the evaluation of host suitability to *P. thornei* and *P. neglectus*, the reproduction factor (*Rf*) was calculated as $Rf = Pf/Pi$, where *Pf* is the final population and *Pi* is the initial population in the tube (400 in these experiments). If no nematodes were extracted from the soil and plant roots, the cultivar were considered resistant. An *Rf* of less than 1 was considered to be moderately resistance and 1 or more as susceptible (Thompson et al., 2008; Keil et al., 2009; Toktay et al., 2012). The experimental data were analyzed using a one-way analysis of variance. The mean number of nematodes was separated using Tukey's test at a significance level of $P < 0.05$ using the SPSS statistics package (SPSS version 20.0 for Windows; IBM, Armonk, NY, USA), the principal component analysis performed using XLSTAT 2016.02.28451 software (Addinsoft, Paris, France).

Results and Discussion

Pratylenchus thornei and *P. neglectus* survived and/or increased on all wheat cultivars, including the check lines. All wheat cultivars tested for resistance to *P. thornei* and *P. neglectus* were moderately resistant or susceptible to both nematode populations, but none of the cultivars were resistant to these nematodes (Table 1). The number of individuals *P. thornei* and *P. neglectus* calculated for each cultivar ranged from 106 to 1,980 and from 100 to 3,495, respectively. There was also a significant difference ($P < 0.001$) in the host suitability of wheat cultivars to *P. thornei* and *P. neglectus*. The *Rf* ranged from 0.43 to 6.06 for across both species. In the susceptible check, Gatcher, the average final numbers of *P. thornei* and *P. neglectus* per plant were 1,116 and 1,826, respectively, whereas in the moderately resistant check (GS50a) they were 194 and 286, respectively.

Table 1. Host suitability of tested wheat cultivars to root lesion nematodes

Cultivar	*Pratylenchus thornei*				*Pratylenchus neglectus*			
	Nematodes*		*Rf*	Phenotype	Nematodes*		*Rf*	Phenotype
Adana99	200 ± 33.5 (140-216)	fg	0.50	MR	172 ± 67.6 (110-280)	d	0.43	MR
Alibey	1032 ± 113.2 (684-1273)	bc	2.58	S	240 ±102.2 (120-400)	d	0.60	MR
Altınbaşak	1622 ± 371.7 (1000-1980)	a	3.83	S	464 ± 35.7 (320-860)	d	1.3	S
Ata89	332 ± 23.9 (298-354)	dg	0.83	MR	232 ± 107.9 (160-420)	d	0.58	MR
Basribey95	592 ± 23.0 (580-628)	dg	1.48	S	1488 ± 626.0 (1110-2600)	bc	3.72	S
Bürküt	340 ± 119.1 (144-427)	dg	0.85	MR	832 ± 486.1 (400-1600)	cd	2.08	S
Ceyhan99	516 ± 58.9 (440-580)	dg	1.29	S	296 ± 101.1 (140-420)	d	0.94	MR
Cumhuriyet75	276 ± 44.9 (204-324)	eg	0.69	MR	552 ± 180.1 (230-640)	d	1.38	S
Gökkan	708 ± 52.8 (653-764)	cd	1.77	S	2152 ± 430.4 (1800-2880)	ab	5.38	S
Gönen98	324 ± 80.6 (285-468)	dg	0.81	MR	674 ± 282.0 (360-1110)	cd	1.68	S
İzmir85	1404 ± 303.9 (869-1621)	ab	3.51	S	560 ± 448.3 (320-1360)	d	1.40	S
Kakliç88	1612 ± 482.8 (756-1928)	a	4.03	S	416 ± 270.3 (140-860)	d	1.64	S
Kaşifbey95	644 ± 80.2 (542-765)	dg	1.61	S	2316 ± 635.4 (1600-3290)	ab	5.79	S
Marmara86	180 ± 46.9 (120-228)	g	0.45	MR	472 ± 238.1 (120-700)	d	1.18	S
Menemen	756 ± 12.5 (564-894)	cd	1.89	S	504 ± 225.3 (330-870)	d	1.26	S
Meta2002	392 ± 26.7 (362-426)	dg	0.98	MR	548 ± 190.1 (410-860)	d	1.37	S
Troya	372 ± 108.3 (258-524)	dg	0.93	MR	821 ± 249.0 (512-1134)	cd	2.94	S
Uludağ	186 ± 61.2 (106-276)	g	0.46	MR	198 ± 87.7 (100-340)	d	0.49	MR
Ziyabey98	1344 ± 116.3 (1200-1463)	ab	3.36	S	2424 ± 794.1 (1600-3495)	a	6.06	S
GS50a (R control)	194 ± 42.2 (160-265)	fg	0.58	MR	286 ± 70.6 (160-320)	d	0.74	MR
Gatcher (S control)	1116 ± 419.9 (423-1421)	bc	2.79	S	1826 ± 33.8 (624-2326)	ab	3.02	S

* Mean ± SD (range). Means followed by the same letter within columns are not significantly different ($P < 0.05$, Tukey's test), *Rf,* reproduction factor*;* MR, moderately resistant; S, susceptible.

Cvs Marmara86 and Uludağ had the lowest average number of *P. thornei* at 180 and 186, respectively and cvs Altınbaşak and İzmir85 had the highest average at 1,622 and 1,612, respectively (Table 1). The *Rf* of *P. thornei* on the 19 cultivars ranged from 0.45 (Marmara86) to 4.03 (Kakliç88). Nine cultivars (Adana99, Ata89, Bürküt, Cumhuriyet75, Gönen98, Marmara86, Meta2002, Troya and Uludağ) had *Rf* of <1, with Marmara86 having the lowest value.

Three groups were apparent among the 21 cultivars assessed (including the two check lines) based on the resistance to *P. thornei* (Figure 1). The first group (moderately resistant) included GS50a and nine moderately resistant cultivars: Adana99, Ata89, Bürküt Cumhuriyet75, Gönen98, Marmara86, Meta2002, Troya and Uludağ. The second group (S-I) included Gatcher and six susceptible cultivars: Gökkan, Basribey95, Kaşifbey95, Ceyhan99, Menemen and Alibey. The *Rf* of S-I was >1 but, lower than the *Rf* of Gatcher (2.79). The final group (S-II) included four susceptible cultivars: Altinbaşak, İzmir85, Kakliç88, and Ziyabey98. The *Rf* of S-II was higher than the *Rf* of Gatcher (Figure 1).

Cvs Adana99, Marmara86 and Uludağ had lower numbers of *P. thornei* than GS50a (Figure 1) (Table 1). Cvs Ata89, Bürküt, Cumhuriyet75, Gönen98, Meta2002 and Troya had significantly lower *Rf* (<1) and higher numbers than GS50a (*Rf* = 0.58). Cvs Basribey, Ceyhan99, Gökkan, Kaşifbey95, Menemen, grouped in S-I for *P. thornei*, had *Rf of* >1 and lower nematodes than Gatcher. Cvs Ziyabey98. Altinbaşak, İzmir85 and Kakliç88 had a higher number of nematodes than Gatcher and were grouped in S-II for *P. thornei.*

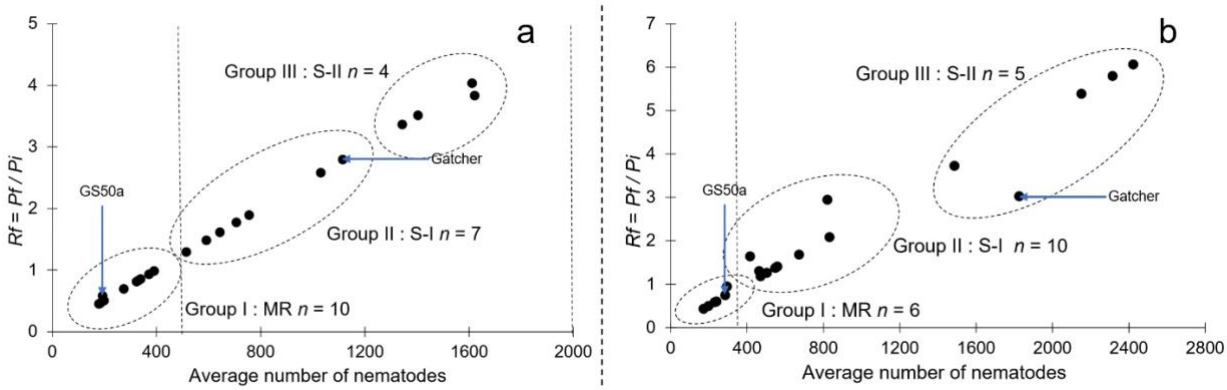

Figure 1. Principal component analysis (Kendall type) showing the plant population structure for a set of GS50a and Gatcher replicates based on their resistance reaction to a) *Pratylenchus thornei* and b) *Pratylenchus neglectus*.

For *P. neglectus,* the lowest numbers extracted where for cvs Adana99 and Uludağ were 172 and 198, respectively (Table 1) and the highest at 2,424 and 2,316 for cvs Ziyabey98 and Kaşifbey95, respectively. The *Rf* for *P. neglectus* ranged from 0.43 (Adana99) to 6.06 (Ziyabey98). The *Rf* values of the five cultivars were <1 and was the lowest for cv. Adana 99 (*Rf* = 0.43).

The phenotyping of *P. neglectus* revealed three groups of cultivars (Figure 1). The first group included GS50a and five moderately resistant cultivars: Ata89, Alibey, Adana99, Ceyhan99 and Uludağ. The second group (S-I) included 9 susceptible cultivars: Altınbaşak, Bürküt, Cumhuriyet75, Gönen98, İzmir85, Kakliç88, Marmara86, Menemen, Meta2002 and Troya. The *Rf* of these was >1 but, lower than the *Rf* value for Gatcher (3.02). The final group (S-II) included Gatcher and four susceptible cultivars: Basribey95, Gökkan, Kaşifbey95 and Ziyabey98. The *Rf* of the S-II group was higher than the *Rf* of Gatcher.

Cvs Ata89, Adana99, Alibey, and Uludağ had lower numbers of *P. neglectus* than GS50a (Table 1). Ceyhan99 had a significantly lower *Rf* (<1) and higher number of nematodes than GS50a. Cvs Altinbaşak, Bürküt, Cumhuriyet75, Gönen98, İzmir85, Kakliç88, Marmara86, Menemen, Meta2002 and Troya had significantly higher *Rf* (>1) and less nematodes than Gatcher. Cvs Basribey, Gökkan, Kaşifbey95 and Ziyabey98 had more nematodes than Gatcher and were grouped as S-II for *P. neglectus*.

The present study determined the host responses of 19 spring wheat cultivars to the two RLNs as moderately resistant and susceptible. RLN resistance in wheat has been studied in either the field or in pots (greenhouse and growth chamber) (Thompson et al., 2015; Dababat et al., 2016). A certain degree of resistance to *P. thornei* has been identified in India (Kranti & Kanwar, 2012), Australia (Thompson & Seymour, 2011) and Turkey (Toktay et al., 2012). For example, several lines were found to be resistant to the Indian population of *P. thornei* among 20 wheat lines (Kranti & Kanwar, 2012), and consistent with the present studies, some wheat lines were moderately resistant to both *P. thornei* and *P. neglectus*. In particular, three cultivars (Ata89, Adana99 and Uludağ) had moderately resistant reactions both of RLN species. This is useful as these species often occur together in wheat fields (Thompson et al., 2010). Cvs Adana99 and Ceyhan99 showed useful resistance to the tested nematodes, which confirms the result of the studies of Toktay et al. (2012), who found resistance in these cultivars. This information allows growers to choose tolerant/resistant crops when both RLN species are present.

As the result of the current study, three wheat cultivars (Marmara86, Adana99 and Uludağ) reduced *P. thornei* densities below that with GS50a showing moderate resistance to *P. thornei.* Also, four wheat cultivars (Ata89, Adana99, Alibey and Uludağ) reduced *P. neglectus* densities below that with GS50a, which is moderately resistant to *P. neglectus.* Numerous sources of resistance to *P. thornei* and *P. neglectus* have been described in wheat germplasm (Thompson & Haak, 1997; Vanstone et al., 1998; Thompson et al., 1999, 2009; Taylor et al., 2000; Toktay et al., 2012). For example, GS50a was reported to be the first source of resistance to *P. thornei* in Australia, which was primarily selected from the cultivar of Gatcher (Thompson & Clewett, 1986). Ten times less RLN reproduction was found in GS50a in comparison to local control lines (Thompson et al., 1999). A reasonable number of Iranian landraces of wheat were assessed for resistance to RLN and 25 of those accessions showed more resistance than GS50a (Sheedy & Thompson, 2009). Similarly, Thompson et al. (2009) performed the screening experiment with wheat accessions from North Africa and West Asian regions and found some additional sources resistance to *P. thornei*.

Mapping of QTLs and phenotypic identification of resistance sources have been used to identify resistance sources to RLNs. The QTLs linked to resistance to *P. thornei* resistance are mapped on different chromosomes of bread wheat (Schmidt et al., 2005; Zwart et al., 2005). The *Rlnn1* locus which is located on the 7A chromosome offers substantial resistance to *P. neglectus* at the seedling stage (Williams et al., 2002). According to Williams et al. (2002), *Rlnn1* originated from an Australian wheat cv. Excalibur, which has been validated for its better resistance to *P. neglectus*. Similarly, another locus conferring resistance to *P. neglectus* has been characterized and identified on the 4D chromosome (Zwart et al., 2005). The relationships between resistance reactions and markers were adequately constant to demonstrate the value of using the marker selection to increase *Pratylenchus* resistance in wheat. The *Rlnn1* marker has been successfully used in this way and is actively implemented as part of international wheat breeding programs in CIMMYT at a global level and in Australia (Williams et al., 2002).

Wheat breeding routinely aims to increase the level of durable resistance of wheat to gain a reasonable yield even in soils with high nematode population densities. To date, several genotypes having RLN-resistance have been identified from the International Winter Wheat Improvement Program (www.iwwip.org) sources, but the genetic basis of resistance is still unknown. Thus, it is important to understand the novelty of the resistance of these cultivars and to use this resistance in different genetic backgrounds by crosses and pyramids to achieve new resistance and high yields that increase grain yield for food security. This study has determined the resistance of some Turkish commercial wheat cultivars, with superior agronomic properties have already, to two RLNs. Therefore, it is suggested that the potentially useful resistance sources determined in the study should be included in breeding studies.

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References

Cook, R. & K. Evans, 1987. "Resistance and Tolerance, 179-231". In: Principles and Practice of Nematode Control in Crops (Eds. R. H. Brown & B. R. Kerry). Academic Press, Sydney, Australia, 447 pp.

Dababat, A. A., G. B. H. Ferney, G. Erginbas-Orakci, S. Dreisigacker, M. Imren, H. Toktay, H. Elekçioğlu, T. Mekete, J. M. Nicol, O. Ansari & F. Ogbonnaya, 2016. "Association analysis of resistance to cereal cyst nematodes (*Heterodera avenae*) and root lesion nematodes (*Pratylenchus neglectus* and *P. thornei*) in CIMMYT advanced spring wheat lines for semi-arid conditions. Breed Science, 66 (5): 692-702.

Dababat, A. A. & H. Fourie, 2018. "Nematode Parasites of Cereals, 163-221". In: Plant Parasitic Nematodes in Subtropical and Tropical Agriculture (Eds. R. A. Sikora, D. Coyne, J. Hallmann & P. Timper). CAB International, Wallingford, UK, 877 pp.

Dababat, A., Ş. Yıldız, V. Ciftci, N. Duman & M. Imren, 2019. Occurrence and seasonal variation of the root lesion nematode *Pratylenchus neglectus* on cereals in Bolu, Turkey. Turkish Journal of Agriculture and Forestry, 43 (1): 21-27.

FAOSTAT, 2020. Food and agriculture organization statistical database. (Web page: http://www.fao.org/faostat/en/#data/QC) (Date accessed: March 2020).

Gheysen, G., W. Van der Eycken, N. Barhels, M. Karimi & M. Van Montagu, 1996. The exploitation of nematode-responsive genes in novel nematode control methods. Pesticide Science, 47 (1): 95-101.

Hooper, D. J., 1986. "Handling, Fixing, Staining and Mounting Nematodes, 59-80". In: Laboratory Methods for Work with Plant and Soil Nematodes (Ed. J. F. Southey). Her Majesty's Stationery Office, London, 202 pp.

Keil, T., E. Laubach, S. Sharma & C. Jung, 2009. Screening for resistance in the primary and secondary gene pool of barley against the root-lesion nematode *Pratylenchus neglectus*. Plant Breeding, 128 (5): 436-442.

Kranti, K. V. V. S. & R. S. Kanwar, 2012. Evaluation of wheat varieties for resistance against *Pratylenchus thornei* and effect of sowing dates on its reproduction. Indian Journal of Nematology, 42 (1): 34-37.

Moens, M. & R. N. Perry, 2009. Migratory plant endoparasitic nematodes: a group rich in contrasts and divergence. Annual Review of Phytopathology, 47 (1): 313-332.

Mokrini, F., L. Waeyenberge, N. Viaene, F. A. Andaloussi & M. Moens, 2016. Diversity of root-lesion nematodes (*Pratylenchus* spp.) associated with wheat (*Triticum aestivum* and *T. durum*) in Morocco. Nematology, 18 (7): 781-801.

Moody, E. H., B. F. Lownsbery & J. H. Ahmed, 1973. Culture of root-lesion nematode *Pratylenchus vulnus* on carrot discs. Journal of Nematology, 5 (3): 226-255.

Nicol, J., H. Elekçioğlu, N. Bolat & R. Rivoal, 2004. The global importance of the cereal cyst nematode (*Heterodera* spp.) on wheat and international approaches to its control. Communications in Agricultural and Applied Biological Sciences, 72 (3): 677-686.

Nicol, J. & I. Ortiz-Monasterio, 2004. Effects of the root-lesion nematode, *Pratylenchus thornei*, on wheat yields in Mexico. Nematology, 6 (4): 485-493.

O'Brien, P. C., 1982. A study on the host range of *Pratylenchus thornei*. Australasian Plant Pathology, 11 (3): 3-5.

OEPP/EPPO, 2013. PM7/119. Nematode extraction. OEPP/EPPO Bulletin, 43 (3): 471-495.

Owen, K. J., T. G. Clewett, K. L. Bell & J. P. Thompson, 2014. Wheat biomass and yield increased when populations of the root-lesion nematode (*Pratylenchus thornei*) were reduced through sequential rotation of partially resistant winter and summer crops. Crop and Pasture Science, 65 (3): 227-241.

Riggs, R. D. & R. P. Schuster, 1997. "Management, 388-416". In: The Cyst Nematodes (Ed. S. B. Sharma). Kluwer Academic Publishers, Dordrecht, The Netherlands, 452 pp.

Sahin, E., J. M. Nicol, A. Yorgancilar, I. H. Elekcioglu, A. Tulek, A. F. Yildirim & N. Bolat, 2008. Seasonal variation of field populations of *Heterodera filipjevi*, *Pratylenchus thornei* and *P. neglectus* on winter wheat in Turkey. Nematologia Mediterranea, 36 (1): 51-56.

Schmidt, A. L., C. L. McIntyre, J. Thompson, N. P. Seymour & C. J. Liu, 2005. Quantitative trait loci for root lesion nematode (*Pratylenchus thornei*) resistance in Middle-Eastern landraces and their potential for introgression into Australian bread wheat. Australian Journal of Agricultural Research, 56 (10): 1059-1068.

Sheedy, J. G. & J. P. Thompson, 2009. Resistance to the root-lesion nematode *Pratylenchus thornei* of Iranian landrace wheat. Australasian Plant Pathology, 38 (5): 478-489.

Sher, S. A. & M. W. Allen, 1953. Revision of the genus Pratylenchus (Nematoda: Tylenchidae). University of California Publications in Zoology, 57 (6): 441-470.

Shroyer, J. P., J. Ryan, M. A. Monem & M. E. Mourid, 1990. Production of fall-planted cereals in Morocco and technology for its improvement. Journal of Agronomic Education, 19 (1): 32-40.

Sirat, A. & İ. Sezer, 2014. Samsun ilinde arpa üretim potansiyeli. Güfbed/Gustij, 4 (2): 183-192.

Smiley, R. W., 2010. Root-Lesion Nematodes: Biology and Management in Pacific Northwest Wheat Cropping Systems. PNW Extension Bulletin Oregon State University, Corvallis, 617 pp.

Smiley, R. W., R. G. Whittaker, J. A. Gourlie, S. A. Easley & R. E. Ingham, 2005. Plant-parasitic nematodes associated with reduced wheat yield in Oregon: *Heterodera avenae*. Journal of Nematology, 37 (3): 297-307.

Taylor, S. L., M. E. Payton & W. R. Raun, 1999. Relationship between mean yield, coefficient of variation, mean square error, and plot size in wheat field experiments. Communications in Soil Science and Plant Analysis, 30 (9-10): 1439-1447.

Taylor, S. P., G. J. Hollaway & C. H. Hunt, 2000. Effect of field crops on population densities of *Pratylenchus neglectus* and *P. thornei* in southeastern Australia; Part 1: *P. neglectus*. Journal of Nematology, 32 (4S): 591-599.

Thompson, J. P., 2008. Resistance to root-lesion nematodes (*Pratylenchus thornei* and *P. neglectus*) in synthetic hexaploid wheats and their durum and *Aegilops tauschii* parents. Australian Journal of Agricultural Research, 59 (5): 432-446.

Thompson, F. J., G. L. Barker, L. Hughes & M. E. Viney, 2008. Genes important in the parasitic life of the nematode *Strongyloides ratti*. Molecular and Biochemical Parasitology, 158 (2): 112-119.

Thompson, J. P., P. S. Brennan, T. G. Clewett, J. G. Sheedy & N. P. Seymour, 1999. Progress in breeding wheat for tolerance and resistance to root-lesion nematode (*Pratylenchus thornei*). Australasian Plant Pathology, 28 (1): 45-52.

Thompson, J. P. & T. G. Clewett, 1986. Research on root lesion nematode: Occurrence and wheat varietal reaction. Queensland Wheat Research Institute Biennial Report, 24 (3): 32-35.

Thompson, J. P., T. G. Clewett & M. M. O'Reilly, 2015. Temperature response of root-lesion nematode (*Pratylenchus thornei*) reproduction on wheat cultivars has implications for resistance screening and wheat production. Annals of Applied Biology, 167 (1): 1-10.

Thompson, J. P., T. G. Clewett, J. G. Sheedy, R. A. Reen, M. M. O'reilly & K. L. Bell, 2010. Occurrence of root-lesion nematodes (*Pratylenchus thornei* and *P. neglectus*) and stunt nematode (*Merlinius brevidens*) in the northern grain region of Australia. Australasian Plant Pathology, 39 (3): 254-264.

