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Original article (Orijinal araştırma)

Karyotype analysis of *Phauloppia lucorum* (Koch, 1841) (Oribatida: Oribatulidae)

Phauloppia lucorum (Koch, 1841)'un karyotip analizi (Oribatida: Oribatulidae)

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Abstract

Currently, about 11,000 oribatid mites have been described, but chromosome numbers have been reported for only a few species. Furthermore, the oribatid mites are a valuable model for holocentric chromosomes in cytogenetic studies. The diploid chromosome number is generally 2*n* = 18 in oribatid mites, though some have 2*n* = 16 or 30. Although there are many studies on the morphology and systematics of *Phauloppia lucorum* (Koch, 1841) (Oribatida: Oribatulidae), there is no information about its karyotype or chromosome numbers. The aim of this study is to investigate the chromosome number, monoploid ideogram and detailed chromosomal measurements of *P. lucorum*. The diploid chromosome number of *P. lucorum* was found to be 2*n* = 12. The total haploid chromosome length and the average chromosome length were 7.39 µm and 1.23 µm respectively. The chromosome lengths varied from 0.91 to 1.67 µm. In conclusion, these results are the first for the chromosome numbers and karyotype analysis for *P. lucorum*.

Keywords: Acari, holocentric chromosome, karyotype, Oribatida, *Phauloppia lucorum*

Öz

Günümüzde, 11,000 oribatid akar türü tanımlanmıştır fakat çok az sayıda türün kromozom sayısı bildirilmiştir. Ayrıca oribatid akarlar sitogenetik çalışmalarda holosentrik kromozomlar için değerli bir modeldir. Genellikle oribatid akarlarda diploid kromozom sayısı 2*n* = 16, 30 gibi bazı istisnalar dışında 2*n* = 18 şeklindedir. *Phauloppia lucorum* (Koch, 1841) (Oribatida: Oribatulidae) üzerine çok sayıda morfolojik ve sistematik çalışmalar bulunmasına rağmen karyotip ve kromozom sayısı hakkında bilgi yoktur. Bu çalışmanın amacı *P. lucorum*'un kromozom sayısı, monoploid ideogram ve detaylı kromozom ölçümlerini araştırmaktır. *P. lucorum*'un diploid kromozom sayısı 2*n* = 12 olarak bulundu. Toplam haploid kromozom uzunluğu ve ortalama kromozom uzunluğu sırasıyla 7.39 µm, 1.23 µm'dir. Kromozom uzunluğu 0.91-1.67 µm aralığında değişmektedir. Sonuç olarak, *P. lucorum*'un kromozom sayısı ve karyotip analizi ilk kez bildirilmiştir.

Anahtar sözcükler: Acari, holosentrik kromozom, karyotip, Oribatida, *Phauloppia lucorum*

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Introduction

Oribatid mites are the dominant microarthropod group in forest soil-litter habitats. They play an important role in the decomposition of the soil organic matter. Oribatid mites are compose of 11,036 described species and subspecies found throughout the world (Subías, 2004, updated 2018; Gulvik, 2007; Ayyıldız & Toluk, 2016).

The species, *Phauloppia lucorum* (Koch, 1841) (Oribatida: Oribatulidae), is distributed in the Holarctic Region (and frequently in the Western Palearctic) and in Nepal (Subías, 2004, updated 2017). The diagnostic characteristics of this species are: integument color yellowish brown, length of body 690 (680-710) µm, width 480 (460-510) µm (*n* = 6), rostrum rounded, rostral setae setiform, lamella reduced as a weak line, costula thin, sensillus short and head claviform, pteromorpha not developed, notogaster with four pairs of porose areas and 14 pairs of setae, epimeral setal formula 3-1-3-3, four pairs of genital setae, two pairs of anal setae, all legs tridactylous (Figure 1).

Although many oribatid mites have been described, chromosome numbers have been reported for only a few species. Furthermore, the oribatid mites are a valuable model with holocentric chromosomes in cytogenetic studies, although not the only model, as about 800 species have been reported to have holocentric chromosomes including plants, nematodes, arachnids and insects (Melters et al., 2012). In general, the diploid chromosome number in oribatid mites is 2*n* = 18 (Oliver, 1977; Norton et al., 1993) with some exceptions having 2*n* = 16 or 30 (Heethoff et al., 2006; Eroğlu & Per, 2016). Although there are many studies on morphologicy and systematics of *P. lucorum*, there is no information about the karyotype or chromosome numbers in the literature. The aim of this study was to investigate the chromosome number, monoploid ideogram and detailed chromosomal measurements of *P. lucorum*.

Material and Methods

Material

The specimens were collected from Turkey: Sakarya, Kılıçkaya Hill, 40º28.214' N, 30º25.027' E, 551 m, in soil under *Pinus* sp., 22.IV.2015, 2 exs (mounted on aluminum stubs and gold-coated for scanning electron microscopy); 40º30.257' N, 30º28.830' E, 1302 m, in soil, 19.VI.2015, 3 exs; 40º30.204' N, 30º27.484' E, 1170 m, in soil, 19.VI.2015, 1 ex.; 40º29.200' N, 30º25.860' E, 896 m, in soil under *Pinus* sp., 21.VI.2015, 1 ex.; 40º29.388' N, 30º23.028' E, 463 m, in lichen on *Pinus* sp., 01.XI.2015, 1 ex.; 40º28.809' N, 30º23.872' E, 721 m, in lichen on *Pinus* sp., 06.XI.2015, 1 ex.; 40º29.207' N, 30º23.763' E, 660 m, in soil under *Pinus* sp., 06.XI.2015, 2 exs; 40º28.940' N, 30º23.523' E, 606 m, in lichen on *Pinus* sp., 06.XI.2015, 1 ex.; 40º28.900' N, 30º23.510' E, 598 m, in lichen on *Pinus* sp., 06.XI.2015, 1 ex. All materials were collected by Sedat Per (Figure 2).

Cytogenetic procedure

The cytogenetic procedure was conducted using the method developed by Imai et al. (1988) with substantial modifications by Gokhman & Quicke (1995). The procedure used on 10 specimens for which sex was not determined: (i) the hypotonic sodium citrate solution (1%) with colchicine (0.005%) for pretreatment and crushing; (ii) the fresh hypotonic solution for incubation; (iii) the fixative series for fixation, fixative 1 (glacial acetic acid-ethanol-distilled water, 3-3-4), fixative 2 (glacial acetic acid-ethanol 1-1), fixative 3 (glacial acetic acid); (iv) Giemsa staining.

At least 10 mitotic plates were assessed to determine the number of diploid chromosomes. A qualified photomicrograph was taken using a DP72 digital camera mounted on an Olympus BX-53 light microscope. The holocentric chromosomes were measured in micrometers using KaryoType software (Altınordu et al., 2016). The ideogram was drawn based on total chromosome lengths in order from largest to smallest.

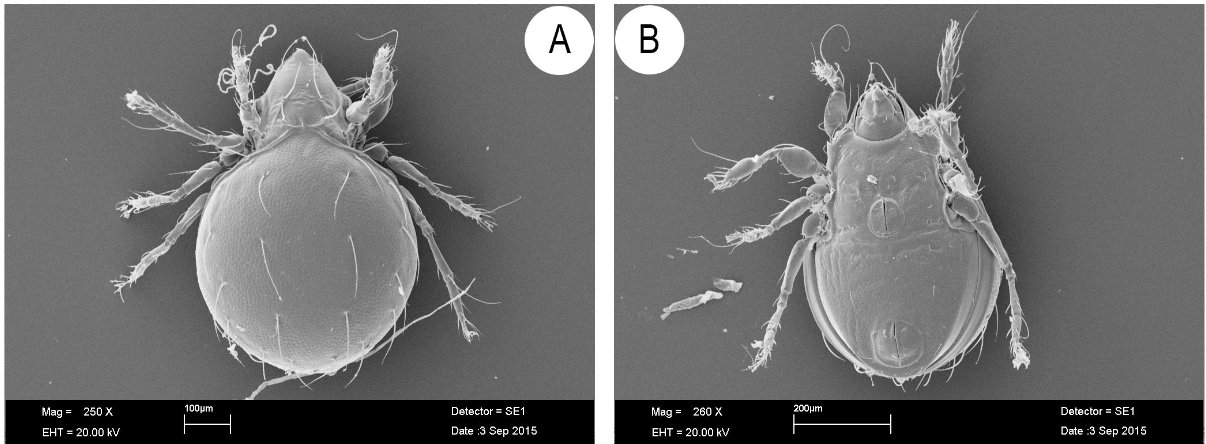

Figure 1. *Phauloppia lucorum*: A) dorsal view, and B) ventral view.

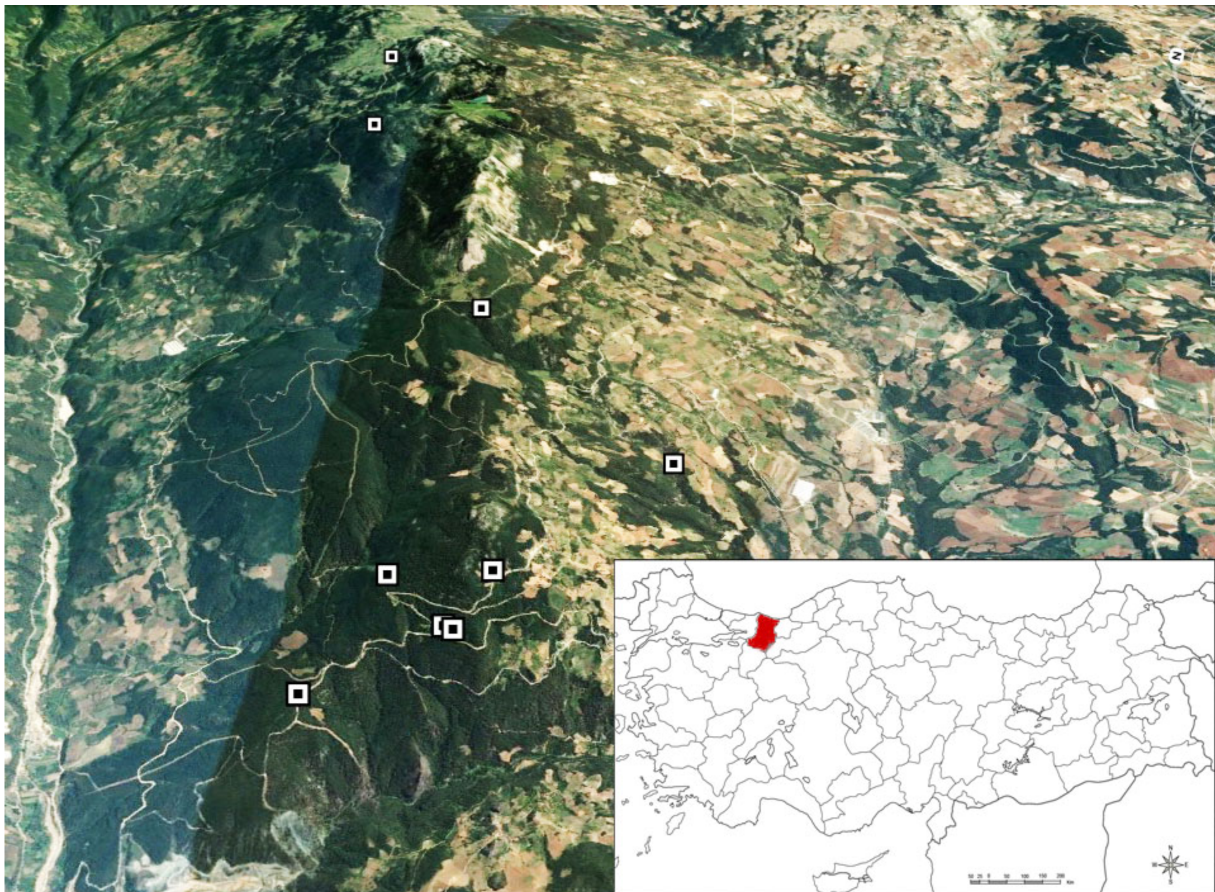

Figure 2. Collection locations of *Phauloppia lucorum* on the Kılıçkaya hill (Sakarya Province, Turkey).

Results

The diploid chromosome number of *P. lucorum* was determined to be 2*n* = 12 and the mitotic metaphase chromosomes are shown in Figure 3. The chromosome lengths and monoploid ideogram are given in Table 1 and Figure 4, respectively. The karyotype consists of holocentric chromosomes. The karyotype formula could not be determined due to the holocentric chromosomes.

The total haploid chromosome length and the average chromosome length were 7.39 and 1.23±0.27. The chromosome lengths varied from 0.91 to 1.67. The satellite was not observed in the chromosomal observations.

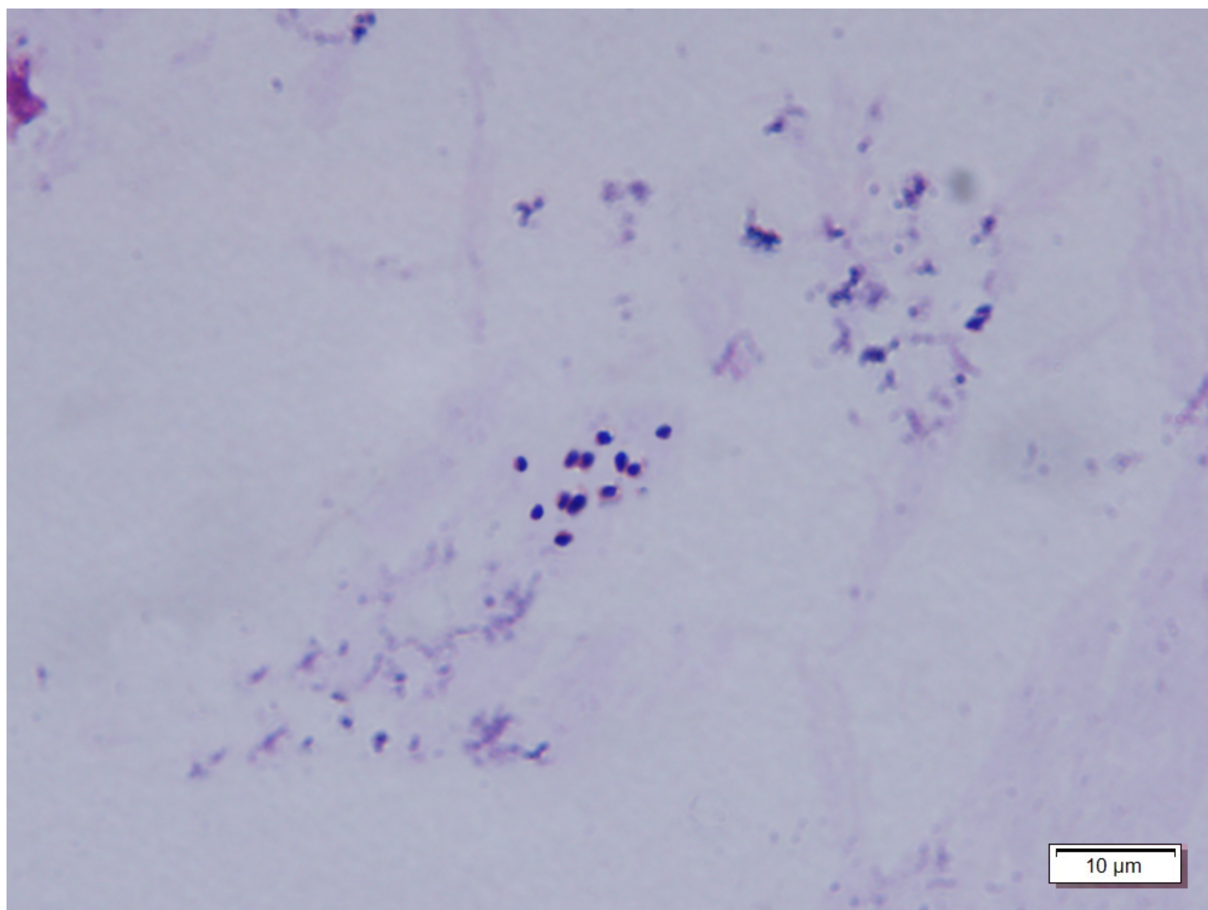

Figure 3. Photomicrograph of mitotic metaphase chromosomes in *Phauloppia lucorum*.

Table 1. The total chromosome lengths of *Phauloppia lucorum*

Chromosome Pair	1	2	3	4	5	6
Length (μm)	1.67	1.40	1.24	1.14	1.03	0.91

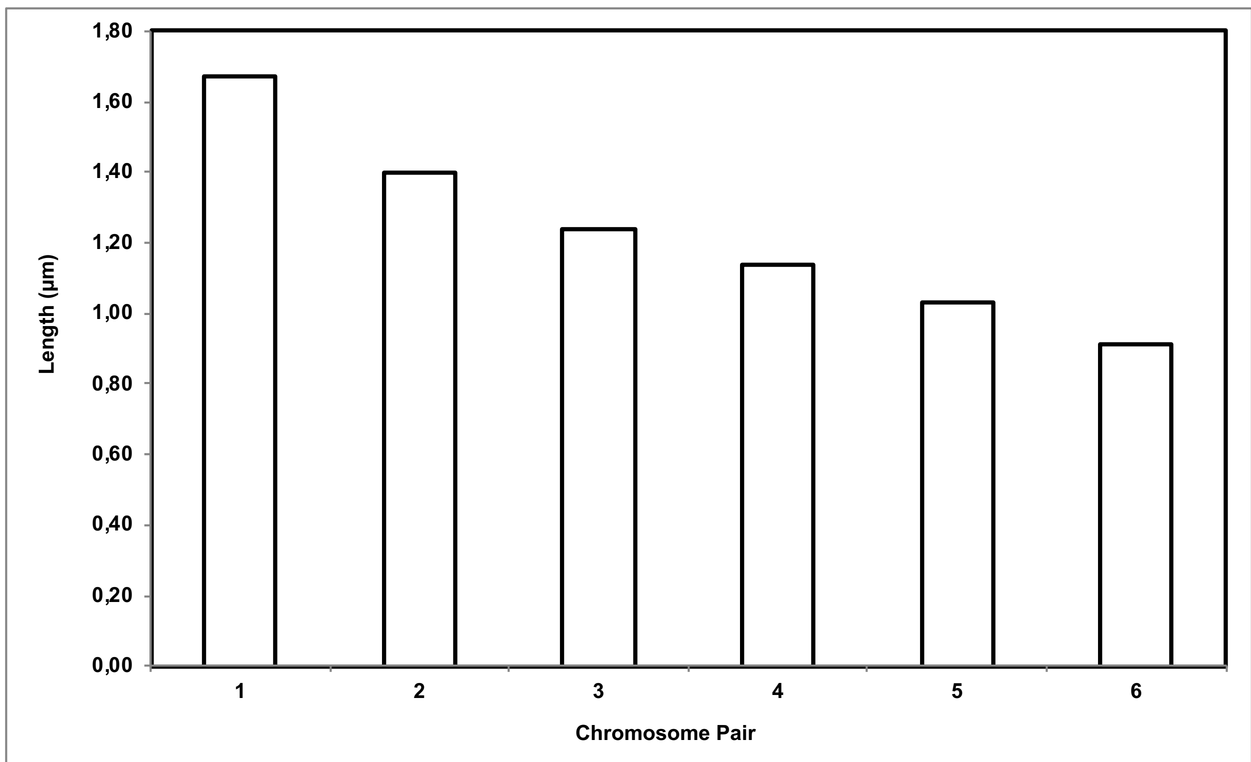

Figure 4. The monoploid ideogram of *Phauloppia lucorum*.

Discussion

Chromosomal parameters are important contributors to the understanding of evolutionary relationships, when used in conjunction with morphological or molecular techniques. Some important chromosomal parameters are basic chromosome number, diploid chromosome number (2*n*), karyotype formula, total haploid length and karyotype asymmetry. The S/AI formula is used to calculate karyotype asymmetry in highly organized animals (Eroğlu, 2015). The karyotype formula and the karyotype asymmetry could not be determined due to the holocentric chromosomes in *P. lucorum*. They are parameters specific to monocentric chromosomes. Although the diploid chromosome number of *P. lucorum* is 2*n* = 12, this not common in the oribatid mites, it is close to the general karyotype reports of the order as *Archegozetes longisetosus* Aoki, 1965, *Galumna* sp. It is reported that the common chromosome number is 2*n* = 18 in oribatid mites (Norton et al., 1993; Heethoff et al., 2006). As a broader example, mites and ticks have two to 36 chromosomes (Oliver, 1977). Eroğlu & Per (2016) found that the chromosome number of one oribatid mite, *Zygoribatula cognata* (Oudemans, 1902), is 2*n* = 30.

The chromosomes of *P. lucorum* are small holocentric chromosomes. The holocentric chromosomes do not show a localized centromere, which is the thin waist-like structure seen in eukaryotic chromosomes. Although monocentric chromosomes are much more common, the holocentric chromosomes have the broad phylogenetic distribution. Melters et al. (2012) reported that the holocentric chromosomes evolved at least nine different times in animals and four different times in plants. The holocentric chromosomes are small-sized chromosomes ranging from 0.5 to 2.0 µm (Wrensch et al., 1994). *Phauloppia lucorum* has small holocentric chromosomes (range 0.91-1.67). Many other arthropods, such as Lepidoptera, Hemiptera and Odonata, also have holocentric chromosomes (White, 1973; Heethoff et al., 2006). Holocentric chromosomes can provide some advantages. For example, sensitivity to radiation infertility is lower in butterflies compared to other insect groups. The main reason for this durability is that butterflies have holocentric chromosomes (North, 1967). After radiation, each fragment that separates from the holocentric chromosomes acts as a separate chromosome and will not

be lost in the anaphase (Lachange, 1967). Another advantage is that very different meiotic adaptations are needed for organisms to adopt holocentric chromosomes. Some of these adaptations are restriction of kinetochore activity, inverted meiosis and asymmetric meiosis (Melters et al., 2012).

The sex chromosomes could not be determined in *P. lucorum*. In generally, the oribatid mites have weak sexual dimorphism in size; and strong dimorphism is rare (Behan-Pelletier, 2015). The sexual dimorphism is a physical difference between two sexes of the same species other than in the sexual organs. There are three reproduction models in the Acari; thelytoky, haplodiploidy and diplodiploidy. It has been reported that the diplodiploidy is the ancestoral reproduction model in mites (Norton et al., 1993; Wrensch et al., 1994). Generally, in diplodiploidy, the young mites (male and female) are produced from fertilized eggs and the sex ratio is almost equal (1:1). However, the order Oribatida is characterized by a similar karyotype with the absence of sex chromosomes (Heethoff et al., 2006). Unlike sexual oribatid mites, the male proportion is very low (males rare) in parthenogenetic species and the rare males are generally sterile. Some factors may affect the proportion of rare male in parthenogenetic species. Environmental conditions are one of the most important of these factors and may induce the production of males (Chang et al., 2017).

This study reports for the first time the chromosome numbers and karyotype of *P. lucorum*. As there are many species for which chromosome data is unknown among oribatid mites, more chromosomal data are needed to support to the cytotaxonomy of oribatid mites.

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The authors would like to thank Mrs. Kübra Çubukçu and Mr. Arif Çubukçu for their contributions and assistance during the collection of samples in the field.

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Original article (Orijinal araştırma)

Effect of different phosphine gas concentrations against *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae) on tomato and green pepper fruit, and determination of fruit quality after application under low-temperature storage conditions[1]

Farklı fosfin gaz konsantrasyonlarının düşük sıcaklık depolama koşullarında domates ve yeşil biberde karantina zararlısı *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae)'e karşı etkileri ile uygulama sonrası meyve kalitesi üzerine etkisinin belirlenmesi

Sait ERTÜRK[2*] **Fatih ŞEN[3]** **Mustafa ALKAN[2]** **Murat ÖLÇÜLÜ[4]**

Abstract

In this study, we investigated the control of western flower thrips [*Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae)], which is an important pest in tomato and green pepper growing areas in Turkey, under cold storage conditions with phosphine fumigation. The trials were conducted in Mersin in 2015, due to infestations of *F. occidentalis* damaging the export quality of tomatoes and green peppers. The experiments were conducted by using $ECO_2FUME^{®}$ with different phosphine gas concentrations including 500, 1000 and 2000 ppm. Fumigation chambers were kept at 4˚C. Lowest mortality was seen at 500 ppm gas concentration with 72% for larval stage on tomatoes and 73.75% mortality for pupal stage on green pepper. The data demonstrate that the minimum requirement of phosphine gas concentration 100% mortality of all stages of *F. occidentalis* was at 2000 ppm. The quality of treated fruit was investigated by analyzing weight loss, sensory quality, fruit firmness, fruit skin color, total soluble solids, titratable acidity contents, physiological and pathological disorders. After treatment, there were no changes in physiologic, pathological and shelf-life properties of the products. The results indicated that, phosphine is a suitable fumigant at low temperature for disinfestion of *F. occidentalis* from tomatoes and green peppers before shipment.

