

**COLOR PREFERENCE AND TEMPORAL PATTERN OF HOST
PARASITIZATION BY THE FEMALE PARASITOID,
*Trichogramma evanescens***

Asmaa G. T. Abd-El Sater*, Abd-Elalim G. Ali*, Hamdy H. Mahmoud*, Doaa
S. Mohammed*, and Medhat M. Sadek**

*Plant Protection Research Institute, Agricultural Research Center, Assiut
Branch, Egypt.

**Department of Zoology, Faculty of Science, Assiut University, Assiut 71516,
Egypt.

E-mail: m.sadek@aun.edu.eg

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The temporal pattern of parasitization of the Angoumois grain moth, *Sitotroga cerealella* eggs by female *Trichogramma evanescens*, and the color preference by the parasitoid, were examined in laboratory. Under light-dark conditions, an obvious rhythmic parasitization activity was observed. Parasitization rate was high during the light phase and extremely low during the dark phase. Under continuous light a similar rhythmicity was observed, suggesting that the activity is controlled by an endogenous circadian clock mechanism. In both cases the number of parasitized eggs was maximal on the first day of contact between the parasitoid and the host eggs, and then gradually decreased. Under continuous darkness, however, much fewer eggs were parasitized and no rhythmicity could be discerned. It is thus concluded that although parasitization behavior is probably shaped by an internal oscillator mechanism, light is necessary for the mechanism to be manifested, and some other environmental factors, e.g., darkness can directly modify the behavior, thus reflecting remarkable plasticity. When given host eggs on card pairs of different colors, the female *T. evanescens* exhibited the following preferences: yellow over green, red or blue; green over red or blue; and blue over red. Given the choice among five different colors, the female *T. evanescens* exhibited a significant color preference ranking of yellow > green > white and blue > red. The disappearance of such preference in darkness indicates that the parasitoids discriminate between colors. Implications of these findings are discussed.

1. INTRODUCTION

Trichogramma evanescens Westwood (Hymenoptera: Trichogrammatidae) is one of about 180 species of minute egg parasitoids belonging to the genus *Trichogramma* (Querino and Zucchi, 2005). These parasitoids are effectively used around the world as biological control agents against a variety of Lepidopteran pests (Smith, 1996). Like other parasitic wasps, *Trichogramma* species are generally thought to use both long-range and short-range cues for host location, including volatile chemicals emitted by

moths or their host plants (Reddy *et al.*, 2002; Scholler and Prozell, 2002), oviposition-induced change of leaf surface chemicals (Fatouros *et al.* 2005), and signals from moth scales or trail pheromones (Thomson and Stinner, 1990; Garnier-Geoffroy *et al.*, 1996).

In contrast to the many studies dealing with chemical and tactile cues, relatively few studies focused on the use of visual cues by *Trichogramma* to locate their host eggs. The use of visual information by parasitic wasps has been investigated in many Hymenoptera species, e.g., *Psytalia concolor* (Benelli and Canale, 2012), *Venturia canescens* (Desouhant *et al.*, 2010), *Diachasmimorpha juglandis* (Henneman *et al.*, 2002), and *Cotesia vestalis* (Kugimiya *et al.*, 2010). However, it is also possible that *Trichogramma* species rely on visual stimuli in their search for host eggs. It is known that eggs of Lepidoptera differ largely in their color, ranging from greenish white to yellow or even orange (Lobdell *et al.*, 2005). Sometimes moths of the same species lay eggs of various colors at different times (du Merle and Brunet, 1991). In addition, eggs usually get darker as they proceed toward hatching. It is also known that most Hymenoptera are able to perceive green, blue, and ultraviolet light, with a few species able to perceive red (Peitsch *et al.*, 1992). Therefore, parasitic wasps could possibly use the color of host eggs or their background color as a means to locate their hosts and/or assess the age and conditions of eggs. For instance, *Trichogramma* wasps were found to preferentially parasitize eggs in areas with higher ultraviolet radiation (van Atta *et al.*, 2015). Begum *et al.* (2004) have found that nectar foraging by *Trichogramma* spp. is influenced by flower color. Trap color has also been shown to affect the patterns of *Trichogramma* capture on sticky-traps (Romeis *et al.*, 1998). In addition, differential response to certain colors was exhibited by female *Trichogramma* when presented to eggs or egg models of different colors (Pak and de Jong, 1987; Calvin *et al.*, 1997; Lobdell *et al.*, 2005).