Thompson, J. P. & M. I. Haak, 1997. Resistance to root-lesion nematode (*Pratylenchus thornei)* in *Aegilops tauschii* Coss., the D-genome donor to wheat. Australian Journal of Agricultural Research, 48 (5): 553-560.

Thompson, J. P., M. M. O'Reilly & T. G. Clewett, 2009. Resistance to the root-lesion nematode *Pratylenchus thornei* in wheat landraces and cultivars from the West Asia and North Africa (WANA) region. Crop and Pasture Science, 60 (12): 1209-1217.

Thompson, J. P., H. E., Rostad & J. P. M. Whish, 2017. Survival of root-lesion nematodes (*Pratylenchus thornei*) after wheat growth in a vertisol is influenced by rate of progressive soil desiccation. Annals of Applied Biology, 170 (1): 78-88.

Thompson, J. P. & N. P. Seymour, 2011. Inheritance of resistance to root-lesion nematode (*Pratylenchus thornei*) in wheat landraces and cultivars from the West Asia and North Africa (WANA) region. Crop and Pasture Science, 62 (1): 82-93.

Toktay, H., 2008. Resistance of Some Spring Wheat against *Pratylenchus thornei* Sher Et Allen Tylenchida: Pratylenchidae. Institute of Nature of Science of University of Cukurova, (Unpublished) PhD Thesis, Adana, Turkey, 131 pp (in Turkish with abstract in English).

Toktay, H., E. Yavuzaslanoğlu, M. İmren, J. Nicol, İ. H. Elekcioğlu & A. Dababat, 2012. Screening for resistance to *Heterodera filipjevi* (Madzhidov) Stelter (Tylenchida: Heteroderidae) and *Pratylenchus thornei* (Sher & Allen) (Tylenchida: Pratylenchidae) sister lines of spring wheat. Turkey Journal of Entomology, 36 (4): 455-461.

TUIK, 2019. Turkish Statistical Institute. (Web page: https://www.tuik.gov.tr) (Date accessed: 15 March 2020).

Urwin, P. E., C. J. Lilley, M. J. McPherson & H. J. Atkinson, 1997. Resistance to both cyst and root-knot nematodes conferred by transgenic *Arabidopsis* expressing a modified plant cystatin. The Plant Journal, 12 (2): 455-461.

Vanstone, V. A., A. J. Rathjen, A. H. Ware & R. D. Wheeler, 1998. Relationship between root lesion nematodes (*Pratylenchus neglectus* and *P. thornei*) and performance of wheat varieties. Australian Journal of Experimental Agriculture, 38 (2): 181-188.

Vanstone, V. A. & M. H. Russ, 2001a. Ability of weeds to host the root lesion nematodes *Pratylenchus neglectus* and *P. thornei* II*. Broad-leaf weeds. Australasian Plant Pathology, 30 (3): 251-258.

Vanstone, V. A. & M. H. Russ, 2001b. Ability of weeds to host the root lesion nematodes *Pratylenchus neglectus* and *P. thornei* I. Grass weeds. Australasian Plant Pathology, 30 (3): 245-250.

Whitehead, A. G., 1998. Plant Nematode Control. CAB International, UK, 384 pp.

Williams, K. J., S. P. Taylor, P. Bogacki, M. Pallotta, H. S. Bariana & H. Wallwork, 2002. Mapping of the root lesion nematode (*Pratylenchus neglectus*) resistance gene Rlnn1 in wheat. Theoretical and Applied Genetics, 104 (5): 874-879.

Yu, L. X., A. Morgounov, R. Wanyera, M. Keser, S. K. Singh & M. Sorrells, 2012. Identification of Ug99 stem rust resistance loci in winter wheat germplasm using genome-wide association analysis. Theoretical and Applied Genetics, 125 (4): 749-758.

Zwart, R. S., J. P. Thompson & I. D. Godwin, 2005. Identification of quantitative trait loci for resistance to two species of root-lesion nematode (*Pratylenchus thornei* and *P. neglectus*) in wheat. Australian Journal of Agricultural Research, 56 (4): 345-352.

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Original article (Orijinal araştırma)

Determination of Zygaenidae (Lepidoptera) species by morphological and molecular methods in the Eastern Mediterranean Region of Turkey[1]

Türkiye'nin Doğu Akdeniz Bölgesi'ndeki Zygaenidae (Lepidoptera) türlerinin morfolojik ve moleküler yöntemler kullanılarak tanılanması

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Abstract

Morphological identification of the majority of Zygaenidae species in the Eastern Mediterranean Region of Turkey was undertaken and a phylogenetic tree of these species was generated. Sampling was performed between May and September in 2017 and 2018 in Adana, Hatay, Kahramanmaraş, Mersin and Osmaniye Provinces. This area is a zoogeographical junction between central Anatolia and the Levant. The species were collected by sweep net and visual control methods. Thirteen Zygaenidae species identified: *Theresimima ampellophaga* (Bayle-Barelle, 1808), *Adscita* (*Adscita*) *obscura* (Zeller, 1847), *Jordanita* (*Tremewania*) *notata* (Zeller, 1847), *Jordanita* (*Praviela*) *anatolica* (Naufock, 1929), *Jordanita* (*Solaniterna*) *subsolana* (Staudinger, 1862) (Procridinae), *Zygaena* (*Mesembrynus*) *diaphana* Staudinger, 1887, *Zygaena* (*Mesembrynus*) *graslini* Lederer, 1855, *Zygaena* (*Mesembrynus*) *punctum* Ochsenheimer, 1808, *Zyganea* (*Agrumenia*) *olivieri* Boisduval, 1828, *Zygaena* (*Agrumenia*) *carniolica* (Scopoli, 1763), *Zygaena* (*Agrumenia*) *viciae* (Denis & Schiffermüller, 1775), *Zygaena* (*Agrumenia*) *loti* (Denis & Schiffermüller, 1775) and *Zygaena* (*Zygaena*) *filipendulae* (L., 1758). Macherey-Nagel NucleoSpin kit was used for DNA extraction and PCR was performed with LCO1490/HCO2198 primer pair for the mtCOI gene region. This analysis effectively separated these genera and species in the phylogenic tree, and these data supported the morphological identification.

Keywords: Eastern Mediterranean Region, Lepidoptera, phylogeny, systematic, Zygaenidae

Öz

Doğu Akdeniz Bölgesi'ndeki Zygaenidae türlerinin çoğunluğunun morfolojik tanılaması yapılmış ve bu türlerin filogenetik ağacı oluşturulmuştur. Örneklemeler Adana, Hatay, Kahramanmaraş, Mersin ve Osmaniye illerinde, 2017 ve 2018 yıllarının mayıs-eylül aylarında gerçekleştirilmiştir. Bu bölge Orta Anadolu ile Levant arasında zoocoğrafik ilişki kuran bir konumdadır. Atrap ve gözle kontrol metotları kullanılarak toplam 13 tür, *Theresimima ampellophaga* (Bayle-Barelle, 1808), *Adscita* (*Adscita*) *obscura* (Zeller, 1847), *Jordanita* (*Tremewania*) *notata* (Zeller, 1847), *Jordanita* (*Praviela*) *anatolica* (Naufock, 1929), *Jordanita* (*Solaniterna*) *subsolana* (Staudinger, 1862) (Procridinae), *Zygaena* (*Mesembrynus*) *diaphana* Staudinger, 1887, *Zygaena* (*Mesembrynus*) *graslini* Lederer, 1855, *Zygaena* (*Mesembrynus*) *punctum* Ochsenheimer, 1808, *Zyganea* (*Agrumenia*) *olivieri* Boisduval, 1828, *Zygaena* (*Agrumenia*) *carniolica* (Scopoli, 1763), *Zygaena* (*Agrumenia*)) *viciae* (Denis & Schiffermüller, 1775), *Zygaena* (*Agrumenia*) *loti* (Denis & Schiffermüller, 1775) ve *Zygaena* (*Zygaena*) *filipendulae* (L., 1758) belirlenmiştir. DNA ekstraksiyonunda Macherey-Nagel NucleoSpin Insect DNA izolasyon kiti ve PCR analizlerinde ise mtCOI gen bölgesinden LCO1490/HCO2198 primer çifti kullanılmıştır. Bu primer çifti ile türleri cins ve tür düzeyinde ayırmanın uygun olduğu ve elde edilen verilerin morfolojik tanımlamayı desteklediği belirlenmiştir.

Anahtar sözcükler: Doğu Akdeniz Bölgesi, Lepidoptera, filogeni, sistematik, Zygaenidae

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Introduction

Turkey, situated at the nexus of Europe, the Middle East, Central Asia and Africa is one of the most species rich countries in the Western Palearctic (Çıplak, 2003). Also, it comprises three biogeographical regions: Mediterranean, Euro-Siberian and Irano-Turanian. This location supplies a natural pathway for the movement of organisms in every direction. Each region has endemic species and a variety of ecosystems (Çıplak, 2003). Consequently, it has the character of a small continent from the standpoint of biological diversity with forest, mountain, steppe, wetland, coastal and marine ecosystems (FAO, 2019).

Zygaenidae is a species rich family of predominantly diurnal moths with a worldwide distribution, being most diverse in tropical and subtropical Asia and Palearctic regions (Epstein, 1996). This group, commonly known as burnet, forester and smoky moths is split up into five subfamilies: Chalcosiinae Walker, 1865 (Palearctic, Oriental), Callizygaeninae Alberti, 1954 (Oriental), Inouelinae Efetov & Tarmann, 2017 (Oriental), Procridinae Boisduval, 1828 (Holarctic, Afrotropical, Oriental, Australian, Neotropical) and Zygaeninae Latreille, 1809 (Palearctic, Oriental, Afrotropical) (Alberti, 1954, 1958, 1959; Tarmann, 1984; 1994, 2004; Hofmann & Tremewan, 1996; Efetov & Tarmann, 2012, 2014, 2017; Efetov et al., 2014, 2015, 2019; Yen, 2003). The family contains more than 1,000 species distributed worldwide and the number of described species increases annually (Efetov et al., 2021). The first studies on this family were conducted by international researchers in Turkey (Zeller, 1847; Mann, 1862). There are currently 56 Zygaenidae species recognized in Turkey, 25 belong to Procridinae and the others to Zygaeninae subfamilies. Five are endemic to Turkey: *Jordanita* (*Jordanita*) *chloronota* (Staudinger,1871), *Zygaena* (*Agrumenia*) *formosa* (Herrich-Schäffer, 1852), *Zygaena* (*Agrumenia*) *peschmerga* Eckweiler & Gorgner, 1981, *Zygaena* (*Mesembrynus*) *lydia* Staudinger, 1887 and *Zygaena* (*Zygaena*) *problematica* Naumann, 1966 (Efetov et al., 2010a, 2019; Hofmann & Tremewan, 2017; Can Cengiz et al., 2018; Okyar et al., 2018; Can et al., 2019).

Determination of the biological properties and the distribution of the species is important for the protection of the zygaenid fauna of Turkey. This family also contains several pest species (Tarmann, 2003). Distribution of many zygaenid species is limited and they need specific ecological conditions. In terms of ecology, zygaenids, along with the lepidopterans, are significant umbrella groups (Efetov et al., 2019). These groups will provide base line information for ecological conservation efforts. This information might also be useful for other subgenera (Nazarov & Efetov, 1993; Schmitt & Seitz, 2004; Tarmann, 2009; Efetov et al., 2019).

Although, many moths are identical morphologically and possess similar wing patterns, they can be classified as different species. In addition, seasonal and sexual dimorphism, color and pattern variations in populations of the same species can make the morphological identification of species difficult (Hausmann, 2001; Mironov, 2003; Sihvonen & Nupponen, 2005; Can, 2009; Spalding et al., 2013; Hofmann & Tremewan, 2017). In order to identify species, molecular methods supporting morphological identification are needed for cryptic cases. Recently, molecular techniques have been used to identify almost all organism and determine their phylogenetic features, but there are few studies conducted on Lepidoptera in Turkey **(**Can, 2009; Serdar, 2014; Can et al., 2018; Kuyulu & Genç, 2020). The first study on determining the molecular characterization of lepidopteran species was conducted by Can (2009) using COI gene region and the first DNA barcode profiles of the Geometridae species of Turkey. Later other studies were conducted on the family Geometridae (Serdar, 2014) and *Cydia pomonella* (L., 1758) (Tortricidae) (Kuyulu & Genç, 2020) using the same gene region. As with many organisms, to determine the DNA sequence of an insect barcoding is performed by revealing the DNA sequence using mostly the mitochondrial COI gene region. This method helps morphological studies by revealing unknown species or to identify the species whose taxonomic position was uncertain (Hebert et al., 2003; Wilson, 2012).

There is some literature on the molecular phylogeny of Zygaenidae (Niehuis et al., 2006a, b, 2007; Efetov et al., 2010b, 2019, 2021). Along with RNA secondary structure variation, some selected morphological and biological characters were examined (Niehuis et al., 2006a) and also using various mitochondrial and nuclear markers four subfamilies were examined except Inouelinae (Niehuis et al., 2006b, 2007). The “DNA barcoding of Zygaenidae moths (ZYGMO)" project began in 2009 (Efetov et al., 2010b). The molecular studies were conducted according to Hebert et al. (2003a, b) and Ratnasingham & Hebert (2007) using the universal gene region mtCOI and standard DNA barcoding protocols, respectively. The purpose of these studies was to create a new databank for this group with new techniques (Efetov et al., 2019). In this study, the morphological characteristics and phylogeny of selected species of the Zygaeninae and Procridinae were investigated in the Eastern Mediterranean Region of Turkey.

Materials and Method

Taxon sampling and morphological studies

The Zygaenidae fauna of the Eastern Mediterranean Region of Turkey, which comprises Adana, Hatay, Kahramanmaraş, Mersin and Osmaniye Provinces, was studied by sweep net in various habitats (Figure 1). This area is a zoogeographical junction between central Anatolia and the Levant. Seventy-two sites of different altitudes, climatic, vegetation and geographical conditions were sampled between May and September in both 2017 and 2018. During these years, 716 specimens were collected. Genital slides were prepared according to standard procedures Doğanlar (2003).

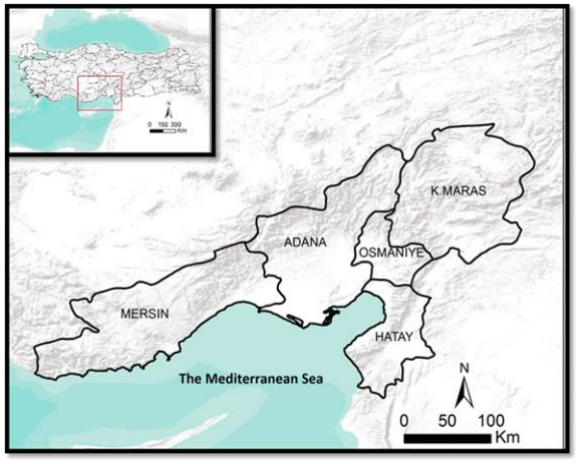

Figure 1. Sampling area; Eastern Mediterranean Region of Turkey.

Molecular studies

Location, altitude and habitat differences were taken into consideration in selecting the samples used in molecular studies, DNA isolation was performed on 69 specimens belonging to 11 species: seven Zygaeninae and four Procridinae. The list and sampling details of the species used in the molecular analyses are presented in Table 1.

DNA was extracted from a single fresh leg. DNA extraction was performed using a NucleoSpin tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The PCR product was purified using the DreamTaq Thermo Scientific PCR Master Mix PCR kit following the manufacturer’s protocol. PCR reaction components and final concentrations were master mix 12.5 µl, primers 1 µl, ddH_20 5.5 µl and 5 µl DNA template in a final volume of 25 µl. DNA fragments were amplified using following primer pairs: HCO2198 (5’-TAAACTTCAGGGTGACCAAAAAATCA-3’) and LCO1490 (5’-GGTCAACAAATCATAAAGATATTGG-3’) (Folmer et al. 1994). The initial PCR cycling profile was at 95ºC for 7 min, 40 cycles at 95ºC for 30 s, 50ºC for 30 s and 72ºC for 1 min followed by a final extension period at 72ºC for 10 min.

Phylogenetic analyses

The PCR products were investigated by Sanger sequencing. Sequences were checked by the Chromas computer program. Sequence alignments were performed manually by using Mega10 (Kumar et al., 2018). Then all sequences data were checked on National Center for Biotechnology Information (NCBI) again to correct the manually cleaned data and created the phylogenetic tree (Anonymous, 2021). Finally, the genetic distance of the species was evaluated, and appropriate parameters, chosen according to the maximum likelihood and general time reversible models, were used to construct the phylogenetic tree (Nei & Kumar, 2000). Support for nodes was evaluated with 1000 bootstrap replicates of the data. For outgroup comparison, *Sesia bembeciformis* (Hübner, 1806) (Lepidoptera: Sesiidae) was chosen and the sequences data were obtained from NCBI (GenBank accession JN279255).

Table 1. Zygaenidae samples collected and molecularly analyzed from the Eastern Mediterranean Region of Turkey

Species	Province	Location	Latitude (N)	Longitude (E)	Altitude (m)	Date	Code
Adscita obscura	Adana	Pozantı	37°28'66"	34°54'42"	1090	19.05.2017	66
			37°28'48"	34°54'23"	1150	18.05.2018	508
	Mersin	Çamlıyayla	37°06'09"	34°40'41"	1150	18.06.2017	206
		Silifke	36°42'69"	33°52'66"	218	19.05.2018	552
	Hatay	Alahan	36°22'46"	36°10'17"	350	07.04.2018	502
		Yayladağı	36°07'25"	36°09'86"	451	18.05.2017	22
	Kahramanmaraş	Fırnız	36°45'21"	36°41'07"	717	14.05.2017	18
			37°45'56"	36°39'25"	717	04.05.2018	709
		KSU campus	37°01'12"	34°21'96"	513	20.05.2018	693
Jordanita anatolica	Adana	Pozantı	37°26'62"	34°54'42"	1090	18.06.2017	326
			37°28'48"	34°54'23"	1150	18.05.2018	517
		Tufanbeyli	38°13'22"	36°01'32"	1370	29.06.2018	661
	Mersin	Erdemli	36°44'87"	34°10'99"	681	28.06.2017	308
		Gülnar	36°24'55"	36°24'58"	1070	19.05.2018	523
		Kurtçukuru	37°09'14"	34°44"91"	460	08.04.2017	473
		Merkez ilçe	37°01'26"	34°25'32"	1356	27.06.2017	299
		Mut	36°41'51"	33°37'74"	1076	19.05.2018	531
		Silifke	36°42'69"	33°52'66"	1422	19.05.2018	551
		Tarsus	37°09'14"	34°44'91"	522	18.05.2018	522

Table 1. Continued

Species	Province	Location	Latitude (N)	Longitude (E)	Altitude (m)	Date	Code
Jordanita anatolica	Hatay	Alahan	36°22'46"	36°10'17"	230	25.04.2018	481
		Belen	36°29'47"	36°09'13"	500	21.07.2017	457
		Dikmece	36°17'85"	36°08'27"	309	29.05.2018	604
		İskenderun	36°33'37"	36°13'51"	617	01.06.2018	616
		Yayladağı	36°07'43"	36°08'43"	281	22.06.2017	266
	Kahramanmaraş	Başkonuş	37°33'58"	36°33'41"	1210	20.05.2018	580
		Fırnız	38°00'54"	36°33'49"	650	24.06.2017	275
		Göksun	38°01'08"	36°34'19"	1380	24.06.2017	270
	Osmaniye	Zorkun	37°01'54"	36°16'40"	730	09.07.2017	418
Jordanita subsolana	Kahramanaraş	KSU campus	37°35'22"	36°49'07"	513	24.04.2018	692
Jordanita notata	Adana	CU campus	37°02'50"	36°22'44"	96	20.05.2018	698
	Kahramanmaraş	Fırnız	36°45'21"	36°41'07"	717	01.05.2017	16
Theresimima ampellophaga	Hatay	Yayladağı	36°01'29"	36°01'49"	470	06.07.2017	247
	Osmaniye	Hasanbeyli	37°07'40"	36°34'33"	847	08.07.2018	668
Zygaena loti	Adana	Pozantı	37°28'48"	34°54'23"	1130	03.06.2017	93
			37°28'48"	34°54'23"	1130	03.06.2017	97
	Mersin	Çamlıyayla	37°10'14"	34°39'15"	1150	10.06.2017	148
		Merkez	36°56'14"	34°33'52"	470	16.05.2018	503
	Hatay	Yayladağı	36°07'25"	36°09'86"	451	18.05.2017	30
	Kahramanmaraş	Andırın	37°33'58"	36°33'41"	1210	02.07.2017	389
		Fırnız	37°45'29"	36°40'55"	700	14.05.2017	20
Zygaena punctum	Adana	Pozantı	37°26'62"	34°54'42"	1130	12.04.2018	194
	Kahramanmaraş	Kılavuzlu	37°37'29"	36°49'50"	567	12.04.2018	682
Zygaena filipendulae	Adana	Pozantı	37°26'62"	34°54'42"	1130	18.06.2017	313
	Mersin	Çamlıyayla	37°10'14"	34°39'15"	1150	10.06.2017	166
			37°10'14"	34°39'15"	1160	26.09.2017	386
			37°10'14"	34°39'15"	1150	10.06.2018	658
	Hatay	Yayladağı	36°07'25"	36°09'86"	451	18.05.2017	27
	Kahramanmaraş	Andırın	37°33'58"	36°33'41"	1210	01.07.2018	666
		Göksun	38°02'12ı	36°36'65ı	1398	02.07.2017	388

Table 1. Continued

Species	Province	Location	Latitude (N)	Longitude (E)	Altitude (m)	Date	Code
Zygaena graslini	Adana	Pozantı	37°26'62"	34°54'42"	1130	18.06.2017	398
	Mersin	Çamlıyayla	37°10'14"	34°39'15"	1150	03.06.2017	102
		Kurtçukuru	37°06'09"	34°40'41"	460	18.06.2017	198
			37°09'10"	34°44'94"	460	18.05.2018	520
		Merkez	36°56'14"	34°33'52"	470	07.04.2018	480
	Hatay	Alahan	36°20'14"	36°10'31"	350	08.05.2017	4
			36°22'46"	36°10'17"	350	07.04.2018	491
		Samandağ	36°06'13"	35°56'54"	40	08.04.2018	474
	Kahramanmaraş	Başkonuş	37°33'57"	36°35'00"	1210	06.04.2018	678
		Fırnız	36°20'14"	36°10'31"	550	01.05.2017	7
		Karacasu	37°23'07"	37°03'15"	582	17.04.2018	681
		KSÜ campus	37°35'22"	36°49'07"	513	24.04.2018	695
Zygaena olivieri	Kahramanmaraş	Andırın	37°33'58"	36°33'41"	1210	02.07.2017	394
Zygaena carniolica	Adana	Karageçit	37°9'10"	34°44'57"	460	10.06.2017	143
		Tufanbeyli	38°19'13"	36°19'86"	1750	17.07.2017	453
	Mersin	Çamlıyayla	37°06'09"	34°40'41"	1150	08.07.2017	409
			37°06'09"	34°40'41"	1150	08.07.2017	411
Zygaena diaphana	Adana	Pozantı	37°28'48"	34°54'23"	1130	18.05.2018	504
			37°28'48"	34°54'23"	1130	18.05.2018	507
	Hatay	Antakya	36°20'24"	36°11'33"	350	01.05.2018	711

Results and Discussion

Thirteen Zygaenidae species, five Procridinae and eight Zygaeninae, were identified: *Theresimima ampellophaga* (Bayle-Barelle, 1808), *Adscita (Adscita) obscura* (Zeller, 1847), *Jordanita (Tremewania) notata* (Zeller, 1847), *Jordanita (Praviela) anatolica* (Naufock, 1929), *Jordanita (Solaniterna) subsolana* (Staudinger, 1862) (Procridinae); *Zygaena (Mesembrynus) diaphana* Staudinger, 1887, *Zygaena (Mesembrynus) graslini* Lederer, 1855, *Zygaena (Mesembrynus) punctum* Ochsenheimer, 1808, *Zygaena (Agrumenia) olivieri* Boisduval, 1828, *Zygaena (Agrumenia) carniolica* (Scopoli, 1763), *Zygaena (Agrumenia) viciae* (Denis & Schiffermüller, 1775), *Zygaena (Agrumenia) loti* (Denis & Schiffermüller, 1775) and *Zygaena (Zygaena) filipendulae* (L., 1758). All species and their genitalia images were presented (Figures 2-4).