Keywords: Fumigation, phosphine, quality analyze, quarantine, storage

Öz

Bu çalışma ile Türkiye'de domates ve yeşil biber alanlarında önemli bir zararlı olan batı çiçek tripsi [*Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae)]'nin soğuk hava depolama koşullarında fosfin fümigasyonu ile kontrolü araştırılmıştır. Domates ve yeşil biberin ihracat kalitesine zarar yapan *F. occidentalis* bulaşmasına bağlı olarak, denemeler 2015 yılında Mersin'de gerçekleştirilmiştir. Denemeler $ECO_2FUME^{®}$ preparatı ile 500, 1000 ve 2000 ppm'lik farklı fosfin gaz konsantrasyonunda test edilmiştir. Fümigasyon birimleri 4˚C tutulmuştur. En düşük ölüm oranı 500 ppm gaz konsantrasyonunda %72 ile domateste larva döneminde, yeşil biberde ise %73.75 ölüm oranı ile pupa döneminde görülmüştür. Çalışmadan elde edilen verilere göre batı çiçek tripsi'nin bütün dönemlerinin ölümü için en az 2000 ppm'lik gaz konsantrasyonunun gerekli olduğu ortaya konmuştur. Uygulama yapılan meyvenin kalitesi, ağırlık kaybı, duyusal kalite, meyve sıkılığı, meyve rengi, toplam çözünebilir katı madde, titrasyon asitliği içeriği, fizyolojik ve patolojik bozukluklar analiz edilerek incelenmiştir. Fosfin uygulamasından sonra ürünlerde fizyolojik, patolojik ve raf ömrü üzerinde herhangi bir değişim olmamıştır. Sonuçlar, taşıma öncesinde düşük sıcaklıkta *F. occidentalis*'in domates ve yeşil biberden dezenfekte edilmesi için fosfinin uygulanabileceğini ortaya koymuştur.

Anahtar sözcükler: Fümigasyon, fosfin, kalite analizi, karantina, depolama

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Introduction

Fresh vegetables such as tomatoes (*Solanum lycopersicum* L., Solanaceae) and green pepper (*Capsicum annuum* L., Solanaceae) are important agricultural export products in Turkey with about 12.6 kt of tomatoes and 2.3 kt of green pepper exported annually (Anonymous, 2016). The major pest of these vegetables in Turkey is western flower thrips, *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae) (Kütük et al., 2016).

Treatment of fruit with aluminum phosphide formulation has a negative effect on fruit quality, including discoloration, softening of the fruit flesh and shortened shelf life under low-temperature storage conditions (Desmarchelier et al., 1998; Ferizli et al., 2004). Therefore, it is also important to determine the quality of the applied product as well as the effect of the application of the phosphine gas on the target pest. For agricultural products to be exported, in addition to freedom from quarantine pests, fruit quality is another important parameter. For fumigant applications, the effects of fumigant to the pests are mostly investigated, while quality studies are done less frequently. If quality is affected, the market value of treated products falls and causes the exporter economic loss.

Frankliniella occidentalis is a cosmopolitan and polyphagous pest (Do Bae et al., 2015). The damage this pest either directly by feeding on plant tissue or indirectly by transmitting plant viruses, for example, tomato spotted wilt virus (Allen & Broadbent, 1986) and impatiens necrotic spot virus (Daughtrey et al., 1997). According to Tunç et al. (2012), *F. occidentalis* was reported for the first time in the Mediterranean Region (Burdur Province in 1992 and Antalya Province in 1993) of Turkey. The first appearance of western flower thrips in the Aegean region was observed on glasshouse tomatoes in Izmir at 1995. The presence of *F. occidentalis* on tomatoes and green pepper is a problem which negatively impacts international trade of fresh vegetables. Due to infestations of western flower thrips in exported products, tomatoes and green peppers are rejected from time to time, this is particularly the case for countries whose quarantine regulations are very strict (Seaton & Joyce, 2010). Also, chemical control of this pest under field conditions is very difficult because it is a polyphagous and multivoltine pest. For the last few decades, either pure phosphine gas alone or combination with CO_2, has been tested for postharvest fumigation of fresh food products such as apple, broccoli, cut flowers, lettuce, grape, peach, pear, plum, paprika, and white chrysanthemum under cold weather conditions (Liu, 2008; Zhang et al., 2012; Rogers et al., 2013; Emekci et al., 2014).

Adults of this pest are most commonly located in the flowers of the host plants, and may also enter closed buds. Eggs are laid in the parenchymatous tissues of leaves, fruit and flower parts. Therefore, fumigation is very important for control of thrips, especially when fresh fruit and vegetables are stored in warehouses. As a postharvest insect control agent, phosphine is usually used as an alternative to methyl bromide. In the atmosphere, methyl bromide depletes the ozone layer and allows increased ultraviolet radiation to reach the earth's surface. Methyl bromide is a Class I ozone-depleting substance defined by the Montreal Protocol. In Turkey, methyl bromide has been banned since 2008. $ECO_2FUME^{®}$ (2% phosphine and 98% CO_2 w/w) cylindered gas formulation of phosphine is safe for commercial use, effective and ready to apply with user-friendly fumigation of food and other commodities. It is used to a relatively limited extent for quarantine treatment of imported grain, flour, fresh fruit and vegetables, oil seeds and nuts that come in shipping containers. For this reason, researchers are searching for alternative methods for postharvest pest management.

There little research on the effect of low-temperature phosphine fumigation for against western flower thrips on tomatoes and green pepper. The aim of this work is to evaluate the efficiency of different phosphine gas concentration under cold storage conditions on the mortality of western flower thrips eggs, larvae, pupae and adults. Also, the effect of phosphine on the quality of the treated tomatoes and green pepper were investigated in vitro.

Material and Methods

Mass rearing of insects

Initial cultures of western flower thrips were established with *F. occidentalis* adults originally collected from greenhouses in Mersin and Adana Districts of Turkey between March and June in 2015. About 50 female and 10 male western flower thrips were collected with an aspirator and transferred to ventilated jars. Glass jars (1 L, 18 cm height x 10 cm diameter) were used to mass rear the thrips mass. For ventilation, the center of the jar lids was drilled with a 7-cm hole and covered with thrips-proof cloth. Adult insects, french bean [*Phaseolus vulgaris* (L.) (Fabaceae)] pods and an amount of vermiculite were put in the jars. French bean pods served as food and oviposition sites for the thrips. Cattail, *Typha* sp. (Typhaceae), pollens were added as additional food source for adults. Vermiculite was used as a suitable pupation site. First instar larvae (L_1) emerged in a few days and started feeding with bean pods and pollens. Every second day fresh bean pods were added to jars and dry pods were removed when needed. Mature second instar larvae (L_2) turned into pupae into vermiculite media and adults emerged in a few days. Insect rearing was done in climate-controlled rooms under 25±1ºC, 60±10% RH and long day (16:8 h L:D photoperiod) conditions Biological Control Research Institute, Adana.

Experimental design

Experiments were performed at the premises of PackErman Logistics Centre (Mersin, Turkey) at 4°C and 75±5% RH in June 2015. With tomato and green pepper fruit, the fumigation treatments against eggs, larvae, pupae and mixed-sex adults of *F. occidentalis* were performed as three (500, 1000 and 2000 ppm) phosphine concentrations ($ECO_2FUME^{®}$, Cytec Industries B.V., Netherlands) on the same day. All stages of western flower thrips (except for eggs) were placed between tomato and green pepper fruit in a special container. In the trials, four replicates were used and each replicate had 25 individual larvae, pupae and mixed-sex adults. Untreated tomato and green pepper fruit were used as controls. In the experiment, we could not use positive control because there was no registered fumigant that has same formulation and properties as $ECO_2FUME^{®}$ in Turkey. Three kg of tomato and green pepper fruit were separated for post-treatment quality analyses and the rest of tomato and green pepper fruit were stored for 7 d at 5±0.5ºC and 7±0.5ºC, respectively, at 85-90% RH. In addition to the post storage period, shelf-life samples were kept for 2 d at 20±1ºC and 65-70% RH. Some quality analyses were performed on samples taken both after storage and shelf life. The quality analyses were conducted according to randomized block experimental design with three replicates each of 3 kg fruit.

Fumigation procedure

For the experiments, a special 1.55 m^3 (1.55 x 1 x 1 m) volume fumigation chamber was made from PVC. Plastic boxes (40 x 25 x 30 cm) containing tomatoe and green pepper fruit were put into each fumigation chamber and all life stages of *F. occidentalis* (eggs, larvae, pupae and adults) were placed at the bottom, center and upper level of the chamber. The chambers were kept in the cold rooms (4°C) and sealed with virtually impermeable film (VIF) (Plastika Kritis, ORGASUN®) using insulation duct tape. Phosphine gas was delivered to each chamber via Parker-Parflex tube attached to a $ECO_2FUME^{®}$ cylinder. Tested phosphine concentrations were 500, 1000, 2000 ppm, which are equivalent to 34.8, 69.6 and 139.2 g/m^3 $ECO_2FUME^{®}$, respectively. Each experimental unit was fumigated for 24 h. During the trial, phosphine concentrations in the chamber were determined using a $CertiPH_3os$ 2240 gas monitor (Messtechnik GmbH, Kirchseeon, Germany) and gas leakage in the room was controlled by the Dräger Pac 7000 (Drägerwerk AG & Co. KGaA, Lübeck, Germany). Temperature of the room was continuously recorded with a Dixell CoolMate XLH260 (Pieve d'Alpago, Italy) during the experiments. Desired phosphine gas concentration was calculated as follows.

$$ECO_2FUME(g) = \frac{50g \text{ x desired phosphine gas concentration } (ppm)}{718\ ppm}$$

After fumigation, the cold room was ventilated and test insects were taken out from each fumigation unit. For mortality assessment, the insects were transferred to a climate chamber and kept at 25±1ºC, 60±5% RH and 16:8 h L:D photoperiod. After 2 d, adults, larvae and pupae mortality were assessed, and mortality of the eggs were determined after incubation for 7 d.

Quality analysis

Tomato and green pepper samples were weighed before and after storage and shelf life on an electronic scale (XB 12100; Presica Instruments Ltd., Switzerland, 0.05 g accuracy), and percentage weight loss determined. Tomato fruit firmness was determined using a penetrometer (Effegi FT 011, Fujihira. Industry Co., Ltd., Tokyo, Japan) with a 7.9-mm diameter head and conical-shaped spear; the results are expressed in Newton (N).

The external skin color was measured at the equatorial area on both sides of 15 fruit using a colorimeter (CR-300; Minolta Co., Osaka, Japan), and the average scores were recorded in terms of CIE-L* a* b* values. These values were then used to calculate chroma ($C^* = [a^{*2} + b^{*2}]^{1/2}$), which indicates the intensity or color saturation and hue angle ($h^o = \tan^{-1} [b^*/a^*]$) that is expressed in degrees and represents: 0° (red-purple), 90º (yellow), 180º (bluish-green), and 270º (blue) (McGuire, 1992).

The total soluble solid (TSS) content of the tomato and green pepper fruit juice was determined by using a digital reflectometer (PR-1; Atago, Tokyo, Japan) and expressed as a percentage. The titratable acidity (TA) was determined by titrating 10 ml of juice with 0.1 N NaOH up to pH 8.1. The results are expressed as g malic acid/100 ml of fruit juice, in accordance with the AOAC standards (1984).

Physiological and pathological disorders over fruit surfaces were examined and their rates were expressed as a percentage. Sensory quality evaluation of appearance was conducted by an experienced six-member panel and a five-point hedonic scale: overall appearance scale (1, extremely poor; 3, poor; 5, moderate and limit of marketability; 7, good; and 9, excellent). Each assessor tasted and evaluated three fruit per replicate. The procedures for sensory evaluation of horticultural crops described by Heintz & Kader (1983) were follow by the panel.

Statistical analysis

Fumigation assays were performed with randomized block experimental design with four replicates. Variance analysis was performed with transformed data. In addition, the differences among treatments were analyzed by means of Tukey multiple comparison test ($P < 0.05$). All statistical analyses were conducted with MINITAB® Release 16 package program. Data obtained from quality analyses were subjected to analysis of variance (ANOVA) by using the IBM® SPSS® Statistics ver. 19 statistical software (IBM, New York, NY, USA). Significant differences between the means for each vegetable (tomato and green pepper) were determined by Duncan's multiple range tests at $P < 0.05$. Standard deviation of the mean was also calculated from the replicates.

Results and Discussion

The lowest effect after 24 h of fumigation was seen in the larval stage at 500 ppm phosphine. At 2000 ppm, 100% mortality was recorded from all stages of western flower thrips after 24 h at 4ºC (Table 1). All treatments were statistically different ($F = 11.57$; $df = 2, 11$; $P < 0.05$).

Table 1. Mortality effects of different phosphine concentrations on different stages of *Frankliniella occidentalis* in tomatoes

Phosphine concentration (ppm)	Mortality (%)±SE*							
	Egg		Larvae		Pupae		Adult	
500	77.29±8.75	b**	72.50±15.00	b	78.75±2.50	b	82.99±14.18	a
1000	86.39±7.68	b	100.00±0.00	a	100.00±0.00	a	97.22±5.56	a
2000	100.00±0.00	a	100.00±0.00	a	100.00±0.00	a	100.00±0.00	a

* SE: Standard error;

** Same letters within columns are not significantly different according to Tukey's multiple range test (P < 0.05).

In green pepper there was a linear relationship between the concentration of phosphine gas and its effect. The lowest mortality of 74% was at 500 ppm for the pupal stage. The highest mortality of 100% rate was recorded for all stages of *F. occidentalis* (Table 2) at 2000 ppm.

Table 2. Mortality effects of different phosphine concentrations on different stages of *Frankliniella occidentalis* in green pepper

Phosphine concentration (ppm)	Mortality (%)±SE*							
	Egg		Larvae		Pupae		Adult	
500	90.00±7.98	b**	85.00±12.91	a	73.75±9.46	b	77.08±11.55	b
1000	100.00±0.00	a	95.00±5.77	a	95.00±10.00	a	100.00±0.00	a
2000	100.00±0.00	a	100.00±0.00	a	100.00±0.00	a	100.00±0.00	a

* SE: Standard error;
** same lower-case letters within columns are not significantly different according to Tukey's multiple range test ($P < 0.05$).

Liu (2008) reported 100% mortality of the *F. occidentalis* was after 18-h exposure period with ≥250 ppm phosphine. In a previous study, cooled iceberg lettuce (3ºC) fumigated with phosphine gas (484 ppm) at room temperature for 18 h, bioassay resulted in complete control of this pest (Liu, 2011). Similarly, Emekci et al. (2014) reported total mortality of all stages of *F. occidentalis* and mites, *Tetranychus cinnabarinus* (Boisduval, 1867), at minimum of 1000 ppm (70 g ECO_2FUME/m^3) after 24 h at 4ºC. Pre-adult insect stages are known to be more tolerant to fumigants because their respiration rates are much lower than adult insects. The results of this study showed that insects collected from different regions have different responses to different doses of the same chemical. Therefore, it is thought that the differences in the mortality rates of the green pepper and tomato were caused by this. Eggs and pupae of several species are reported as the most phosphine tolerant stages (Chaudhry, 1997). Shorter fumigation period with lower phosphine gas level would also help prevent injury to vegetables. The treated fruit were compared with untreated controls to determine potential injury from the fumigant. No negative effects of the fumigation treatments were found. Phosphine fumigation applications at low temperature could be used effectively to control *F. occidentalis* on fresh tomato and green pepper fruit for export. The effect of different phosphine doses on weight, firmness and color (hº) of the tomato fruit is shown in Table 3. The weight loss was similar after 7 d of storage and 3 d of shelf life, and ranged from 1.09-1.20% and 2.37-2.57%, respectively. The no significant effect of the different doses of phosphine on tomato firmness of fruit was found and after shelf life, firmness varied between 18.43 and 20.23 N, respectively. The effect of phosphine treatments on color (hº) of tomato fruit was found to be minimal and after shelf life varied between 44.1 and 49.2.

Table 3. The effect of different concentrations of phosphine on weight, color and firmness of tomato fruit

Phosphine concentration (ppm)	Weight loss (%)			Firmness (N)			h° value	
	AS	ASL	AT	AS	ASL	AT	AS	ASL
Control	1.20^{ns}	2.37^{ns}	21.05^{ns}	20.95^{n}	18.43^{ns}	47.44^{ns}	44.89^{ns}	44.07^{ns}
100	1.12	2.46	21.61	21.82	20.08	47.06	45.82	48.40
200	1.09	2.57	22.27	22.38	20.23	46.44	48.94	45.09
300	1.11	2.40	21.36	21.37	18.98	43.97	47.52	49.24

^{ns}: Not significant; AS: after storage; ASL: after shelf life; AT: after treatment.

The variation of TSS, TA and sensory evaluation of tomato fruit according to phosphine treatments is given in Table 4. The effect of the phosphine doses on TSS, TA and sensory evaluation of tomato fruit were similar. After shelf life, TSS, TA and sensory evaluation ranged between 4.20-4.60%, 0.29-0.37 g/100 ml and 4.2-4.6, respectively. No negative effect of phosphine treatments was observed for these parameters.

Table 4. Effects of different concentrations of phosphine on TSS (%), TA content (g citric acid/100 ml) and sensory analysis of tomato fruit after treatment, storage and shelf life

Phosphine concentration (ppm)	TSS content			TA content			Sensory analysis	
	AT	AS	ASL	AT	AS	ASL	AS	ASL
Control	4.45^{ns}	4.45^{ns}	4.20^{ns}	0.33^{ns}	0.28^{ns}	0.29^{ns}	4.8^{ns}	4.4^{ns}
100	4.15	4.25	4.50	0.29	0.30	0.37	5.0	4.6
200	4.50	4.35	4.50	0.31	0.27	0.31	4.8	4.2
300	4.45	4.35	4.60	0,29	0.29	0.31	4.8	4.4

^{ns} : Not significant; AS: after storage; ASL: after shelf life; AT: after treatment.

The variation in weight loss and color values (C* and hº) of green pepper fruit according to phosphine treatments is given in Table 5. The effect of phosphine treatments on weight and color of green pepper fruit were similar. After 7 d of storage followed by 2 d of shelf life the weight loss ranged between 1.62-1.76%, C* and hº color values ranged between 37.18-40.84 and 112.63-113.47, respectively.

Table 5. The effect of different concentrations of phosphine treatments to weight loss and color of green pepper fruit

Phosphine concentration (ppm)	Weight loss (%)		C* value			hº value		
	AS	ASL	AT	AS	ASL	AT	AS	ASL
Control	0.71^{ns}	1.76^{ns}	39.54^{ns}	39.70^{ns}	38.35^{ns}	112.71^{ns}	112.93^{ns}	112.73^{ns}
100	0.80	1.62	39.59	39.08	40.84	112.66	112.71	112.63
200	0.67	1.63	39.16	41.00	38.79	113.02	112.66	112.97
300	0.87	1.72	37.29	40.46	37.18	113.06	112.28	113.47

^{ns}: Not significant; AS: after storage; ASL: after shelf life; AT: after treatment.

The effect of the phosphine treatments to TSS, TA and sensory evaluation of green pepper fruit were similar. After shelf life the TSS, TA and sensory evaluation of green pepper samples were on average 4.53%, 0.12 g/100 ml and 4.7, respectively (Table 6).

Table 6. Effects of different concentrations of phosphine on TSS (%), TA content (g citric acid/100 ml) and sensory analysis of green pepper fruit after treatment, storage and shelf life

Phosphine concentration (ppm)	TSS content			TA content			Sensory	
	AT	AS	ASL	AT	AS	ASL	AS	ASL
Control	4.23^{ns}	4.50^{ns}	4.60^{ns}	0.13^{ns}	0.14^{ns}	0.13^{ns}	5.0^{ns}	4.8^{ns}
100	4.30	4.45	4.60	0.12	0.14	0.11	5.0	4.6
200	4.20	4.40	4.45	0.12	0.13	0.12	5.0	4.6
300	4.17	4.50	4.45	0.13	0.14	0.10	5.0	4.8

^{ns}: Not significant; AS: after storage; ASL: after shelf life; AT: after treatment.

After the storage and shelf life, physiological and pathological changes were not determined for the phosphine treated tomato and green pepper fruit. When using this fumigant or any other product, the efficiency in controlling the pests and toxicity should be determined along with and effects on product quality (Desmarchelier et al., 1998). After storage and shelf life, no physiological and pathological disorders were found in the phosphine treated tomato and green pepper fruit and the reason for this was there was not skin damage. Skin damage causes both decay and color alteration. For example, long exposure to a high phosphine dose (2.0 g/t) in dry fig caused darkening due to skin damage, but phosphine treatment had limited effect on the chemical composition of fruit according with previous studies (Meyvaci & Sen, 2007; Sen et al., 2009, 2015). Consequently, considering the physical, chemical and sensory analysis, no negative effects were identified to fruit quality after the treatment, storage and shelf life in green pepper fruit treated with phosphine (ECO_2FUME®) for 24 h at 500, 1000 and 2000 ppm.

The results indicate that ECO_2FUME® is a suitable fumigant for *F. occidentalis* disinfestation of tomatoes and green peppers at low temperature before shipment, with no negative effects on quality parameters.

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Original article (Orijinal araştırma)

New records for the horse fly (Diptera: Tabanidae) fauna of Turkey and description of *Hybomitra tanatmisi* sp. nov.[1]

Türkiye at sineği (Diptera: Tabanidae) faunası için yeni kayıtlar ve *Hybomitra tanatmisi* sp. nov.'nin deskripsiyonu

Ferhat ALTUNSOY[2*]

Abstract

Due to the geographical location of Turkey and its special geological, geomorphological and climatic features, Turkish Tabanidae fauna (Insecta: Diptera) have great species richness with 171 known species and 15 subspecies. The main purpose of the study was to contribute to the Tabanidae fauna of Turkey. Specimens were collected from Black Sea Region of Turkey between 2009 and 2015 with Malaise and Nzi traps which were baited with 1-octen-3-ol. As a result of the study, new records for Turkey, *Hybomitra arpadi* (Szilady, 1923), *Hybomitra aterrima* (Meigen, 1820), *Hybomitra montana* (Meigen, 1820) and *Hybomitra morgani* (Surcouf, 1912), are presented and *Hybomitra tanatmisi* sp. nov. is described. Distribution, adult and larval habitats and zoogeography of these species are briefly discussed.

Keywords: Anatolia, fauna, horse fly, Hybomitra, Tabanidae, Turkey

Öz

Türkiye'nin coğrafik lokasyonu ve jeolojik, jeomorfolojik ve klimatik özellikleri sayesinde, Türkiye Tabanidae faunası (Insecta: Diptera) 171 tür ve 15 alt tür ile büyük tür çeşitliliğine sahiptir. Çalışmanın temel amacı, Türkiye'nin Tabanidae faunasına katkıda bulunmaktır. Örnekler 2009 ve 2015 yılları arasında Türkiye'nin Karadeniz Bölgesi'nden, 1-octen-3-ol'e batırılmış Malezya ve Nzi tuzakları kullanılarak toplanmıştır. Çalışmanın sonucu olarak, Türkiye faunası için yeni kayıt olan *Hybomitra arpadi* (Szilady, 1923), *Hybomitra aterrima* (Meigen, 1820), *Hybomitra montana* (Meigen, 1820) ve *Hybomitra morgani* (Surcouf, 1912) türleri sunulmuştur ve *Hybomitra tanatmisi* sp. nov.'nin tanımlaması yapılmıştır. Bu türlerin dağılımları, ergin ve larva habitatları ile zoocoğrafik özellikleri kısaca tartışılmıştır.

Anahtar sözcükler: Anadolu, fauna, at sineği, Hybomitra, Tabanidae, Türkiye

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Introduction

Female horse flies cause considerable problems both medically and economically during their blood-feeding period. Their importance is associated with both transmission of diseases, their economic impact when they occur in large numbers and their persistent and painful bites which irritate grazing animals considerably with resulting weight loss, decreased milk production and allergic responses. (Chvala et al., 1972; Olsufjev, 1977; Foil, 1989).

Due to its geographical location, the territory of Turkey has special geological, geomorphological and climatic features that make it one of the most important places in the Palearctic Region for studying the horse fly fauna. Asia Minor, one of the most remote areas of the Eastern Mediterranean, has a large number of faunistic elements that congregate from the three main parts of the Palearctic region, Mediterranean, Asian and European. Almost all of these species can find suitable habitats in the main part of Asia Minor because of the high diversity of various landscapes, climatic conditions, mountain chains with vertical zonation, plains along the rivers and seashores, all of which are found in Turkey. Based on previous studies, Turkish Tabanidae fauna (Insecta: Diptera) includes 171 species and 15 subspecies (Kılıç, 2006; Andreeva et al., 2009; Altunsoy & Kılıç, 2010, 2014) that represents almost 30% of Palearctic fauna of 610 species (Chvala, 1988; Andreeva, 2004). Earlier studies on the horse fly fauna of Turkey were made by international researchers between 1850 and 1960 (Walker, 1854; Loew, 1856; Austen, 1925; Leclercq, 1966a, b, 1967a, b). More recent studies have been more comprehensive being mostly conducted by local researchers, and include many new records and new species in the horse fly fauna of Turkey (Schacht, 1983, 1984, 1985, 1987; Yücel, 1987; Erdoğmuş, 1992; Hayat & Özbek, 1992; Kılıç, 1992, 1995, 1996a, b, c, 1997, 1999, 2001a, b, c, 2003, 2004, 2005; Kılıç & Schacht, 1995; Kılıç & Öztürk, 2002; Altunsoy et al., 2010; Altunsoy & Kılıç, 2010, 2011; Büber et al., 2011). In this study, four species were added to the horsefly fauna of Turkey and one new species is described.