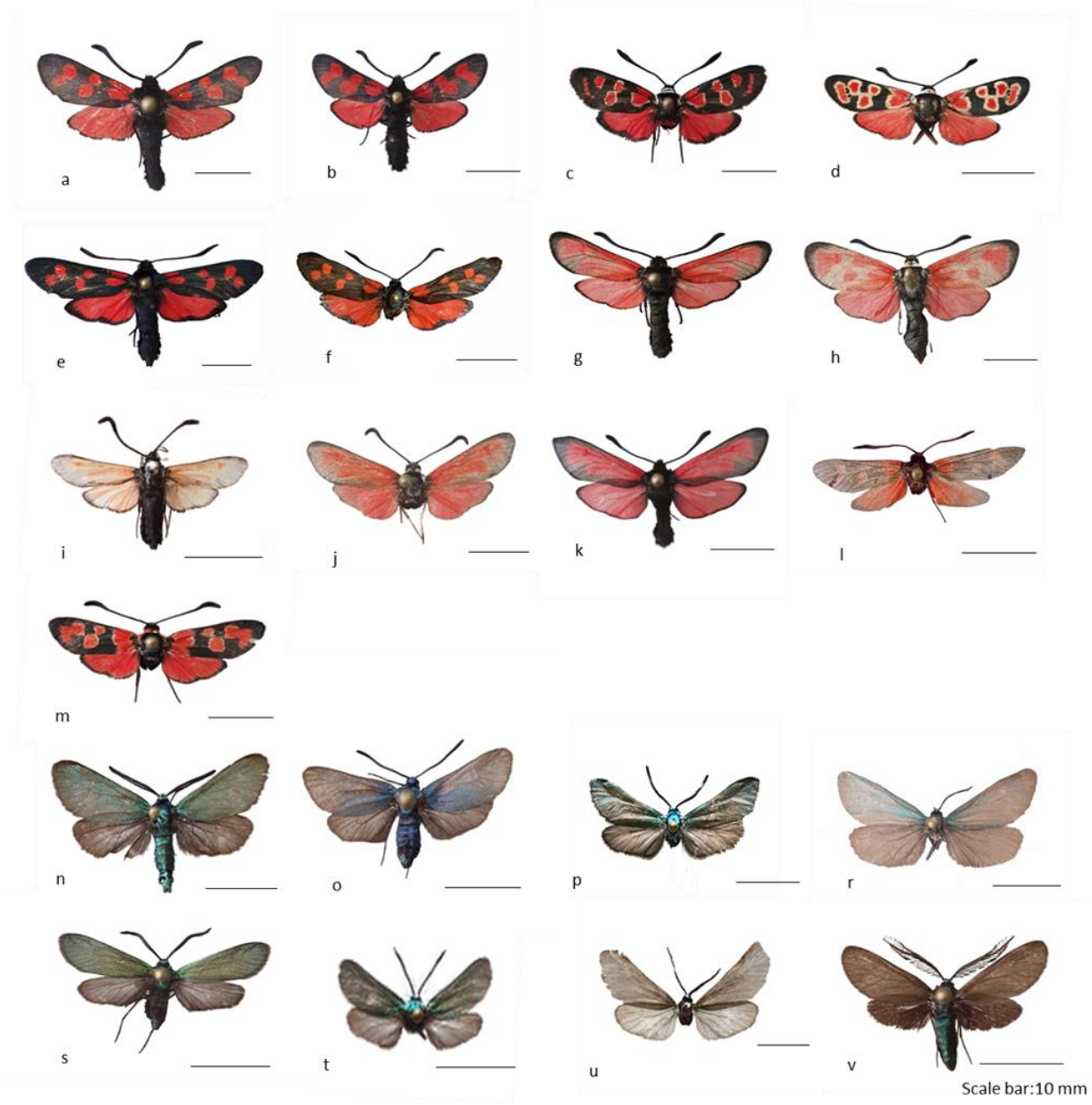

Figure 2. Wing pattern of species in the Zygaenidae: a) *Zygaena graslini* ♂, b) *Z graslini* ♀, c) *Z. carniolica* ♂, d) *Z. carniolica* ♀, e) *Z. filipendulae* ♂, f) *Z. filipendulae* ♀, g) *Z. loti* ♂, h) *Z. loti* ♀, i) *Z. punctum* ♂, j) *Z. punctum* ♀, k) *Z. diaphana* ♂, l) *Z. viciae* ♂, m) *Z. olivieri* ♂, n) *Adscita obscura* ♂, o) *A. obscura* ♀, p) *Jordanita notata* ♂, r) *J. notata* ♀, s) *J. anatolica* ♂, t) *J. anatolica* ♀, u) *J. subsolana* ♂, and v) *Theresimima ampellophaga* ♂.

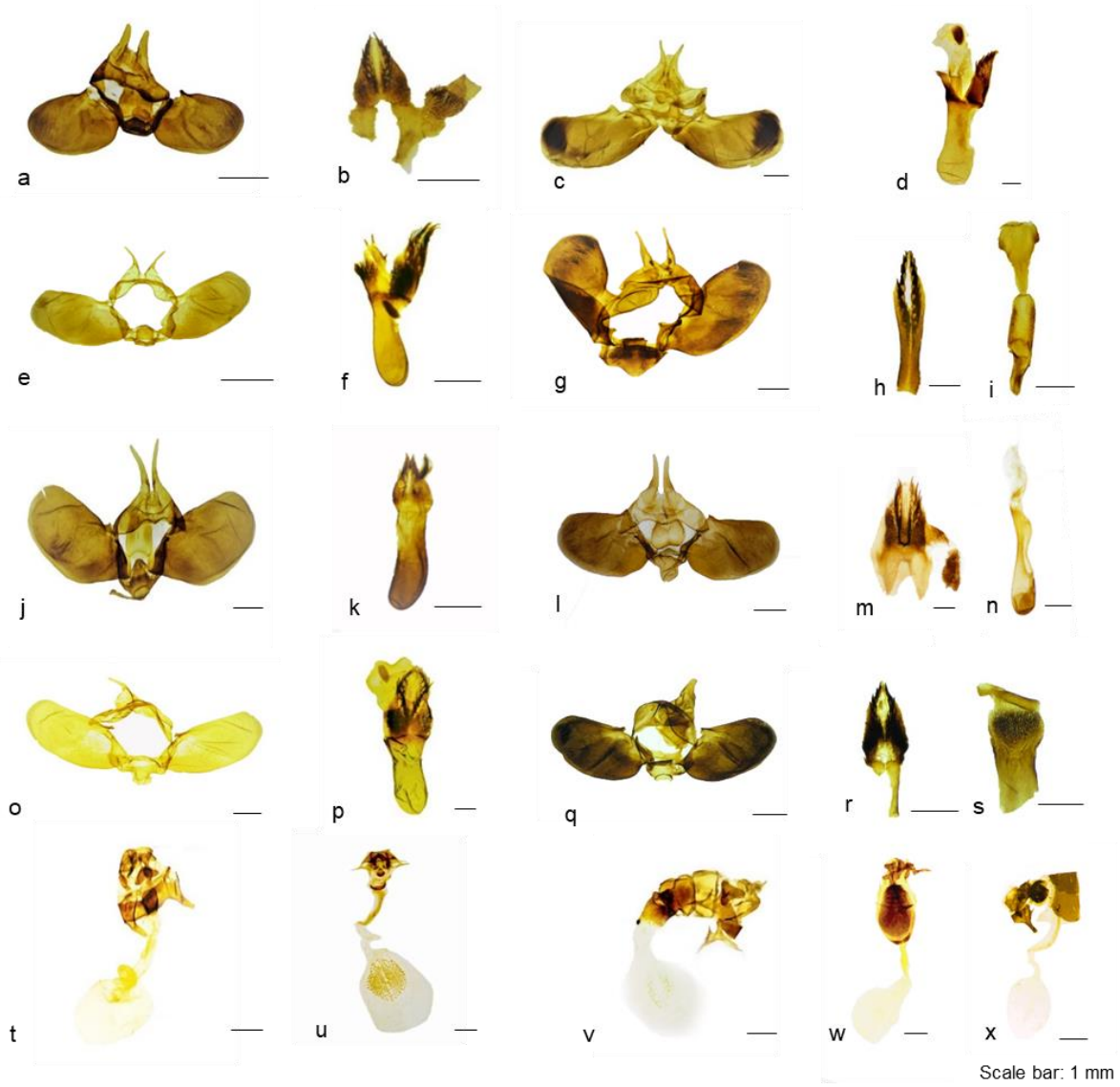

Figure 3. Male and female genitalia of species in the Zygaeninae: a) genitalia of *Zygaena graslini* ♂, b) lamina ventralis and lamina dorsalis, c) *Z. carniolica* ♂, d) aedeagus, e) *Z. filipendulae* ♂, f) aedeagus g) *Z. loti* ♂, h) lamina ventralis, i) lamina dorsalis, j) *Z. olivieri* ♂, k) aedeagus, l) *Z. punctum* ♂, m) lamina ventralis and dorsalis, n) aedeagus, o) *Z. viciae* ♂, p) aedeagus, q) *Z. diaphana* ♂, r) lamina ventralis, s) lamina dorsalis, t) *Z. graslini* ♀, u) Z. *carniolica* ♀, v) *Z. filipendulae* ♀, w) *Z. loti* ♀, and x) *Z. punctum* ♀.

Figure 4. Male and female genitalia of species in the Procridinae: a) *Adscita obscura* ♂, b) aedeagus, c) *Jordanita notata* ♂, d) aedeagus, e) *J. anatolica*, ♂, f) aedeagus g) *J. subsolana* ♂, h) aedeagus, i) *Theresimima ampellophaga* ♂, j) aedeagus, k) *A. obscura* ♀, l) *J. notata* ♀, and m) *J. anatolica* ♀.

This is the first study that investigated the phylogeny and systematic of the Zygaenidae in Turkey. The molecular identification of the species and phylogenetic trees are presented in Figures 5 and 6. The original tree of the samples located the upper left of the figures. The tree was divided into two sections according to subfamilies, so was investigated in more detail. The DNA of 36 of 37 zygaenid samples collected from the Eastern Mediterranean Region of Turkey in 2017 and 28 of 32 zygaenid samples collected in 2018 were amplified by PCR and the expected band (658 bp) was obtained at the same level as the positive control. However, the DNA of 308 (*J. anatolica*) coded sample from 2017 and 692 (*J. subsolana*), 504 (*Z. diaphana*), 711 (*Z. diaphana*) and 681 (*Z. graslini*) from 2018 were not been able to amplified.

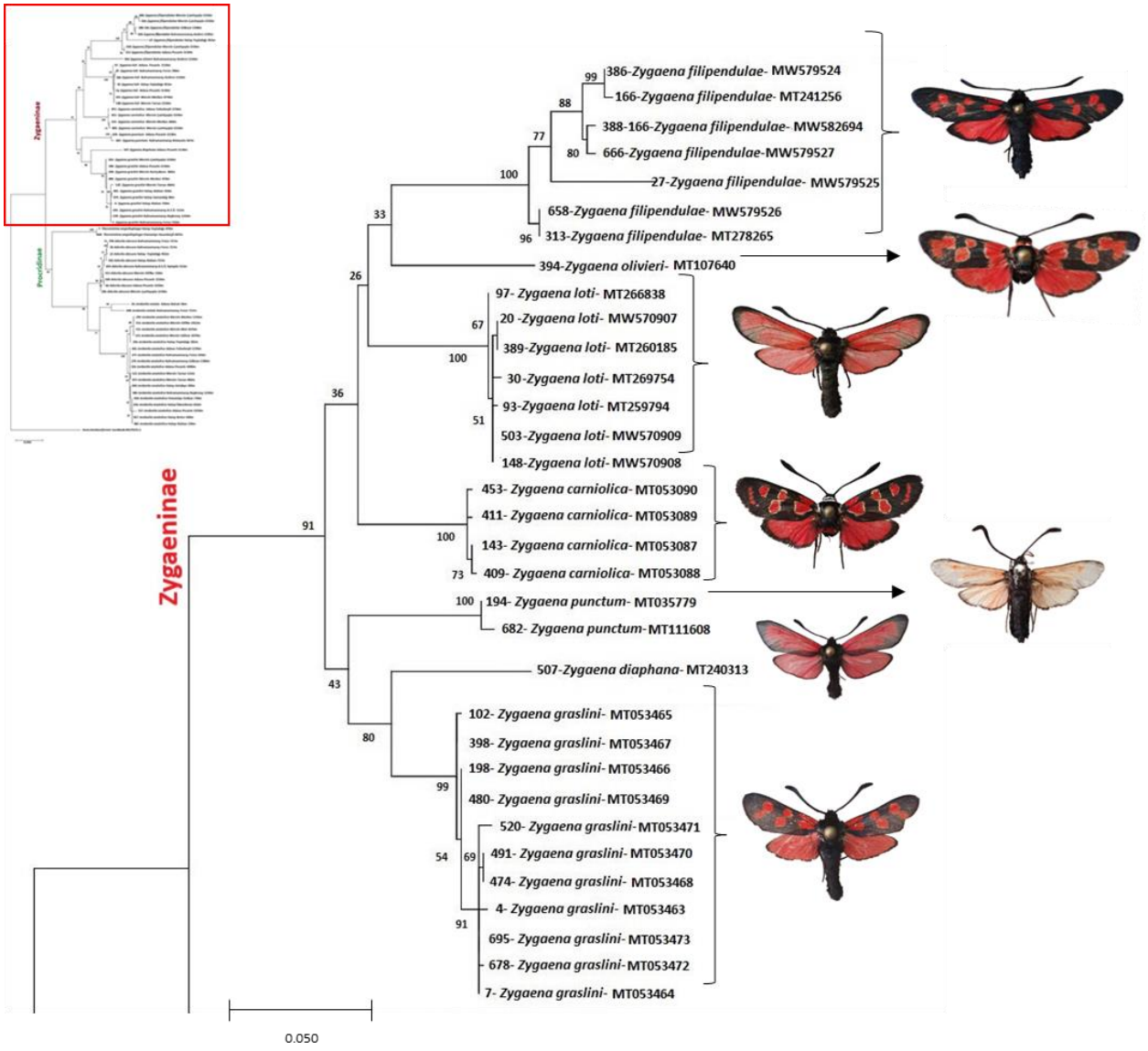

Figure 5. Phylogenetic tree of the mitochondrial COI gene region of species in the Zygaeninae with GeneBank accession numbers based on maximum likelihood and general time reversible models.

When *Sesia bembeciformis*, which is the outer group in this study, is evaluated separately, the phylogenetic tree is divided into two main branches as Zygaeninae and Procridinae. All species were clustered into their own distinct groups. *Zygaena filipendulae*, *Z. olivieri* and *Z. loti* species were grouped on one branch. *Zygaena olivieri* and *Z. filipendulae* were also grouped on one branch and *Z. loti* were on separate branch (Figure 5). No genital or external morphological differences were observed. However, according to the study of DNA barcoding of world zygaenids, *Z. filipendulae* and *Z. loti* species are closer to each other than *Z. olivieri* with the same primer pair and more samples in that study (Efetov et al., 2019). The phylogeny of *Z. graslini* was previously studied with many molecular markers from both nuclear and mitochondrial genes (Niehuis et al., 2007), and the mtCOI gene was also discussed in this study. In the present study, only one specimen of *Z. olivieri* was found, so its phylogenetic analysis is limited and therefore more specimen to be compared for this species.

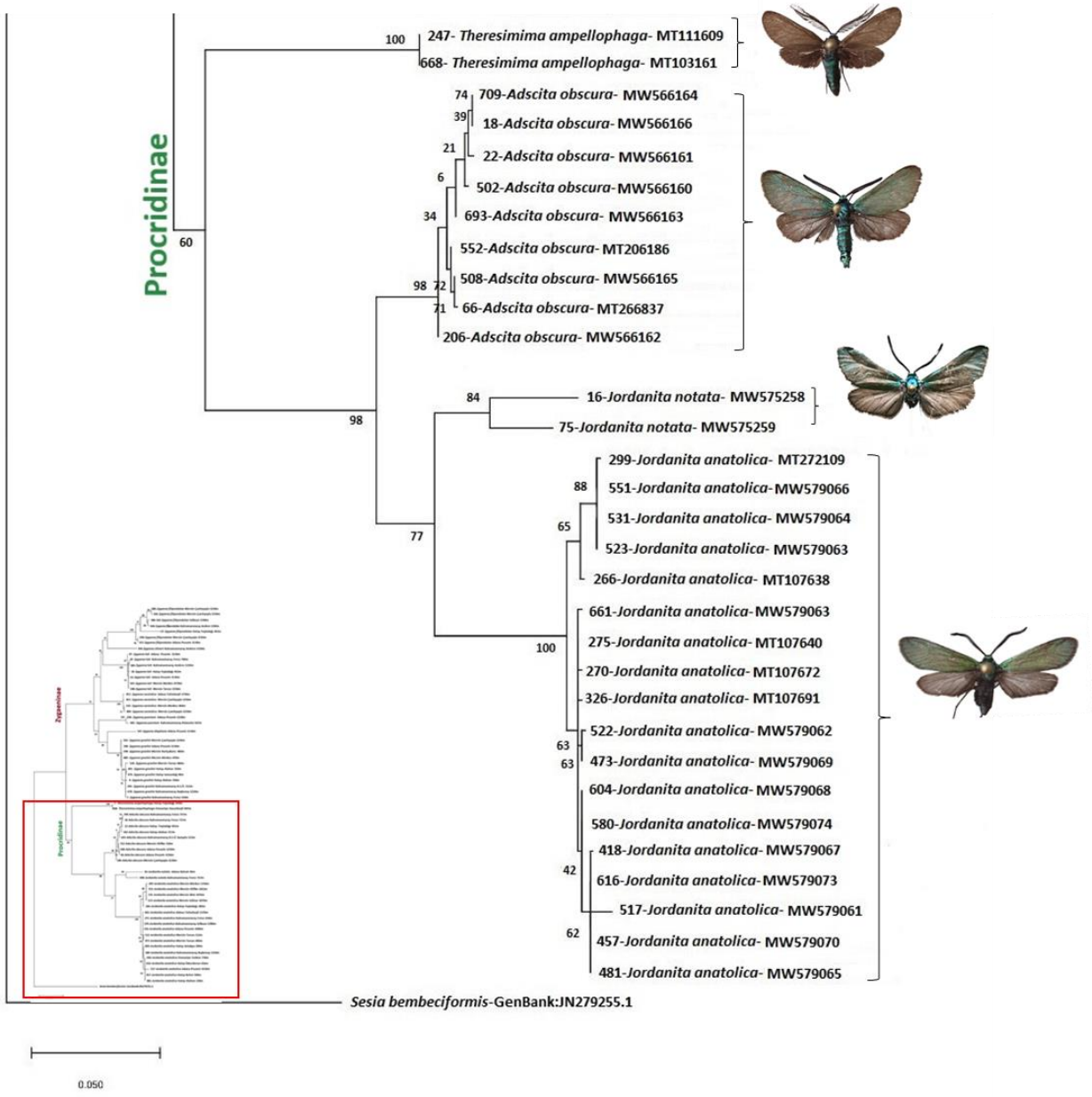

Figure 6. Phylogenetic tree of the mitochondrial COI gene region of species in the Procridinae with GeneBank accession numbers based on maximum likelihood and general time reversible models.

According to Hofmann & Tremewan (2017), several species (e.g. *Z. brizae*, *Z. ephialtes*, *Z. formosa*, *Z. laeta*, *Z. lydia*, *Z. problematica* and *Z. purpuralis*) recorded in the Eastern Mediterranean Region were not found in this study. Although, *Z. viciae* was found, it was not included in the phylogenetic tree.

Within the *Z. graslini* samples, only small nucleotide differences were found, and *Z. diaphana* was determined as the closest species. *Zygaena diaphana* belongs to the *purpuralis complex* group, Z. *purpuralis* and *Z. minos*, and has recently been reinstated to species rank (Nahirnic, 2019). Therefore, while scanning the GeneBank records with the sequence results obtained from this species, it was found that no *Z. diaphana* sequences have been submitted. Also, since they were in the same complex group

with *Z. diaphana*s, sequences of *Z. purpuralis* and *Z. minos* are listed in NCBI as the most similar gene sequences. As an outcome of the present study, the first submission of *Z. diaphana* was lodged in GeneBank (accession JN279255.1).

Four species in three genera of the Procridinae were studied (Figure 6). While the samples in the genera *Jordanita* and *Adscita* were located on one branch of the tree, *Theresimima* was on a separate branch. Therefore, this subfamily was divided across two branches. In the present study, there was no variation observed between genetic differences and geographical distribution of the species.