Material and Methods

Sampling and collecting method

Adult specimens of the horse fly were collected with Malaise and Nzi traps, baited with 1-octen-3-ol, and water traps. The water trap used for male tabanids was based on Barnes (2018). Octenol is well known as an attractant for horse flies as reported by Altunsoy & Afacan (2014), Mihok et al. (2007) and Krčmar et al. (2005, 2006, 2009). Trapping was done during daylight from 8 am to 7 pm. Collection and preparation of specimens were done according to the methods of Chvala et al. (1972) and Olsufjev (1977).

Identification and morphological study

Specimens were identified according to Chvala et al. (1972), Olsufjev (1977), Peus (1980), Schacht (1987), Leclercq (1966a, b; 1967a, b) and Rubio (2002). The identification of the specimens was made using Leica MZ7,5 stereo microscope.

The taxonomic status of the species was checked according to the recent updates of Fauna Europaea, Global Species and Chvala (1988).

The Atlas of |nsect Morphology (Steinmann & Zombory, 1985) was used for description of adult tabanids and specific terminology for Tabanidae was used according to Chvala et al. (1972). Identified specimens are preserved in the Zoological Museum of Anadolu University (AUZM).

Study area

Study was conducted in Ardahan, Artvin, Bartın and Bolu Provinces in the central and northeastern parts of Black Sea Region of Turkey between 2009 and 2015. Localities, coordinates, altitude, habitats and dates are presented in Table 1. Distribution of each species is summarized according to Chvala et al. (1972), Chvala (1988) and the Catalogue of Life: Systema Dipterorum (Anonymous, 2018).

Table 1. Study areas and habitats

Locality		Coordinates	Altitude (m)	Habitat	Date
Province	District				
Ardahan	Pasof	41°29'52'' N, 42°44'24'' E	1360	*Abies-Picea* forest	30 July 2011
Artvin	Maçahel	41°29'36'' N, 41°56'57'' E	976	*Abies-Picea*-Pinus forest	28 July 2011
	Maçahel	41°29'36'' N, 41°59'57'' E	1276	*Abies-Picea* forest	28 July 2011
	Borçka	41°23'32'' N, 41°51'13'' E	1470	*Abies-Picea* forest	25 June 2010
	Borçka	41°23'32'' N, 41°51'13'' E	1470	*Abies-Picea* forest	15 June 2012
Bolu	Gölcük	40°32'38'' N, 31°36''10'' E	1380	*Pinus* forest	18 June 2009
Bartın	Kurucaşile	41°47'51'' N, 32°34'43'' E	320	*Quercus* forest	20 June 2015
	Kurucaşile	41°48'21'' N, 32°35'24'' E	250	*Quercus* forest	22 June 2011

Results and Discussion

In total, 24 specimens belonging to the Tabaninae subfamily, were examined and four species are determined as new records for Turkey: *Hybomitra arpadi* (Szilady, 1923), *H. aterrima* (Meigen 1820), *H. montana* (Meigen, 1820) and *H. morgani* (Surcouf, 1912). *Hybomitra tanatmisi* sp. nov. is described.

Order: Diptera

Family: Tabanidae

Subfamily: Tabaninae

Tribe: Tabanini

Genus: Hybomitra Enderlein, 1922

Hybomitra arpadi (Szilady, 1923) (Figure 1)

Material examined: Turkey - 1 ♀ (AUZM), Pasof District, Ardahan Province, 41°29'52'' N, 42°44'24'' E, 1360 m, 30.VII.2011, Leg. F. Altunsoy; 2 ♀♀, Maçahel-District, Artvin Province, 41°29'36'' N, 41°56'57'' E, 976 m, 28.VII.2011, Leg. F. Altunsoy.

Type locality: Alaska; chorotype: A Holarctic species that commonly known from Scandinavia especially Sweden and Finland, it has a wide area of distribution from northern parts of America, occurs in regions of Moscow, Kamtchatka and Southwest Germany, and rarely in Japan. This is the first record for the Turkey. Three female specimens were collected in the early to late afternoon in July 2011.

Comments: This species occurring mostly in taiga and forests in the north in Central Europe on peat-bogs and hilly countries. Specimens (adult and larvae) were collected from marshy habitats in pine forests. *Hybomitra arpadi* is not a highly variable species but as reported by Dvorák and Petrašiunas (2010) identification of this species is difficult and requires more experience and accurate identified material for comparison. Some members of the *H. bimaculata* [*H. bimaculata* (Macquart, 1826), *H. distinguenda* (Verrall, 1909), *H. muehlfeldi* (Brauer, 1880)] and *H. montana* (especially *H. lundbecki* Lyneborg, 1959) groups are quite similar to *H. arpadi* (Szilady, 1923).

Figure 1. *Hybomitra arpadi* : a) dorsal view; b) antenna and palp ♀; and c) antenna and palp ♂.

Hybomitra aterrima (Meigen, 1820) (Figure 2)

Material examined: Turkey - 3 ♀♀ (AUZM), Borçka District, Artvin Province, 41°23’32’’ N, 41°51’13’’ E, 1470 m, 25.VI.2010, Leg. F. Altunsoy; 4 ♀♀, 2 ♂♂, Gölcük District, Bolu Province, 40°32’38’’ N, 31°36’’10’’ E, 1380 m, 18.VI.2009, Leg. A. Y. Kılıç; 2 ♀♀, Maçahel District, Artvin Province, 41°29’36’’ N, 41°59’57’’ E, 1276 m, 28.VII.2011, Leg. F. Altunsoy.

Type locality: Sweden, chorotype: A boreo-mountainous species known from North Europe from Scandinavia (Norway, Sweden and Finland) and can also be observed in mountains of Central and South Europe. It is known from the Alps up to 2400 m from France, South Germany and Central Austria. This is the first record for Turkey. In total, 11 specimens were collected in the afternoon from three different locations in three different years.

Comments: *H. aterrima* easily distinguishable from other closely related species with abdominal pubescence and entirely black abdomen, legs, antennae, face and slender palpi black haired. Larvae were collected from moist habitats between pine and oak forests. Adults are found near edaphobiotic habitats.

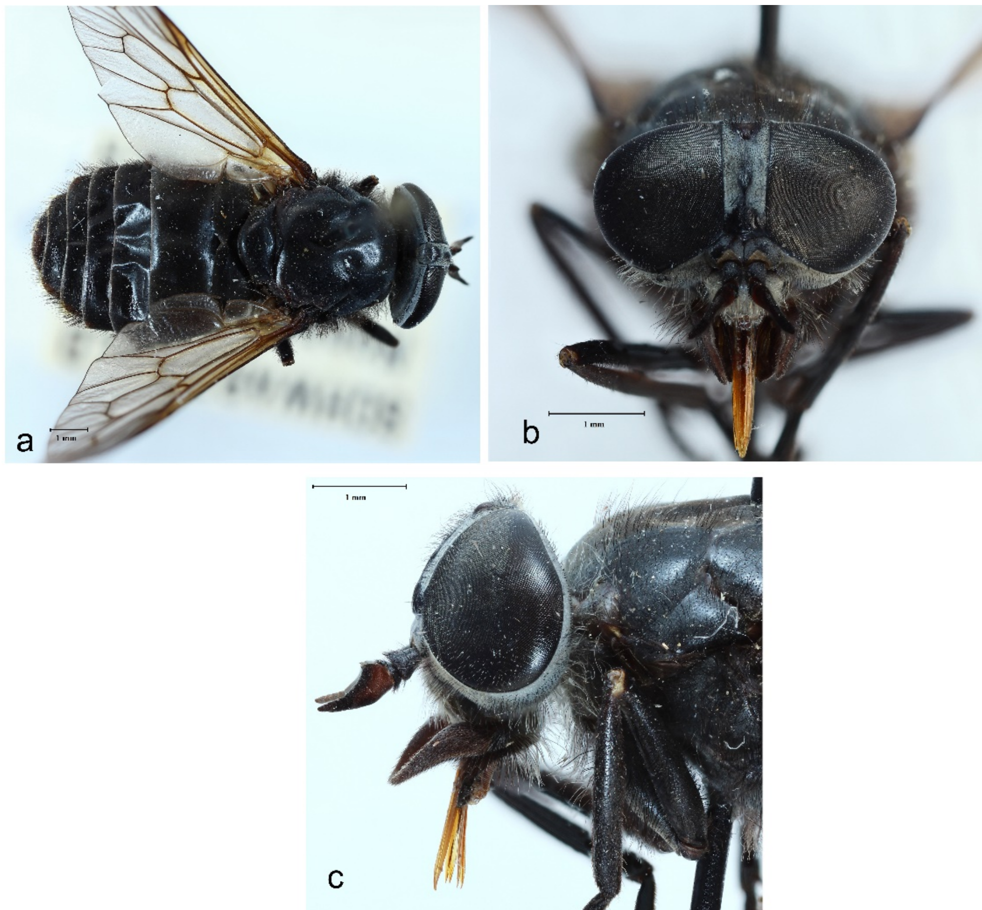

Figure 2. *Hybomitra aterrima* ♀: a) dorsal view; b) frons; and c) antenna and palp.

Hybomitra montana (Meigen, 1820) (Figure 3)

Material examined: Turkey - 4 ♀♀ (AUZM), Pasof District, Ardahan Province, 41°29'52'' N, 42°44'24'' E, 1360 m, 28.VII.2011, Leg. A. Y. Kılıç; 6 ♀♀, Karagöl, Borçka District, Artvin Province, 41°23'32'' N, 41°51'13'' E, 1470 m, 15.VI.2012, Leg. F. Altunsoy.

Type locality: Germany, chorotype: It is a widely distributed species in the Palearctic region especially in British Isles including Ireland and Scandinavia. It has also been recorded from West Austria, North Italy, Germany, Netherlands, Spain, France, Slovenia, China, Korea and Japan. This is the first record for Turkey. In total, 10 female specimens were collected in two different locations in 2011 and 2012.

Comments: This species is mainly found in hilly to mountainous areas; but is not restricted to such places. Larvae were collected from moist habitats between pine and oak forests. Adults were found near edaphobiotic habitats. *Hybomitra montana* (Meigen, 1820) is an extensively darkened species and some variability can be seen in brownish side markings on the first tergites. However, the antennae are more slender and darker than the nominate form.

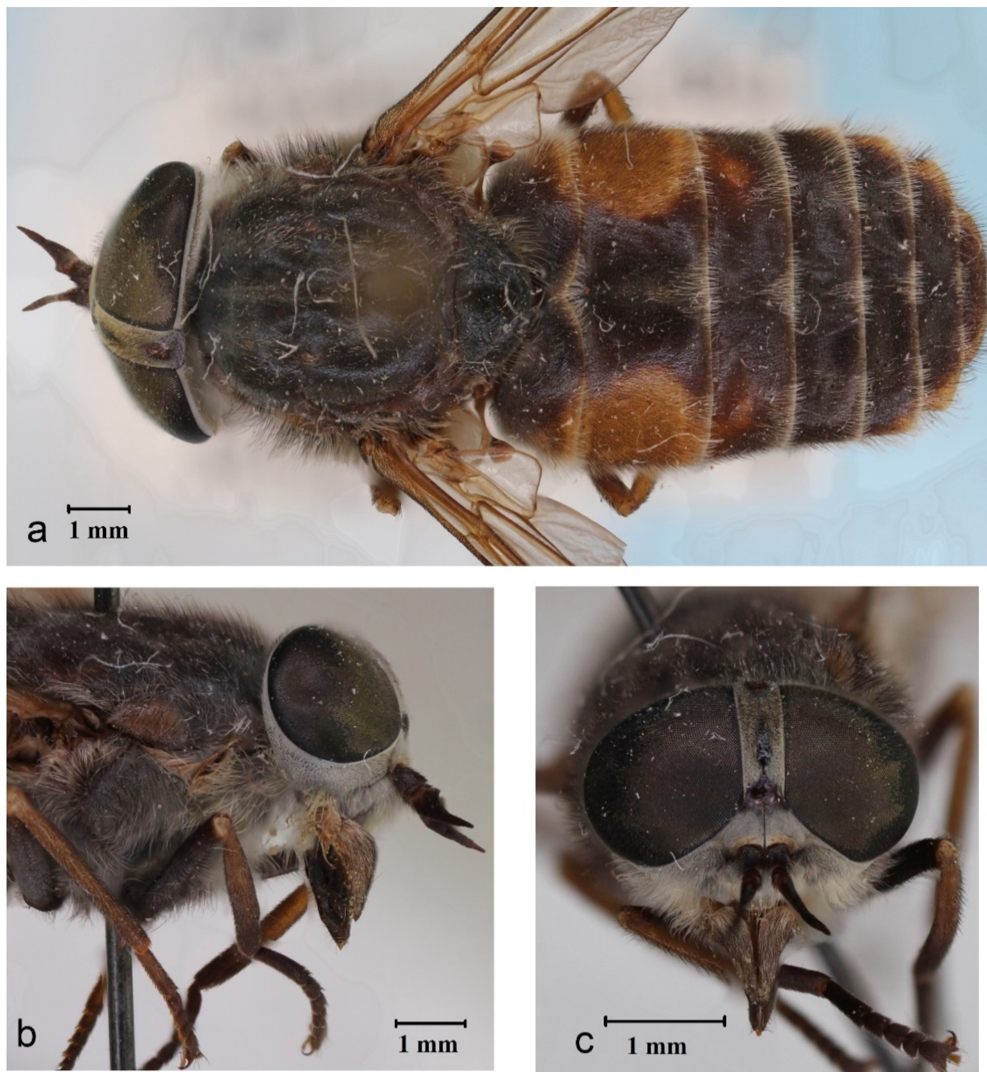

Figure 3. *Hybomitra montana* ♀: a) dorsal view; b) antenna and palp; and c) frons.

Hybomitra morgani (Surcouf, 1912) (Figure 4)

Material examined: Turkey - 2 ♀♀ (AUZM), Karagöl, Borçka District, Artvin Province, 41°23’32’’ N, 41°51’13’’ E, 1470 m, 12.VI.2012, Leg. A. Y. Kılıç; 5 ♀♀, Maçahel District, Artvin Province, 41°29’36’’ N, 41°56’57’’ E, 976 m, 18.VI.2012, Leg. F. Altunsoy.

Type locality: Iran, chorotype: The area of distribution of this species is not well known but confirmed from Denmark, Russia, Middle Asia, Iran, Georgia, Armenia, Mongolia, Azerbaijan and China. This is the first record for Turkey. In total, seven female specimens were collected during late afternoon from two different locations in June of 2011 and 2012.

Comments: This species inhabits various types of biotopes, but it occurs most frequently in forest regions, especially pine forests. Larvae and pupae were collected from moist habitats between pine and oak forests. Adults were found near edaphobiotic habitats.

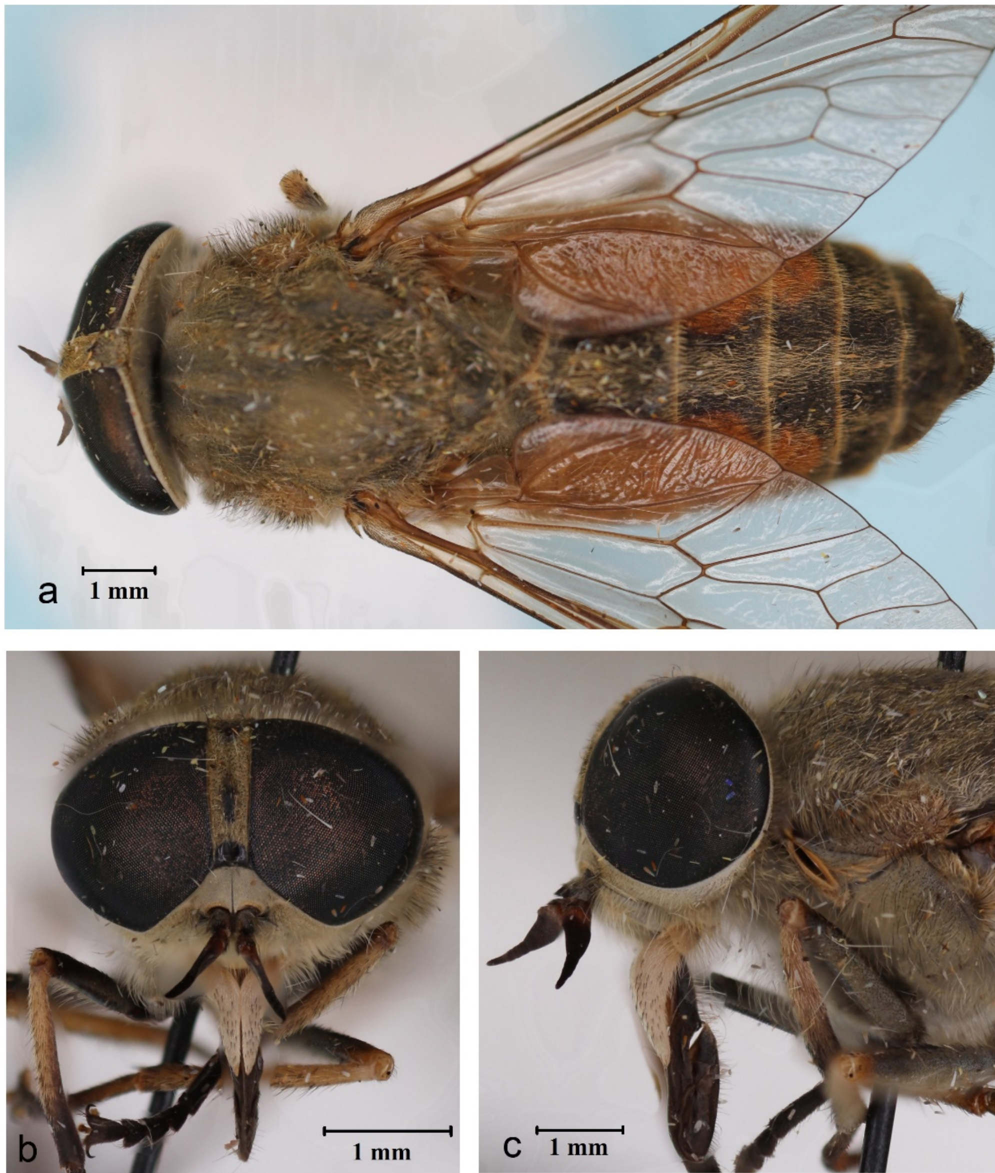

Figure 4. *Hybomitra morgani* ♀ : a) Dorsal view; b) frons; and c) Antenna and palp.

Hybomitra tanatmisi sp. nov. (Figures 5 & 8)

Material examined: Holotype: 1 ♀ Turkey (AUZM), Kanatlı Village, Kurucaşile District, Bartın Province, (41°48’22’’ N, 32°35’24’’ E), 250 m, 22.VI.2011, Leg. F. Altunsoy; Paratypes: 1 ♀, Kanatlı Village, Kurucaşile District, Bartın Province, (41°48’21,74’’ N, 32°35’24,27’’ E), 250 m, 22.VI.2011, Leg. F. Altunsoy; 4 ♀♀, Meydan Village, Kurucaşile District, Bartın Province, (41°47’52’’ N, 32°34’44’’ E), 320 m, 20.VI.2015, Leg. F. Altunsoy.

Etymology: The new species is dedicated to Mustafa Tanatmış, a Turkish entomologist who is respected colleague of the authors. We are thankful to his important research and valuable contributions to entomology.

Diagnosis: The new species has characteristic features of the *H. bimaculata* group, which are frontal index 1:4-6, distinctly wrinkled above, lower callus small and distinctly wrinkled, not polished (Figures 5 & 8). Smaller to medium sized length, 13-16 mm, brownish species with chestnut brown side markings on the anterior three tergites. Notopleural lobes are black and long yellowish haired. Frons narrow, lower callus small and distinctly wrinkled, antennae reddish-brown, basal segment long black haired. Closely related with *H. muehlfeldi, H. distinguenda*, *H. bimaculata* and *H. ciureai* (Seguy, 1937). Distinctive characters of this species with closely related species are given in Table 2.

Figure 5. *Hybomitra tanatmisi* sp. nov. ♀: a) dorsal view; b) frons; and c) antenna and palp.

Table 2. Distinctive characters of *Hybomitra tanatmisi* sp. nov. compared to four closely related *Hybomitra* spp.

Features	*H. tanatmisi*	*H. ciureai*	*H. muehlfeldi*	*H. distinguenda*	*H. bimaculata*
Frons index	1:5-5.5	1:4-4.5	1:5-6	1:5-6	1:4.5-5
Antenna	reddish-brown	reddish-brown	reddish-brown	brown to reddish-brown	Reddish-brown to blackish-brown
Basal antennal segment	reddish-brown and gray dusted	reddish-brown and black haired	brown, densely grayish dusted and short black haired	densely grayish-black dusted and mostly pale haired	blackish-gray covered and gray dusted and densely black pubescent
Palpus	yellowish-brown, long yellow short black haired	light brown to yellowish-gray, mostly pale haired and some black haired	yellowish-brown to grayish-brown, apical segment clothed with pale and black hairs	grayish brown to brown apical segment stout at base, covered with short black hairs and longer pale ones	grayish-yellow, apical segment stout at base, clothed with mostly short black hairs
Notoplaural lobes	black and short black haired	yellowish-brown	partly brown	brown to blackish	blackish-gray
Wings	saliently brownish colored along costal margin	slightly tinted yellowish along costal margin	slightly brownish clouded along costal margin and in basal cell	distinctly brownish clouded along costal margin	clear costal margin and especially costal cell slightly brownish
Cerci	Slender eliptic shape (Figure 6a)	rectangular with oblique lateral edges (Figure 6b)	conspicuously high and narrow, apically rounded (Figure 6c)	broadly rectangular with rounded lateral edges (Figure 6d)	conspicuously slender, rectangular (Figure 6e)
Subgenital plate	Equal length (Figure 7a)	with a deep excision on lower margin, upper part regularly semiglobular (Figure 7b)	higher than broad (Figure 7c)	straight on lower margin upper part rather broad with only a slight excision above (Figure 7d)	broad, upper margin broadly semiglobular with a small indication of median excision, lower margin straight (Figure 7e)

Description: ♀

Head: Eyes; long yellowish-brown haired with three purple bands. Frons; yellowish-brown dusted, long yellowish and black haired, narrow index 1:5-5.5, widened towards vertex, Lower callus large, blackish-brown, square and connected with median callus. Ocellar tubercule shining dark brown and triangular. Subcallus gray dusted and naked. Face and cheek grayish dusted and long yellowish haired. Vertex short yellow haired. Scape and pedicel segments reddish-brown in ground color. Scape gray dusted and long black haired. Pedicel black and reddish-brown haired. Third segment reddish-brown dorsal tooth not well developed and short minute black haired. Flagellar segments blackish-brown. Apical segment of palpi long and slender, about three, five times as long as deep, yellowish-brown, long yellow and short black haired. First segment of palpi blackish and long yellow haired.

Thorax: Black, mesonotum and scutellum blackish grey dusted with yellowish-grey haired. Pleura black ground color, gray pubescent long yellowish haired. Mesonotum with indefinite grayish longitudinal stripes, short black and yellowish haired. Notopleural lobes black and short black haired. Legs; femur grayish-black and long yellow haired. Femur black, short yellowish haired. Tibia light brown, at posterior black and at anterior yellowish haired. Tarsus dark brown and black haired. Wings; veins, blackish-brown to black, saliently brownish clouded along the costal margin and basal cells. Halters dark brown and knop slightly grayish at the tip.