As a result of this study, it was found to be effective to phylogenically distinguish these genera and species with the primer used, and the data obtained support the morphological identification. Considerable morphological and molecular reference material and data were obtained and uploaded in to GeneBank, and these data should be useful for future studies. Therefore, the data obtained has made a contribution to the “DNA barcoding of Zygaenidae moths (ZYGMO)” project and all accession numbers were added on the phylogenetic tree. Lepidoptera systematic taxonomy studies in Turkey have been morphological studies. However, for species identification the combining morphological and molecular methods will help clarify earlier findings.

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References

Alberti, B., 1954. Über die stammesgeschichtliche Gliederung der Zygaenidae nebst Revision einiger Gruppen (Insecta, Lepidoptera). Mitteilungen aus dem Zoologischen Museum der Humboldt-Universität Berlin, 30: 115-480.

Alberti, B., 1958. Über den stammesgeschichtlichen Aufbau der Gattung Zygaena und ihrer Vorstufen (Insecta, Lepidoptera). Mitteilungen aus dem Zoologischen Museum der Humboldt-Universität Berlin, 34: 245-396.

Alberti, B., 1959. Über den stammesgeschichtlichen Aufbau der Gattung Zygaena F. und ihrer Vorstufen (Insecta, Lepidoptera). Mitteilungen aus dem Zoologischen Museum der Humboldt-Universität Berlin,35: 203-242.

Anonymous, 2021. National center for biotechnology information (NCBI). (Web page: https://www.ncbi.nlm.nih.gov) (Date accessed: 01.02.2020).

Can Cengiz, F., K. A. Efetov, K. Kaya, E. E. Kucherenko, Z. Okyar & G. M Tarmann, 2018. Zygaenidae (Lepidoptera) of Thrace Region of Turkey. Nota Lepidopterologica, 41 (1): 23-36.

Can, F., 2009. DNA barcoding confirms species rank for a cryptic geometrid species from Turkey and Bulgaria (Lepidoptera: Geometridae: Sterrhinae). Zootaxa, 2314 (1): 63-68.

Can, F., K. A. Efetov, J. Burman, K. Kaya, E. E. Kucherenko, B. Ulaşlı & G. M. Tarmann, 2019. A study of the Zygaenidae (Lepidoptera) fauna of Central Anatolia Turkey. Turkish Journal of Entomology, 43 (2): 189-199.

Can, F., B. Ulaşlı & A. Hausmann, 2018. An integrative approach to understand the biogeography, taxonomy and ecology of the Macroheteroceran fauna of the Amanos Mountains in Southern Turkey. Journal of the Entomological Research Society, 20 (2): 91-101.

Çıplak, B., 2003. Distribution of Tettigoniinae (Orthoptera, Tettigoniidae) bush-crickets in Turkey: the importance of the Anatolian Taurus Mountains in biodiversity and implications for conservation. Biodiversity & Conservation, 12 (1): 47-64.

Doğanlar, F., 2003. Doğu Akdeniz Bölgesi Geometridae (Lepidoptera) Familyası Üzerinde Faunistik ve Sistematik Araştırmalar. Çukurova Üniversitesi, Fen Bilimleri Enstitüsü, Bitki Koruma Ana Bilim Dalı, (Unpublished) PhD Thesis, Adana, Turkey, 295 pp (in Turkish with abstract in English).

Efetov, K. A., F. Can, T. B. Toshova & M. Subchev, 2010a. New sex attractant for Jordanita anatolica (Naufock) (Lepidoptera: Zygaenidae: Procridinae). Acta Zoologica Bulgarica, 62 (3): 315-319.

Efetov, K. A., A. Hofmann, G. M. Tarmann & W. G. Tremewan, 2014. Taxonomic comments on the treatment of the Zygaenidae (Lepidoptera) in volume 3 of Moths of Europe, Zygaenids, Pyralids 1 and Brachodids (2012). Nota Lepidopterologica, 37 (2): 123-133.

Efetov, K. A., A. V. Kirsanova, Z. S. Lazareva, E. V. Parshkova, G. M., Tarmann & R. Rougerie, 2010b. "Early results in DNA barcoding of Zygaenidae (Lepidoptera), pp. 7-8. 7". XII. International Symposium on Zygaenidae, (5-9 May 2010, Hatay-Turkey) 26 pp.

Efetov, K. A., A. V. Kirsanova, Z. S. Lazareva, E. V. Parshkova, G. M. Tarmann, R. Rougerie & P. D. N. Hebert, 2019. DNA barcoding of Zygaenidae (Lepidoptera): results and perspectives. Nota Lepidopterologica, 42 (2): 137-150.

Efetov, K. A., Z. S. Lazareva, E. V. Parshkova & G. M. Tarmann, 2021. Molecular genetic characters of species of the genus *Jordanita* Verity, 1946 (Lepidoptera: Zygaenidae, Procridinae): DNA barcodes and corresponding amino acid sequences. Russian Journal of Genetics, 57 (1): 61-69.

Efetov, K. A., E. V.Parshkova, L. G. Tarasova & G. M. Tarmann, 2015. The karyotypes of Procridinae (Lepidoptera: Zygaenidae), with the first record of the karyotype of *Pollanisus commoni* Tarmann, 2004, a representative of the tribe Artonini. Entomologist's Gazette, 66 (2): 121-125.

Efetov, K. A. & G. M. Tarmann, 2012. A Checklist of the Palearctic Procridinae (Lepidoptera: Zygaenidae). CSMU Press, Simferopol & Innsbruck, 108 pp.

Efetov, K. A. & G. M. Tarmann, 2014. *Illiberis* (*Alterasvenia*) *banmauka* sp. nov. (Lepidoptera: Zygaenidae, Procridinae) from China and Myanmar. Entomologist's Gazette, 65 (1): 62-70.

Efetov, K. A. & G. M. Tarmann, 2017. The hypothetical ground plan of the Zygaenidae, with a review of the possible autapomorphies of the Procridinae and the description of the Inouelinae subfam. nov. Journal of the Lepidopterists' Society, 71 (1): 20-49.

Epstein, M. E., 1996. Revision and Phylogeny of the Limacodid group Families, with Evolutionary Studies on Slug Caterpillars (Lepidoptera, Zygaenoidea). Smithsonian Institution Press, Washington D. C., 112 pp.

FAO, 2019. Türkiye'nin Biyoçeşitliliği: Genetik Kaynakların Sürdürülebilir Tarım ve Gıda Sistemlerine Katkısı. Ankara. 222 pp (in Turkish).

Folmer, O., M. Black, W. Hoeh, R. Lutz & R. Vrijenhoek, 1994. DNA primers for amplification of mitochondrial cytochrome *c*oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology & Biotechnology, 3 (5): 294-299.

Hausmann, A., 2001. The Geometrid Moths of Europe. Apollo Books, Strenstrup-Denmark, Volume I, 282 pp.

Hebert, P. D. N., A. Cywinska, S. L. Ball & J. R. de Waard, 2003a. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B, Biological Sciences, 270 (1512): 313-321.

Hebert, P. D. N., S. Ratnasingham & J. R. deWaard, 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal (Supplement 1), 270: S96-S99.

Hofmann, A. & W. G. Tremewan, 1996. A Systematic Catalogue of the Zygaeninae (Lepidoptera: Zygaenidae). Harley Books, Colchester, 251 pp.

Hofmann, A. & W. G. Tremewan, 2017. The Natural History of Burnet Moths, Part I. Museum Witt Munich and Nature Research Center Vilnius, 630 pp.

Kumar, S., G. Stecher, M. Li., C. Knyaz & K. Tamura, 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology & Evolution, 35 (6):1547-1549.

Kuyulu, A. & H. Genç, 2020. Genetic diversity of codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) populations in Turkey. Turkish Journal of Zoology, 44 (5): 462-471.

Mann, J. J., 1862. Verzeichniss der im Jahre 1851 bei Brussa in Kleinasien gesammelten Schmetterlinge, Tafel 3. Wiener Entomologische Monatsschrift, 6: 373-409.

Mironov, V., 2003. The Geometrid Moths of Europe. Apollo Books, Strenstrup-Denmark, Volume 4, 464 pp.

Nahirnic, A., 2019. *Zygaena diaphana* Staudinger, 1887 bona species (Lepidoptera: Zygaenidae). SHILAP Revista de lepidopterología, 47 (186): 341-347.

Nazarov, V. V. & K. A. Efetov, 1993. On the role of Zygaenidae (Lepidoptera) in pollination of *Anacamptis pyramidalis* (Orchidaceae). Zoologichesky Zhurnal, 72 (10): 54-67.

Nei, M. & S. Kumar, 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York, 333 pp.

Niehuis, O., A. Hofmann, C. M. Naumann & B. Misof, 2007. Evolutionary history of the burnet moth genus *Zygaena* Fabricius, 1775 (Lepidoptera: Zygaenidae) inferred from nuclear and mitochondrial sequence data: phylogeny, host-plant association, wing pattern evolution and historical biogeography. The Linnean Society of London, Biological Journal of the Linnean Society, 92 (3): 501-520.

Niehuis, O., C. M. Naumann & B. Misof, 2006a. Phylogenetic analysis of Zygaenoidea small-subunit rRNA structural variation implies initial oligophagy on cyanogenic host plants in larvae of the moth genus *Zygaena* (Insecta: Lepidoptera). Zoological Journal of the Linnean Society, 147 (3): 367-381.

Niehuis, O., S. H. Yen, C. M. Naumann & B. Misof, 2006b. Higher phylogeny of zygaenid moths (Insecta: Lepidoptera) inferred from nuclear and mitochondrial sequence data and the evolution of larval cuticular cavities for chemical defence. Molecular Phylogenetics & Evolution, 39 (3): 812-829.

Okyar, Z., K. A. Efetov, K. Kaya & G. M. Tarmann & F. Can, 2018. "Preliminary list of the Zygaenidae (Lepidoptera) of Turkey, 37". XVI. International Symposium on Zygaenidae (1-5 May 2018, İzmir, Turkey), 39 pp.

Ratnasingham, S. & P. D. N. Hebert, 2007. The barcode of life data system. Molecular Ecology Notes, 7 (3): 355-364.

Schmitt, T. & A. Seitz, 2004. Low diversity but high differentiation: the population genetics of *Aglaope infausta* (Zygaenidae: Lepidoptera). Journal of Biogeography 31 (1): 137-144.

Serdar, M., 2014. Erzurum İli Geometridae (Lepidoptera) Türlerinin DNA Barkodlama Yöntemiyle Karakterizasyonu. Atatürk Üniversitesi, Fen Bilimleri Enstitüsü, Bitki Koruma Ana Bilim Dalı, (Unpublished) Master Thesis, Erzurum, Turkey, 74 pp. (in Turkish with abstract in English).

Sihvonen, P. & K. Nupponen, 2005. Taxonomy of *Rhodostrophia jacularia* (Hubner, 1813) a Sterrhinae moth with variable female wing shape (Lepidoptera: Geometridae). Nota Lepidopterologica, 28 (2): 113-122.

Spalding, A., I. Fukova & H. R. Constant-Ffrench, 2013. The genetics of *Luperina nickerlii* Freyer, 1985 in Europes (Noctuidae). Nota Lepidopterologica, 36 (1): 35-46.

Tarmann, G. M., 1984. Generische Revision der amerikanischen Zygaenidae mit Beschreibung neuer Gattungen und Arten (Insecta: Lepidoptera). Entomofauna (Zeitschrift für Entomologie) Supplemantary, 2 (1): 176 pp.

Tarmann, G. M., 1994. A preliminary review of the classification of the zygaenid subfamily Procridinae (Lepidoptera). Nota Lepidopterologica, 5: 115-123.

Tarmann, G. M., 2003. "Zygaenidae as pest species, 151-229". In: Proceedings of the 7th International Symposium on Zygaenidae (Lepidoptera), (4-8 September 2000, Innsbruck) (Eds. K. A. Efetov, W. G. Tremewan & G. M. Tarmann). Simferopol. Crimean State Medical University Press, 360/151-229 pp.

Tarmann, G. M., 2004. "Zygaenid moths of Australia: A revision of the Australian Zygaenidae (Procridinae: Artonini)". CSIRO Publishing, Collingwood, 248 pp.

Tarmann, G. M., 2009. Die Vinschgauer Trockenrasen-ein Zustandsbericht auf Basis der Bioindikatoren Tagfalter und Widderchen (Lepidoptera: Rhopalocera, Zygaenidae). Wissenschaftliches Jahrbuch der Tiroler Landesmuseen 2: 306-350.

Wilson, J. J., 2012. "DNA Barcodes for Insects, 17-46". In: DNA Barcodes. Methods in Molecular Biology: Methods and Protocols (Eds. W. Kress & D. Erickson), Humana Press, Totowa, Vol 858, 470 pp.

Yen, S. H., 2003. Phylogeny and systematics of the major lineages of Chalcosiinae s.l. (Zygaenidae s.l.): preliminary observations on morphological characters. Systematic Entomology, 30 (3): 358-397.

Zeller, P. C., 1847. Verzeichniss der vom Prof. Dr Loew in der Türkeij und Asien gesammelten Lepidoptera. Isis von Oken, 40: 3-39.

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Original article (Orijinal araştırma)

Effects of cold storage on the developmental biology of *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae) at different larval stages

Soğukta depolamanın farklı dönemlerdeki *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae) larvalarının gelişimsel biyolojisine etkileri

Evrim SÖNMEZ[1*]

Abstract

Being able to store insects at low temperature is important in the mass breeding of insects for commercial purposes. The aim of this study is to investigate the effect of cold storage on pupal and adult weight, adult emergence time and proportion and adult deformation proportion of different weight *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae) larvae. This study was conducted between 2018-2019 in the Biology Laboratory of Science Teaching Department, Sinop University. Trials were performed at 27 ± 2 and 4ºC, 65 ± 5% RH and continuous darkness. After the larvae began to form, they were weighed and divided into three groups according to their weight. They were exposed to 4ºC for 5, 10, 20, 30 and 60 d in separate Petri dishes. With increased cold storage time, the proportion of deformation increased and the proportion of adult emergence decreased. Adult weight, pupal weight and longevity depended on both larval weight and cold exposure time. In conclusion, it is recommended that mass producers or researchers pay attention to the size of the larvae and the cold exposure times to obtain the best quality product and high production efficiency.

Keywords: Cold exposure, deformation rate, insect, insect development, temperature, *Tenebrio molitor*

Öz

Böcekleri düşük sıcaklıklarda depolamak, böceklerin ticari amaçla kitlesel olarak yetiştirilmesinde önemli rol oynamaktadır. Bu çalışmadaki amaç düşük sıcaklıkta depolamanın farklı ağırlıktaki *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae) larvalarının pupa ve ergin ağırlığı, ergin çıkış süresi ve yüzdesi ve ergin deformasyon yüzdelerine etkisini araştırmaktır. Bu çalışma 2018-2019 yılları arasında Sinop Üniversitesi, Fen Bilgisi Eğitimi, Biyoloji Laboratuvarında yapılmıştır. Denemeler 27 ± 2 ve 4ºC, %65 ± 5 bağıl nem ve devamlı karanlık şartlarında yapılmıştır. Larvalar oluşmaya başladıktan sonra tartılarak ağırlıklarına göre 3 gruba ayrılmıştır. Gruplara ayrılan larvalar ayrı petrilerde 5, 10, 20, 30 ve 60 gün olmak üzere 4ºC'ye soğuğa maruz bırakılmıştır. Sonuç olarak, soğukta depolama süresi arttıkça deformasyon yüzdesinin arttığı ve ergin çıkış yüzdesinin azaldığı görülmüştür. Ergin ağırlığı, pupa ağırlığı ve ömür uzunluğu ise larva ağırlıklarına ve soğuğa maruz kalma süresine bağlı olarak değişmiştir. Kitlesel üretim yapacak üreticiler veya araştırıcıların en iyi kaliteyi ve verimi alabilmek için larvaların büyüklüklerine dikkat etmeleri önerilmektedir.

Anahtar sözcükler: Soğuğa maruz bırakma, deformasyon oranı, böcek, böcek gelişimi, sıcaklık, *Tenebrio molitor*

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Introduction

Temperature is an important factor affecting the developmental biology of insects, as in all organisms (Sinclair et al., 2003; Arbab, 2019). Large larvae and pupae of insects are more resistant to adverse climatic conditions than eggs or young larvae (Gagneâ & Coderre, 2001; Khaliq et al., 2014; Arbab, 2019). As temperature decreases, insects lose some of their ability to feed, move and fly. Prolongation of exposure to cold may cause irreversible loss of these activities (Rathee & Ram, 2018). Costa et al. (2016) found in a study of eggs and nymphs of *Podisus nigrispinus* Say, 1832 (Hemiptera: Pentatomidae) that most embryos died at 5ºC storage. They suggested that eggs can be stored at 15ºC for a maximum of 15 d.

Being able to store insects at low temperature is important in commercial breeding of insects or biological control methods (Kim et al., 2015). Insect embryos get the energy required to maintain their development directly from vitellogenic reserves (Denlinger & Lee, 2010; Chapman, 2013; Klowden, 2013). When exposed to low temperature, they slow down their metabolic activities and they can survive longer by means of the reserves they previously stored. Conversely, developmental times are prolonged. However, if exposure to the cold is prolonged, these reserves are exhausted and eventually they die (Chown & Nicolson, 2004; Kostal et al., 2004; Colinet et al., 2015). Many studies have found that cold storage can have negative effects on insect biology. For example, when *Trissolcus basalis* Wollaston, 1858 (Hymenoptera: Platygastridae) and *Telenomus podisi* Ashmead, 1893 (Hymenoptera: Scelionidae) pupae were stored at different temperatures and at different periods (12 and 15ºC, 4-7 months), and no adult emergence was observed at 12ºC (Foerster & Doetzer, 2006). In another study, Liu and Tian (1987) found that when *Encarsia formosa* was stored between 3 and 12ºC, the adult growth rate increased with the storage temperature.

The larvae of some insects such as *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae), a species that damage stored products, are used as livestock feed and even as human food in some countries. However, the last instar of this insect larvae is also used as feed in aquaculture and poultry breeding because they contain high quality protein and fat (Li et al., 2013; Shockley & Dossey, 2014; Çalışlar, 2017; Arbab, 2019; Selaledi et al., 2020). The biology of the *T. molitor* has been well studied and its optimal conditions are 27ºC at >60% RH (Li et al., 2013). According to Li et al. (2014), the egg period lasts 3-9 d, the larval stage 26-76 d, and the pupal stage 5-17 d. Before emergence, most of the larvae go through typically 15 to 20 instars (Park et al., 2014). However, developmental rate and size of insects is affected by factors such as temperature, humidity, light, food and density of samples in breeding tanks (Koo et al., 2013).

Tenebrio molitor stands out as a promising species suitable for mass production among insects. They are in high demand for use as natural feed sources for poultry and fish. For this reason, producers keep the *T. molitor* in the refrigerator until supplied to costumers because cold storage extends the shelf life of the farmed insects (Levie et al., 2005; Koo et al., 2013; Bovera et al., 2015; Sihyeon et al., 2016). However, this can cause deformed adults with low egg quality or nutritional value to reach the customers depending on the length of the waiting period. Also, when the culture is very intense in the laboratory, the researchers keep the *T. molitor* larvae or pupae in the refrigerator to study later. This also affects the parameters of the adults emerging from these larvae or pupae, such as deformed, low quality eggs and longevity. The use of these adults in other studies may also cause false results. Also, prolonged exposure time to low temperatures may have negative effects on the life cycle of insects. Especially, it can negatively affect many parameters such as developmental time, pupal emergence rate, adult emergence time and deformation percentages (Liu et al., 2014). In order to prevent these negative effects, the biology of the insect must be well known.

Previously Sönmez & Koç (2019) determined that as exposure of *T. molitor* pupae to low temperature increases, the pupal period and deformation rates increase. It is known that resistance to low temperatures is lower for larvae than for pupae (Punzo & Mutchmor, 1980). Studies on the effects of low temperature

development of larvae and pupae on rate of pupation, rate of adult emergence and deformation rate of adult are increasing (Arbab, 2019). How larvae in different larval stages respond to low temperature has become increasingly important in recent years. In this study, *T. molitor* larvae of different weight (different larval instars) were exposed to cold for different periods. The hypothesis tested was that increasing exposure time to low temperature differentially affects the development of larvae of different weight. Due to reduced lipid and protein contents, small larvae were predicted to be affected more than medium and large larvae in terms of pupal period, adult emergence time, proportion of adult emergence and proportion of adult deformation.

Materials and Methods

Tenebrio molitor cultures were grown at the Sinop University, Biology Laboratory of Science Teaching Department for five generations were used. Trials were performed at 27 ± 2 and 4ºC, 65 ± 5% RH. Flour and whole meal flour were used as food in 1:1 ratio. The insects were reared in plastic containers (30 × 20 × 5 cm). Sawdust was added in the containers to ease movement on the foodstuff. Small pieces (2 for each container, 4 × 4 × 6 cm) were cut from egg boxes for providing convenience for adults to mate and lay eggs. The containers were covered to prevent entrance of other invertebrates, but small holes were opened on the top side to enable gas exchange. Potato was used for humidity (3 × 3 × 3 cm), wrapped into aluminum foil to prevent contact, moisturizing and decay of the food. The potatoes were changed every 3 d for the food layer not to get moldy. The food layer was adjusted to 4-5 cm thickness. The food was renewed with intervals of 10 d. The larvae in the old food were separated by using a sifter and they were transferred on the new food. The containers were checked every day (Figure 1).

Figure 1. *Tenebrio molitor* rearing containers, experimental study establishment and deformed individuals.

Tenebrio molitor larvae show plasticity in the larval stage. Larval instar number may vary depending on environmental factors such as nutrients, temperature and humidity (Graham et al., 2000; Morales-Ramos, 2010; Park et al., 2014). Larval stages are therefore very difficult to distinguish. When selecting larvae, control groups were used and the methods of Graham et al. (2000) and Park et al. (2014) were used. While selecting the larvae and determining the instar difference (age), their weights were checked and the photographs of Park et al. (2014) were used. In the control group, larvae pupated after ~210 mg. Therefore, larvae larger than 210 mg were not included in the study. The larvae of 180-210 mg were

included in the study as the last larval instar. Larval weights were limited both because it was difficult to distinguish larval stages and larvae less than 100 mg were likely to be damaged during transfer (Graham et al., 2000). Therefore, while small larvae were selected, the larvae under 40 mg were not included in the trials (Graham et al., 2000; Morales-Ramos et al., 2012, 2015; Park et al., 2014). In order to set a standard, those lighter than 100 mg were divided into small larvae (7-11th larval instar), and those heavier than 180 mg were divided into large groups (17-20th last larval instar). Larvae of 100-180 mg (12-16th instar) were grouped as medium larvae (Graham et al., 2000; Park et al., 2014).