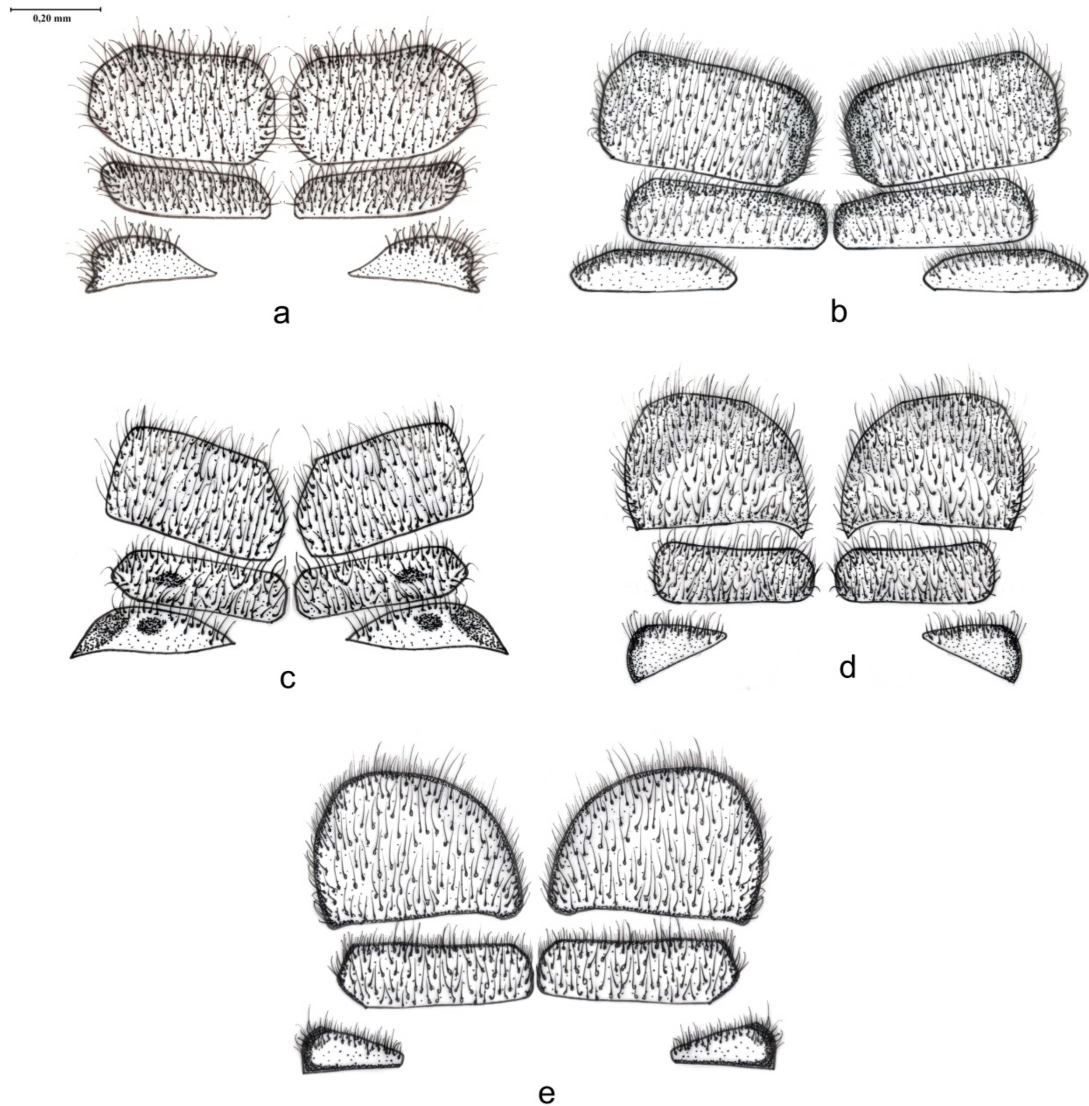

Figure 6. Cerci of species: a) *Hybomitra tanatmisi* sp. nov.; b) *Hybomitra ciureai*; c) *Hybomitra muehlfeldi*; d) *Hybomitra distinguenda*; and e) *Hybomitra bimaculata.*

Abdomen: Black anterior three tergites chestnut brown at sides. Brown side marking distinctly yellow haired. Black median stripes broader, occupying at least third of the tergite on segment 2, slightly with a row of none or least distinct colored and silvery pubescent median triangles. Vertex black, anterior four sternite chestnut brown. Subgenital plate, U-shaped 0,86 mm long and 0,86 mm broad. Apical part of subgenital yellowish-brown. Subgenital densely long and pale brown haired. Subgenital plate with excision on lower margin and upper part semi globular (Figure 7a). Cerci 0,54 mm long and 0,84 mm broad, yellowish-brown colored and slightly black haired. Cerci rectangular with oblique sides (Figure 6a). Spermatheca (Figure 9) W-shaped and oblique at tip, three of ejection apparatus are 0.23 mm and with valve shaped in the distal end. Three of them same length and shape. Reservoir elliptic shape and dark brown color with 4/3 part of distal tubes.

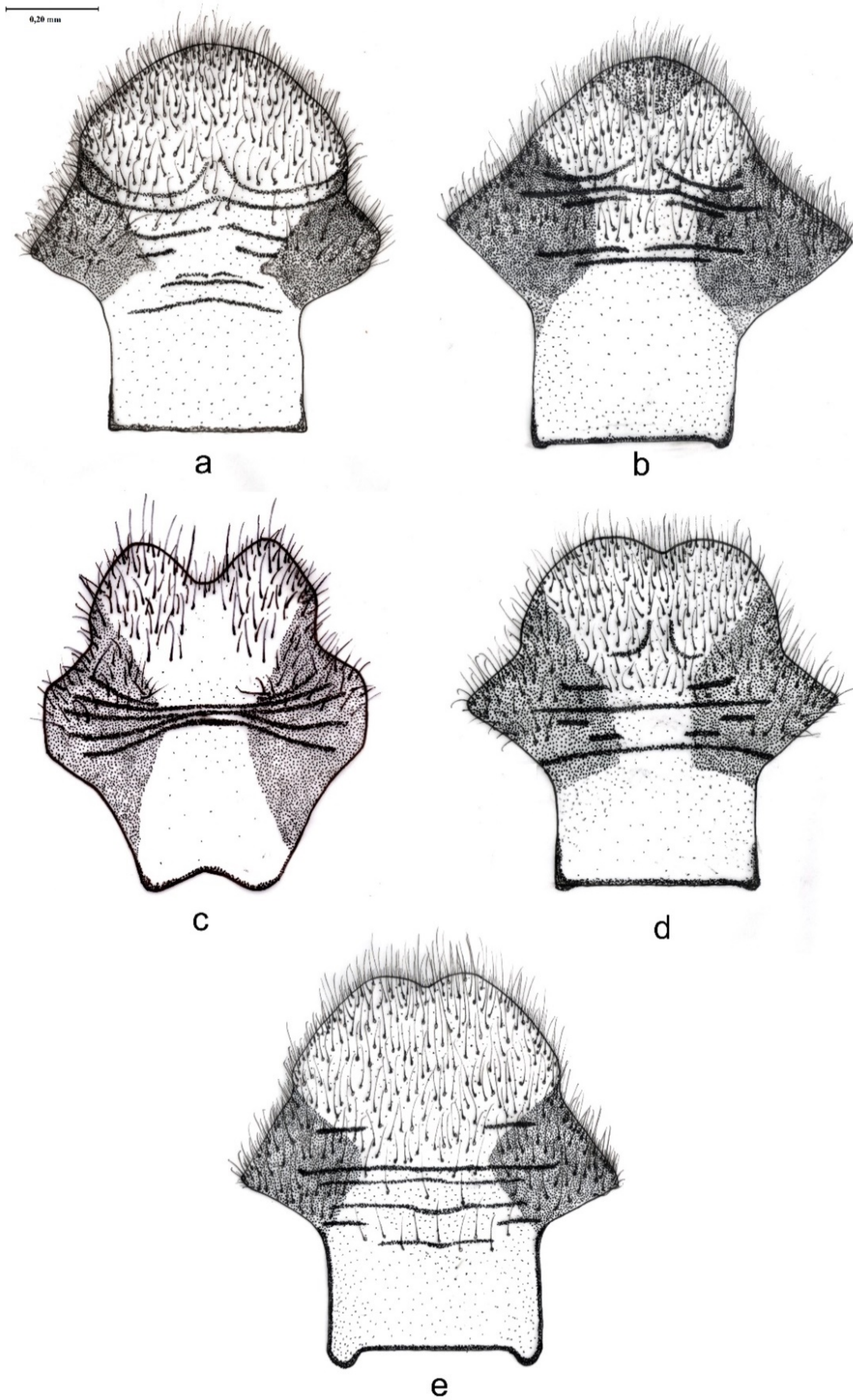

Figure 7. Subgenital plates of species: a) *Hybomitra tanatmisi* sp. nov.; b) *Hybomitra ciureai*; c) *Hybomitra muehlfeldi*; d) *Hybomitra distinguenda*; and e) *Hybomitra bimaculata.*

Variability: This species is not highly variable, but side markings on the abdomen of same samples extensively darkened and blackish-red colored. In contract, the third segment of antennae blackish-brown colored and light gray dusted and dorsal tooth little developed and minute black and dark gray haired. Subgenital plate and cerci are not variable, but coloration and shape of the reservoir of spermatheca is quite variable.

Comments: Generally, species of *Hybomitra* are found between 1100-1900 m altitude near pine and oak forests, but this species was found at 200-500 m altitude in moist pine forests. Probably this species is endemic to Turkey especially for the Middle Black Sea Region. Adults are edaphobiotic, and probably females lay eggs in semi-hydrobiotic habitats in these pine forests. Seasonal activity of the species is extremely short compared to other species of *Hybomitra,* and was from mid-June to the first week of July in 2011. Specimens were collected in the early afternoon.

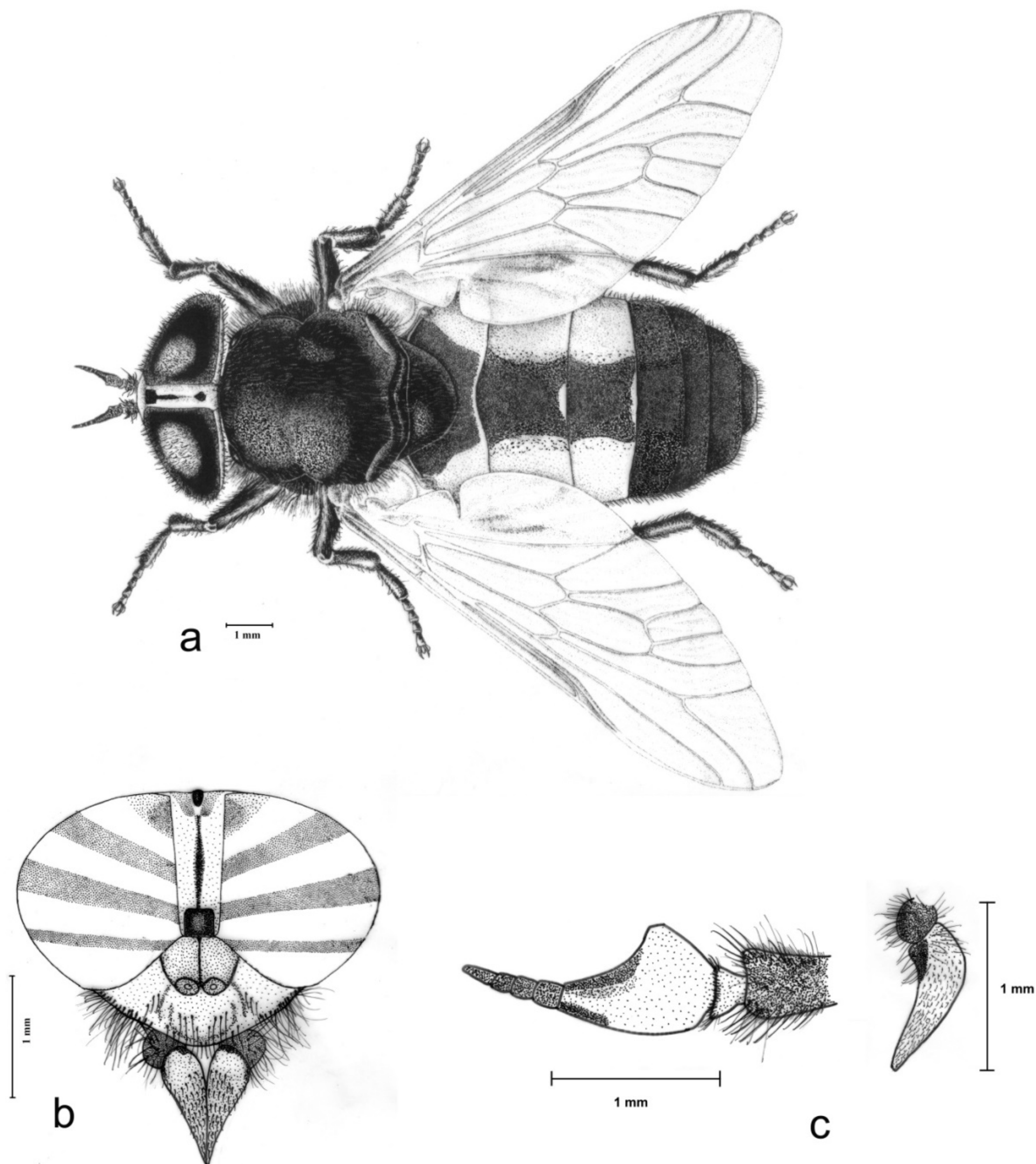

Figure 8. *Hybomitra tanatmisi* sp. nov. ♀: a) dorsal view; b) frons; and c) antenna and palp.

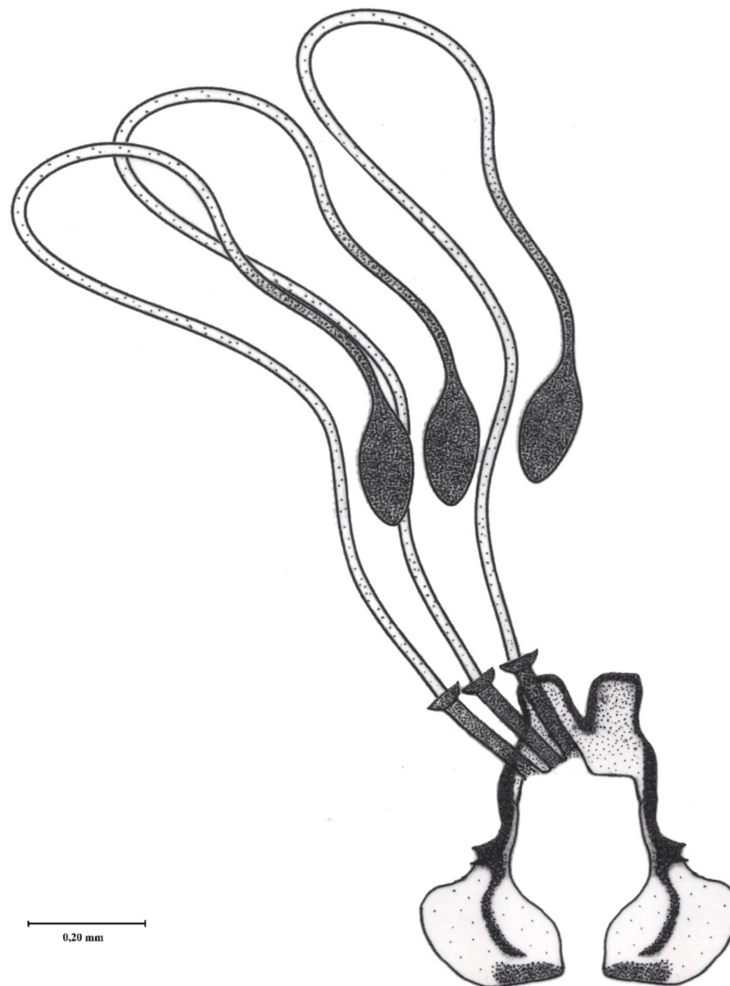

Figure 9. Spermatheca of *Hybomitra tanatmisi* sp. nov.

Turkey has a significant position in terms of biodiversity due to its geographic and climatic features, with three major parts of the Palearctic Region, Mediterranean, Asian and European, meeting in Turkey. Nevertheless, high mountain chains, such as Anatolian Diagonal and Black Sea Mountain lines have blocked entry to Turkey and obstructed distributions of many species. Therefore, *H. arpadi* (Szilady, 1923), *H. montana* (Meigen, 1820) and *H. morgani* (Figures 1, 3 & 4) have been determined in the northern part of the Eastern Black Sea Mountains. According to the data obtained, these species cannot reach beyond the inner part of the mountain ranges of Turkey, they can only spread amongst the northern side of these mountain ranges at 3000-4000 m altitude. Therefore, distribution of this species is limited to the eastern side of Black Sea Region of Turkey. Whereas, *H. aterrima* (Figure 2) was found to the North of Middle and Eastern Black Sea Mountain chains, and *H. tanatmisi* sp. nov. was only found in moist pine forests at 200-500 m altitude in the Middle Black Sea Region.

Especially these three species, *H. montana, H. morgani, H. arpadi,* cannot pass from the north to the south side of the Black Sea Mountains because of altitude and habitat structure where species has been found. The northern side of the Black Sea Mountains has a high precipitation rate but the southern side is colder and drier. These three species need wetland habitats to complete larval development which are always available on the north side of the Black Sea Mountains. Adults can find appropriate places to deposit eggs in forests which are full of wetland habitats, such as rivers and lakes. Whereas, the ecological and environmental conditions on the south side are generally not suitable for this species.

Descriptions of two new species, one new subspecies and eight new records for Turkey have been published (Timmer, 1984; Andreeva et al., 2009; Altunsoy & Kılıç, 2010; Kılıç et al., 2014). Nevertheless, faunal complex and distributions of horsefly species in Turkey are not completely known. The comparative diversity of the Turkish Tabanidae to that of neighboring countries leads us to conclude that the actual diversity of this family in Turkey could be expected to be higher than that currently known. Therefore, we expect that further records for this family in Turkey will made in the future.

From this study, four species have been added to Turkish horsefly fauna, and one new species described. Therefore, the Tabanidae now consists of 176 species and 15 subspecies in Turkey. However, taking into account all previous literature, the fauna of Tabanidae of the Central Turkey region is still inadequately known.

Acknowledgments

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Original article (Orijinal araştırma)

Spermathecae morphology of some Tephritinae (Diptera: Tephritidae) species: A scanning electron microscope study[1]

Bazı Tephritinae (Diptera: Tephritidae) türlerinin spermateka morfolojisi: Bir taramalı elektron mikroskop çalışması

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Abstract

Spermathecal structure of five species [*Campiglossa producta* (Loew, 1844), *Campiglossa tessellata* (Loew, 1844), *Euaresta bullans* (Wiedemann, 1830), *Tephritis formosa* (Loew, 1844), *Tephritis nigricauda* (Loew, 1856)] from subfamily Tephritinae were examined using light and scanning electron microscopy. The specimens were collected between 1999 and 2013 from various provinces of Turkey. Spermathecae were coated in gold/palladium with a Emitech SC 7620 Sputter Coater and examined with a Jeol 6390 LV scanning electron microscope (SEM) operated at 10 kV. Spermathecal structures were characterized as spermathecal bulb, pumping region and spermathecal channel. Descriptions of the spermathecal structures, size of spermathecal bulb, aspect ratio of spermathecal bulb and SEM micrographs of spermathecae are presented for each species.

Keywords: SEM, spermathecae, Tephritidae, Tephritinae

Öz

Tephritinae altfamilyasına ait beş türün [*Campiglossa producta* (Loew, 1844), *Campiglossa tessellata* (Loew, 1844), *Euaresta bullans* (Wiedemann, 1830), *Tephritis formosa* (Loew, 1844), *Tephritis nigricauda* (Loew, 1856)] spermateka yapıları ışık ve taramalı elektron mikroskopu (SEM) kullanılarak incelenmiştir. Türler 1999 ve 2013 yılları arasında Türkiye'nin çeşitli illerinden toplanmıştır. Spermateka örneklerine Emitech SC 7620 Sputter Coater ile altın/paladyum kaplaması yapılarak Jeol 6390 LV SEM ile 10 kV' da incelendi. Spermateka yapıları spermatekal bulb, pompalama bölgesi ve spermateka kanalı olarak karakterize edilmiştir. Makalede, her bir türün spermateka yapılarının tanımlamaları, spermatekal bulbun boyutları ve en-boy oranı ve SEM mikrografları sunulmuştur.

Anahtar sözcükler: SEM, spermateka, Tephritidae, Tephritinae

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Introduction

Worldwide, fruit flies (Diptera: Tephritidae) include 4792 species in 497 genera and many additional taxa are still to be discovered (Freidberg, 2006; Pape et al., 2011). Tephritinae is the most specialized subfamily of the Tephritidae. Predominantly, the larvae of Tephritinae infest the inflorescences of the Asteraceae, the largest, and the most advanced and widespread angiosperm family. With few exceptions, the tephritines are small to medium-sized flies, often with whitish, thickened post ocular setae, dark wing pattern with hyaline spots, oval epandrium and two spermathecae (Korneyev, 1999).

Sperm storage organs allow females to temporally separate insemination from fertilization, manipulate ejaculates and control fertilization. In the reproductive tract of female fruit flies, sperm are found in two different organs a pair or triplet of spermathecae, and a fertilization chamber (Twig & Yuval, 2005).

Generally, insects have multiple sperm storage organs and it has been suggested these structures provide a means for females to influence paternity by differential filling or emptying to favor sperm of one male over another (Hellriegel & Ward, 1998; Pitnick et al., 1999; Hellriegel & Bernasconi, 2000; Fritz & Turner, 2002).

For a long time, researchers have tried to understand the reproductive structures and reproductive systems of insects due to their important role in insect biodiversity and evolution. In this study, we aimed to describe surface morphology of the spermathecal structures of five species in two tribes (Tephritini and Euarestini) of the subfamily Tephritinae. Spermathecae were examined in scanning electron microscopy (SEM) micrographs. Spermathecal structures, aspect ratio of spermathecal bulb and spermathecal duct were defined. Also, the similarities and differences between these species were considered.

Material and Methods

Specimens (Table 1) from Entomology Museum of Gaziantep University that had been collected between 1999 and 2013 from different regions of Turkey were examined. The specimens were boiled for 30-35 min in 10% KOH and dissected to obtain spermathecae for examination under a light microscope (Olympus SZX12, Tokyo, Japan). These spermathecae were cleaned in 96% alcohol and stored in glycerin. The preparation of the specimens followed Candan & Erbey (2006).

Table 1. Taxa, collection locations and number of specimens examined

Species	Collection location	Specimens
Campiglossa producta (Loew, 1844)	Demirtaş, Alanya, Antalya, 36º26' N, 32º12' E, 80 m, 16.V.1999	3 ♀♀
Campiglossa tessellata (Loew, 1844)	Besni, Adıyaman, 37º42' N, 38º00' E, 687 m, 07.VI.2009	2 ♀♀
Euaresta bullans (Wiedemann, 1830)	Boyalı, Eğirdir, Isparta, 38º03' N, 30º50' E, 950 m, 12.V.2001	4 ♀♀
Tephritis formosa (Loew, 1844)	Sarız, Kayseri, 38º27' N, 36º28' E, 1610 m, 08.VII.2005	5 ♀♀
Tephritis nigricauda (Loew, 1856)	Güzelyurt, Aksaray, 38º15' N, 34º25' E, 1789 m, 27.V.2013	4 ♀♀

For SEM observation, spermathecal structures were dried with air for about 10 min then placed on SEM stubs. These samples were coated in gold/palladium with an Emitech SC7620 Sputter Coater (Quorum Technologies Ltd, Laughton, East Sussex, UK) and examined with a Jeol 6390LV SEM (Joel Ltd, Tokyo, Japan) operated at 10 kV, at Gaziantep University Entomology Laboratory and Electron Microscopy Unit.

The spermathecal terminology used follows that of Mcalpine (1981). The spermathecae consists of spermathecal bulb, valve, pumping region and spermathecal duct. In addition, during the designation process the aspect ratio was determined as it is having diagnostic value.

Results

Spermathecal structures of five species in three genera of Tephritinae were photographed using SEM and compared. Characteristic features of spermathecal morphology, surface of spermathecal bulb, glands and pores on the bulb, spermathecal channel and valves were identified for each species. Width, length and aspect ratio are given in the Table 2.

Table 2. Width, length and aspect ratio of spermathecal bulb

Species	Spermathecal bulb		
	Width (µm)	Length (µm)	Aspect ratio
Campiglossa producta	77.80	124.83	0.62
Campiglossa tessellata	55.48	90.70	0.61
Euaresta bullans	58.03	130.81	0.44
Tephritis formosa	63.64	330.42	0.19
Tephritis nigricauda	46.55	181.25	0.25

Campiglossa producta (Loew, 1844)

Spermathecal structure consists of three parts; spermathecal bulb, spermathecal channel and pumping region (valve). Spermathecal bulb papillose, intense papillose shape and formed like pyriform. In addition, spermathecal channel is formed thin and long. Size of spermathecal bulb is 77.80/124.83 (width/length, µm); Aspect ratio of spermathecal bulb is 0.62. Glands located on spermathecal surface one by one or clustered. Spermathecal channel is cylindrical and consist many lateral muscle fibers (Figure 1).

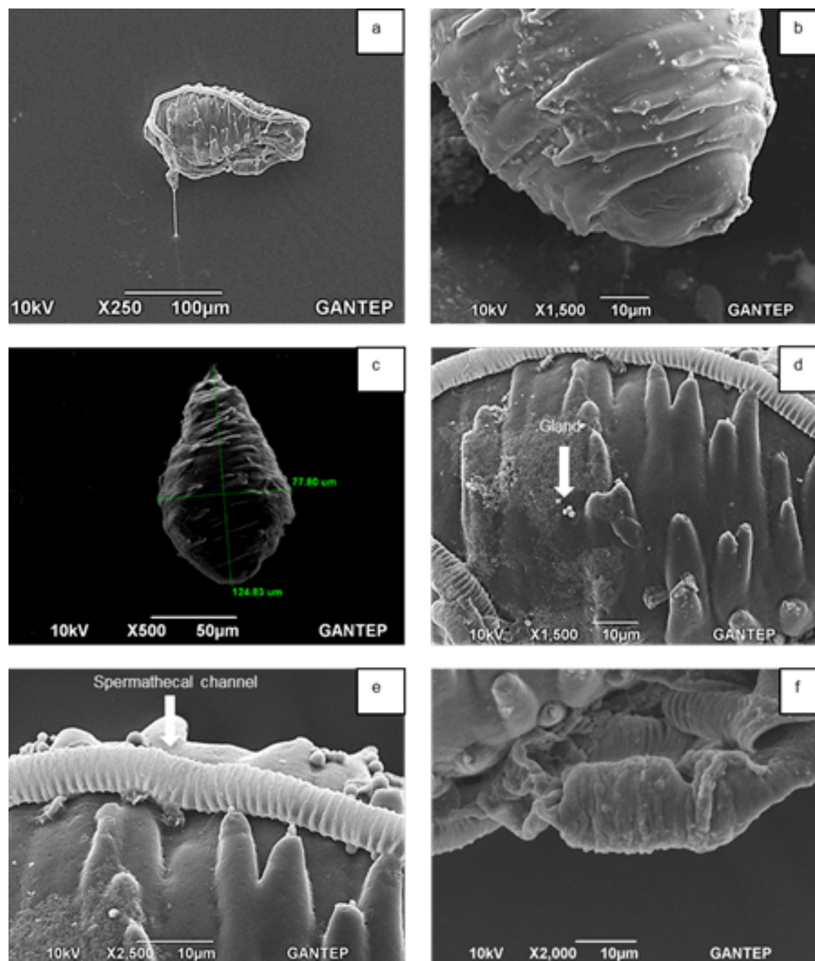

Figure 1. SEM micrographs of the spermathecae of *Campiglossa producta*: a) SEM photo of spermathecal bulb and distal flange of pump; b) apical part of spermathecal bulb; c) length/width of spermathecal bulb; d) spermathecal gland; e) spermathecal channel; and f) pumping region.

Campiglossa tessellata (Loew, 1844)

Spermathecae consists of three parts; spermathecal bulb, spermathecal channel and pumping region (valve). Spermathecal bulb generally seems oval and size is 55.48/90.70 (width/length, µm) and aspect ratio is 0.61. Spermathecal bulb has thin papillose structure. Apex of spermathecal bulb is flat not papillose. Spermathecal channel distinct and consist many lateral muscle fibers and turbinated (Figure 2).

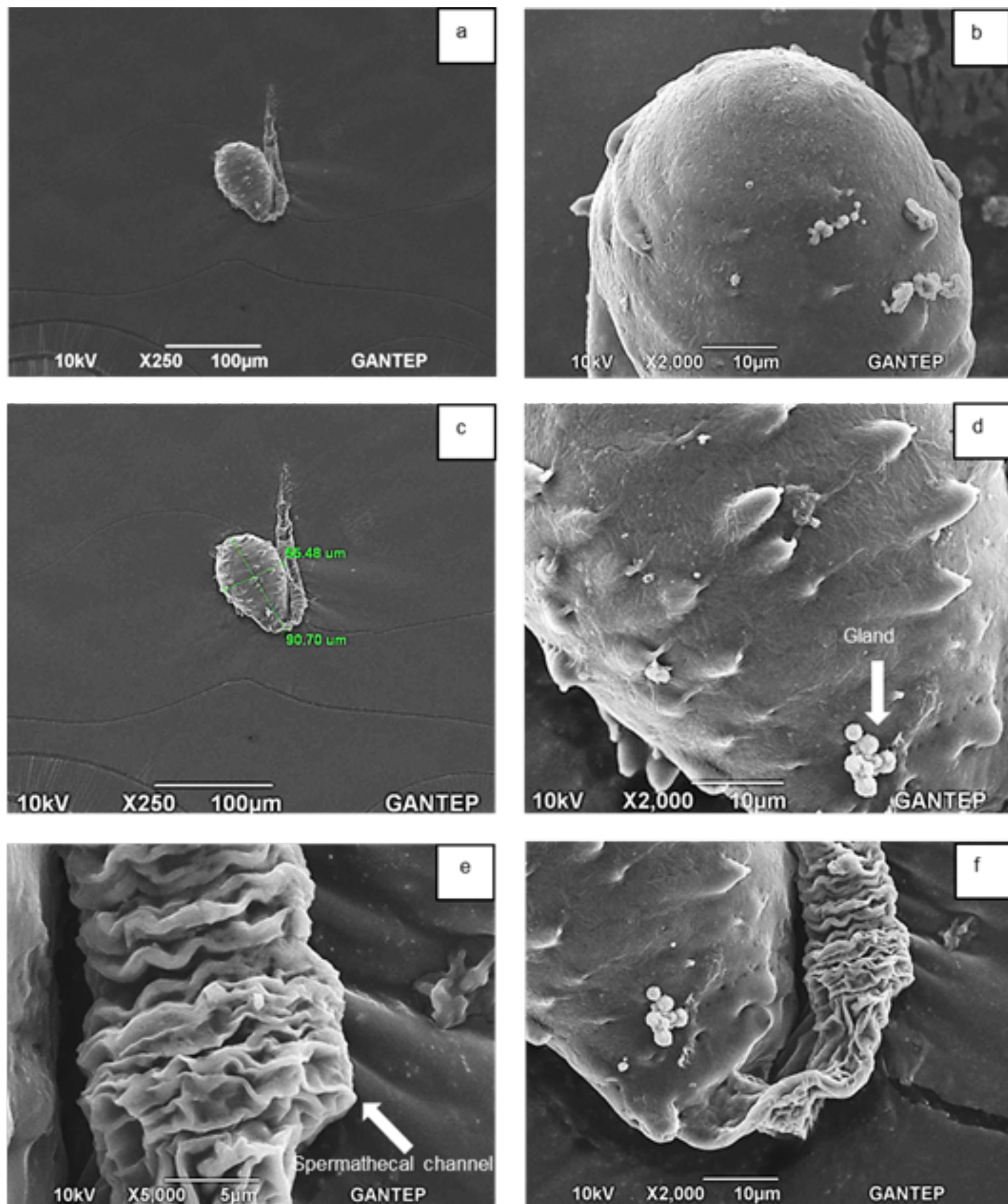

Figure 2. SEM micrographs of the spermathecae of *Campiglossa tessellata*: a) SEM photo of spermathecal bulb and distal flange of pump; b) apical part of spermathecal bulb; c) length/width of spermathecal bulb; d) spermathecal gland; e) spermathecal channel; and f) pumping region.

Euaresta bullans (Wiedemann, 1830)

Spermathecae consist of three parts; spermathecal bulb, pumping region (valve) and spermathecal channel. Spermathecal bulb is regular from basal to apical and formed as a saccate. Size of spermathecal bulb is 58.03/130.81 (width/length, µm) and aspect ratio is 0.44. Surface morphology of spermathecal bulb is dense papillose form and it has rare ducts. Papillose structure is spiral form. Spermathecal channel distinct and consist many lateral muscle fibers (Figure 3).

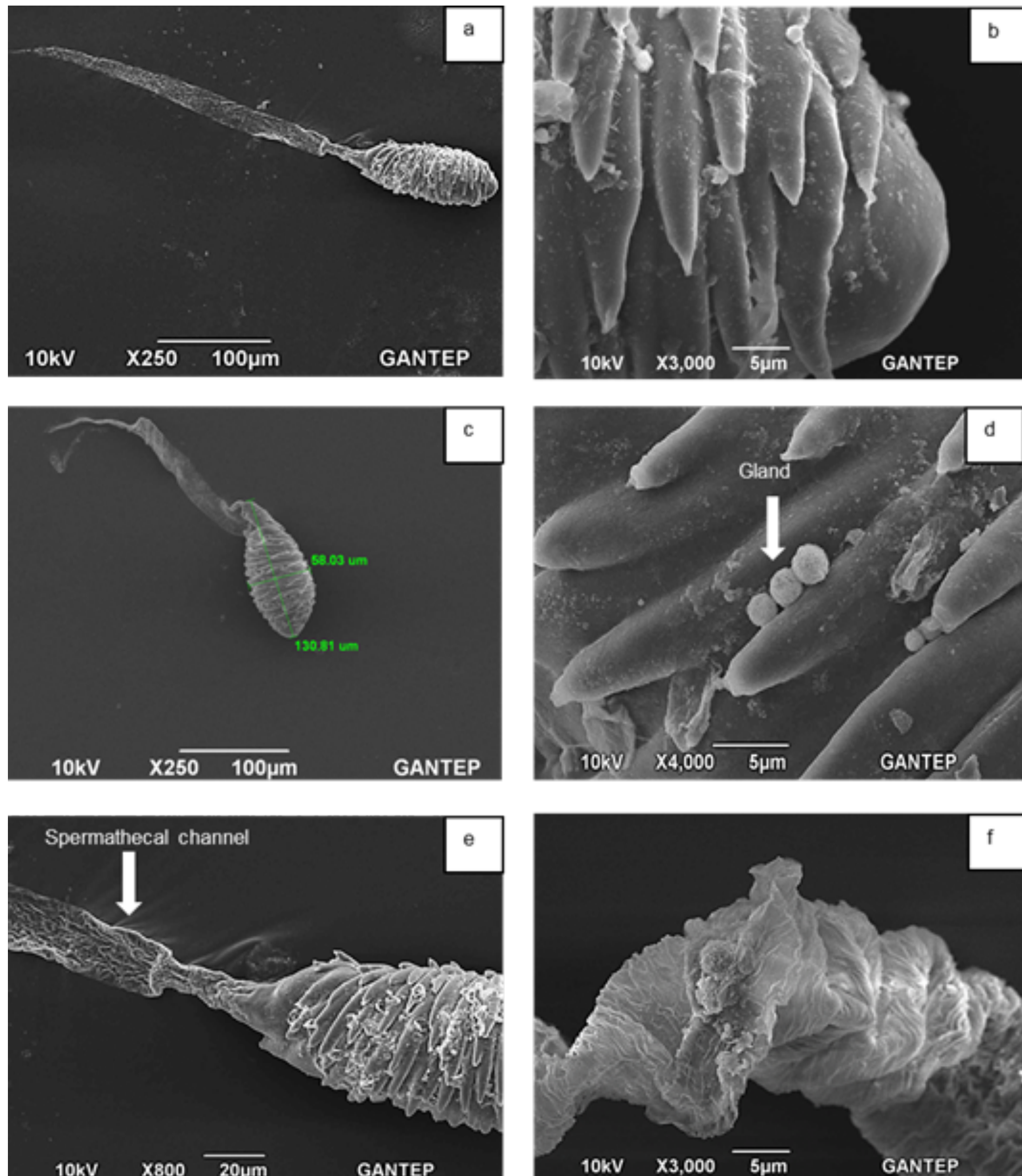

Figure 3. SEM micrographs of the spermathecae of *Euaresta bullans*: a) SEM photo of spermathecal bulb and distal flange of pump; b) apical part of spermathecal bulb; c) length/width of spermathecal bulb; d) spermathecal gland; e) spermathecal channel; and f) pumping region.

Tephritis formosa (Loew, 1844)

Spermathecae consists of three parts; spermathecal bulb, pumping region (valve) and spermathecal channel. Spermathecal bulb formed as corncob. Apical part of spermathecal bulb is oval, middle part is swollen and basal part is formed in a J-shape and connected to spermathecal channel. Size of spermathecal bulb is 63.64/330.42 (width/length, µm) and aspect ratio is 0.61. Spermathecal bulb consists dense papillose and glands clustered (Figure 4).

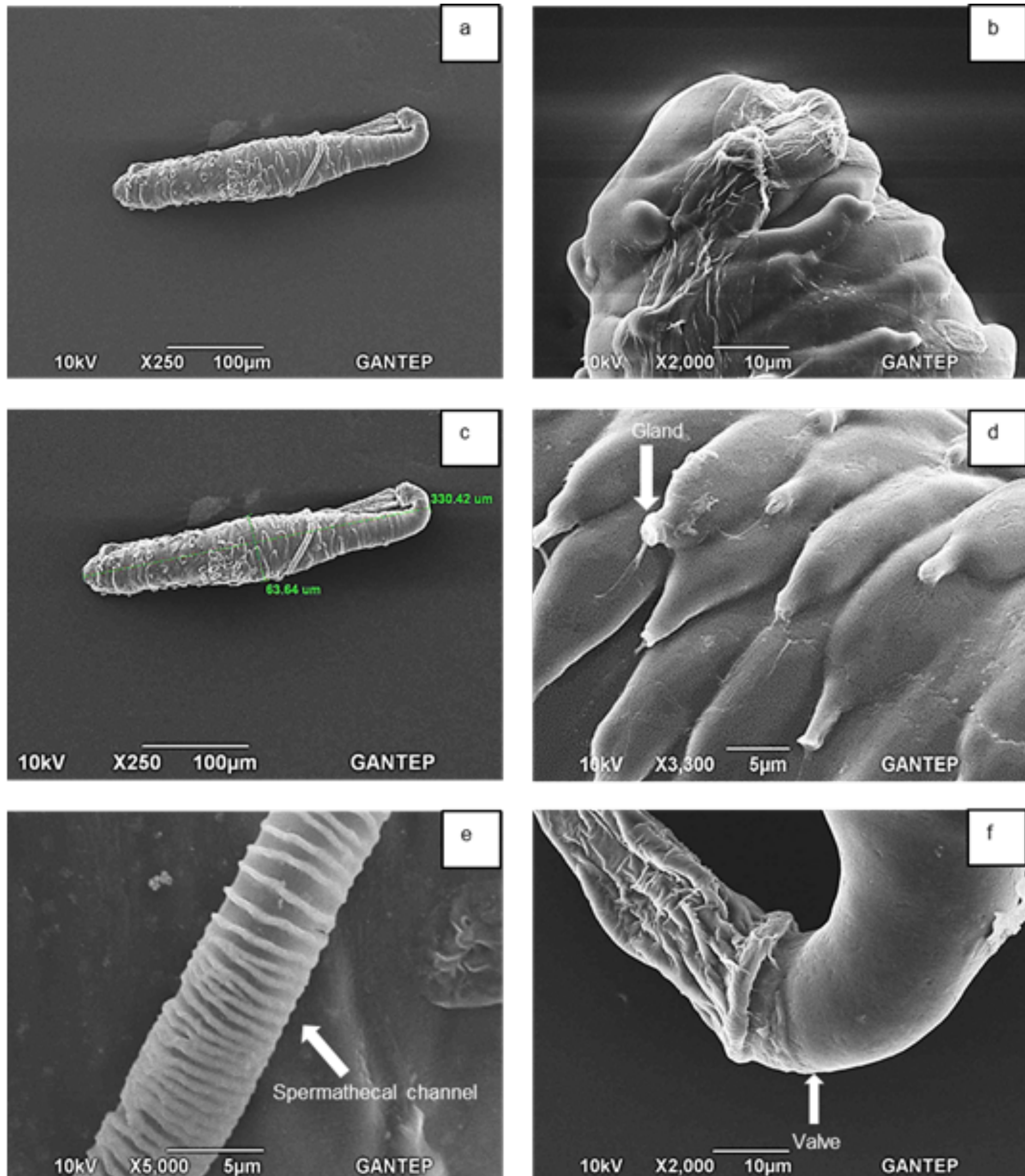

Figure 4. SEM micrographs of the spermathecae of *Tephritis formosa*: a) SEM photo of spermathecal bulb and distal flange of pump; b) apical part of spermathecal bulb; c) length/width of spermathecal bulb; d) spermathecal gland; e) spermathecal channel; and f) pumping region.

Tephritis nigricauda (Loew, 1856)

Spermathecae consist of three parts; spermathecal bulb, pumping region (valve) and spermathecal channel. Spermathecal bulb formed as corncob, there is a stenosis in its central parts towards base from ends, and it becomes thicken and then thinner towards base. General size of spermathecal bulb is 46.55/181.25 (width/length, µm) and aspect ratio 0.25. Gland canaliculus and glands extend outward from pores taking part at the end of digitate bulges. Spermathecal bulb formed in a J-shape at its base and connected to channel. Muscle fibrils in the spermathecal channel are distinct and fibrous in structure (Figure 5).

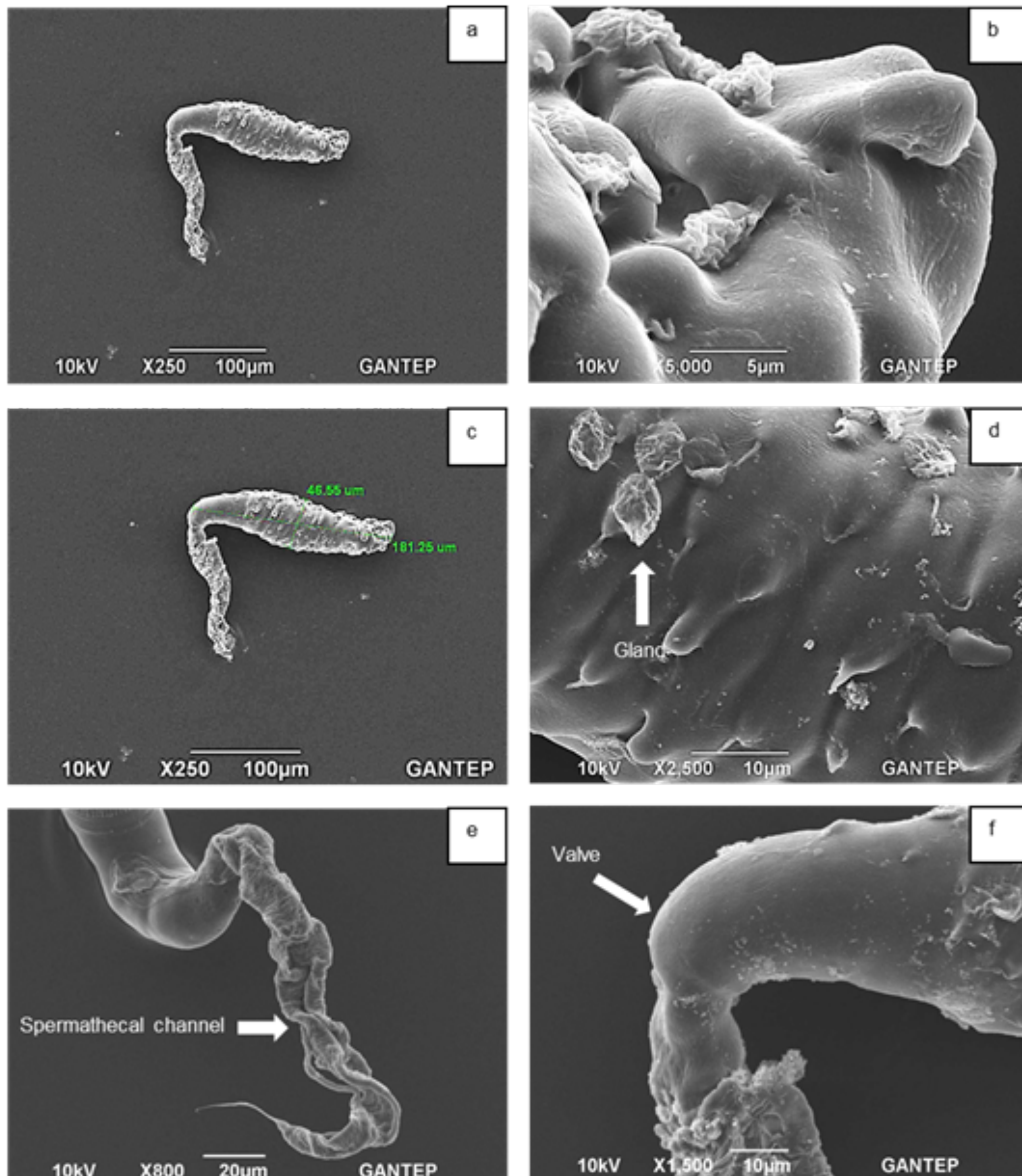

Figure 5. SEM micrographs of the spermathecae of *Tephritis nigricauda*: a) SEM photo of spermathecal bulb and distal flange of pump; b) apical part of spermathecal bulb; c) length/width of spermathecal bulb; d) spermathecal gland; e) spermathecal channel; and f) pumping region.

Discussion

Studies on the morphology of the spermathecae have increased in recent years, however, there are few studies on the spermathecal morphology of fruit flies. Knowledge of spermathecal morphology may be useful to elucidate taxonomic and phylogenetic relationships between genera of Tephritinae.

Spermathecae structures of five species were identified using by SEM micrographs. The spermathecal bulb of *Campiglossa* and *Euaresta* are pyriform and differ from *Tephritis* with this feature. In the two *Tephritis* spp., the spermathecal bulb resembles a corncob in appearance. *Euaresta bullans* clearly differs from *C. producta* and *C. tessellata* with its long papillose shape. Also, the aspect ratio of *E. bullans* was determined to be 0.44 (Table 1). In *C. producta* and *C. tessellata,* the aspect ratio was determined to be 0.61 and 0.62, respectively (Table 1). The spermathecal bulb of *C. producta* is longer than that of *C. tessellata*, being 124.83 and 90.70 µm, respectively. *Campiglossa producta* clearly differs from *C. tessellata* with intense papillose shape. In *C. tessellata*, papillose rare and short. The spermathecal bulb of *Tephritis formosa* has intense and long papillose shape, and *T. nigricauda* has short and rare papillose shape. Also, aspect ratio of spermathecal bulb was determined to be 0.19 and 0.25 in *T. formosa* and *T. nigricauda,* respectively. The spermathecal bulb of *T. formosa* is longer than that of *T. nigricauda* being 330.42 and 181.25 µm, respectively (Table 1).

Based on these observations, it is evident that spermathecae morphology is useful for the identification of species and genera of Tephritinae; especially the size of spermathecal bulb for species and the aspect ratio of the spermathecal bulb for genera. Therefore, the findings of this study make a significant contribution by demonstrating characters that can be used to distinguish similar species and genera.

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Original article (Orijinal araştırma)

Reproductive performance and mating behavior of the predatory bug, *Anthocoris minki* Dohrn, 1860 (Hemiptera: Anthocoridae)

Predatör böcek *Anthocoris minki* Dohrn (Heteroptera: Anthocoridae)'nin üreme performansı ve çiftleşme davranışları

Abdalla E. T. ADAM[1*] **Ertan YANIK[2]** **Denis Tange ACHIRI[3]**

Abstract

Anthocoris minki Dohrn, 1860 (Hemiptera: Anthocoridae) is a potential predator for biological control of the pistachio psyllid, *Agonoscena pistaciae* Burckhardt & Lauterer, 1989 (Hemiptera: Psyllidae) in pistachio orchards. This study aimed to examine the impact of mating behavior on the reproductive output of *A. minki*. The study was conducted under the laboratory conditions in 2015 at Harran University. Males and females of *A. minki* used in the experiments were obtained from laboratory-reared population and female mating behavior and fertilization capacity of males were investigated. In the multiple mating, matings made after the first copulation had an adverse effect on egg deposition. Mated females deposited significantly fewer eggs in the presence of males than in their absence. Male fertilization capability was evaluated by enabling a single virgin male to mate sequentially with three virgin females in different intervals between mating events. Regardless of the length of interval between mating events, the number of eggs laid by the first-mated female was significantly greater than the number of eggs laid by the second- or third-mated females. The study suggests that achievement of economic and large-scale rearing of *A. minki* can be done if the post-mating interactions that negatively influence the female reproduction and longevity are understood and handled appropriately.

Keywords: *Anthocoris minki*, mating, reproductive performance

Öz

Anthocoris minki Dohrn, 1860 (Heteroptera: Anthocoridae), antepfıstığı bahçelerinde yaygın olan antepfıstığı psillidi *Agonoscena pistaciae* Burckhardt & Lauterer, 1989 (Hemiptera: Psyllidae)'nin biyolojik mücadelesinde potensiyel bir predatördür. Bu çalışmada, *A. minki*'nin çiftleşme davranışlarının, üremesi üzerine olan etkisinin araştırılması amaçlanmıştır. Çalışma, 2015 yılında Harran Üniversitesi'nde laboratuvar koşullarında yürütülmüştür. Denemelerde kullanılan *A. minki*'nin erkek ve dişi bireyleri, laboratuvarda üretilen popülasyondan elde edilmiştir ve dişilerin çiftleşme davranışları ve erkeklerin dölleme kapasitesi incelenmiştir. Çoklu çiftleşme denemelerinde, ilk çiftleşmeden sonra olan çiftleşmelerin yumurta bırakma üzerine olumsuz bir etki gösterdiği görülmüştür. Çiftleşmiş dişilerin erkek varlığında bıraktığı yumurta sayısı, erkek yokluğuna göre daha az olduğu saptanmıştır. Erkeklerin dölleme kapasitesini değerlendirmek amacıyla çiftleşmeler arasında farklı aralıklarla ilk kez çiftleşecek erkekler, sırayla hiç çiftleşmemiş üç dişi ile çiftleştirilmiştir. Çiftleşmeler arasında verilen süreye bakılmaksızın, ilk çiftleşen dişi tarafından bırakılan toplam yumurta sayısı, ikinci veya üçüncü çiftleşen dişi tarafından bırakılan yumurta sayısından daha fazla olduğu belirlenmiştir. Çalışma, *A. minki* dişilerinin üremesini ve yaşam süresini olumsuz etkileyen çiftleşme sonrası etkileşimlerinin anlaşılıp, uygun bir şekilde yönetildiğinde bu böceklerin ekonomik ve geniş çapta üretilmesinin başarılabileceği önermektedir.