Larvae were randomly selected and weighed (Radwag AS 220.R2) and divided into three groups according to their weight. Each of the larvae separated into was exposed to the cold at +4ºC for 5, 10, 20, 30 and 60 d in a separate Petri dish with added food (9 × 1.5 cm) (for each cold group: 10 larvae × 10 Petri dishes × 3 trial groups). The groups whose cold exposure ended were brought to the laboratory where further experiments were performed (27 ± 2ºC and 65 ± 5% RH). The nutritional conditions given above were provided until the larvae pupated. The Petri dishes were checked every day. When larvae pupated, they were weighed. Date was recorded on the day they pupated. Pupae were checked every day and the date of the adult was recorded. The days between the day they become pupa and the day of adult emergence were calculated as the adult emergence time. Adult insects were weighed, those with deformation were identified and placed in separate Petri dishes (with food added) for longevity trials. The day insects died was recorded. The days between the day of became adult and the day until death was calculated as the longevity. Deformation was defined as adults without wings, with curved wings or with deformations of elytra. The deformation rates were determined by dividing the deformed individuals first by the number of pupae placed in Petri dish. The nutritional conditions as given above were provided. Trials were repeated three times for each group of larvae (<100, 100-180 and >180 mg). A total of 30 (10 × 3) larvae were used for each weight of larva groups. For example, for <100 mg cold application of 5 d, a total of 30 larvae were used, 10 larvae in three replicates. There are six cold groups together with the control groups. A total of 540 larvae were used for the whole study. In the trials, no adult emergence was observed in the <100 mg larva group that was stored in cold for 60 d only, the all pupae were dead. Therefore, adult weight, adult emergence time, adult emergence and deformation rate and longevity were not shown in the tables.

Data analysis

All the data were analyzed with the SPSS 21 statistical package program. Descriptive statistics of the data were given first in the study. Secondly, the normality assumption of the data was checked by the Kolmogorov-Smirnov test and it was observed that the data were distributed normally ($p > 0.05$). Analysis of groups and their interactions was done using generalized linear models (two-way ANOVA). Analysis of different groups was also investigated by Tukey HSD test. Tukey HSD test was used to determine the differences among the small (<100 mg), medium (100-180 mg) and large larvae (>180 mg) and their relationship to each other. Since no adult emergence was observed after pupation in 60-day groups less than 100 mg, the data in this group were not evaluated, and only five groups were compared and control groups. Independent t-test was performed for paired comparisons according to larval weights. For statistical analysis, average data of three trials (10 insects in each trial with three repeats giving 30 insects in total) for each cold application were obtained.

Results

The differences between the weights of the pupae ($F_{4,524} = 2.45$, $p < 0.001$; $F_{2,524} = 115$, $p < 0.001$; $F_{8,524} = 12.5$, $p < 0.001$) and adults ($F_{4,499} = 11.8$, $p < 0.001$; $F_{2,499} = 32.4$, $p < 0.001$; $F_{8,499} = 8.16$, $p < 0.001$) (including control groups) for small, medium and large larvae stored in the cold for 5-60 d were statistically significant (Table 1). No adults developed from small larvae stored in the cold for 60 d.

Table 1. Two-way ANOVA-Tukey HSD test results of the pupal and adult weight obtained from *Tenebrio molitor* larvae stored in cold for 5-60 d

Larval group	Periods of cold storage (d)					
	5	10	20	30	60	Control
Pupae (mg, mean ± SD)						
<100 mg	144 ± 23.2 Aa*	126 ± 20.4 Ba	128 ± 27.8 ABa	123 ± 17.5 Ba	126 ± 23.5 Ba	152 ± 19.6 Ca
100-180 mg	119 ± 18.8 Ab	141 ± 16.7 Bb	124 ± 17.3 Ba	131 ± 22.8 ABa	132 ± 19.6 ABa	158 ± 17.1 Ca
>180 mg	163 ± 26.0 Ac	164 ± 26.7 Ac	165 ± 8.8 Ab	151 ± 15.8 Ab	164 ± 24.9 Ab	156 ± 18.3 Ba
Adults (mg, mean ± SD)						
<100 mg	122 ± 16.9 Aa	112 ± 17.6 Aa	119 ± 17.8 Aa	118 ± 21.0 Aa	—	130 ± 14.3 Ba
100-180 mg	100 ± 18.3 Ab	117 ± 23.0 Ba	109 ± 15.6 ABb	113 ± 23.2 ABb	116 ± 16.7 Bb	129 ± 15.6 Ca
>180 mg	104 ± 12.6 Ab	143 ± 19.7 Bb	140 ± 8.4 Bc	123 ± 26.1 Ca	133 ± 16.5 BCa	128 ± 13.2 Da

* Means followed by the same uppercases letter within rows or lowercase letter with columns are not statistically significant ($p > 0.05$).

There was a difference between the emergence times of adults between all larval groups exposed to cold for 5-60 d ($F_{4,534}$ =5.90, $p < 0.001$; $F_{2,526} = 3.84$, $p = 0.022$; $F_{8,526} = 8.16$, $p < 0.001$) (Table 2). There were statistical differences between the proportion of adult emergence compared to the control ($F_{4,46} = 14.7$, $p < 0.001$; $F_{2,46} = 10.3$, $p < 0.001$; $F_{8,46} = 1.71$, $p = 0.014$) (Table 2).

Table 2. Two-way ANOVA-Tukey HSD test results of the adult emergence time and proportion of adult emergence obtained from *Tenebrio molitor* larvae stored in cold for 5-60 d

Larval group	Periods of cold storage (d)					
	5	10	20	30	60	Control
Adult emergence time (d, mean ± SD)						
<100 mg	7.3 ± 0.9 Aa*	6.4 ± 1.5 Aa	7.4 ± 2.5 Aa	7.5 ± 0.8 Aa	—	5.9 ± 0.6 Ba
100-180 mg	6.8 ± 0.9 Aa	7.5 ± 1.5 Ab	7.2 ± 0.8 Aa	7.3 ± 0.7 Aa	9.0 ± 1.5 Bb	5.6 ± 1.6 Ca
>180 mg	7.9 ± 0.8 Aa	7.5 ± 1.1 Ab	6.9 ± 1.1 Aa	7.6 ± 0.7 Aa	7.0 ± 0.9 Aa	5.0 ± 1.2 Ba
Proportion of adult emergence (%, mean ± SD)						
<100 mg	53.3 ± 15.2 Aa	40.0 ± 13.2 Aa	35.0 ± 12.9 Ba	25.0 ± 11.7 Ca	—	94.1 ± 12.4 Da
100-180 mg	41.6 ± 7.6 Aa	45.0 ± 13.5 Aa	38.3 ± 12.3 Aa	40.0 ± 18.0 Aa	8.0 ± 5.0 Ba	95.2 ± 9.8 Ca
>180 mg	68.3 ± 12.5 Ab	62.6 ± 6.3 Ab	60.6 ± 7.5 Ab	56.3 ± 10.4 Ab	10.6 ± 1.5 Bb	98.5 ± 13.5 Ca

* Means followed by the same uppercases letter within rows or lowercase letter with columns are not statistically significant ($p > 0.05$).

The differences between the longevity ($F_{4,524} = 2.76$, $p = 0.027$; $F_{2,524} = 3.33$, $p = 0.037$; $F_{8,524} = 1.54$, $p < 0.001$) and proportion of deformation ($F_{4,44} = 148$, $p <0.001$; $F_{2,44} = 9.53$, $p < 0.001$; $F_{8,44} = 112$, $p < 0.001$) between all larval groups were statistically significant (Table 3).

The proportion of deformation increased in direct relationship as the period of exposure to low temperature. The proportion of adult deformation of adults developing from larvae exposed to cold increased up to 90% especially for 30 and 60 d (in medium and large larvae).

Table 3. Two-way ANOVA-Tukey HSD test results of the adult longevity (d, mean ± SD) and proportion of adult deformation (%, mean ± SD) obtained from *Tenebrio molitor* larvae stored in cold for 5-60 d

Larval group	Periods of cold storage (d)					
	5	10	20	30	60	Control
	Longevity (d, mean ± SD)					
<100 mg	19.2 ± 5.0 Aa*	16.1 ± 4.7 ABa	14.0 ± 6.5 Ba	14.1 ± 3.8 Ba	—	25.1 ± 6.8 Ca
100-180 mg	15.2 ± 4.8 Ab	14.6 ± 6.9 Aa	14.2 ± 3.0 Aa	14.6 ± 4.6 Aa	15.1 ± 4.6 Aa	27.8 ± 3.2 Ba
>180 mg	16.1 ± 1.7 Ab	15.6 ± 7.4 Aa	15.7 ± 5.6 Aa	16.2 ± 7.3 Aa	16.6 ± 4.4 Aa	28.2 ± 7.7 Ba
	Proportion of deformation (%, mean ± SD)					
<100 mg	32.6 ± 5.0 Aa	45.3 ± 2.5 Ba	48.3 ± 3.5 Ba	62.3 ± 5.5 Ca	—	9.7 ± 3.1 Da
100-180 mg	10.6 ± 2.5 Ab	40.3 ± 5.0 Bbc	42.3 ± 5.0 Bbc	48.3 ± 2.0 Bb	90.6 ± 4.3 Ca	8.9 ± 4.3 Da
>180 mg	12.1 ± 2.0 Ab	30.3 ± 4.3 Bc	33.1 ± 1.5 Bc	45.6 ± 3.7 Cb	92.3 ± 2.5 Da	7.3 ± 3.7 Ea

* Means followed by the same uppercases letter within rows or lowercase letter with columns are not statistically significant ($p > 0.05$).

Discussion

This study showed that as duration of cold exposure increased, especially in adults the proportion of deformation increased. In addition, the weights of pupa and adult obtained from the larvae in cold storage changed as the storage period increased. Although, no change was observed in the adult emergence time groups except for the 60-d-cold group and the control. The proportion of adult emergence was clearly lower in the 60-d-cold group. In terms of longevity, only the small larva group was affected and all groups were found to be significantly different from the control. Therefore, the initial hypothesis was confirmed that proportion of deformation increases with cold storage time. Although food was placed in Petri dishes during the cold storage process, the larvae remained inactive and either stopped feeding completely or fed only a little. However, the larvae were returned to laboratory conditions after cold storage recommenced feeding. For this reason, the survival of these larvae during and after cold storage depends on the proteins and lipids they can store for surviving the cold storage period. The data obtained and the increase in the deformation rate in parallel with the cold storage time show that long-term storage affects these reserves considerably (Graham et al., 2000; Kalyoncu et al., 2005; Geng et al., 2019).

In the study of Sönmez & Koç (2019), it was determined that the adult emergence times were prolonged as the time of cold exposure of the pupae increased. The proportion of deformation in emerged adults increased with duration of cold exposure. Adult weights did not change, while adult emergence rate decreased. The proportion of adult emergence of adults decreased and the deformation rate of emerging adults increased with the exposure to low temperature. However, as the exposure time to cold increased, there was a tendency was for no change in adult emergence time except when compared to control. Therefore, from a mass production point of view, larvae are more tolerant to low temperature exposure than pupae (Ludwig, 1956; Punzo & Mutchmor, 1980; Riberio, 2017). In other words, it is evident from this study that larvae kept in cold are more resistant than those kept in the pupal stage (Paul et al., 2017). According to Sönmez & Koç (2019), these differences in the proportion of adult emergence and adult emergence time could indicate a positive correlation between the lipid content stored by the larvae in their bodies and their tolerance to low temperatures (Chown & Gaston, 2010; Mitchella et al., 2017). Especially the proportion of adult emergence of the large larvae was higher than others (for example, when 5 d cold storage, small, medium and large larvae were compared). Therefore, large larvae have more stored lipids and use it for pupa formation, and have a greater tolerance to low temperatures (Packer & Corbet, 1989; Smith, 2002; Beukeboom, 2018). Since smaller larvae store less lipids, it has been reported that the generations from these may cause a decrease in fecundity and longevity, and delays in development (Mirth & Riddiford, 2007).

Acclimation is an adaptation to temperature changes and can cause deformations in some insects. Insects develop different adaptations to survive at low temperature. One of these is water loss, which reduces changes in biology at low temperatures (Punzo & Rosen, 1984; Taşkın & Ergin, 2013; Mitchella et al., 2017). In the present study, it was determined that as the exposure time of the larvae to low temperature increases, the proportion of adult deformation increases in direct proportion. Especially in medium and large larvae, the proportion of deformation in larvae exposed cold for 60 d has increased up to 90% (Table 3). It was determined that all pupae obtained from the small larvae that were exposed to cold for 60 d died and no adult emergence. It can be argued that the group most resistant to cold in terms of the resulting proportion of adult emerging was the large larvae. However, the deformation proportion of the emerging adults also increased as the duration of exposure to cold increased. The high resistance of medium and large larvae to low temperature is probably enabled by the lipids and proteins stored by the insect during embryological development, and allows the larval stage to reach adult. However, the rate of deformation of adults may be due to their inability to resist dehydration due to the period of time at low temperature (Punzo & Rosen, 1984; Selaledi et al., 2020). Large larvae appear to be more tolerant of low temperatures because they contain more lipids and proteins, but the emerging adult will not be able to have a healthy life because of higher deformation rates.

Kalyoncu et al. (2005) exposed the pupae of *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae) to cold and found that the adults obtained from these pupae were small and some of them had curved wings. Pupae hatching percentage decreased depending on application time. Smith (2002) found that *Nicirophorus investigator* (Zetterstedt, 1824) (Coleoptera: Silphidae) adults that spent the winter as larvae were larger than those that spent the winter as adults and had higher survival rates. Geng et al. (2019) found that when *Phyllonorycter ringoniella* Matsumura, 1931 (Lepidoptera: Gracillariidae) egg, larva and pupa were cold stored (4ºC, 0-105 d), the highest deformation rate was observed in those in the pupal stage. The deformation rate increased in direct proportion to the duration of exposure to low temperature. Similar to the present study, in a study conducted with *T. molitor*, Graham et al. (2000) separated the larvae by their weight: 11-13, 100-120 and t>190 mg. They found that exposed the larvae to 4ºC for 4 weeks, thermal hysteresis proteins increased regardless of the larval size, but growth and development continued. In addition, they found that in the pupal stage, these proteins decreased twentyfold, and development and growth stopped. For this reason, it may be more appropriate for *T. molitor* to keep larvae of >180 mg rather than pupae. Also, adult deformation rates should be taken into account and larvae should not be kept for more than 20 d in the cold. Temperature changes can also affect the longevity of insects. In a study they conducted on *Trichogramma evanescens* Westwood, 1833 (Hymenoptera: Trichogrammatidae), Karabörklü & Ayvaz (2007) stored egg, larvae and pupae at 4ºC for 10, 20, 30 and 40 d. As the exposure time increased, the number and longevity of adults obtained from all stages significantly decreased. In the present study, the proportion of adults and longevity decreased with storage period, especially for small and medium larvae. Ayvaz & Karabörklü (2008) found the mortality close to 50% after 6 weeks and 100% after 10 weeks in *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) exposed to 10ºC for 1-10 weeks. In the present study, small larvae, in particular, could not tolerate 60 d of cold storage and there was no adult emergence. Adults obtained from medium and large larvae exposed to cold for only 5 d had shorter survival than small larvae. While the longevity of small larvae decreased as cold exposure time increased, no change was observed between medium and large larvae (Mirth & Riddiford, 2007). Gülel (1982) found that the lifespan of *Dibrachys boarmiae* Walker, 1863 (Hymenoptera: Pteromalidae) adults kept at 4ºC for 15 d increased, whereas *Apanteles galleria* Wilkinson, 1932 (Hymenoptera: Braconidae) adults died at 6ºC. In contrast, adults of *Panolis flammea* (Denis & Schiffermüller, 1775) (Lepidoptera: Noctuidae) emerging from the pupa exposed to 2°C for 5-20 d had decreasing longevity with increased exposure (Leather, 1990).

Tunca et al. (2014) studying *Venturia canescens* Gravenhorst, 1829 (Hymenoptera: Ichneumonidae) found that the decrease in temperature and extended storage time (5, 10 and 15ºC for 1, 3, 5, 7 and 15 d) did not affect longevity or adult weight. A study conducted by Tunca et al. (2014) gave similar results in terms of longevity and adult weight. Low temperature exposure of larvae can directly affect the size of pupae and adults (Mirth & Riddiford, 2007; Arbab, 2019). Insects consume lipids when kept at low temperatures, which can result in weight loss (Nurullahoğlu & Kalyoncu, 2000). In the present study, it was determined that the weight of pupae obtained from small larvae decreased compared to the control group. It was found that adults decreased compared to the control group, but no change was observed between the cold treatment groups. Temperature changes affect the life cycles of insects and vary according to their developmental stages (Ludwig, 1956; Bowler, 1967; Mirth & Riddiford, 2007; Costa et al., 2016; Mitchella et al., 2017; Arbab, 2019). This may be due to an increase in weight with the increase in tolerance to low temperature, as well as an increase in lipid storage (Mitchella et al., 2017; Paul et al., 2017; Beukeboom, 2018).

Conclusions

Tenebrio molitor larvae or pupae are typically cold stored during laboratory studies or during commercial mass production when the population has reached the desired density. However, low storage temperature and long exposure times will change the biology of this insect. Such exposure will particularly increase the number of deformed insects. Given that the amount of protein and lipid stored in larvae will decrease during cold storage, it may not be appropriate to use cold-stored insects in biological studies or as a commercial live-feed product. In this study, it was determined that as the storage period increases, the proportion of deformation particularly increases, and the weight of pupae and adults is decreases particularly for small larvae. Consequently, producers or researchers need to pay attention to the size of the larvae in order to obtained the best quality product and highest yield. Therefore, it is recommended that larvae are not stored for more than 20 d. Future studies should also investigate to what extent eggs of *T. molitor* are affected by low temperature exposure.

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References

Arbab, A., 2019. Effect of temperature on pupal development in meal worm *Tenebrio molitor* L. Indian Journal of Entomology, 81 (4): 640-646.

Ayvaz, A. & S. Karabörklü, 2008. Effect of cold storage and different diets on Zeller (Lep: Pyralidae). Journal of Pest Science, 81 (1): 57-62.

Beukeboom, L. W., 2018. Size matters in insects-an introduction. Entomologia Experimentalis et Applicata, 166 (1): 2-3.

Bovera, F., G. Piccolo, L. Gasco, S. Marono, R. Loponte, V. Vassalotti, G. Mastellone, P. Lombardi, Y. A. Attia & A. Nizza, 2015. Yellow mealworm larvae (*Tenebrio molitor*, L.) as a possible alternative to soybean meal in broiler diets. British Poultry Science, 56 (5): 569-575.

Bowler, K., 1967. Changes in temperature tolerance with adult age in *Tenebrio molitor*. Entomologia Experimentalis et Applicata, 10 (1): 16-22.

Çalışlar, S., 2017. Nutrient content of Mealworms (*Tenebrio molitor* L.) and the utilization possibilities in poultry nutrition. Nevşehir Bilim ve Teknoloji Dergisi, 6 (ICAFOF 2017 Special Issue): 226-232.

Chapman, R. F., 2013. The Insects: Structure and Function (Ed. S. J. Simpson). Cambridge University Press, New York, USA, 929 pp.

Chown, S. L. & K. J. Gaston, 2010. Body size variation in insects: a macroecological perspective. Biological Reviews, 85 (1): 139-169.

Chown, S. L. & S. W. Nicolson, 2004. Insect Physiological Ecology: Mechanism and Patterns. Oxford University Press Inc., New York, USA, 256 pp.

Colinet, H., B. J. Sinclair, P. Vernon & D. Renault, 2015. Insects in fluctuating thermal environments. Annual Review of Entomology, 60 (1): 123-140.

Costa, V. H. D., M. A. Soares, S. A. Jr Assis, R. D. Silveira, T. C. Reis & E. M. Pires, 2016. Egg storage of *Podisus nigrispinus* (Hemiptera: Pentatomidae) predators at low temperatures. Revista Árvore, Viçosa-MG, 40 (5): 877-884.

Denlinger, D. L. & R. E. Lee, 2010. Low Temperature Biology in Insects. Cambridge University Press, Cambridge, UK, 404 pp.

Foerster, L. A. & A. K. Doetzer, 2006. Cold storage of the egg parasitoids *Trissolcus basalis* (Wollaston) and *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae). Biological Control, 36 (2): 232-237.

Gagneâ, I. & D. Coderre, 2001. Cold storage of *Coleomegilla maculata* larvae. Biocontrol Science and Technology, 11 (3): 361-369.

Geng, S., H. Hou & C. Jung, 2019. Effect of diets and low temperature storage on adult performance and immature development of *Phyllonorycter ringoniella* in laboratory. Insects, 10 (11): 2-13.

Graham, A. L., V. K. Walker & P. L. Davies, 2000. Developmental and environmental regulation of antifreeze proteins in the mealworm beetle *Tenebrio molitor.* Europan Journal of Biochemistry, 267 (21): 6452-6458.

Gülel, A., 1982. Studies on the biology of the *Dibrachys boarmiae* (Walker) (Hymenoptera: Pteromalidae) parasitic on *Galleria mellonella* (L.). Zeitschrift Angewandte Entomologie, 94 (1-5): 138-149.

Kalyoncu, L., T. Üstüner & A. Aktümsek, 2005. Effects of different temperature degrees on ratio of adult emergence of *Galleria mellonella* (L.) pupae. Selçuk Üniversitesi Fen Edebiyat Fakültesi Fen Dergisi, 26 (1): 71-74.

Karabörklü, S. & A. Ayvaz, 2007. Effects of cold storage on the life stages of *Trichogamma evanescens* Westwood (Hym: Trichogrammatidae) reared on different host eggs. Erciyes Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 23 (1): 30-36.

Khaliq, A., M. Javed, M. Sohail & M. Sagheer, 2014. Environmental effects on insects and their population dynamics. Journal of Entomology and Zoology Studies, 2 (2): 1-7.

Kim, S. Y., Y. B. Lee, J. B. Park & N. J. Kim, 2015. Growth characteristics of mealworm *Tenebrio molitor*. Journal of Sericultural & Entomological Science, 53 (1): 1-5.

Klowden, M., 2013. Physiological Systems in Insects. 3th Edition. Elsevier Inc., London, UK, 696 pp.