Anahtar sözcükler: *Anthocoris minki*, çiftleşme, üreme performansı

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Introduction

In simple terms, the biological control of insects is an essential component of any integrated management program as an ecosystem-based strategy focuses on long-term prevention of insects or their damage. Successful biological control significantly depends on the natural enemies and their ability to adapt to the habitat and to their hosts. Good understanding of the factors affecting the ability of biological control agents to rapidly increase their own populations in the ecosystem is also considered an important step. Post-mating interactions, such as multiple mating and male annoyance, can have negative effects. In addition, this kind of interactions may also markedly decrease the ability to mass rear of biological control agents (Hardy et al., 2005; Li et al., 2014). There are many species of bugs that have been used as natural enemies in different regions, and their feeding habits, population establishment and interaction with other predators have been studied intensively (Tavella et al., 2000). *Anthocoris* are an insect group of natural enemies which are widely used as biological control agents of pests, including psyllids, thrips, mites and aphids (Lattin, 1999). The predatory insects, *Anthocoris nemoralis* (Fabricius, 1794) and *Anthocoris nemorum* (Linnaeus, 1761), have an important role in biological control programs, and are commercially produced because of their potential to be used in the management of common pistachio psyllids (Sigsgaard et al., 2006). The predatory bug, *Anthocoris minki* Dohrn, 1860, is another important species used to manage the common pistachio psyllid, *Agonoscena pistaciae* Burckhardt & Lauterer, 1989, which is considered a serious pest causing severe yield loss in pistachio production in Asia, the Middle East and Mediterranean regions, including Turkey (Burckhardt & Lauterer, 1993; Mart et al., 1995; Lauterer et al., 1998). In a field study conducted in pistachio growing areas in the Southeastern Anatolia Region of Turkey, *A. minki* was reported as a beneficial insect (Bolu, 2002). Another field study conducted by (Yanik & Unlu, 2015), where *A. minki* was released to control the pistachio psyllid in pistachio orchards in Şanlıurfa, Turkey and was found to give a remarkable reduction (82%) in the population density of *A. pistaciae*. Consequently, *A. minki* is considered an effective native natural enemy for biological control of this insect pest. The chemical control of common pistachio psyllid is problematic because of decreased effectiveness of the pesticides used, and some studies conducted in Iran have shown the existence of pistachio psyllids resistant to many insecticides (Mehrnejad, 2001; Alizadeh et al., 2011). In Turkey, there have been no investigations on the development of insecticide resistance in *A. pistaciae*. Therefore, gaining a comprehensive understanding of what can affect the mating and reproduction behavior of *A. minki* may help in assessing their effectiveness in both natural and agricultural ecosystems. This study aims to characterize the mating, reproduction and the intersexual interactions of *A. minki* species and thereby offer an opportunity to address specific questions related to male and female reproduction biology: How many copulations can successfully occur per unit time? Does the mating status (mated or unmated female) have a bearing on male acceptance and female longevity? Can male density and copulation duration affect egg lay? The results may also highlight many ways in which we could make breakthroughs the cost-effectiveness and convenience of mass production systems for *A. minki*.

Material and Methods

Material

A population of predatory insect *A. minki* was cultured under laboratory conditions at 25±1ºC, 60±10% RH and 16:8 h L:D photoperiod, and fed on eggs of Mediterranean flour moth, *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) (Schmidt et al., 1995). Fresh bean pods were added to the culture to provide moisture, supplementary food and an egg laying sites. Fifth instar nymphs were separated into ventilated vials (70 mm) with prey *(E. kuehniella* eggs) and wet cotton sheet composed of two layers until they reached adulthood stage, then they transferred in groups into well ventilated plastic containers. All bugs used in this study were those that had newly reached the adulthood stage (1-2 d for males and 2-3 d for females). Sex separation was done using the well-known sexual dimorphism in the abdominal and antennal characters which is common in Anthocoridae. The bean pods were replaced every 3 d. Eggs laid on the bean pods were checked daily under a microscope and counted. The study was conducted in a controlled-environment chamber at Department of Plant Protection, Faculty of Agriculture, Harran University in 2015.

Oviposition of virgin females

Thirty virgin females of *A. minki* were individually placed in Petri dishes (50 mm). Females were fed on prey eggs. Bean pods were also placed in the Petri dishes to provide oviposition sites and replaced every 3 d. The pods were regularly checked under a microscope to determine if eggs had been laid.

Female tendency to multiple mating

Female tendency to multiple mating was determined by testing the tendency of once mated females to re-mating. In this experiment, four virgin females (2- to 3-d-old) were placed separately in the courtship arena (50-mm Petri dishes) and one virgin male (1- to 2-d-old) was added to each virgin female to achieve the first copulation, and the copulation periods were recorded in minutes. After the first mating, the males were immediately removed with an aspirator without disturbing the mating and the female were left into their experimental arenas. Fresh bean pods and flour moth eggs were provided to females to lay their eggs. To examine the tendency of the mated females to re-mating with other virgin males, except the female in the first arena, other females were given an opportunity to mate again with a virgin male 1 d after the first mating. Seven days later, except the females in the first and second arenas, the rest of the females were allowed to mate for a third time with virgin males. Lastly, only the female in the fourth arena was given a chance to mate for the fourth time; 14 d after the first mating. Therefore, the females in the first to fourth arenas were given the opportunity to mate one to four times, respectively. The lifespan of each female was calculated in days to estimate the effect of the copulation number on female lifespan. In order to compare the longevity of mated and unmated females, a parallel experiment was set up where five adult virgin females were kept individually and the longevity recorded daily. The experiment was laid out in a completely randomized design with ten replicates. Additionally, egg laying rate was assessed for once mated females. Egg number was counted every 5 d, throughout their lifespan. The first count was made 5 d after mating (referred to as day 1-5), the second count was made 10 d after mating (day 6-10), and counts were made nine times. Data were also recorded for the total number of eggs laid per female per day.

Effect of male density on female reproduction

The effect of male density on female reproduction was investigated by placing once mated female with 0, 1, 3 and 5 males in the same experimental arena. In this experiment, five virgin females (2- to 3-d-old) were individually placed in Petri dishes, then one virgin male (1- to 2-d-old) was introduced to each Petri dish. The mating behavior was observed and the copulation duration was recorded. Once the first mating was over, the males were removed and the females each transferred to a new Petri dish (i.e. five new Petri dishes in total) and new moth eggs and bean pods were provided. The effect of male density on once mated female reproduction was evaluated by adding 0, 1, 3 and 5 virgin males into the first to fourth Petri dish, respectively. The once mated female in the fifth Petri dish was given five virgin females; and thus, saved as an experiment control to determine whether the male-female or female-female interactions would affect the reproductive performance. The total number of eggs and egg hatch percentage were recorded over the lifespan of each female. If bugs used in this experiment died, especially males or virgin females, were immediately replaced. The experiment was conducted in a completely randomized design with sixteen replicates.

Male fertilization capacity

In order to examine the mating behavior and fertilization capacity of single males, males (2- to 3-d-old) were given the opportunity to mate with three females sequentially with 0, 1 or 2 d between mating events. In this experiment, three virgin males were individually placed in Petri dishes and the male in the first arena was allowed to mate with three virgin females, sequentially within the same day; the male in the second arena was allowed to sequentially mate with three virgin females, with 1-d interval between the mating sessions; and the male in the third arena was allowed to sequentially mate with three females, with 2-d intervals between the mating. The duration of all copulations was recorded. For each male, the first to third copulation was separated by intervals of 0, 1 or 2 d. At the end of the experiment, all males

were kept in ventilated plastic arenas with prey eggs and their lifespan was recorded daily. As a control, 22 virgin males were kept in separated arenas with food and bean pods, and their lifespan recorded. Concurrently, all mated females were kept in Petri dishes with prey eggs and bean pods, then the total eggs and percentage hatch were recorded. Throughout the experiment, the males that died were substituted by males that newly reached adulthood, so that the number of adult males was consistent throughout the experiment. The experiment was replicated eight times in a completely randomized design.

Statistical analysis

In data analysis, the variance analysis was performed, and the difference between the averages was determined by Duncan's multiple comparison test at $P < 0.05$. Student's t-tests were used to compare the mean differences in longevity between mated and unmated males and females. To explore the relationship of the longevity with egg laying of female, a correlation analysis was performed.

Results

Oviposition by virgin females

During this study, we did not observe any eggs laid by virgin female used in the experiments at any stage during their lifespan. Thus, we conclude that any oviposition event was as a successful mating.

Female tendency to multiple mating

All females (n = 40) in this experiment oviposited within 1-2 d after their first mating. Regardless of the interval between mating events, the successfully mated females showed a clear tendency to refuse any additional copulation. However, the Petri dishes had been shaken hand, therefore the females were forced to have additional mating. A significant reduction in the number of eggs after the second mating was observed with the increasing number of copulations (Table 1). Statistically, the average number of eggs laid by the four-times-mated females was significantly fewer when compared with the eggs laid by once-, twice- or three-times-mated females ($F = 1.53$, $P < 0.05$). The effect of multiple mating on female lifespan was not statistically different ($F = 0.92$, $P > 0.05$).

Table 1. The effect of multiple mating events on egg production and female lifespan

Mating events	Egg number (mean±SEM)*	Lifespan (d) (mean±SEM)
1	102.1±0.69	41.8±0.47 a
2	125.1±0.65 a	43.1±0.62 a
3	99±0.52 b	39.6±0.52 a
4	61±0.33 b	32.8±0.06 a

* Means in the same column labeled with the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).

The total number of eggs laid per female per day was 3-4 eggs during the first 4 weeks of oviposition, after which oviposition dropped markedly (Table 2). Throughout the oviposition period, females lay an average three eggs per day, but on some days, they did not lay any eggs.

Table 2. Oviposition rate throughout the lifespan of once mated female of the predator bug, *Anthocoris minki*

Days after the first mating	Average eggs/female/d (mean±SEM)*
1-5	3.2±0.26 a
6-10	4.0±0.38 a
11-15	4.2±0.36 a
16-20	3.7±0.48 a
21-25	3.5±0.41 a
26-30	3.3±0.50 a
31-35	1.2±0.36 b
36-40	0.4±0.36 c
41-45	0.0±0.00 c

* Means in the same column labeled with the same letter are not significantly different according to Duncan's multiple range test (P < 0.05).

There was a significant difference between the mated and unmated females in term of longevity (t = 2.16, df = 53, P = 0.02). On average, the longevity of virgin females (47.6±2 d, n = 20), was found to be 25% longer than of mated females (37.0±2 d, n = 40) (Figure 1).

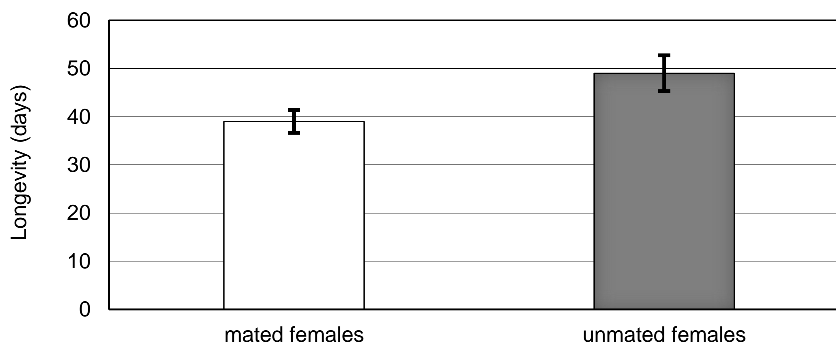

Figure 1. The effect of mating on female lifespan (bars represent the SEM).

Effect of male density on female reproduction

There was a significant steady decrease (F = 40.2, P < 0.05) observed in the number of eggs in parallel with the increasing number of males (Table 3). This may be an indicator of the negative effect of male presence on the egg deposition of mated females. There was no significant difference between the females held alone and those held with one male in the total number of eggs (F = 2.78, P = 0.08). In addition, the total eggs laid by the once mated females held with five virgin females was significantly (F = 7.37, P = 0.02) fewer than those held alone. The eggs laid by the females held with five males was 38% fewer than by the females held with five virgin females. However, the percentage hatch (91.06±1.23%) was not significantly affected by male density. There was no significant effect (F = 2.44, P = 0.09) of male density on the longevity of mated females. In addition, there was no significant difference (P = 0.06) in longevity of females held alone and those held with five males, but there was a significant difference (F= 9.15, P = 0.03) in longevity between females held with three or five males and those held with five virgin females.

Table 3. Effect of the male density on mated female egg production, percentage of hatching eggs and longevity

Male density	Number of eggs per female (mean±SEM)*	Percent hatch (mean±SEM)	Female longevity (d) (mean±SEM)
No males (lone female)	82.6±0.73 a	90.6±0.11 a	41.8±0.02 a
One male	79.6±0.30 a	89.6±0.39 a	39.7±0.88 a
Three males	52.6±0.71 c	90.2±0.24 a	38.3±0.46 a
Five males	44.0±0.99 d	91.4±0.39 a	38.5±0.93 a
Five virgin females with a mated female	71.8±0.38 b	93.2±0.03 a	37.8±0.32 a

* Means in the same column labeled with the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).

Female longevity was positively correlated with the total number of eggs per female ($R^2 = 0.53$, $F = 44.64$, $df = 10$, $P = 0.02$). Egg production ranged from 41 to 120 eggs per female and averaged 90.82±0.75 (mean±SE) (Figure 2).

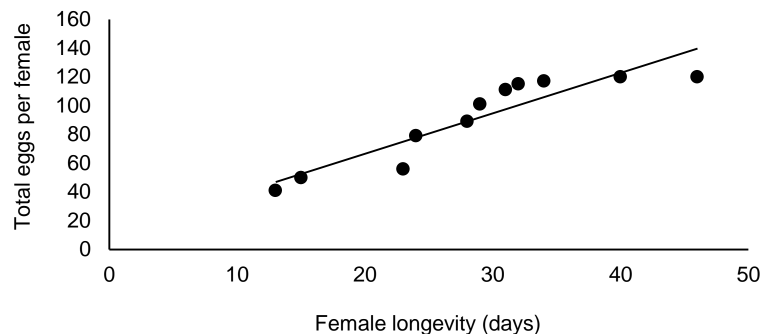

Figure 2. Relationship between longevity and total egg production of once mated *Anthocoris minki* females (data are fit with a linear regression).

Male fertilization capacity

There was no significant difference ($F = 0.63$, $P > 0.05$) between the copulation periods when the same male was sequentially inseminated three different females with 2 d between matings (Table 4). However, when the three mating events were on the same day or separated by 1 d, there was a significant difference ($F = 18.2$, $P < 0.05$ and $F = 4.64$, $P < 0.05$, respectively) in the time spent in the first compared to the second and third matings.

Table 4. Effect of time (0, 1 and 2 d) between mating attempts on the copulation period (same day: one male mated with three females within 1 d; 1-d interval: one male mated with three females by giving 1-d interval between 1st, 2nd and 3rd copulation; 2-d interval: one male mating with three females by giving 2-d interval between 1st, 2nd and 3rd copulation)

Interval (d)	Copulation periods (min)*		
	1st Copulation	2nd Copulation	3rd Copulation
0 (same day)	15.35 aA	6.14 cB	4.13 cB
1	15.16 aA	11.31 bB	8.35 bB
2	16.23 aA	15.30 aA	14.14 aA

* Means labeled with the same letter (lowercase within columns and uppercase within rows) are not significantly different according to Duncan's multiple range test at ($P < 0.05$).

Furthermore, when the time interval between the copulations was 1 d, a steady decrease in copulation duration has been observed among the three mating events (15.16, 11.31 and 8.35 min, respectively). Significantly fewer (P < 0.05) eggs were laid by the third female than that laid by the first or second females (Figure 3). When the three copulations were on the same day, a significant difference (F = 15.8, df = 4, P = 0.02) in the average of eggs laid was observed. Moreover, with 1 d (F = 7.40, df = 4, P < 0.05) or 2 d (F = 9.64, df = 2, P < 0.05) intervals, the third mated female laid significantly fewer eggs.

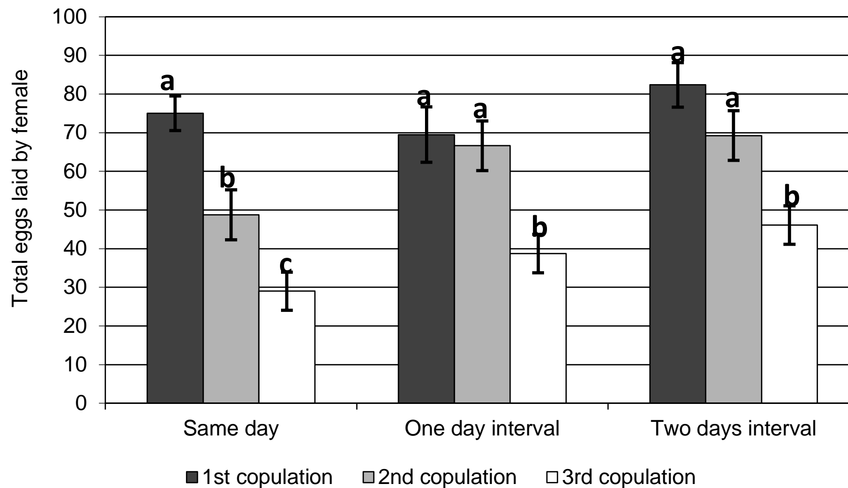

Figure 3. Effect of time (0, 1 and 2 d) between mating attempts on the total number of eggs laid. Bars with the same letter means that there is no significant difference according to Duncan’s multiple range test at (P < 0.05).

The interval between copulations were not observed to have any negative effect on the fertilization capability of *A. minki* males. The number of matings and the interval between the first, second and third mating had no significant effect on the egg hatch rate. No significant differences (P > 0.05) in the longevity of females mated at different intervals were observed (Figure 4). According to the statistical analysis, copulations on the same day (F = 0.69, P > 0.05), or at 1-d interval (F = 0.36, P > 0.05) or 2-d interval (F = 0.12, P > 0.05) had no significant effect on female longevity.

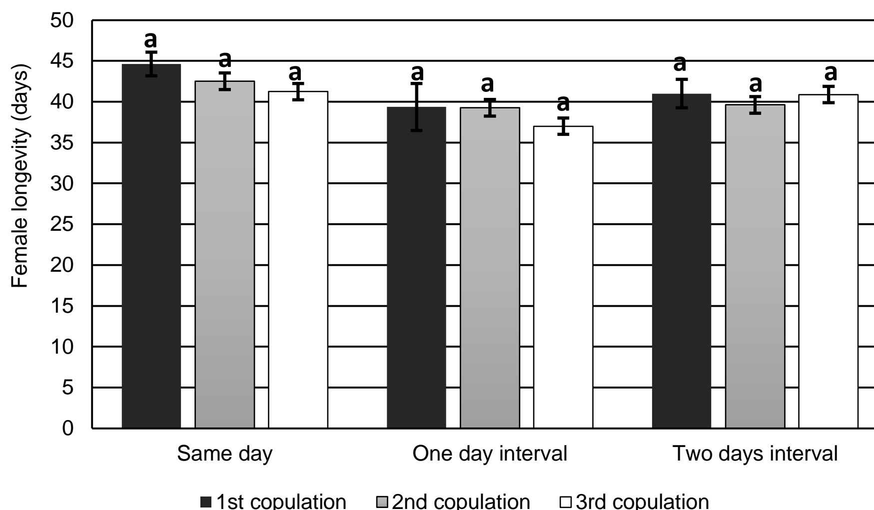

Figure 4. Effect of time (0, 1 and 2 d) between mating attempts on the female longevity (same day: one male mating with three females within 1 d; 1-d interval: one male mating with three females by giving 1-d interval between 1st, 2nd and 3rd copulation; 2-d interval: one male mating with three females by giving 2-d interval between 1st, 2nd and 3rd copulation). Bars with the same letter do not differ significantly according to Duncan’s multiple range test at (P < 0.05).

The average longevity of mated and unmated males was not significantly different (t = 0.69, df = 44, P = 0.69) (Figure 5).

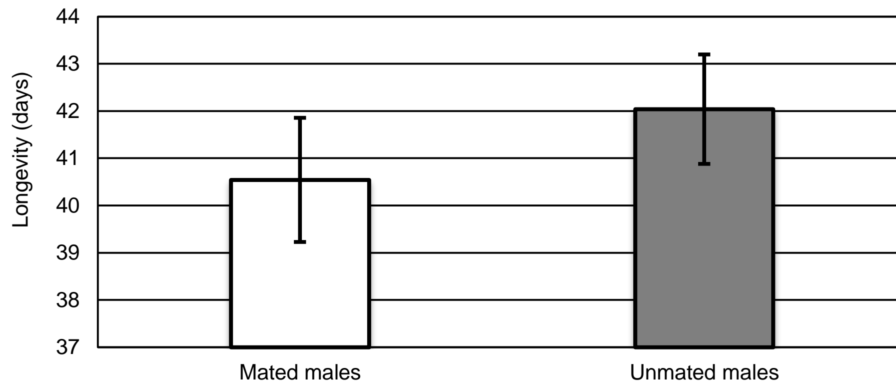

Figure 5. Effect of mating on male lifespan (bars represent the SEM).

Discussion

In this study, females tended to mate only once and most of them avoided the multiple mating regardless of length of the period given between mating events. The females did not lay eggs unless they were mated and once mated, they deposited eggs throughout their lives. This situation occurs because after mating, females retain sperm in their sperm storage tubules and use it to fertilize the eggs over a long period of time (Scudder, 1971; Leon-Beck & Coll, 2009). There are significant differences in longevity between mated and virgin females; this can probably be attributed to energy that has been depleted in the mating or in the egg laying process (Thornhill & Alcock, 1983; Arnqvist & Nilsson, 2000; Simmons & Kotiaho, 2007; Leon-Beck & Coll, 2009). A clear relationship between male density and female reproduction was found. Fewer eggs were deposited by females kept with the larger numbers of males compared to those kept with the smaller numbers of males. Multiple mating appeared to have had a negative effect on egg deposition. It is likely that male density is only influencing the oviposition process, not the viability of eggs, because the male density did not affect egg hatch. In addition, female longevity decreased as male density increase. Egg laying was positively correlated with female lifespan, therefore, the reduction in offspring with increased male density may indicate the impact of male intensity on female lifespan. As reported by (Li et al., 2014), the post-mating intersexual interactions may negatively influence both males and females in terms of reproductive performance. Most common male-female interactions occur in *A. minki* are male annoyance and female reluctance. These interactions clearly reduced female longevity and fecundity. However, multiple mating and post-mating interactions had no effect on male longevity. Males of *A. minki* are generally adapted to multiple mating, since every single male successfully inseminated three females without affecting their lifespan. When three mating events occurred on the same day, the time spent in the second and third mating was less, while this period was similar to when the interval was 1 or 2 d. These results suggest that male fertilization success does not depend on the male resting period as much as it depends on the number of copulations achieved. As reported by Linley & Hinds (1974), Cook (1999), Leon-Beck & Coll (2009) for *Culicoides melleus* (Coq., 1901) (Diptera: Ceratopogonidae), *Plodia interpunctella* (Hubner, 1813) (Lepidoptera; Pyralidae) and *Orius laevigatus* (Fieber, 1860) (Hemiptera: Anthocoridae), the gradual decrease in the sperm is probably the main reason for the gradual decline of the number of eggs laid by subsequent or later mated females as a result of deficient fertilization. There were different responses between males and females in their tendencies towards multiple matings. Females did not all start laying eggs on the same day, nor did they continue laying for the same length of time. Our results suggest that the mated females do not need to be kept in an insect rearing facility for more than 1 month because the number of eggs laid rose from the 10th day and declined after the 30th day, so mass production of *A. minki* can be achieved at low cost and over a short period of time.

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Original article (Orijinal araştırma)

Formulation and physicochemical characterization of neem oil nanoemulsions for control of *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae)

Sitophilus oryzae (L., 1763) (Coleoptera: Curculionidae) ve *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae)'un kontrolünde neem yağının nano emülsiyonlarının ve fizikokimyasal tanımlanması ve formülasyonu

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Abstract

This report describes the development of environmentally-benign nanoemulsion formulations for control of adult *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae). The formulations were emulsions of neem [*Azadirachta indica* A. Juss (Sapindales: Meliaceae)] oil, nonionic surfactant (alkylpolyglucoside or polysorbate 80), and water using low-energy emulsification method. Four formulations were chosen and physicochemical characterization showed a particle size range of 208-507 nm in diameter. Bioassays were conducted using food and filter paper impregnation methods. The formulation comprised of polysorbate surfactant with 2.0 mL/kg azadirachtin caused a 100% mortality of *S. oryzae* adults after only 24 h of exposure, with the food impregnation method. The toxicological studies carried out at Universiti Putra Malaysia toxicology laboratory between November 2014 and December 2015 indicated that *S. oryzae* adults were more susceptible than *T. castaneum* adults to nanoemulsion formulations including 2.0 mL/kg azadirachtin according to median lethal times.

Keywords: Biopesticide, nanoemulsion formulation, neem oil, *Sitophilus oryzae, Tribolium castaneum*

Öz

Bu çalışmada, *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) ve *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) erginlerin mücadelesinde kullanmak için çevre dostu nano emülsiyon formülasyonlarının geliştirilmesini amaçlanmıştır. Emülsiyon formülasyonu, düşük enerjili emülsiyonlaşma yöntemi kullanılarak neem yağından [*Azadirachta indica* A. Juss (Sapindales: Meliacear)], iyonik olmayan yüzey aktif maddeden (alkilpoliglukosid veya polisorbat 80) ve sudan oluşturulmuştur. Dört formülasyon seçilmiş ve fizikokimyasal çapı 208-507 nm olan partikül boyutu aralığı ile karakterize edilmiştir. Biyoassay çalışmaları, besine ve filtre kağıdına emdirme yöntemleri kullanılarak yürütülmüştür. Besine emdirme yöntemi ile, 2.0 mL/kg azadirachtin konsantrasyonlu polisorbat yüzey aktif maddeden oluşan formülasyona sadece 24 saatlik uygulamada *S. oryzae* erginlerinin %100 ölümü gerçekleşmiştir. Kasım 2014 ile Aralık 2015 tarihleri arasında Universiti Putra Malaysia toksikoloji laboratuvarında yürütülen toksikolojik çalışmalar, *S. oryzae* erginlerinin ölüm zamanlarının medyan değerlerine göre 2.0 mL/kg neem yağı içeren nano-emülsiyon formülasyonlarına *T. castaneum* erginlerinden daha duyarlı olduklarını göstermiştir.

Anahtar sözcükler: Biyopestisit, nanoemülsiyon formülasyonu, neem yağı, *Sitophilus oryzae, Tribolium castaneum*

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Introduction

The rice weevil, *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) is a major primary pest, and the red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) is a major secondary pest of stored grain-based products, including maize, rice and wheat, particularly in the tropic regions (Howe, 1965; Gonzalez et al., 2013; Wagan et al., 2016). These pests can cause considerable damage to stored grains and completely destroy kernels. Moreover, the product quality is affected by presence of eggs and dead insects, and holes on the grains (Lu & He, 2010). According to the global economic estimation, the related costs of pests to stored food stuffs could reach to about 500 million USD per year (Domínguez Umpiérrez & Marrero Artabe, 2010).

One of the most common control measures in stored-product insect pest management is chemical control, which is mainly dependent on the use of synthetic insecticides, such as fumigants [mostly phosphine (PH_3) or methyl bromide (MeBr)], residual contact insecticides such as organophosphates and pyrethroids (Kljajic & Peric, 2006; Islam et al., 2010). Since these chemicals are easy-to-use and cost-effective, their intensive and repeated use has resulted in several problems, such as the development of insecticide resistance, effectiveness of the ecosystem and toxic effects on humans and mammals (Bell & Wilson, 1995). Many researchers have been seeking for new alternatives to replace synthetic pesticides such as biological control, control by natural plant products (inert dusts and essential oils), behavioral control using insect pheromones and microbial control, to control insect pests (Mazzonetto & Vendramim, 2003). In recent years, one of the alternatives to control insect pests is to use bio insecticides including natural substances derived from the plants in forms of powders, extracts and oils. The secondary metabolites of these plants, known as botanical pesticides, have repellent, antifeedant, growth inhibition and fumigant effects on insect pests (Weaver et al., 1994).

Neem [*Azadirachta indica* A. Juss (Sapindales: Meliaceae)] oil is a broad spectrum botanical pesticide commonly used for stored grain protection worldwide (Medeiros et al., 2007; Costa et al., 2014; Choupanian et al., 2017). In a recent study, the contact toxicity of neem oil nanoemulsions, were evaluated against *S. oryzae* and *T. castaneum* adults by filter paper impregnation method (Choupanian et al., 2017). In order to understand better the performance of the nanoemulsion formulations when applied by different methods, it is also relevant to study the effect of the product when mixed with the commodity. It is known that insecticidal efficacy of neem oil is affected by its application method (Gahukar, 2014).

Therefore, the aims of the study were to (a) evaluate the insecticidal properties of four neem oil nanoemulsion formulations in management of two common insect pests of stored products, *S. oryzae* and *T. castaneum* adults by two different application methods (filter paper and food impregnation method) and (b) evaluate the median lethal time (LT_{50}) of the nanoemulsion formulations against the target pests.

Material and Methods

Chemicals

The nonionic surfactants, APG (Agnique® MBL 510H), and Polys (Tween 80) were supplied by Cognis Oleochemicals (M) Sdn Bhd (Selangor, Malaysia) and Duchefa Biochemie (Haarlem, The Netherlands), respectively. Neem oil was received from VM Consolidated (M) Sdn Bhd (Seri Kembangan, Malaysia) and Neemix® (EC formulation) was provided from Zeenex AgroScience (M) Sdn Bhd (Kuala Lumpur, Malaysia).

Insects

The initial populations of the rice weevil (*S. oryzae*) and the red flour beetle (*T. castaneum*) were obtained from the Entomology Laboratory of Universiti Putra Malaysia in January 2015. *Sitophilus oryzae* and *T. castaneum* were cultured in clear plastic containers of rice grain and wheat germ, respectively, under laboratory conditions of 27±1ºC, 75±1% RH and 12:12 h L:D photoperiod. Adult insects, 7-14 d old, were used for the experiments.

Preparation and characterization of the neem oil nanoemulsions

Pseudoternary phase behavior study was conducted in previous study in October 2014 (Choupanian et al., 2017). Four formulations were selected from the stable region of the emulsion system based on the features of being optically clear and single-phase, and substantially stable at 25°C for 30 d (Flanangan et al., 2006). The selected formulations were physicochemically characterized with respect to stability (at room temperature for a period of 90 d), thermostability (at 54ºC for a period of 14 d), particle size, and zeta potential analysis in November 2014.

Toxicity of the neem oil nanoemulsions using food impregnation method

The toxicity of the formulated neem oil nanoemulsions against the adults of tested insect species was studied using food impregnation method according to Talukder & Howse (1994) with some modifications in March 2015. The nanoemulsion formulations were diluted to four azadirachtin concentrations (5.0, 6.0, 7.5 and 10.0 mL/kg). One mL of each formulation was loaded into 5 g disinfested rice grain for *S. oryzae* and broken wheat grain for *T. castaneum*, to bring the amount of azadirachtin in the food media to 1.0, 1.2, 1.5 and 2.0 mL/kg, where Neemix® and neem oil alone were used as positive control and distilled water as negative control. The treated food media were air-dried to evaporate the solvent and placed into glass vials. Afterwards, 20 adults of *S. oryzae* and *T. castaneum* were released into rice kernel and broken wheat grain, respectively. The experiment was repeated five times, and adult mortality was recorded after 24, 48 and 72 h of exposure.

Toxicity of the nanoemulsion formulations using filter paper impregnation method

The contact toxicity of the nanoemulsion formulations was evaluated using filter paper impregnation method (Hameed et al., 2012) in March 2015. The nanoemulsion formulations were diluted to four azadirachtin concentrations (5.0, 6.0, 7.5 and 10.0 mL/kg) and then 1 mL of each formulation was loaded into a filter paper (5 cm ø), to bring the amount of azadirachtin on the filter paper to 2.6, 3.1, 3.8 and 5.1 mL/m^2. The treated filter papers were left at room temperature for 5 min to evaporate the solvent and then, were placed into Petri dishes. Twenty adults of *S. oryzae* or *T. castaneum* were released into each set of treatment and the Petri dishes were covered with lid so that the adults could not escape. The experiment was repeated five times and adult mortality was recorded after 24, 48 and 72 h of exposure.

LT_{50} determination

The LT_{50} for *S. oryzae* and *T. castaneum* adults at 2.0 mL/kg (in food media) and 5.1 mL/m^2 (on filter paper) of neem oil nanoemulsion, and the controls, was evaluated using the same bioassays, food impregnation method (Talukder & Howse, 1994) and filter paper impregnation method (Hameed et al., 2012), with five replicates. The treatments were checked every 2-3 h to record the number of dead adults. The experiment was done in June 2015.

Statistical analysis

The mortality data were tested for normality using Bartlett's test and, where necessary, transformed using Box-Cox transformation (Osborne, 2010). Repeated measure analysis of variance (ANOVA) was used to analyze mortality data with considering exposure time as the repeated variable (because the same Petri dishes were tested after 24, 48 and 72 h of exposure) and formulation with concentration as the main effects, using SAS 9.2 software (SAS Institute, Carey, NC, USA). Mean values were separated by Tukey's multiple comparison test ($P < 0.05$).

The LT_{50} values of the neem oil nanoemulsions and controls at 2.0 mL/kg (in food media), and 5.1 mL/m^2 (on filter paper) were determined for 50% mortality with confidence intervals of 95% using Probit analysis with PoloPlus software version 1.0 (LeOra Software, El Cerrito, CA, USA) according to the classical maximum likelihood procedure of Finney (1971).

Results

Composition and physicochemical characteristics of the nanoemulsion formulations

Four formulations were selected from the isotropic region of the phase diagram plots with 48:30:22 ($NeemPolys_1$ and $NeemAPG_1$) and 40:27:33 ($NeemPolys_2$ and $NeemAPG_2$) percentage of neem oil, surfactant and water (Table 1). The formulations were visually clear and transparent and physically stable after storage at room temperature (25ºC) for 90 d and subsequently at 54ºC for 14 d, except $NeemAPG_2$. All the selected compositions unequivocally exhibited long term and well stabilized properties. However, $NeemAPG_2$ showed phase separation at 54°C after 14 d. The formulations contained Polys surfactant showed smaller particle size of 208 ($NeemPolys_1$) and 253 nm ($NeemPolys_2$), but the formulations comprised APG exhibited bigger particle size of 328 ($NeemAPG_1$) and 507 nm ($NeemAPG_2$). The data of zeta potential analysis demonstrates that $NeemPolys_1$ and $NeemPolys_2$ comprised of Polys surfactant are more stable (39.1-37.9 mV) than $NeemAPG_1$ and $NeemAPG_2$ containing APG surfactant (32.5-31.3 mV) (Table 1).

Table 1. Physicochemical characteristics of the neem oil nanoemulsions for stability, thermostability, particle size, and zeta potential analysis

Formulation	Component (%) [a]	S [b]	T [c]	Particle size (nm)±SE [d]	Zeta potential (mV)±SE [d]
$NeemPolys_1$	30:48:22	√	√	208±1.2 d	39.1±1.5 a
$NeemPolys_2$	27:40:33	√	√	253±1.6 c	37.9±1.1 ab
$NeemAPG_1$	30:48:22	√	√	328±2.3 b	32.5±1.9 b
$NeemAPG_2$	27:40:33	√	×	507±2.7 a	31.3±1.3 b

[a] Surfactant (Polys or APG): neem oil: water;
[b] stability at 25ºC for 90 d;
[c] thermostability at 54ºC for 14 d;
[d] each value is the mean of three replicates (n = 3),
Within the column means followed by the same letter are not significantly different (Tukey's test, P < 0.05); √= stable; ×= not stable.

Bioassay

Mortality of *S. oryzae* and *T. castaneum*, via food and filter paper impregnation methods, were significantly affected by the exposure time, all main effects (formulations and concentrations) and associated interactions (Table 2). There were significant differences between toxicity of controls (Neemix® and neem oil) and nanoemulsions against the tested insect species for both application methods (P < 0.05) (Tables 3-6).

After 24 h of exposure, the highest mortality was recorded for *S. oryzae*, with food impregnation method, exposed to 2.0 mL/kg of $NeemPolys_1$, where all exposed adults were dead (Table 3). After 48 h of exposure in both application methods, 2.0 mL/kg (in food media) and 5.1 mL/m^2 (on filter paper) of the nanoemulsion formulations caused over 75% mortality of exposed adults of both insect species (Tables 3-6). After 72 h of exposure in both application methods, all concentration rates resulted in, more than 50% mortality of the exposed adults, and the proportion was lower for filter paper impregnation method (Tables 3-6). The mortality of *S. oryzae* reached to 100% after 72 h of exposure at all nanoemulsion formulation concentrations, with food impregnation method (Table 3).

Among the nanoemulsion formulations, $NeemAPG_2$ showed lower toxicity than other formulations in some cases, but despite showing less mortality there was trend toward enhanced efficiency throughout the assay. The mortality on the control treatment with neem oil and Neemix® were significantly lower than that of the nanoemulsion formulations. Neem oil exhibited the lowest mortality in each assessment and also on the total mortality. Mortality of *S. oryzae* and *T. castaneum* for neem oil alone treatment, after 72 h of exposure and all concentration rates, ranged between 8.2 and 33.5%, and 0 and 27.7%, respectively, via application of food impregnation method (Tables 3 and 4). A similar trend for the application of filter paper impregnation method was observed by mortality range of 0 to 21.7% (*S. oryzae*) and 0 to 15.5% (*T. castaneum*) (Tables 5 & 6).

Table 2. Parameters of repeated measure ANOVA for main effects and associated interactions for mortality of *Sitophilus oryzae* and *Tribolium castaneum* adults, through application of food and filter paper impregnation methods (error *df* = 360)

Source	*df*	Food impregnation method				Filter paper impregnation method			
		S. oryzae		*T. castaneum*		*S. oryzae*		*T. castaneum*	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Exposure time	2	6.35	0.019	8.73	0.005	6.73	0.009	5.95	0.012
Formulation	5	63.17	0.000	32.04	0.000	11.77	0.001	10.89	0.001
Concentration	4	18.76	0.000	24.54	0.000	14.27	0.001	13.88	0.001
Exposure time × formulation	10	1.52	0.167	2.87	0.009	5.39	0.000	8.22	0.000
Exposure time × concentration	8	6.25	0.000	4.90	0.000	6.22	0.000	9.23	0.000
Formulation × concentration	20	6.87	0.000	8.01	0.000	6.80	0.000	11.08	0.000
Exposure time × Formulation × concentration	40	53.37	0.000	40.74	0.000	42.50	0.000	25.75	0.000

Table 3. Mean mortality (%±SE) of *Sitophilus oryzae* adults exposed for 24, 48 and 72 h on rice kernels treated with four nanoemulsion formulations of neem oil, Neemix® and neem oil (crude extract) at five concentrations

Exposure time (h)	Formulation [a]	Mortality (%)±SE [b]				
		Concentration (mL/kg)				
		0.0	1.0	1.2	1.5	2.0
24	$NeemAPG_1$	0.0±0.0a	15.0±1.1c	42.2±0.4c	80.0±0.7bc	88.0±0.8b
	$NeemAPG_2$	0.0±0.0a	0.0±0.0d	29.4±0.3d	73.2±0.4c	81.2±0.4b
	$NeemPolys_1$	0.0±0.0a	26.2±0.4a	76.3±0.6a	95.0±0.4a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	19.3±1.2b	57.2±0.7b	88.7±0.2ab	96.3±0.4a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0e	13.5±0.4d	21.8±0.7c
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0e	0.0±0.0e	0.0±0.0d
48	$NeemAPG_1$	0.0±0.0a	46.2±0.4b	87.6±1.2b	100.0±0.0a	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	34.0±0.5c	63.2±1.3c	100.0±0.0a	100.0±0.0a
	$NeemPolys_1$	0.2±0.3a	86.3±0.3a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	59.2±1.2b	100.0±0.0a	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0d	20.3±0.6b	34.7±0.4b
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0d	8.2±0.8c	18.5±0.6c
72	$NeemAPG_1$	0.3±0.9a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemPolys_1$	0.4±0.7a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.2±0.5a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	12.5±1.3b	25.2±0.6b	35.4±0.4b	52.5±0.7b
	Neem oil	0.0±0.0a	8.2±0.5c	17.5±0.5c	24.5±0.8c	33.5±0.6c

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Each value is the mean of five replicates (n = 5);
For each exposure time, within each concentration (columns), means followed by the same letter are not significantly different (Tukey HSD test at $P < 0.05$).

Table 4. Mean mortality (%±SE) of *Tribolium castaneum* adults exposed for 24, 48 and 72 h on broken wheat grains treated with four nanoemulsion formulations of neem oil, Neemix® and neem oil (crude extract) at five concentrations

Exposure time (h)	Formulation [a]	Mortality (%)±SE [b]				
		Concentration (mL/kg)				
		0.0	1.0	1.2	1.5	2.0
24	$NeemAPG_1$	0.0±0.0a	5.5±0.4b	27.0±0.7b	59.5±0.4bc	73.5±0.8ab
	$NeemAPG_2$	0.0±0.0a	0.0±0.0c	17.2±0.4c	51.2±0.8c	68.5±0.4b
	$NeemPolys_1$	0.0±0.0a	12.5±0.9a	51.5±0.6a	73.5±0.9a	86.2±0.8a
	$NeemPolys_2$	0.0±0.0a	7.5±0.7b	34.5±0.9b	68.7±0.5ab	80.7±0.5ab
	Neemix®	0.0±0.0a	0.0±0.0c	0.0±0.0d	8.5±0.4d	15.7±0.8c
	Neem oil	0.0±0.0a	0.0±0.0c	0.0±0.0d	0.0±0.0d	0.0±0.0d
48	$NeemAPG_1$	0.0±0.0a	31.5±0.6c	70.0±1.1b	87.5±0.6b	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	0.0±0.0d	48.2±0.7c	76.7±0.5c	95.0±0.7a
	$NeemPolys_1$	0.0±0.0a	72.5±0.4a	88.2±0.4a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	41.0±1.8b	79.5±0.6b	93.7±0.4a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0d	15.5±0.9d	27.7±0.4b
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0d	0.0±0.0e	9.8±0.3c
72	$NeemAPG_1$	0.3±0.5a	63.7±0.8c	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	44.0±0.4d	79.5±0.4b	100.0±0.0a	100.0±0.0a
	$NeemPolys_1$	0.2±0.2a	91.5±0.6a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	78.7±1.3b	100.0±0.0a	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	6.2±0.4e	14.7±0.8c	23.5±0.6b	46.5±0.6b
	Neem oil	0.0±0.0a	0.0±0.0e	9.6±0.5d	19.7±0.4b	27.7±0.3c

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Each value is the mean of five replicates (n = 5);
For each exposure time, within each concentration (columns), means followed by the same letter are not significantly different (Tukey HSD test at $P < 0.05$).

Table 5. Mean mortality (%±SE) of *Sitophilus oryzae* adults exposed for 24, 48 and 72 h on filter paper treated with four nanoemulsion formulations of neem oil, Neemix® and neem oil (crude extract) at five concentrations

Exposure time (h)	Formulation [a]	Mortality (%)±SE [b]				
		Concentration (mL/m²)				
		0.0	2.6	3.1	3.8	5.1
24	NeemAPG1	0.0±0.0a	0.0±0.0a	10.2±0.4b	24.5±0.7bc	46.7±0.7b
	NeemAPG2	0.0±0.0a	0.0±0.0a	0.0±0.0c	15.7±0.8c	37.5±1.2c
	NeemPolys1	0.0±0.0a	0.0±0.0a	21.2±0.6a	42.5±1.3a	65.2±0.7a
	NeemPolys2	0.0±0.0a	0.0±0.0a	16.2±0.5ab	31.7±0.5ab	52.2±0.9ab
	Neemix®	0.0±0.0a	0.0±0.0a	0.0±0.0c	0.0±0.0d	0.0±0.0d
	Neem oil	0.0±0.0a	0.0±0.0a	0.0±0.0c	0.0±0.0d	0.0±0.0d
48	NeemAPG1	0.0±0.0a	16.5±0.8c	49.5±0.9c	79.2±0.6b	100.0±0.0a
	NeemAPG2	0.0±0.0a	0.0±0.0d	32.2±1.4d	61.5±0.8c	82.5±0.6b
	NeemPolys1	0.0±0.0a	42.2±0.4a	78.2±0.8a	100.0±0.0a	100.0±0.0a
	NeemPolys2	0.0±0.0a	27.2±1.5b	64.5±0.6b	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0e	6.5±0.9d	17.5±0.6c
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0e	0.0±0.0d	0.0±0.0d
72	NeemAPG1	0.0±0.0a	32.2±0.4c	59.5±0.5b	100.0±0.0a	100.0±0.0a
	NeemAPG2	0.0±0.0a	21.5±0.6d	47.2±0.4b	100.0±0.0a	100.0±0.0a
	NeemPolys1	0.0±0.0a	56.2±0.8a	81.5±0.7a	100.0±0.0a	100.0±0.0a
	NeemPolys2	0.4±0.2a	41.5±0.6b	73.5±0.5a	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0e	7.75±1.5c	16.5±1.9b	25.8±1.4b
	Neem oil	0.0±0.0a	0.0±0.0e	0.0±0.0c	11.5±1.3c	21.7±1.7b

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Each value is the mean of five replicates (n = 5);
For each exposure time, within each concentration (columns), means followed by the same letter are not significantly different (Tukey HSD test at P < 0.05).

Table 6. Mean mortality (%±SE) of *Tribolium castaneum* adults exposed for 24, 48 and 72 h on filter paper treated with four nanoemulsion formulations of neem oil, Neemix® and neem oil (crude extract) at five concentrations

Exposure time (h)	Formulation [a]	Mortality (%)±SE [b]				
		Concentration (mL/m^2)				
		0.0	2.6	3.1	3.8	5.1
24	$NeemAPG_1$	0.0±0.0a	0.0±0.0a	0.0±0.0b	19.5±0.6bc	38.5±0.8bc
	$NeemAPG_2$	0.0±0.0a	0.0±0.0a	0.0±0.0b	11.7±1.6c	29.5±1.3c
	$NeemPolys_1$	0.0±0.0a	0.0±0.0a	17.5±1.4a	37.5±1.3a	59.5±1.7a
	$NeemPolys_2$	0.0±0.0a	0.0±0.0a	12.5±0.6a	28.5±0.9ab	45.5±0.8b
	Neemix®	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0d	0.0±0.0d
	Neem oil	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0d	0.0±0.0d
48	$NeemAPG_1$	0.0±0.0a	11.5±0.5c	41.5±0.8c	70.7±1.5c	90±0.0b
	$NeemAPG_2$	0.0±0.0a	0.0±0.0d	24.5±0.5d	53.5±2.4d	75.5±2.6c
	$NeemPolys_1$	0.0±0.0a	37.5±1.2a	70.8±2.3a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	21.5±1.5b	58.5±1.8b	92.5±0.8b	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0e	0.0±0.0e	10.4±0.8d
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0e	0.0±0.0e	0.0±0.0e
72	$NeemAPG_1$	0.0±0.0a	27.2±1.1bc	53.3±0.8bc	89.5±2.1b	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	16.5±0.8c	41.5±1.6c	78.3±1.1c	100.0±0.0a
	$NeemPolys_1$	0.0±0.0a	51.5±0.6a	76.0±0.9a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	32.7±1.5b	61.5±1.8b	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0d	13.5±1.4d	19.6±1.1b
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0d	0.0±0.0e	15.5±1.9b

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Each value is the mean of five replicates (n = 5);
For each exposure time, within each concentration (columns), means followed by the same letter are not significantly different (Tukey HSD test at $P < 0.05$).

Lethal time determination (LT_{50} and LT_{90})

Results of Probit analysis and lethal time determination are shown in Tables 7 and 8. According to the LT_{50} and LT_{90} values, regardless formulation type and application method, it is concluded that *S. oryzae* adults are more susceptible (LT_{50} 9.6 to 140.4 h) while *T. castaneum* adults (LT_{50} 11.3 to 159.9 h) are more tolerant. The time required for the nanoemulsion formulations to give 50% mortality of *S. oryzae* adults ranged from 9.6 to 17.4 h (food impregnation method) and 17.0 to 32.4 h (filter paper impregnation method). However, LT_{50} values for *T. castaneum* ranged from 11.3 to 23.1 h (food impregnation method at 2.0 mL/kg azadirachtin) and 19.2 to 35.8 h (filter paper impregnation method at 5.1 mL/m^2 azadirachtin). Therefore, it is concluded that food impregnation method is the more effective method for this assay. Generally, the nanoemulsion formulations had lower LT_{50} values compared to neem oil and Neemix® alone as controls. Among the nanoemulsion formulations, $NeemPolys_1$ acted as the most efficient formulation. In all cases, increased susceptibility of both insect species was directly associated with particle size of the formulation and exposure time.

Table 7. LT_{50} and LT_{90} (Lethal Time) (hour) of *Sitophilus oryzae* and *Tribolium castaneum* adults exposed to the formulations at 2.0 mL/kg azadirachtin via food impregnation method

Insect species	Formulation [a]	LT_{50} (lower-upper CL) [b] hours	LT_{90} (lower-upper CL) [b] hours	Slope±S.E	Chi square
S. oryzae	NeemAPG$_1$	14.6 (13.8-15.1)	35.5 (28.8-49.1)	2.7±0.3	4.4
	NeemAPG$_2$	17.4 (16.0-18.2)	42.3 (33.7-59.9)	3.1±0.4	4.5
	NeemPolys$_1$	9.6 (8.1-11.0)	27.7 (22.3-31.2)	3.5±0.4	2.9
	NeemPolys$_2$	11.6 (9.6-13.6)	28.8 (25.1-35.7)	2.8±0.3	3.1
	Neemix®	46.2 (41.3-54.4)	91.8 (84.1-97.3)	1.6±0.2	1.2
	Neem oil	92.2 (79.9-106.4)	156.0 (151.9-182.2)	2.4±0.3	0.8
T. castaneum	NeemAPG$_1$	17.9 (15.7-19.4)	55.4 (41.7-88.1)	2.4±0.3	0.3
	NeemAPG$_2$	23.1 (20.9-25.6)	62.6 (56.0-123.6)	2.5±0.3	2.8
	NeemPolys$_1$	11.3 (9.7-12.0)	35.3 (27.1-54.4)	1.1±0.2	3.6
	NeemPolys$_2$	13.9 (12.4-15.2)	45.1 (34.8-67.0)	2.9±0.3	0.1
	Neemix®	54.7 (52.4-57.6)	109.4 (106.7-126.7)	1.3±0.2	1.2
	Neem oil	112.7 (105.3-129.6)	177.1 (170.0-185.8)	2.2±0.4	4.4

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Upper and lower 95% confidence limits.