Koo, H., K. Seon-Gon, O. Hyung-Keun, K. Jung-Eun, C. Duck-Soo, K. Do-Ik & K. Iksoo, 2013. Temperature-dependent development model of larvae of mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Korean Journal of Applied Entomology, 52 (4): 387-394.

Kostal, V., J. Vambera & J. Bastl, 2004. On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. Journal of Experimental Biology, 207 (9): 1509-1521.

Leather, S. R., 1990. Life spain and ovarian dynamics of the pine beauty moth, *Panolis flammea* (D&S): The effect of low temperatures after adult emergence on reproductive success. Physiological Entomology, 15 (3): 347-353.

Levie, A., P. Vernon & T. Hance, 2005. Consequences of acclimation on survival and reproductive capacities of cold-stored mummies of *Aphidius rhopalosiphi* (Hymenoptera: Aphidiinae). Journal of Economical Entomology, 98 (3): 704-708.

Li, K., B. Fu, J. Lin, Y. Fu, Z. Peng & Q. Jin, 2014. Effect of temperature and cold storage on performance of *Tetrastichus brontispae* (Hymenoptera: Eulophidae), a parasitoid of *Brontispa longissima* (Coleoptera: Chrysomelidae). Journal of Insect Science, 14 (257): 1-5.

Li, L. Y., Zhao, Z. R. & Liu, H, 2013. Feasibility of feeding yellow mealworm (*Tenebrio molitor* L.) in bioregenerative life support systems as a source of animal protein for humans. Acta Astronautica, 92 (1): 103-109.

Liu, J. J. & Y. Tian, 1987. Cold storage of *Encarsia formosa* Gahen. Chinese Journal of Biological Control, 3 (1): 4-6.

Ludwig, D., 1956. Effects of temperature and parental age on the life cycle of the mealworm, *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). Annals of the Entomological Society of America, 49 (1): 12-15.

Mirth, C. K. & L. M. Riddiford, 2007. Size assessment and growth control: how adult size is determined in insects. BioEssays, 29 (4): 344-355.

Mitchella, K. A., L. Boardmana, S. Clusella-Trullas & J. S. Terblanchea, 2017. Effects of nutrient and water restriction on thermal tolerance: a test of mechanisms and hypotheses. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 212 (4): 15-23.

Morales-Ramos, J. A., 2010. Developmental plasticity in *Tenebrio molitor* (Coleoptera: Tenebrionidae): analysis of instar variation in number and development time under different diets. Journal of Entomological Science, 45 (2): 75-90.

Morales-Ramos, J. A., S. Kay, M. G. Rojas, D. I. Shapiro-Ilan & W. L. Tedders, 2015. Morphometric analysis of instar variation in *Tenebrio molitor* (Coleoptera: Tenebrionidae). Annals of the Entomological Society of America, 108 (2): 146-159.

Morales-Ramos, J. A., M. G. Rojas, S. Kay, D. I. Shapiro-Ilan & W. L. Tedders, 2012. Impact of adult weight, density and age on reproduction of *Tenebrio molitor* (Coleoptera: Tenebrionidae). Journal of Entomological Science, 47 (3): 208-220.

Nurullahoğlu, Z. Ü. & L. Kalyoncu, 2000. Effects of low temperature on total lipid and fatty acid percentages of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) pupae. Selçuk Üniversitesi Fen Edebiyat Fakültesi Fen Dergisi, 1 (16): 91-93.

Packer, M. J. & P. S. Corbet, 1989. Size variation and reproductive success of female *Aedes punctor* (Diptera: Culicidae). Ecological Entomology, 14 (3): 297-309.

Park, B. J., W. H. Choi, S. H. Kim, H. J. Jin, Y. S. Han, Y. S. Lee & N. J. Kim, 2014. Developmental characteristics of *Tenebrio molitor* larvae (Coleoptera: Tenebrionidae) in different instars. International Journal of Industrial Entomology, 28 (1): 5-9.

Paul, A., M. Frederich, R. C. Megido, T. Alabi, P. Malik, R. Uyttenbroeck, F. Francis, C. Blecker, E. Haubruge, G. Lognay & S. Danthine, 2017. Insect fatty acids: a comparison of lipids from three Orthopterans and *Tenebrio molitor* L. larvae. Journal of Asia-Pacific Entomology, 20 (2): 337-340.

Punzo, F. & J. A. Mutchmor, 1980. Effects of temperature, relative humidity and period of exposure on the survival capacity of *Tenebrio molitor* (Coleoptera: Tenebrionidae). Journal of the Kansas Entomological Society, 53 (2): 260-27.

Punzo F. & L. Rosen, 1984. Comparative temperature and water relations of *Tenebrio obscurus* larvae (Coleoptera: Tenebrionidae). Comparative Biochemistry and Physiology Part A: Physiology, 77 (4): 779-784.

Rathee, M. & P. Ram, 2018. Impact of cold storage on the performance of entomophagous insects: an overview. Phytoparasitica, 46 (6): 421-449.

Riberio, N. T. G. M., 2017. *Tenebrio molitor* for food or feed. Rearing conditions and the effect of pesticides on its performance. Escola Superior Agraria, Politecnica de Coimbra, 1 (1): 1-70.

Selaledi, L., C. A. Mbajiorgu & M. Mabelebele, 2020. The use of yellow mealworm (*T. molitor*) as alternative source of protein in poultry diets: a review. Tropical Animal Health and Production, 52 (1): 7-16.

Shockley, M. & A. T. Dossey, 2014. Insects for Human Consumption, 617-652. In: Invertebrates and Entomopathogens: Mass Production of Beneficial Organisms (Eds. J. A. Morales-Ramos, M. G. Rojas & D. I. Shapiro-Ilan). Academic Press, London, UK, 764 pp.

Sihyeon, K., S. J. Lee, J. C. Lim & J. S. Kim, 2016. Establishment of optimal rearing conditions for the production of *Tenebrio molitor* larvae. Korean Journal of Applied Entomology, 55 (4): 421-429.

Sinclair, B., P. Vernon, J. Klok & S. L. Chown, 2003. Insects at low temperature: an ecological perspective. Trends in Ecology & Evolution, 18 (5): 257-262.

Smith, J. R., 2002. Effect of larval body size on overwinter survival and emerging adult size in the burying beetle, *Nicrophorus investigator*. Canadian Journal of Zoology, 80 (9): 1588-1593.

Sönmez, E. & Y. Koç, 2019. Effects of cold exposure on *Tenebrio molitor* (Coleoptera: Tenebrionidae) pupal period, proportion of adult emergence, weight and deformation percentage. Entomologica Fennica, 30 (4): 43-48.

Taşkın, D. & E. Ergin, 2013. Effect of low temperature and age on adult emergence ratio, time, number of progeny, and sex ratio of *Itoplectis melanocephala* (Gravenhorst) (Hymenoptera:Ichneumonidae). Adıyaman Üniversitesi Fen Bilimleri Dergisi, 3 (1): 38-48.

Tunca, H., A. N. Yeşil & T. F. Çalışkan, 2014. Cold storage possibilities of a larval parasitoid, *Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae). Turkish Journal of Entomology, 38 (1): 19-29.

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Original article (Orijinal araştırma)

Status of the invasive mosquito species *Aedes aegypti* (L., 1762) and *Aedes albopictus* (Skuse, 1895) (Diptera: Culicidae) in Turkey[1]

İşgalci sivrisinek türleri *Aedes aegypti* (L., 1762) ve *Aedes albopictus* (Skuse, 1895) (Diptera: Culicidae)'un Türkiye'deki durumları

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Abstract

Aedes aegypti (L., 1762) and *Aedes albopictus* (Skuse, 1895) (Diptera: Culicidae) are important vectors of arboviruses. In Turkey, *Ae. albopictus* eggs were detected in the Thrace area of northwestern Turkey for the first time in 2011. In 2015, studies revealed the spread of *Ae. albopictus* and the first detection of *Ae*. *aegypti* within northeastern Turkey was reported. This paper reports the results of a survey of the presence and distribution of *Ae*. *albopictus* and *Ae*. *aegypti* in Turkey conducted over 5 years. As of 2019, monitoring studies were conducted on the presence of *Ae*. *albopictus* and *Ae*. *aegypti* in five geographical regions (Black Sea, Central Anatolia, Marmara, Mediterranean and Aegean Regions). A comprehensive range of potential larval habitats, such as tires, artificial containers, cemeteries, water bottles and natural breeding habitats, were assessed. In addition, standard ovitraps and adults' traps were used in some localities. This study showed that *Ae*. *albopictus*, in particular, expanded its distribution each year and has the potential to extend its range throughout Turkey over the next few years. In Turkey, the distribution of *Ae*. *aegypti* is currently limited to northeastern Turkey. Future work focused on determining more effective surveillance and control studies is discussed.

Keywords: *Aedes aegypti*, *Aedes albopictus*, breeding habitats, mosquito ecology, vector control

Öz

Aedes aegypti (L., 1762) ve *Aedes albopictus* (Skuse, 1895) (Diptera: Culicidae) birçok arbovirüsü taşıyan çok önemli vektörlerdir. Türkiye'de *Ae. albopictus* yumurtaları ilk defa 2011 yılında Türkiye'nin kuzeybatısında bulunan Trakya'da tespit edilmiştir. 2015 yılında ise yapılan çalışmalarla, *Ae. albopictus*'un yayılımını genişlettiği ortaya çıkarılmış ve *Ae*. *aegypti* türü de Türkiye'nin kuzeydoğusunda ilk defa tespit edilmiştir. Bu çalışma *Ae*. *albopictus* ve *Ae*. *aegypti*'nin Türkiye'de 5 yıl boyunca yayılım ve dağılımlarına dair bulgular veren ilk çalışmadır. 2019 yılı itibarıyla *Ae*. *albopictus* ve *Ae*. *aegypti*'nin varlığını tespit etmek için 5 coğrafi bölgede (Karadeniz, İç Anadolu, Marmara, Akdeniz ve Ege Bölgeleri) çalışmalar yapılmıştır. Çalışmada lastikler, yapay konteynerler, mezarlıklar, su şişeleri ve doğal üreme habitatları gibi tüm potansiyel larva üreme alanları kontrol edilmiştir. Ayrıca bazı alanlarda ovitrap ve ergin tuzakları da kullanılarak örneklemeler yapılmıştır. Sonuçlar, *Ae*. *albopictus*'un her yıl yayılımını arttırdığını ve gelecek yıllarda tüm Türkiye'de yayılma potansiyeli taşıdığını göstermiştir. *Aedes aegypti*'nin yayılımı ise şimdilik sadece kuzeydoğu ile sınırlı kalmıştır. Gelecekte yapılabilecek daha efektif sürveyans ve kontrol çalışmaları da tartışılmıştır.

Anahtar sözcükler: *Aedes aegypti*, *Aedes albopictus*, üreme alanları, sivrisinek ekolojisi, vektör kontrolü

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Introduction

The yellow fever mosquito, *Aedes aegypti* (L., 1762) (Diptera: Culicidae), originated in Africa where a domestic form arose through a single sub speciation event and spread throughout the rest of the subtropical world via human movement and trade. While it established throughout southern Europe during from the late eighteenth to the mid twentieth centuries, the mosquito inexplicably disappeared from the Mediterranean, Black Sea and Macaronesian biogeographical regions (Canary Islands, Madeira and the Azores) (Schaffner & Mathis, 2014). *Aedes aegypti* has since colonized Madeira (Almeida et al., 2007), reappeared in Georgia and southern Russia (Krasnodar Krai and Abkhazia) (Yunicheva et al., 2008) and has been reported in the Netherlands (Scholte et al., 2010) and Turkey (Akıner et al., 2016).

The Asian tiger mosquito, *Aedes albopictus* (Skuse, 1895) (Diptera: Culicidae) has a widespread distribution that includes both temperate and tropical climates. However, the mosquito is native to subtropical and tropical parts of Southeast Asia, and has spread to many regions including Africa, Europe, the Middle East and America due to international human travel and the transportation of used tires (Knudsen, 1995; Mitchell, 1995). *Aedes albopictus* has been reported in over 20 European countries and is considered the most settled invasive mosquito species in Europe (Medlock et al., 2015).

Aedes aegypti and *Ae. albopictus* are vectors of several important arboviruses. *Aedes aegypti* is known to be an efficient vector of several arboviruses including chikungunya virus (CHIKV), yellow fever virus (YFV), Zika virus (ZIKV) and dengue virus (DENV). *Ae. albopictus* has a wide host range, and provides important bridge vectors for zoonotic pathogen spread between humans and other hosts. Additionally, studies performed on *Ae*. *albopictus* specimens collected from several locations in Black Sea region of Turkey during 2016-2017 revealed the presence of West Nile virus (WNV), *Aedes* flavivirus (AEFV) and cell fusing agent virus (CFAV) within the species (Akıner et al., 2019).

Aedes albopictus eggs were detected for the first time in 2011 in the Thrace area of northwestern Turkey by Oter et al. (2013). Additional monitoring was conducted in northeastern Turkey and Georgia in September 2015 to get information about the dispersion of these invasive *Aedes* species (Akıner et al., 2016). Knowledge regarding the ecological and behavioral attitudes of mosquito populations is important for enhancing our understanding of the transmission dynamics of mosquito-borne diseases and for developing more efficient vector control programs. Studies of these two invasive species have increased worldwide. Here, we present the results of five years of surveillance of the presence and spread of *Ae*. *albopictus* and *Ae*. *aegypti* and assess their ecological adaptability in Turkey. Additionally, we discuss the future direction of research and promising control strategies with the potential to be employed throughout the country to minimize harmful effects of the vector of humans.

Material and Methods

Aedes aegypti and *Ae. albopictus* were monitored throughout 2016 and 2017 along the entire Black Sea coastline, which includes Black Sea and Marmara Regions (Figure 1). The Black Sea has a temperate, oceanic climate, warm-wet summers and cool to cold-wet winters (Sensoy et al., 2008). The coast areas of the Black Sea Region have the greatest annual rainfall, receiving 2,200 mm rainfall annually (Sensoy et al., 2008). The Marmara Region and Istanbul have transitional climates, with warm, hot and moderately dry summers and cool to cold, rainy winters (Sensoy et al., 2008). However, in the winter temperatures can drop below zero. The Black Sea Region, in particular, has large tire dumps that provide ideal breeding sites for *Aedes* species. Additionally, tires ideal for providing breeding sites for these invasive species have been used as building materials to make handmade elevators that are used to carry tea leaves from hills and for garden beds within yards. Finally, in order to determine potential spread of the two species to other major touristic localities, such as in Antalya, Ankara and Izmir, surveillance studies were expanded to the Central Anatolia, Mediterranean and Aegean Regions in 2018 and 2019 (Figure 1). Central Anatolia has a semiarid

continental climate with hot, dry summers and cold, snowy winters whereas the Mediterranean and Aegean Regions have hot, dry summers and mild, rainy winters. Additionally, depending on the precise location considered, precipitation varies from 580 to 1,300 mm annually in Mediterranean and Aegean Regions (Sensoy et al., 2008).

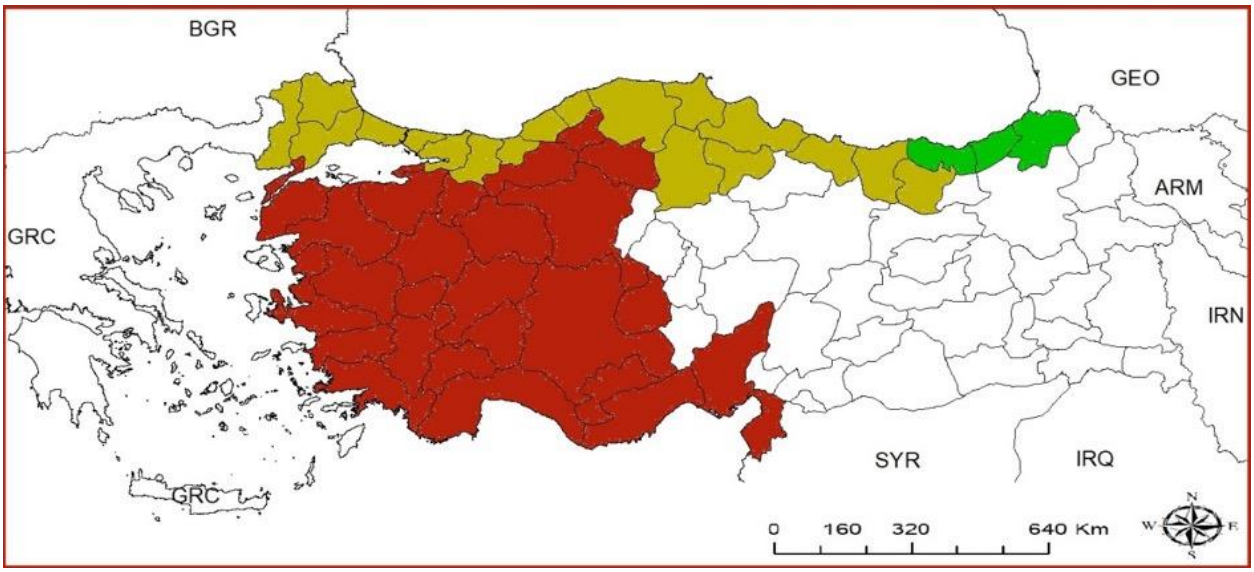

Figure 1. 2015-2019 surveillance area. Green: 2015 surveillance area (East Black Sea Region); Yellow: 2015-2016-2017 surveillance area (West Black Sea and Marmara Regions); Red: 2015-2016-2017-2018 and 2019 surveillance area: (Aegean, Mediterranean and Central Anatolia Regions). ARM: Armenia; BGR: Bulgaria; GE: Georgia; GRC: Greece; IRQ: Iraq; IRN: Iran.

A comprehensive range of larval habitats, such as tires, artificial containers, cemeteries, water bottles and natural breeding habitats, were examined and larvae were collected using a dipper or pipette. In addition, adults were collected using human landing catch (HLC) and BG-Sentinel™ traps in some localities. HLC included two adults with mosquitoes collected after landing on their exposed legs with a Hepa Filter Mouth Aspirator. Collections were performed for 10 min on one day per month from May to October 2017 to 2019. The BG sentinel traps (with BG-Lure) were placed on the ground with the trap mouth opening positioned 40 cm above the ground. The traps were operated for 24-h once a week from May to October 2017 to 2019. In addition, ovitrap surveillance was conducted 5 days per month from May to November 2017 to 2019. Black plastic cups (1 L) filled with water were used as ovitraps. The ovitraps were lined with a strip of filter paper along the water margin where female *Aedes* species could lay their eggs. All collected ovitraps were brought back to laboratory and allowed to develop to adults under standard laboratory conditions at 25ºC and 65 ± 20% RH. The HLC, BG-Sentinel™ traps and ovitraps sampling locations and coordinates are given in Tables 1 and 2. Sampling was performed on private land after obtaining permission of the owners. Sampling locations were georeferenced using GPS and the type of containers from which the species were collected were recorded. The morphological identification of species was performed microscopically and selected samples were confirmed molecularly. Morphological identification of larvae and females was performed using the interactive CD of Schaffner et al. (2001). Molecular confirmation was obtained by the amplification of the cytochrome c oxidase I (COI) gene using LCO1490 and HCO2198 primers (Folmer et al., 1994). Twenty females *Ae*. *albopictus* and four females *Ae*. *aegypti* from different sampling locations were used for molecular confirmation.

Results

Aedes aegypti and *Aedes albopictus* distribution

In total, 33,580 larval stages of *Ae*. *albopictus* and *Ae*. *aegypti* were collected over the 5 years. The COI barcoding was used for confirmation of species identification and the maximum likelihood method, based on a general time reversible model, was used to infer the evolutionary history (Nei & Kumar, 2000). The tree with the highest log likelihood (-1090) is shown in Figure 2. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A maximum composite likelihood approach was used with performing the BioNJ method to obtain initial trees for the heuristic search. Modeling the evolutionary rate differences between sites [(4 categories (+G, parameter = 0.802)] were performed with a distinct gamma distribution. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 33.1% sites). The tree is drawn to scale, with branch lengths as the number of substitutions per site. The analysis involved 28 nucleotide sequences. Codon positions included were the first, second, third and noncoding positions. There was a total of 599 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

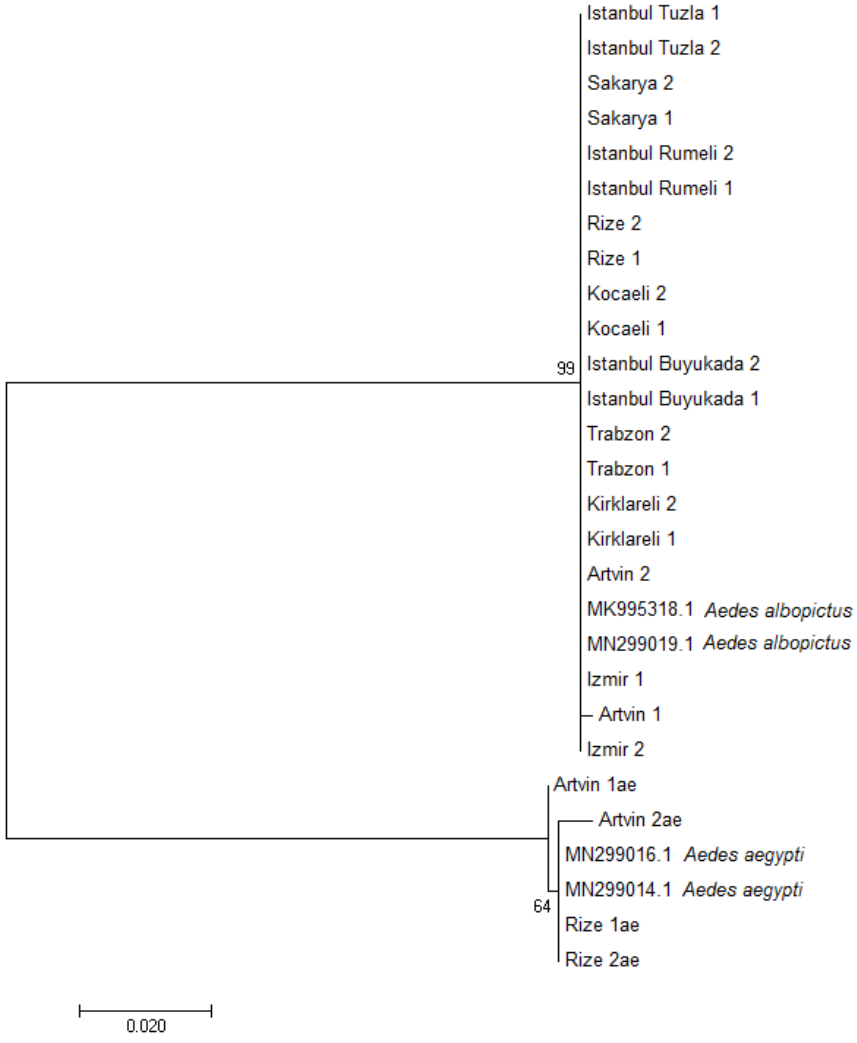

Figure 2. Molecular phylogenetic analysis by maximum likelihood method. The sequences obtained in mosquito species in this study are given with the name of sampling locations and numbers. Reference sequences are shown with GenBank accession number and species.

The distribution of *Ae*. *albopictus* and *Ae*. *aegypti* in northeastern Turkey in 2015 are shown in Figure 3. *Aedes albopictus* was found in 21 (34%) out of 62 monitored areas, *Ae. aegypti* was found 8 (13%) and both *Ae*. a*lbopictus* and *Ae*. *aegypti* were identified in three sites (5%). In all locations in which both species were identified, *Ae*. *albopictus* was the most abundant.

Figure 3. Distribution of *Aedes albopictus* and *Aedes aegypti* in northeastern Turkey in 2015. Blue triangles; monitored sampling sites, yellow circles; positive sampling sites for *Ae. albopictus* and green circles; positive sampling sites for *Ae. aegypti*.

The distribution of *Ae*. *albopictus* and *Ae*. *aegypti* in 2016 and 2017 are shown in Figures 4 and 5, respectively. In 2016, *Ae*. *albopictus* was found in 53 (33.8%) and *Ae*. *aegypti* was found in 21 (13.4%) sites monitored and the both species were identified in 4 (2.5%) of the 157 total sites monitored. In 2017, *Ae*. *albopictus* was found in 208 (51.2%) and *Ae*. *aegypti* in 29 (7.1%) monitored sites and both species were identified from 28 (6.9%) of 406 monitored sites.

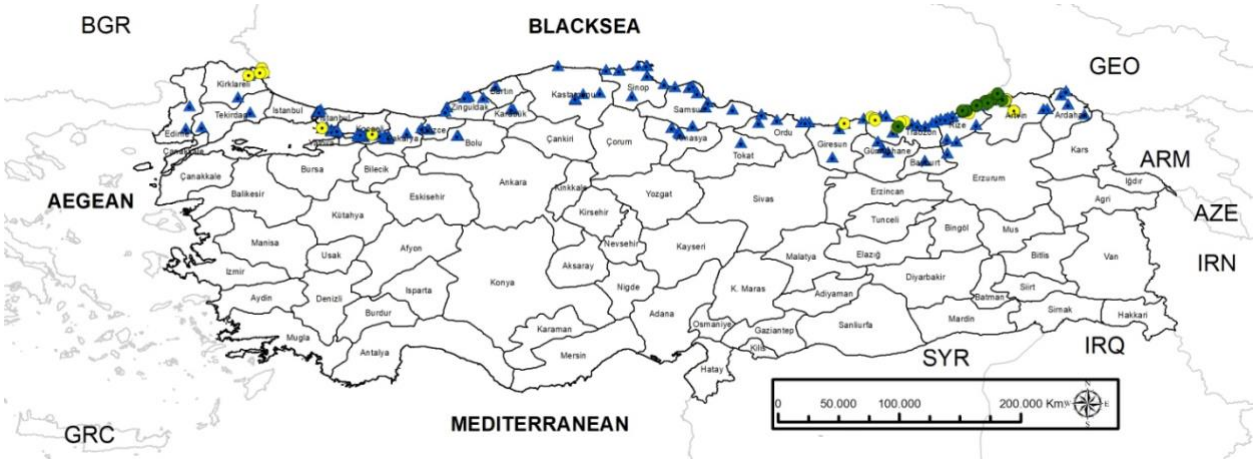

Figure 4. Distribution of *Ae*des *albopictus* and *Ae*des *aegypti* in northeastern Turkey in 2016. Blue triangles; monitored sampling sites, yellow circles; positive sampling sites for *Ae. albopictus* and green circles; positive sampling sites for *Ae. aegypti*.

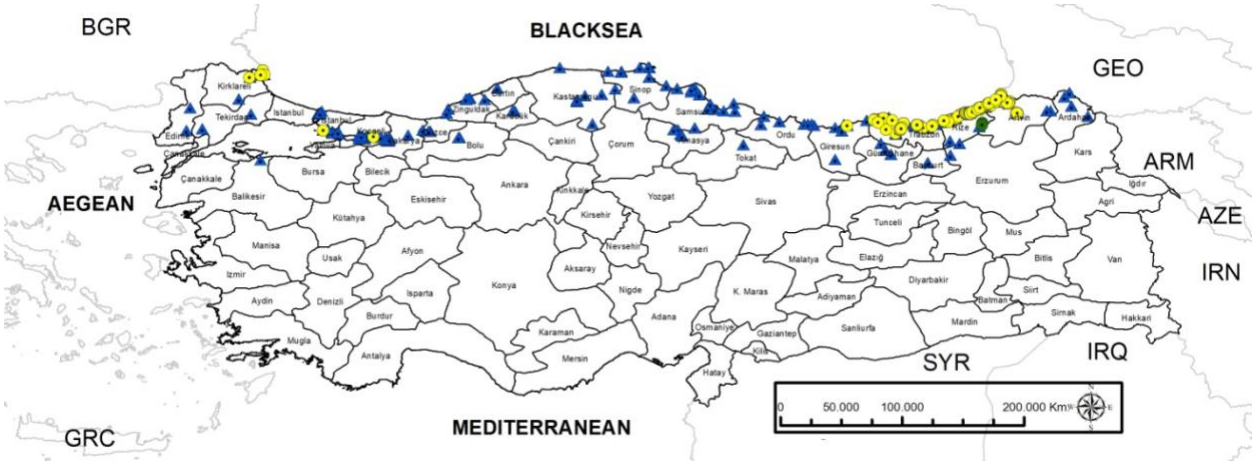

Figure 5. Distribution of *Aedes albopictus* and *Aedes aegypti* in northeastern Turkey in 2017. Blue triangles; monitored sampling sites, yellow circles; positive sampling sites for *Ae. albopictus* and green circles; positive sampling sites for *Ae. aegypti*.

In 2018 and 2019, mosquito sampling areas were expanded, and surveillance also included Central Anatolia, Aegean and Mediterranean Regions. *Aedes albopictus* was identified in three geographical regions (Black Sea, Marmara and Aegean) whereas *Ae*. *aegypti* was only found in northeastern Turkey. The distributions of *Ae*. *albopictus* and *Ae*. *aegypti* in 2018 and 2019 are shown in Figures 6 and 7, respectively. In 2018, *Ae*. *albopictus* was found in 234 (36.9%) and both *Ae*. *albopictus* and *Ae*. *aegypti* were found together in 7 (1.1%) of 635 monitored sites. In 2019, *Ae*. *albopictus* was found in 457 (65.3%) and both *Ae*. *albopictus* and *Ae*. *aegypti* were found together in 9 (1.3%) of 700 monitored areas.

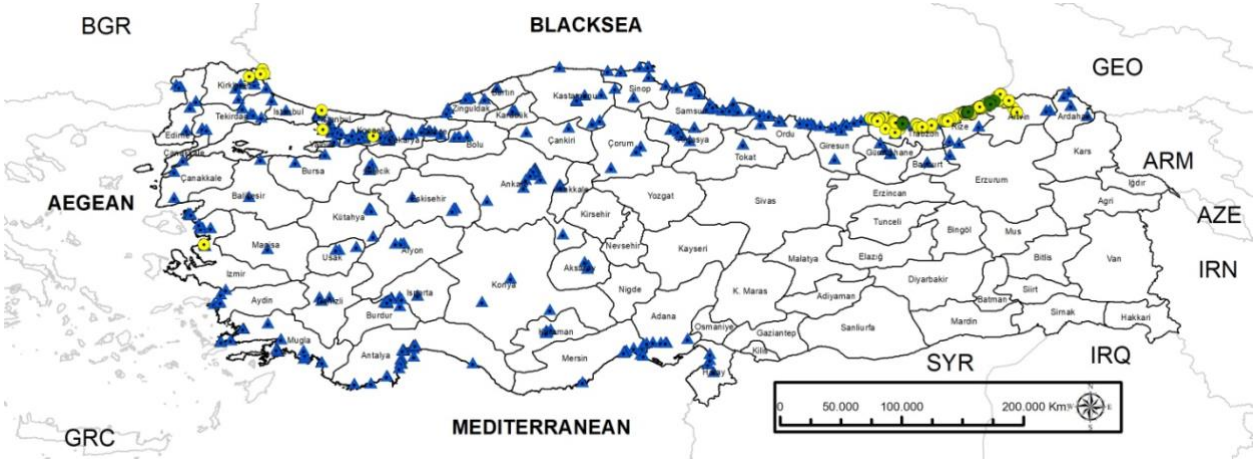

Figure 6. Distribution of *Aedes albopictus* and *Aedes aegypti* in northeastern Turkey in 2018. Blue triangles; monitored sampling sites, yellow circles; positive sampling sites for *Ae. albopictus* and green circles; positive sampling sites for *Ae. aegypti*.

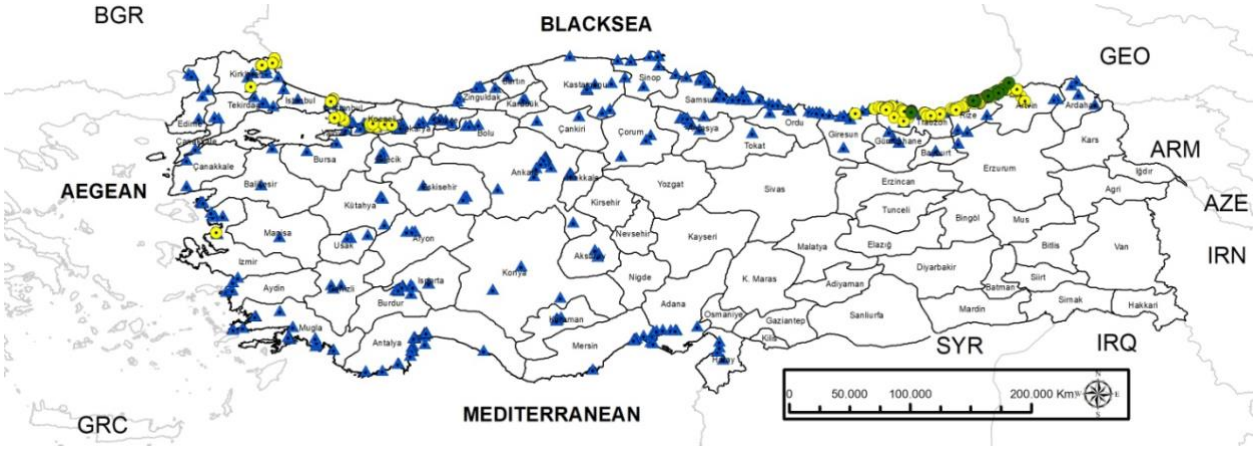

Figure 7. Distribution of *Aedes albopictus* and *Aedes aegypti* in northeastern Turkey in 2019. Blue triangles; monitored sampling sites, yellow circles; positive sampling sites for *Ae. albopictus* and green circles; positive sampling sites for *Ae. aegypti*.

Adult collection was standardized in some localities during 2017 to 2019 and number of adults were collected using HLC, BG-Sentinel™ and also number of eggs collected using ovitraps and number of adults emerged from eggs are given in Tables 1 and 2.

Table 1. Human landing catch, BG-Sentinel™ traps and ovitraps sampling locations and coordinates for *Aedes albopictus* and number of adults emerged from eggs

		2017		2018		2019	
Sampling Sites	Coordinates	OVI (eggs)	HLC	OVI (eggs)	HLC	OVI (eggs)	HLC
Hopa Automobile Industrial Estate (BSR)	41º25'42" N 41º26'00" E	2511	511	2390	501	4410	340
Arhavi (BSR)	41º21'01" N 41º17'54" E	3386	219	3253	183	4830	127
Fındıklı (BSR)	41º16'34" N 41º08'49" E	2836	110	2703	75	7223	70
Hamidiye (BSR)	41º10'37" N 40º57'14" E	1661	141	1559	82	2984	93
Sürmene (BSR)	40º54'44" N 40º06'53" E	1256	196	1162	155	1366	161
Trabzon Automobile Industrial Estate (BSR)	40º59'53" N 39º45'13" E	1740	120	1662	88	2016	104
İstanbul Kartal (MR)	40º56'07" N 29º10'46" E	5150	226	4828	181	4672	176
İstanbul Rumeli Kavağı (MR)	41º11'05" N 29º03'38" E	5487	253	5320	213	4909	215
Kırklareli Beğendik (MR)	41º57'35" N 28º01'12" E	5125	172	4918	151	5248	154
İzmir Aliağa (AR)	38º48'03" N 27º03'19" E	4843	176	4661	161	4515	158
Total		33995	2124	32456	1790	42173	1598
Number of adults emerged from eggs		30935 (90.9%)		29632 (91.3%)		38124 (90.3%)	

*HLC, human landing catch; OVI, ovitrap; BSR, Black Sea Region; and MR, Marmara Region.

Table 2. Human landing catch, BG-Sentinel™ traps and ovitraps sampling locations and coordinates for *Aedes aegypti* and number of adults emerged from eggs

		2017			2018			2019		
Sampling Sites	Coordinates	OVI (eggs)	HLC	BG	OVI (eggs)	HLC	BG	OVI (eggs)	HLC	BG
Hopa Automobile Industrial Estate (BSR)	41º25'42" N 41º26'00" E	2511	511		2390	501		4410	340	
Arhavi (BSR)	41º21'01" N 41º17'54" E	3386	219		3253	183		4830	127	
Fındıklı (BSR)	41º16'34" N 41º08'49" E	1226	38	12	848	24	9	1040	32	17
Pazar (BSR)	41º10'50" N 40º52'56" E	1223	50	16	1020	37	22	1065	42	22
Total		4335	168	57	2932	111	50	3637	138	66
Number of adults emerged from eggs		3641 (84%)			2500 (85.2%)			3033 (83.4%)		

*HLC, human landing catch; OVI, ovitrap; BG, BG-Sentinel trap; BSR, Black Sea Region; and MR, Marmara Region.

Larval habitats assessed at each location

By 2019, a total of 700 potential breeding sites has been examined throughout four geographical regions spanning Turkey. Of these, 466 (66.6%) were determined to be infested with immature *Aedes* spp. *Aedes albopictus* and *Ae*. *aegypti* were found in association with several other mosquito species. These include *Anopheles plumbeus* Stephens 1828, *Anopheles claviger* (Meigen, 1804), *Culex territans* Walker 1856, *Culex pipiens* Linnaeus 1758 and *Aedes geniculatus* (Olivier, 1791). These positive breeding sites were distributed into four groups, as follows: used tires, natural areas and mixed breeding sites such as discarded water bottles, flower pots, drainage pipes and closets. Used tires were the abundant potential breeding site studied (88.6%), followed by mixed breeding sites (7.7%) and natural breeding sites (3.6%) (Table 3). Photos of breeding site types are given in Figure 8.

Table 3. Larval breeding containers and their characteristic

Region	Discarded tires	Natural	Mixed	Total
Black Sea	391 (83.9%)	15 (3.2%) (temporary breeding habitats)	13 (2.8%) discarded containers) 5 (1.2%) (flower pots) 2 (0.4%) (thrown closets) 5 (1.1%) (water bottles) 2 (0.4%) (drainage pipes)	433 (92.9%)
Marmara	22 (4.7%)	2 (0.4%) (tree holes)	4 (0.9%) (discarded containers) 2 (0.4%) (drainage pipes) 2 (0.4%) (water bottles)	32 (6.7%)
Mediterranean	0	0	0	0
Aegean	0	0	1 (0.2%) (water bottle)	1 (0.2%)
Total	413 (88.6%)	17 (3.6%)	36 (7.7%)	466 (100%)

Figure 8. Representative examples of breeding habitats for *Aedes* mosquitos. Breeding site types including temporary (natural) breeding habitats (a), discarded tires (b-d) and mixed breeding habitats (e-h).

Discussion

This is the first study that has performed the widespread profiling of the geographical distribution and prevalence of *Ae*. *albopictus* and *Ae*. *aegypti* mosquitoes in Turkey since both species were reported in 2011 and 2015, respectively. The study revealed that the most infested areas are settlements, permanent populations of the species were detected for the first time (northeastern Turkey) and *Ae*. *albopictus* has been identified in several areas where it was not determined to be present previously. However, the distribution of *Ae*. *aegypti* in Turkey remains limited to northeastern Turkey.

Our study reveals the coexistence of *Ae*. *albopictus* and *Ae*. *aegypti* within the same larval sites. However, a decrease in the distribution of *Ae. aegypti* was observed after 2017. It seems the species has become scarce within areas where it previously had been detected. The distribution of *Ae*. *albopictus* is much wider than that of *Ae*. *aegypti*. Although environmental factors such as vegetation and climate may be responsible for observed differences between the prevalence of the two species, difference may also be a result of competition between the species. While the coexistence of the two species has been documented within the same larval developmental areas (Braks et al., 2003; Simard et al., 2005; Chen et al., 2006), a competitive advantage for *Ae*. *albopictus* over *Ae*. *aegypti* has been suggested in several studies (O'Meara et al., 1995; Barrera, 1996; Daugherty et al, 2000; Juliano et al., 2002, 2004; Lounibos et al., 2002). *Ae*des *albopictus* appears to have a great degree of environmental plasticity, which facilitates the adaptation of the species to different environments (Hawley, 1998). Kobayashi et al. (2002) showed that *Ae*. *albopictus* can synthesize large amounts of lipids, which provides substantial yolk resources to the eggs in diapause which facilitated the enhanced adaptation of the species to cooler climates than *Ae*. *aegypti* and increased the capacity of *Ae*. *albopictus* to distribute throughout both temperate and tropical regions. Also, Otero et al. (2006) showed that *Ae*. *aegypti* eggs have elevated mortality rates when exposed to frost during intense winters. However, while both species have desiccant-resistant eggs, *Ae*. *aegypti* is more tolerant to elevated temperatures than *Ae*. *albopictus,* and thus, *Ae*. *aegypti* is more capable of living within hot and dry environments than *Ae*. *albopictus,* if breeding sites are available (Sota, 1993; Juliano et al., 2004).

In the present study, used tires, in particular, were identified as sites associated with high detection rates for invasive *Aedes* larvae. This is consistent with studies conducted in other countries, including India (Singh & Rahman, 2013; Vijayakumar et al., 2014) Mexico (Lloyd et al., 1992) and Africa (Simard et al., 2005; Kamgang et al., 2010; Ngoagouni et al., 2015). This might be due to the fact that discarded tires are often stored for long periods, which makes them suitable breeding containers for larvae that are not often disturbed (Snr et al., 2011). Also, the attraction of *Ae*. *albopictus* and *Ae*. *aegypti* to tires may be associated with the similarity of tires to natural breeding habitats, such as natural tree holes (Tedjou et al., 2019). Both sites share similar characteristics including dark color and dark interior, and both provide suitable resting and oviposition sites. Laboratory-based studies on the oviposition of *Ae. albopictus* have also revealed that the species is attracted to black colored jars (Yap et al., 1995). Also, the attachment of eggs to the tires is important for the protection of *Aedes* population during the mosquito off season. However, the results showed that both species have the capacity to adapt to different breeding habitats such as discarded tanks, flower pots and water bottles, which is similar to reports of mosquito habits observed in other countries (Eritja et al., 2005; Seidahmed & Eltahir, 2016; Mathias et al., 2017; Stefopoulou et al., 2018). In this present study, tire dumps and used tires were mainly targeted for sampling to increase the possibility that immature stages of the species would be found. Also, no biotic/abiotic factors that may have affected the oviposition preferences of vector species such as water quality, vegetation and microbiota were not examined. Nonetheless, it remains important to focus on the common occurrence of huge tire dumps throughout the Black Sea Region and the presence of discarded tires that are used in tea leaf elevators of the eastern Black Sea Region (Figure 8d). This observation may be useful for raising awareness of the larval habitats of these vector species and for fighting arboviral diseases. Despite the limitations of the study, it represents the first report to characterize the presence of *Aedes* mosquitoes and their preferred breeding habitats in Turkey and to provide baseline data regarding the presence and distribution of the invasive mosquitoes in Turkey.

Aedes albopictus and *Ae*. *aegypti* are known to transmit ZIKV, CHICKV, YFV, all four DENV serotypes and are also potential vectors for Venezuelan equine encephalitis virus, Eastern equine encephalitis virus, Mayaro virus, Potosi virus, Cache Valley virus and La Crosse virus (Fontenille et al., 1997; Gratz, 2004; Turell et al., 2005; Long et al., 2011; da Moura et al., 2015; Seixas et al., 2018). Studies also have demonstrated the independent replication and dissemination of DENV and CHIKV when *Ae*. *aegypti* and *Ae. albopictus* were coinfected with the arboviruses (Vazeille et al., 2010; Nuckols et al., 2015). Also, its known that *Ae*. *albopictus* is able to transmit at least 22 arboviruses, including Rift Valley fever, Japanese encephalitis, WNV, and Sindbis viruses (Mitchell, 1995; Schaffner & Mathis, 2014; Medlock et al., 2015; Xia et al., 2018). Laboratory-based studies have revealed the potential of the species to transmit other arboviruses such as Oropouche, Trivittatus viruses and San Angelo virus (Moore & Mitchell, 1997). In tropical and subtropical countries, the epidemiology of arboviruses, such as CHICKV, ZIKV and DENV, are very different than in Europe due to the existence of the sylvatic cycle between wild animals and mosquitoes that facilitates year-round viral circulation in tropical/subtropical climates (Diallo et al., 1999). Due to the lack of this sylvatic cycle, local transmission throughout European countries only occurs when a competent vector becomes infectious after feeding an imported human case. This occurred throughout chikungunya fever spread in Italy in 2007, West Nile fever outbreaks in Romania and Greece in 2010 and regional dengue fever transmission that occurred in France and Croatia in 2010 (Hubálek & Halouzka, 1999; Lanciotti et al., 1999; Tsai et al., 1998; Papa et al., 2011; Lwande et al., 2015). Additionally, a study performed on *Ae*. *albopictus* and *Ae*. *aegypti* specimens collected from varied locations throughout the Black Sea Region of Turkey throughout 2016-2017 possessed WNV, CFAV and AEFV (Akıner et al., 2019). While there has been reports about imported DENV, CHIKV and ZIKV cases (Yağcı Çağlayık et al., 2012; Sezen et al., 2018) and serologically confirmed sporadic exposure to DENV (Ergünay et al., 2010), there is no information about the local transmission of ZIKV, DENV, YFV or CHIKV in humans in Turkey.