Table 8. LT_{50} and LT_{90} (Lethal Time) (hours) of *Sitophilus oryzae* and *Tribolium castaneum* adults exposed to the formulations at 5.1 mL/m^2 via filter paper impregnation method

Insect species	Formulation [a]	LT_{50} (lower-upper CL) [b] hours	LT_{90} (lower-upper CL) [b] hours	Slope±S.E	Chi square
S. oryzae	NeemAPG$_1$	24.9 (21.1-28.0)	71.3 (48.2-81.1)	3.1±0.3	1.2
	NeemAPG$_2$	32.4 (29.3-35.0)	81.3 (53.8-97.3)	4.1±0.3	4.3
	NeemPolys$_1$	17.0 (14.8-19.4)	59.4 (43.8-96.8)	2.4±0.2	2.1
	NeemPolys$_2$	22.4 (19.8-25.6)	68.2 (52.3-101.9)	2.6±0.2	0.4
	Neemix®	68.2 (62.5-73.1)	161.7 (158.6-174.4)	1.2±0.2	0.4
	Neem oil	140.4 (137.2-143.6)	197.9 (193.0-211.8)	2.7±0.5	0.3
T. castaneum	NeemAPG$_1$	29.4 (28.0-31.9)	73.2 (54.4-103.4)	3.1±0.3	7.9
	NeemAPG$_2$	35.8 (32.0-38.4)	80.4 (66.4-106.6)	3.±0.4	0.4
	NeemPolys$_1$	19.2 (16.1-21.8)	61.6 (46.9-93.8)	2.1±0.2	1.6
	NeemPolys$_2$	24.7 (20.9-27.3)	70.2 (68.9-187.3)	2.2±0.2	1.1
	Neemix®	81.7 (74.2-89.0)	172.6 (169.1-173.2)	2.4±0.3	0.2
	Neem oil	159.9 (156.9-162.0)	227.2 (216.0-251.4)	3.7±0.6	0.5

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Upper and lower 95% confidence limits.

Discussion

The emulsion produced for the present study had a particle size range of below 600 nm, which can be considered as a nanoemulsion formulation (Solans et al., 2003; Shafiq et al., 2007). Consistent with Mishra et al. (2014), a reduction in the particle size and turbidity was observed by increasing the surfactant concentration and decreasing the neem oil concentration. The presence of the surfactant in the nanoemulsion formulations created the reduction of interfacial tension at the oil/water interface leading to a drop in the free energy and thus creates a mechanical obstacle to the disambiguation of the droplets, therefore the nano-droplets showed good stability in the emulsion system (Reiss, 1975). Also, Chen & Tao (2005) stated that the well stabilized properties of the nanoemulsion formulations is due to the enhanced adsorption of surfactant molecules at the oil-water borders. However, $NeemAPG_2$ showed phase separation at 54°C, which is probably due to a lower surfactant concentration in conjunction with more water content compared to $NeemAPG_1$ comprised the same surfactant. Also, $NeemAPG_2$ with the biggest droplet size of 507 nm demonstrated significantly higher capability to self-assemble as the hydrophobic affinity was enlarged while the solubility in water declined and made the hydrophobic chain to self-organize into larger aggregates (Lin & Lin, 2003).

The occurrence of coalescence also increased the droplet size of the nanoemulsion with time, because the liquid film between droplets disrupted and became thin, which led to larger droplet size (Taylor, 2003). Another reason for increasing droplet size is decreasing surfactant/water ratios at fixed amount of oil or increasing of oil/water ratios at constant surfactant (Morales et al., 2003). The particle size and zeta potential characteristics of the nanoemulsions correlated with the type of surfactant. In these circumstances, Polys surfactant preferentially formed better formulations with smaller droplet size and higher zeta potential. The results from zeta sizer analysis confirmed that Polys surfactant acts as the best surfactant compared to APG with less significant difference and thus higher stability (Table 1). As reported in previous studies, the nanoemulsion formulations that contained nonionic surfactants with the smallest particle size were more effective. The lack of phase separation of the nanoemulsion formulations over a long period of time contributed to their extended stability (Solans et al., 2005; Anjali et al., 2012).

The bioassay of the nanoemulsion formulations of neem oil demonstrated toxicity effects on both insect species. The insecticidal effects of the formulations varied with the insect species, concentration of the formulations, exposure time and the method of application. The results of contact toxicity in the filter paper impregnation method showed lower mortality than that with food impregnation method. The possible explanation for these results is the higher absorption of the toxic substance occurs through ingestion of the food into the insect's body. In previous studies (Negahban et al., 2007; Sahaf et al., 2007, 2008; Ogendo et al., 2008; Taghizadeh-Saroukolai et al., 2010), it was found that *S. oryzae* is significantly more susceptible than *T. castaneum,* which is consistent with the results obtained in our experiment. Studies have not previously reported a rapid-acting nanoemulsion formulation of neem oil produced from low-energy emulsification method and low concentrations of azadirachtin for control of stored products insect pests. The effectiveness of neem crude extract with 2.5% azadirachtin against *T. castaneum* has been evaluated by Hameed et al. (2012), which lead to about 46% mortality after 7 d of exposure using filter paper dip method. Among the nanoemulsion formulations, the highest and fastest toxic effect were observed with $NeemPolys_1$ against *S. oryzae* via food impregnation, which justifies the use of Polys surfactant in this formulation as it leads to smaller particle size and therefore more opportunity of the formulation to come in contact with the target insect. Whereas, the lower mortality caused by $NeemAPG_2$ with the biggest particle size indicates that the smaller the particle size, the greater the probability of higher efficacy. This finding is consistent with the studies of Lim et al. (2013) and Asib et al. (2015).

The bioassay results, in most cases, showed that mortality from exposure to the nanoemulsion formulations was not significantly different at 2.0 mL/kg (in food media) and 5.1 mL/m^2 (on filter paper) azadirachtin after 48 h. Therefore, the lethal time experiments were conducted to determine the fastest acting formulation at 2.0 mL/kg (via food impregnation method) and 5.1 mL/m^2 azadirachtin (via filter paper impregnation method). The study showed that oil based nanoemulsion formulations were able to increase the mean mortality rate of *S. oryzae* and *T. castaneum* compared to the commercial EC formulation of neem oil (Neemix®) and crude extract of neem oil. Moreover, the low LT_{50} at 2.0 mL/kg (in food media) and 5.1 mL/m^2 (on filter paper) azadirachtin of the formulated nanoemulsions was due to the

presence of surfactant in the formulations, which increased the opportunity for the toxic substance (azadirachtin) to act more efficiently and stably. Therefore, this study clearly indicates that the formulating of azadirachtin is one of the most effective ways to increase mortality of stored-product insect pests. Generally, these observations evidenced that the formulated neem oil nanoemulsions have suitable properties to be considered as toxic component against insect pests of stored products with rapid and high mortality impacts.

Conclusion

The neem oil, surfactant and deionized water were successfully prepared as nanoemulsion formulation by a low-energy emulsification method. $NeemPolys_1$ containing Polys surfactant with smallest droplet size of 208 nm was the most effective formulation for control of *S. oryzae* and *T. castaneum* adults compared to the other formulations with bigger droplet size. $NeemPolys_1$ also demonstrated the highest mortality against *S. oryzae* via food impregnation method in less than 10 h at 2.0 mL/kg azadirachtin compared to other formulations as well as the conventional emulsifiable formulation (Neemix®) and the unformulated neem oil. Overall, the laboratory studies have shown that the formulated nanoemulsions of neem oil could be a good alternative for the control of the tested species in stored products; however, further research is required to demonstrate the effectiveness of the nanoemulsion formulations on stored products insect pests, in practice through packaging.

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Original article (Orijinal araştırma)

Tachinid (Diptera: Tachinidae) parasitoids of the lucerne beetle, *Gonioctena fornicata* (Brüggemann, 1873) (Coleoptera: Chrysomelidae), with a new parasitoid record and their parasitism rates

Yonca yaprak böceği, *Gonioctena fornicata* (Brüggemann, 1873) (Coleoptera: Chrysomelidae)'nın yeni bir parazitoit kaydı ile Tachinid (Diptera: Tachinidae) parazitoitleri ve parazitleme oranları

Turgut ATAY[1*]

Abstract

The lucerne beetle, *Gonioctena fornicata* (Brüggemann, 1873) (Coleoptera: Chrysomelidae), is a serious pest of lucerne. In this study, tachinid (Diptera: Tachinidae) parasitoids of *G. fornicata*, and their parasitism rates were investigated at one location in Tokat Province, Turkey during 2016 and 2017. For this purpose, the lucerne beetle larvae were collected from an lucerne field and reared in the laboratory. Two tachinid species were obtained from the reared larvae. The species were *Meigenia mutabilis* (Fallén, 1810) and *Macquartia tenebricosa* (Meigen, 1824) (Diptera: Tachinidae). *Macquartia tenebricosa* was recorded first time as parasitoid of *G. fornicata*. The number and sex of the emerged parasitoids were recorded daily. The parasitism rates were 3.61% and 1.07% during 2016, and 3.69% and 0.50% in 2017, for *M. mutabilis* and *M. tenebricosa*, respectively. Based on these data, it was concluded that *M. mutabilis* is more effective parasitoid of *G. fornicata*. This paper reports for the first time detailed information on the natural enemies of *G. fornicata* and their effectiveness both on Turkey and worldwide.

Keywords: *Gonioctena fornicata*, lucerne, parasitoids, Tachinidae, Turkey

Öz

Yonca yaprak böceği, [*Gonioctena fornicata* (Brüggemann, 1873) (Coleoptera: Chrysomelidae)], yoncanın önemli bir zararlısıdır. 2016-2017 yıllarında gerçekleştirilen bu çalışmada, Tokat ilinde belirlenen bir lokasyonda yonca yaprak böceği (*G. fornicata*)'nin Tachinid (Diptera: Tachinidae) parazitotleri ve parazitleme oranları araştırılmıştır. Bu maksat için yonca yaprak böceğinin larvaları yonca arazisinden toplanarak laboratuvarda kültüre alınmıştır. Tachinidae familyasına ait iki tür tespit edilmiş olup bunlar, *Meigenia mutabilis* (Fallén, 1810) ve *Macquartia tenebricosa* (Meigen, 1824) (Diptera: Tachinidae)'dır. *Macquartia tenebricosa*'nın *G. fornicata*'nın parazitoiti olduğu ilk kez bu çalışma ile bildirilmiştir. Çıkan parazitoitlerin sayısı ve cinsiyetleri günlük olarak kaydedilmiştir. *Meigenia mutabilis* ve *M. tenebricosa* için 2016 yılındaki parazitleme oranları, sırasıyla %3.61 ve %1.07; 2017 için ise %3.69 ve %0.50 olarak belirlenmiştir. Elde edilen sonuçlara göre *M. mutabilis*'in *G. fornicata* üzerinde daha etkili bir parazitoit olduğu saptanmıştır. Bu çalışma, *G. fornicata*'nın doğal düşmanlarının ve etkinliklerinin belirlenmesi bakımından hem dünya hem de Türkiye'de gerçekleştirilen ilk detaylı çalışma niteliğindedir.

Anahtar sözcükler: *Gonioctena fornicata*, yonca, parazitoitler, Tachinidae, Türkiye

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Introduction

Forage crops have a very important place in agriculture, and particularly for animal production (Yolcu & Tan, 2008). Lucerne is the most favored forage crop worldwide due to its high adaptability to different climatic and environmental conditions, high feed efficiency and good feed quality (Özyazıcı et al., 2013). The lucerne beetle, *Gonioctena fornicata* (Brüggemann, 1873) (Coleoptera: Chrysomelidae) causes significant damage to lucerne and other species in the Fabaceae. Adults and larvae feed on lucerne leaves, causing significant yield losses depending on their density in the field. Alkan (1946) was the first to record the presence and damage of *G. fornicata* in Turkey. Later, many researchers have published information about the damage, spread and biology of *G. fornicata* in the country (Medvedev, 1970; Kısmalı, 1973; Kovancı, 1982; Kasap, 1988; Yıldırım et al., 1996; Aslan & Özbek, 1999; Efe & Özgökçe, 2014). Coşkuncu & Gençer (2006) reported that the first adults were observed in Bursa at the end of March, and damage was observed in the lucerne fields until end of July. Furthermore, it was reported that the insect has one generation per year. Çam & Atay (2006) also reported that larvae and adults cause heavy damage to lucerne leaves in Tokat Province, Turkey. Grigorov (1976) reported that the larvae and adults feed on leaves, shoots and leaf buds of the plants causing severe losses which may exceed 60% for vegetative mass and 100% for seeds in Central and Southeastern Europe.

Collection of larvae and adults is recommended for control this pest in small areas. Early and frequent cutting or flooding of lucerne stands may lead to mortality of the pest. There have also been some biological control studies. Particularly, entomopathogenic fungi contribute to regulation of its population. Atay et al. (2015) found that 36% of *G. fornicata* overwintered adults in lucerne growing areas of Tokat Province were naturally infested with an entomopathogenic fungus *Beauveria* sp. Despite the significant damage, an effective control strategy against these pests is yet to be developed.

Lucerne is a perennial plant having an average life of 5 years. Many different organisms can establish in lucerne growing areas as it creates habitat for them. Lucerne fields provide a temporary equilibrium in terms of flora and fauna among field crops. In this way, lucerne cultivation contributes to the natural balance in agroecosystems. Therefore, the development of eco-friendly and appropriate methods of control against *G. fornicata* will allow for the provision of production, as well as the protection of this natural balance. All over the world natural enemies, biological control agents, are regarded as important repressive elements to achieve this goal. In this framework, the efficacy of the tachinid species against *G. fornicata* was investigated.

The Tachinidae is a family of parasitoids, which are very important biological control agents. Many of their hosts are significant pests of cultivated crops. Most species parasitize lepidopteran larvae, other hosts are ranged as the orders Coleoptera, Hemiptera, Hymenoptera, Orthoptera and Diptera according to their importance (Grenier, 1988; Stireman et al., 2006). Kara & Tschorsnig (2003) prepared a detailed catalog containing tachinid hosts identified in Turkey. The most recent and comprehensive catalog of Palearctic hosts was prepared by Tschorsnig (2017). No records of Tachinidae in lucerne beetle have been published for Turkey.

Material and Methods

This study was conducted to determine the tachinid parasitoids of *G. fornicata* and parasitism rates at one location in Tokat Province, Turkey during 2016-2017.

Study area: a lucerne field in Ballıdere, Tokat - Merkez (40º21'03" N, 36º38'05" E, 606 m, 0.3 ha).

Lucerne beetle larvae were collected (1200 larvae in each year) from the lucerne field through mid-May to early June in both 2016 and 2017. The larvae were brought to the laboratory with their host plants, which were replaced with new ones every day, transferred to separate cages, kept at 25±2ºC and 60-70% RH, and inspected daily for emergence of the parasitoids. The number and sex of the parasitoids obtained were recorded daily. Parasitism rates for each species were calculated using the following formula (Keçeci et al., 2008).

Parasitism rate (%) = [Number of adult parasitoids / (Number of lucerne beetle larvae + Number of adult parasitoids)] x 100

After adult emergence, the flies were pinned and processed for identification. They were identified using the keys of Mesnil (1962), Tschorsnig & Herting (1994) and Tschorsnig & Richter (1998). Nomenclature and arrangement of tachinids followed Herting & Dely-Draskovits (1993). The specimens were deposited at the Plant Protection Museum in Gaziosmanpaşa University, Agricultural Faculty, Plant Protection Department, Tokat, Turkey.

Results and Discussion

Two tachinids, *Meigenia mutabilis* (Fallén, 1810) and *Macquartia tenebricosa* (Meigen, 1824) (Diptera: Tachinidae), were obtained and their parasitic efficiencies determined. *Macquartia tenebricosa* was recorded for the first time as parasitoid of *G. fornicata*. No hymenopteran parasitoids were encountered during the study. The distribution and some additional information related to tachinid species is as follows.

Subfamily: Exoristinae

Tribe: Blondeliini

Meigenia mutabilis (Fallén, 1810)

Distribution in Turkey: Erzurum (Doğanlar, 1982), Tokat (Kara, 1998), Amasya (Kara, 2001).

Distribution elsewhere: Caucasus, Soviet Middle Asia, East Siberia, Asia (Herting & Dely-Draskovits, 1993), Europe (Tschorsnig et al., 2004).

Remarks: From the middle of April to the middle of October, it has several generations in Central Europe. It can be observed on flowers and leaves. *Meigenia mutabilis* has many Chrysomelidae hosts, rarely Curculionidae and Tenthredinidae (Tschorsnig & Herting, 1994; Tschorsnig, 2017). No host of *M. mutabilis* has previously been reported in Turkey. Tschorsnig (2017) mentions a few old records of *M. mutabilis* being obtained from *G. fornicata.* This parasitoid kills the host and then emerges from the host and turns into pupae (Figure 1a).

Subfamily: Tachininae

Tribe: Macquartiini

Macquartia tenebricosa (Meigen, 1824)

Distribution in Turkey: Tokat (Kara, 1999), Amasya (Kara, 2001), Bartın (Korkmaz, 2007).

Distribution elsewhere: Caucasus, Soviet Middle Asia, East and West Siberia, Asia (Herting & Dely-Draskovits, 1993), Europe (Tschorsnig et al., 2004). Remarks: From the middle of April to the early October, the species has several generations in Central Europe. Only Chrysomelidae are known as reliable hosts (Tschorsnig & Herting, 1994; Tschorsnig, 2017). No hosts of *M. tenebricosa* have been reported in Turkey until this report. *Gonioctena fornicata* is a new host species for this tachinid. This parasitoid kills the host and then turns into pupae inside the host larval skin (Figure 1b).

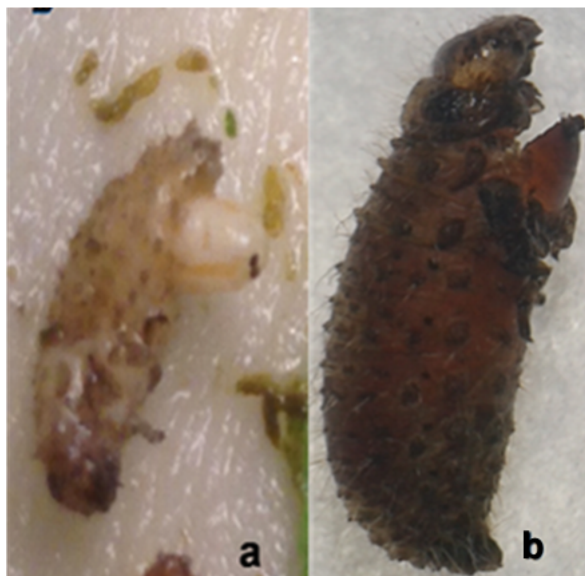

Figure 1. a) *Meigenia mutabilis* larvae emerging from *Gonioctena fornicata* larvae; and b) the puparium of *Macquartia tenebricosa* inside the host larval skin.

Data collected on the parasitoids are presented in Table 1. The parasitism rates were 3.61 and 3.69% for *M. mutabilis,* and 1.07 and 0.50% for *M. tenebricosa* during 2016 and 2017, respectively. The parasitism rate of *M. mutabilis* was higher than *M. tenebricosa* in both years of the study. The total parasitism rate of the two parasitoids was 4.68% in 2016 and 4.19% in 2017 (Table 1).

Table 1. Numbers and parasitism rates of *Meigenia mutabilis* and *Macquartia tenebricosa*

Year	*M. mutabilis*				*M. tenebricosa*			
	Male	Female	Total	Parasitism Rate (%)	Male	Female	Total	Parasitism Rate (%)
2016	26	19	45	3.61	7	6	13	1.07
2017	19	27	46	3.69	0	6	6	0.50

The number and sex of emerged parasitoids were recorded daily. Overall higher number of emerging individuals of *M. mutabilis* than *M. tenebricosa* was recorded during both years of the study (Table 1). Notably, individuals of *M. mutabilis* emerged earlier than those of *M. tenebricosa*, regardless of the year and sex (Figure 2). Early emergence of parasitoids from their hosts increases the impact of the parasitoid on the host population. The emergence of males of *M. tenebricosa* was only recorded in 2016. Both parasitoids yielded higher number of males than females in 2016, while more number of females was recorded during the second year of the study (Table 1 and Figure 2). The total number of males of *M. mutabilis* and *M. tenebricosa* were 45 and seven, respectively, in both years of the study, while the total female numbers were 46 and 12, respectively (Table 1). Consistently female-biased parasitoid sex ratios may benefit biological control because successful parasitization can result from lone females (Heimpel & Lundgren, 2000).

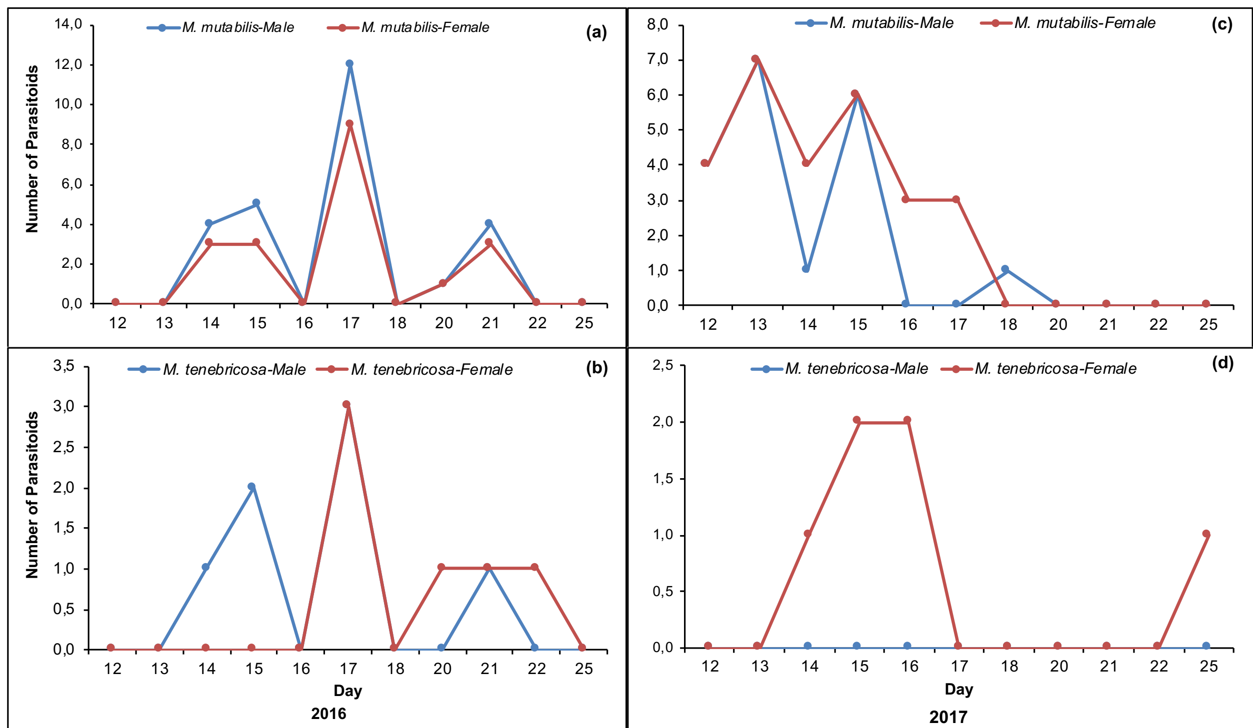

Figure 2. Emergence time of males and females of *Meigenia mutabilis* and *Macquartia tenebricosa* in 2016 (a and b, respectively) and 2017 (c and d, respectively) from the host.

There is limited literature on the natural enemies of *G. fornicata*. This paper reports the first detailed information in terms of determination of natural enemies of *G. fornicata* and their effectiveness. No there are published records of Tachinidae reared from lucerne beetles in Turkey. Elsewhere, it is only known to be parasitized by *M. mutabilis* (Mellini, 1954; Michieli, 1957; Tschorsnig, 2017). Brovdii (1976) considered that the natural enemies of this pest in the Ukraine are insufficiently known, while the larvae are parasitized by *M. mutabilis* in southern Europe.

Meigenia mutabilis was found to be a more effective parasitoid of *G. fornicata*. Tachinids have been used both in agricultural and forest areas in more than 100 biological control programs over the last century (Grenier, 1988). Getting benefit from tachinids within the framework of biological control programs requires determination of natural habitats of species, explaining their biology, illuminating host-parasitoid relations and determining their effectiveness in nature.

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