Over the past 30 years, the global geographical distribution of *Ae*. *albopictus* and *Ae*. *aegypti* has greatly expanded, and the effect of climate change on the range of the species has been shown (Romi et al., 1999; Benedict et al., 2007). Temperature can affect mosquito development and infection rates and may allow vector species to develop pathogens increasingly rapidly, which may facilitate their spread to new areas (Chaves & Koenraadt, 2010; Patz et al., 2003). Other effects of climate change, such as enhanced insecticide resistance, population density, sociodemographic factors, lifestyle, intensive agriculture, improper water storage and used tire trading, have the potential to affect the spread and expansion of vectors and pathogens (Gratz, 2004). The control of *Ae*. *albopictus* and *Ae*. *aegypti* is difficult and could require a combination of different vector control strategies including chemical, biological and genetic methods, along with public education strategies that involve informing individuals about health risks associated with the species and strategies that involve the cleaning or removal of possible larval habitats (O'Meara et al., 1995; Abramides et al., 2011). It has previously been shown that artificial containers on private land make up the majority of reproductive sites for *Aedes* larvae and the reduction of larval breeding habitats by removing water containers may be the most effective *Ae*. *albopictus* control method (Bartlett-Healy et al., 2012). The temporary suppression of immature *Ae*. *albopictus* by reducing larval sources was first reported in the USA (Ali & Nayar, 1997). The phenomenon was also reported in Spain (Abramides et al., 2011) New Jersey (Fonseca et al., 2013) and Grand Cayman Island (Wheeler et al., 2009). Involving the communities in *Aedes* mosquito control using different public awareness campaigns involving various communication channels, such as the internet and media, should be combined with the application of larvicides and adulticides.

In conclusion, our results show that *Ae*. *albopictus*, in particular, has expanded its distribution each year, and has the potential to extend its range throughout Turkey in the next few years. Observed increases in both mosquito distribution as well as detection of imported virus cases have the potential to produce a worrying scenario in the future. Unfortunately, the is no current national strategy for reducing populations and the dispersal of these invasive species in Turkey. Therefore, we strongly recommend the implementation of appropriate control strategies, which should be managed by local, regional and central governments. In Turkey, especially in the eastern Black Sea Region, numerous and large tire dumps currently serve as principal *Aedes* larval habitats, and urgent solutions are needed to mandate the removal/recycle of tires. In addition, tourism is very important in the Black Sea Region. Future arbovirus circulation may be enhanced due to the fact that the majority of tourists that come to the region are of Arabian origin, and the Arabian Peninsula contains large numbers of some arboviruses. In this sense, cooperation between the government, researchers, local administrations and policymakers is necessary for the standardization of invasive *Aedes* surveillance, for performing integrated control studies (public awareness, chemical, biological and genetic methods) and for funding countrywide control strategies.

References

Abramides, G. C., D. Roiz, R. Guitart, S. Quintana, I. Guerreroe & N. Giménez, 2011. Effectiveness of a multiple intervention strategy for the control of the tiger mosquito (*Aedes albopictus*) in Spain. Transactions of the Royal Society of Tropical Medicine & Hygiene, 105 (5): 281-288.

Akıner, M. M., B. Demirci, G. Babuadze, V. Robert & F. Schaffner, 2016. Spread of the invasive mosquitoes *Aedes aegypti* and *Aedes albopictus* in the Black Sea region increases risk of chikungunya, dengue, and Zika outbreaks in Europe. PLoS Neglected Tropical Diseases, 10 (4): e0004664.

Akıner, M. M., M. Öztürk, A. B. Başer, F. Günay, S. Hacioglu & A. Brinkmann, 2019. Arboviral screening of invasive *Aedes* species in northeastern Turkey: West Nile virus circulation and detection of insect-only viruses. PLoS Neglected Tropical Diseases, 13 (5): e0007334.

Ali, A. & J. K. Nayar, 1997. Invasion, spread, and vector potential of *Aedes albopictus* in the USA and its control possibilities. Medical Entomology and Zoology, 48 (1): 1-9.

Almeida, A. P., Y. M. Goncalves, M. T. Novo, C. A. Sousa, M. Melim & A. J. Gracio, 2007. Vector monitoring of *Aedes aegypti* in the Autonomous Region of Madeira, Portugal. Eurosurveillance, 12 (11): E0711156.

Barrera, R, 1996. Competition and resistance to starvation in larvae of container-inhabiting *Aedes* mosquitoes. Ecological Entomology, 21 (2): 112-127.

Bartlett-Healy, K., I. Unlu, P. Obenauer, T. Hughes, S. Healy, T. Crepeau, A. Faraji, B. Kesavaraju, D. Fonseca, G. Schoeler, R. Gaugler & D. Strickman, 2012. Larval Mosquito Habitat Utilization and Community Dynamics of *Aedes albopictus* and *Aedes japonicus* (Diptera: Culicidae). Journal of Medical Entomology, 49 (4): 813-24.

Benedict, M. Q., R. S. Levine, W. A. Hawley & L. P. Lounibos, 2007. Spread of the tiger: Global risk of invasion by the mosquito *Aedes albopictus*. Vector-Borne and Zoonotic Diseases, 7 (1): 76-85.

Braks, M. A., N. A. Honorio, R. Lourenco-De-Oliveira, S. A. Juliano & L. P. Lounibos, 2003. Convergent habitat segregation of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in southeastern Brazil and Florida. Journal of Medical Entomology, 40 (6): 785-794.

Chaves, L. & C. Koenraadt, 2010. Climate change and highland malaria: Fresh air for a hot debate. The Quarterly Review of Biology, 85 (1): 27-55.

Chen, C. D., W. A. Nazni, H. L. Lee, B. Seleena, S. Mohd Masri, Y. F. Chiang & M. Sofian-Azirun, 2006. Mixed breeding of *Aedes aegypti* (L.) and *Aedes albopictus* Skuse in four dengue endemic areas in Kuala Lumpur and Selangor. Tropical Biomedicine, 23 (2): 224-227.

da Moura, A. J., M. A. de Melo Santos, C. M. Oliveira, D. R. Guedes, D. de Carvalho-Leandro, M. L. da Cruz Brito, L. F. Gomez & C. F. Ayres, 2015. Vector competence of the *Aedes aegypti* population from Santiago Island, Cape Verde, to different serotypes of dengue virus. Parasite and Vectors, 8: 114.

Daugherty, M. P., B. W. Alto & S. A. Juliano, 2000. Invertebrate carcasses as a resource for competing *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology, 37 (3): 364-372.

Diallo, M., J. Thonnon, M. Traore-Lamizana & D. Fontenille, 1999. Vectors of chikungunya virus in Senegal: Current data and transmission cycles. American Journal of Tropical Medicine and Hygiene, 60 (2): 281-286.

Ergünay, K., M. B. Saygan, S. Aydoğan, N. Litzba, M. Niedrig, A. Pınar & D. Us, 2010. Investigation of dengue virus and yellow fever virus seropositivities in blood donors from Central/Northern Anatolia. Mikrobiyoloji Bülteni, 44 (3): 415-424.

Eritja, R., R. Escosa, J. Lucientes, E. Marques, D. Roiz & S.Ruiz, 2005. Worldwide invasion of vector mosquitoes: Present European distribution and challenges for Spain. Biological Invasions, 7: 87-97.

Fonseca, D. M., I. Unlu, T. Crepeau, A. Farajollahi, S. P. Healy, K. Bartlett-Healy, D. Strickman, R. Gaugler, G. Hamilton, D. Kline & G. G. 2013. ClarkArea-wide management of *Aedes albopictus*. Part 2: gauging the efficacy of traditional integrated pest control measures against urban container mosquitoes. Pest Management Science, 69 (12): 1351-1361.

Fontenille, D., M. Diallo, M. Mondo, M. Ndiaye & J Thonnon, 1997. First evidence of natural vertical transmission of yellow fever virus in *Aedes aegypti*, its epidemic vector. Transactions of the Royal Society of Tropical Medicine and Hygiene, 91 (5): 533-535.

Gratz, N. G., 2004. Critical review of the vector status of *Aedes albopictus*. Medical and Veterinary Entomology,18 (3): 215-227.

Hawley, W. A., 1998. The biology of *Aedes albopictus*. Journal of the American Mosquito Control Association Supplement, 1 (1): 1-39.

Hubálek, Z. & J. Halouzka, 1999. West Nile fever-a reemerging mosquito-borne viral disease in Europe. Emerging Infectious Diseases, 5 (5): 643-650.

Juliano, S. A., L. P. Lounibos & G. F. O'Meara, 2004. A field test for competitive effects of *Aedes albopictus* on *Aedes aegypti* in south Florida: differences between sites of coexistence and exclusion? Oecologia, 139 (4): 583-593.

Juliano, S. A., G. F. O'Meara, J. R. Morrill & M. M. Cutwa, 2002. Desiccation and thermal tolerance of eggs and the coexistence of competing mosquitoes. Oecologia, 130 (3): 458-469.

Kamgang, B., J. Y. Happi, P. Boisier, F. Njiokou, J. P. Herve, F. Simard & C. Paupy, 2010. Geographic and ecological distribution of the dengue and chikungunya virus vectors *Aedes aegypti* and *Aedes albopictus* in three major Cameroonian towns. Medical and Veterinary Entomology, 24 (2): 132-141.

Knudsen, A. B., 1995. Global distribution and continuing spread of *Aedes albopictus*. Parassitologia, 37 (2-3): 91-97.

Kobayashi, M., N. Nihei & T. Kurihara. 2002. Analysis of northern distribution of *Aedes albopictus* (Diptera: Culicidae) in Japan by geographical information system. Journal of Medical Entomology, 39 (1): 4-11.

Kumar, S., G. Stecher & K. Tamura, 2015. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets.

Lanciotti, R., J. T. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K. E. Volpe, M. B. Crabtree, J. H. Scherret, R. A. Hall, J. S. MacKenzie, C. B. Cropp, B. Panigrahy, E. Ostlund, B. Schmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H. M. Savage, W. Stone, T. McNamara & D. J. Gubler, 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science, 286 (5448): 2333-2337.

Lloyd, L. S., P. Winch, J. Ortega-Canto & C. Kendall, 1992. Results of a community-based *Aedes aegypti* control program in Merida, Yucatan, Mexico. The American Journal of Tropical Medicine and Hygiene, 46 (6): 635-642.

Long, K. C., S. A. Ziegler, S. Thangamani, N. L. Hausser, T. J. Kochel, S. Higgs & R. B. Tesh. 2011. Experimental transmission of Mayaro virus by *Aedes aegypti*. The American Journal of Tropical Medicine and Hygiene, 85 (4): 750-757.

Lounibos, L. P, S. Saurez, Z. Menedez, N. Nishimura, R. L. Escher, S. M. O'Connell & J. R. Rey. 2002. Does temperature affect the outcome of larval competition between *Aedes aegypti* and *Aedes albopictus*? Journal of Vector Ecology, 27 (1): 86-95.

Lwande, O. W., G. Mosomtai & S. Symekher, 2015. West nile virus, a reemerging virus. Precision Medicine, 2 (1): e604.

Mathias, L., V. Baraka, A. Philbert, E. Innocent, F. Francis, G. Nkwengulila & E. J. Kweka, 2017. Habitat productivity and pyrethroid susceptibility status of *Aedes aegypti* mosquitoes in Dar es Salaam, Tanzania. Infectious Diseases of Poverty, 6 (102): 1-10.

Medlock, J. M, K. M Hansford, V. Versteirt, B. Cull, H. Kampen, D. Fontenille, G. Hendrickx, H. Zeller, W. Van Bortel & F. Schaffner, 2015. An entomological review of invasive mosquitoes in Europe. Bulletin of Entomological Research, 105 (6): 637-663.

Mitchell, C., 1995. Geographic spread of *Aedes albopictus* and potential for involvement in arbovirus cycles in the Mediterranean basin. Journal of Vector Ecology, 20 (1): 44-58.

Moore, C. G. & C. J. Mitchell, 1997. *Aedes albopictus* in the United States: Ten-year presence and public health implications. Emerging Infectious Diseases, 3 (3): 329-334.

Nei, M. & S. Kumar, 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York. 333 pp.

Ngoagouni, C., B. Kamgang, E. Nakoune, C. Paupy & M. Kazanji, 2015. Invasion of *Aedes albopictus* (Diptera: Culicidae) into central Africa: what consequences for emerging diseases? Parasite and Vectors, 8 (191): 1-7.

Nuckols, J., Y.-J. Huang, S. Higgs & A. Miller, 2015. Evaluation of simultaneous transmission of chikungunya virus and dengue virus type 2 in infected *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). Journal of Medical Entomology, 52 (3): 447-451.

O'Meara, G. F., L. F. Evans, A. D. Gettman & J. P. Cuda, 1995. Spread of *Aedes albopictus* and decline of *Ae. aegypti* (Diptera: Culicidae) in Florida. Journal of Medical Entomology, 32 (4): 554-562.

Oter, K., F. Gunay, E. Tuzer, Y. M. Linton, R. Bellini & B. Alten. 2013. First record of *Stegomyia albopicta* in Turkey determined by active ovitrap surveillance and DNA barcoding. Vector Borne and Zoonotic Diseases, 13 (10): 753-761.

Otero, M., H. G. Solari & N. Schweigmann, 2006. A stochastic population dynamics model for *Aedes aegypti*: Formulation and application to a city with temperate climate. The Bulletin of Mathematical Biology, 68 (8): 1945-1974.

Papa, A., K. Xanthopoulou, S. Gewehr & S. Mourelatos, 2011. Detection of West Nile virus lineage 2 in mosquitoes during a human outbreak in Greece. Clinical Microbiology and Infection, 17 (8): 1176-1180.

Patz J. A., A. K. Githeko, J. P. McCarty, S. Hussain, U. Confalonieri & N. de Wet, 2003. "Climate Change and Infectious Diseases, 103-132". In: Climate Change and Human Health: Risks and Responses (Eds. A. J. McMichael, D. H. Campbell-Lendrum, C. F. Corvalán, K. L. Ebi, A. K. Githeko, J. D. Scheraga & A. Woodward). World Health Organization, Geneva, Switzerland, 322 pp.

Romi, R., M. Di Luca & G. Marjori, 1999. Current status of *Aedes albopictus* and *Aedes atropalpus* in Italy. Journal of the American Mosquito Control Association, 15 (3): 425-427.

Schaffner, F., A. Guy, G. Bernard, H. Jean-Paul, A. Rhaiem & J. Brunhes. 2001. Les moustiques d'Europe: Logiciel d'identification et d'enseignement = The Mosquitoes of Europe: an Identification and Training Programme. Paris (FRA); Montpellier: IRD; EID, 1 CD ROM (Didactiques). ISBN 2-7099-1485-9.

Schaffner, F. & A. Mathis, 2014. Dengue and dengue vectors in the WHO European region: past, present, and scenarios for the future. Journal of the American Mosquito Control Association,14 (12): 1271-1280.

Scholte, E., W. Den Hartog, M. Dik, B. Schoelitsz, M. Brooks & F. Schaffner, 2010. Introduction and control of three invasive mosquito species in the Netherlands. Euro Surveillance,15 (45): 19710.

Seidahmed, O. M. E. & E. A. B Eltahir, 2016. A sequence of flushing and drying of breeding habitats of *Aedes aegypti* (L.) prior to the low dengue season in Singapore. PLoS Neglected Tropical Diseases 10 (7): e0004842-e0004813.

Seixas, G., H. Jupille, P. S. Yen, B. Viveiros, A. B Failloux & C. A, 2018. Sousa Potential of *Aedes aegypti* populations in Madeira Island to transmit dengue and chikungunya viruses. Parasite and Vectors, 11 (509): 1-8.

Sensoy, S., M. Demircan, U. Ulupinar & I. Balta. 2008. Climate of Turkey. (Web page: http://www.mgm.gov.tr/files/en-US/climateofturkey.pdf) (Date accessed: 31.12. 2012).

Sezen, A. L., M. Yıldırım, M. N. Kültür, F. Pehlivanoğlu & D. Menemenlioğlu. 2018. Cases of Zika virus infection in Turkey: newly married couple returning from Cuba. Mikrobiyoloji Bülteni, 52 (3): 308-315.

Simard, F., E. Nchoutpouen, J. C. Toto & D. Fontenille, 2005. Geographic distribution and breeding site preference of *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae) in Cameroon, Central Africa. Journal of Medical Entomology, 42 (2): 726-731.

Singh, S. & A. Rahman, 2013. Contribution of *Aedes aegypti* breeding by different income group communities of Dehradun city, Uttarakhand, India. Biological Forum International Journal, 5 (1): 96-99.

Snr, S., Y. Norma-Rashid & M. Sofian-Azirun, 2011. Mosquitoes larval breeding habitat in urban and suburban areas, Peninsular Malaysia. World Academy of Science, Engineering & Technology, 58: 569-573.

Sota, T, 1993. Response to selection for desiccation resistance in *Aedes albopictus* eggs (Diptera: Culicidae). Applied Entomology and Zoology, 28 (2): 161-168.

Stefopoulou, A., G. Balatsos, A. Petraki, S. L. LaDeau, D. Papachristos & A. Michaelakis, 2018. Reducing *Aedes albopictus* breeding sites through education: A study in urban area. Plos One, 13 (11): e0202451.

Tedjou, A. N., B. Kamgang, A. P. Yougang, F. Njiokou & C. S. Wondji, 2019. Update on the geographical distribution and prevalence of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae), two major arbovirus vectors in Cameroon. PLOS Neglected Tropical Diseases, 13 (3): e0007137.

Tsai, T. F., F. Popovici, C. Cernescu, G. L Campbell & N. I. Nedelcu, 1998. West Nile encephalitis epidemic in southeastern Romania. Lancet, 352 (9130): 767-771.

Turell, M. J., D. J. Dohm, M. R. Sardelis, M. L. Oguinn, T. G. Andreadis & J. A. Blow, 2005. An update on the potential of north American mosquitoes (Diptera: Culicidae) to transmit West Nile Virus. Journal of Medical Entomology, 42 (1): 57-62.

Vazeille, M., L. Mousson, E. Martin & A-B. Failloux, 2010. Orally co-Infected *Aedes albopictus* from La Reunion Island, Indian Ocean, can deliver both dengue and chikungunya infectious viral particles in their saliva. PLOS Neglected Tropical Diseases, 4 (6): e706.

Vijayakumar, K., T. K. Sudheesh Kumar, Z. T. Nujum, F. Umarul & A. Kuriakose, 2014. A study on container breeding mosquitoes with special reference to *Aede*s (Stegomyia) *aegypti* and *Aedes albopictus* in Thiruvananthapuram district, India. Journal of Vector Borne Diseases, 51 (1): 27-32.

Wheeler, A. S, W. D. Petrie & D. Malone, 2009. Reintroduction of *Aedes aegypti* into Grand Cayman. Journal of the American Mosquito Control Association, 25 (3): 260-264.

Xia, H., Y. Wang, E. Atoni, B. Zhang & Z. Yuan, 2018. Mosquito-Associated Viruses in China. Virologica Sinica, 33 (1): 5-20.

Yağcı Çağlayık, D., Y. Uyar, G. Korukluoğlu, M. Ertek & S. Unal, 2012. An imported Chikungunya fever case from New Delhi, India to Ankara, Turkey: the first imported case of Turkey and review of the literature. Mikrobiyololoji Bülteni, 46 (1): 122-128.

Yap, H. H., C. Y. Lee, N. L. Chong, A. E. S. Foo & M. P. Lim, 1995. Oviposition site preference of *Aedes albopictus* in the laboratory. Journal of the American Mosquito Control Association, 11 (1): 128-132.

Yunicheva, Y. U., T. E. Ryabova, N. Y. Markovich, O. V. Bezzhonova, L. A. Ganushkina & V. B. Semenov, 2008. First evidence on the presence of breeding populations of the *Aedes aegypti* L. mosquito in Greater Sochi and various cities of Abkhazia. Meditsinskaya Parazitologiya i Parazitarnye Bolezni, 3: 40-43.

Türkiye Entomoloji Dergisi Yayın İlkeleri

Derginin yayın ilkeleri aşağıda özet olarak sunulmuştur. Ayrıntılar için web adresine (www.entomoloji.org.tr) bakınız.

1. Dergi, entomoloji ve tarımsal zooloji bilim dallarıyla ilişkili konulara açıktır.
2. Dergide Türkçe veya İngilizce yazılmış orijinal araştırmalar yayımlanır.
3. Yayımlanması istenilen eserlerin kısmen veya tamamen herhangi bir yerde yayınlanmamış veya yayımlanmayacak olması zorunludur.
4. Daha önce Kongre/Sempozyum vs. de sözlü/poster bildiri olarak sunulmuş ancak sadece kısa özet olarak basılmış eserler, dipnotta belirtilmesi koşuşuyla kabul edilir.
5. Lisansüstü tezleri veya TÜBİTAK, DPT, BAP gibi çeşitli kurumlarca desteklenen proje bulgularından kısımlar içeren eserler ilgililerinden gerekli izinler alındıktan sonra hazırlanmalı, ilgi durum dipnotta mutlaka belirtilmelidir.
6. Türkiye veya herhangi bir bölge için, başta karantina listesinde bulunan türler olmak üzere, yeni tür kayıtlarını içeren eserler gönderilmeden önce mutlaka ilgili kurumlara bilgi verilmiş olmalıdır.
7. Dergide yayımlanması istenilen eserler, web sayfasında sunulan “eser başvurusu” bölümünde açıklandığı gibi hazırlanarak, üst yazı, imzalı telif hakları formu ve başvuru ücreti dekontu ile dergi e-posta adresine gönderilmelidir.
8. Yayımlanması istenilen eserler web sayfasında sunulan “örnek makale taslağı” kullanılarak, gereksiz tekrar, şekil ve cetvellerden kaçınılarak, özden uzaklaşmayacak şekilde hazırlanmalı ve 16 sayfadan fazla olmamalıdır.
9. Yayın ilkelerine uygun olmayan eserler istenilen şekle göre yeniden düzenlenmek üzere yazara geri gönderilir. Detaylar için web sayfasında sunulan “eser değerlendirme süreci” ne bakınız.
10. Bir eser yayıma kabul edildiğinde, telif hakları formu tüm yazarlar tarafından imzalanıp dergimize gönderilmeden yayımlanmaz. Sorumlu yazara eserin pdf formatında hazırlanmış hali e-posta ile gönderilir, ayrıca telif ücreti ödenmez. Yayımlanan eserlere ait şekil dışı sorumluluklar yazarlarına aittir.

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