Response of *Trichogramma* species to visual cues implies the importance of light for successful host finding. A study on the ultrastructure of the eye of *T. evanescens* suggests that vision in this wasp is limited to bright light conditions (Fischer *et al.*, 2011). Behavioral studies of the species have shown that the female wasps are more active than males as they disperse over larger areas searching for hosts to deposit their eggs (Martel and Boivin, 2004). These parasitoids are practically used under different light conditions, including complete darkness (e.g. in stored grains), natural light (e.g. in cultivated crops) and artificial lighting (e.g. in greenhouses). Therefore, understanding the temporal pattern of activity of these

parasitoids within the light and dark cycle is critical to successful use as biocontrol agents. In addition, Lepidopterous pests lay their eggs on plant parts with different colors, including leaves (Sadek, 2011), flowers (Bopp and Gottsberger, 2004) and fruits (Blackmer *et al.*, 2001). Color preference may therefore affect the parasitization activity of wasps during their search for host eggs. Studies on the visual system of *Trichogramma* species have focused almost entirely on the visual detection of host eggs (Pak *et al.*, 1991; Bruins *et al.*, 1994; Begum *et al.*, 2004; van Atta *et al.*, 2015). In contrast, color preference has only been touched upon. As far as the authors know, color preference has only been investigated in three studies, and all were on species other than *T. evanescens* (Kadlubowski, 1970; Romeis *et al.*, 1998; Lobdel *et al.*, 2005). *T. evanescens* is among the *Trichogramma* species that occur naturally in Egypt, and has been successfully used against a large number of butterfly and moth species (Abbas, 1990; Hegazi *et al.*, 2005). The present work has thus aimed at investigating the color preference and temporal pattern of parasitization activity of female *T. evanescens*, since information about the behavior of this parasitoid could be essential for improving the control tactics targeting phytophagous Lepidoptera.

MATERIALS AND METHODS

Rearing of insects:

T. evanescens were taken from a laboratory strain that was reared on eggs of *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) for many years in the Entomology Laboratory, Agricultural Research Center in Assiut. The culture was kept at 20°-25°C and 12:12 light-dark cycle. The rearing procedure of *T. evanescens* was modified from the method described by Hassan and Abdelgader (2001), as follow: 1) eggs of the factitious host, *S. cerealella*, taken from a stock culture kept on wheat grains in the laboratory, were glued to 4×4 cm pieces of cardboard. Almost 400-500 eggs were glued on each card, 2) the cards were left in muslin-covered glass jars about 10 cm in diameter and 20 cm high. Similar cards carrying *S. cerealella* eggs previously parasitized by *T. evanescens* were placed in the jars. The parasitized eggs on the latter cards contained pharate adult parasitoids, i.e. parasitoids that were about to emerge, 3) after 1-3 days, a period assumed to be enough for all parasitoids on the old cards to emerge, mate, and parasitize host eggs on the new cards, the cards with freshly parasitized eggs were taken and kept in other jars until the time expected for parasitoid emergence, and 4) these cards were in turn used as a source of parasitoids. So, two types of egg-carrying cards were used in the experiments, "source cards" (i.e. cards carrying old parasitized eggs, where *T. evanescens* are in their pharate

adult stage) and "target cards" (i.e., cards to which fresh unparasitized *S. cerealella* eggs are glued).

Investigation of temporal pattern of parasitization

The parasitization activity of female *T. evanescens* was examined under conditions of light-dark cycle, continuous light, or continuous darkness. To examine the activity under light-dark cycle, two incubators were set to the experimental conditions of 25°C and 12L:12D cycle. In the first incubator, however, the L:D cycle was adjusted to match the laboratory day-night cycle (i.e., the artificial day was generally in sync with the working day), whereas in the second incubator the cycle was reversed (i.e., the incubator's dark phase coincided with the working day) to enable checking the activity of parasitoids during their dark phase in the usual working time. Source cards with roughly 200-250 parasitoids reared in normal, or reversed cycle, were transferred to jars containing target cards in the two incubators, respectively. Sentinel source cards carrying 1-day-old parasitized eggs were kept to monitor the time expected for emergence burst. When the source cards were expected to release the main bulk of their adult parasitoids, they were transferred to the incubators right at the beginning of light phase. With this setting, it is assumed that the source cards will be source of mated females within minutes, since the great majority of *Trichogramma* parasitoids mate on the emergence patch immediately after eclosion (Martel, 2007). Every two hours, the target cards were removed from the jars and replaced by new ones. The parasitized eggs on the removed target cards were later counted under a light microscope. In the case of having insects under the reversed cycle, the incubator was kept in a dark room and red light was used to discriminate between source and target cards.

To examine the parasitization activity under continuous light or continuous dark, the same procedure was followed except that the experiment was either carried out in an incubator with continuous light or in an incubator with continuous dark. Either regime was kept unchanged along the three days of investigation. The same precautions for handling the insects in dark were also adopted. However, the insects were checked, i.e. the target cards were removed and replaced with new ones, every 4 hours rather than every 2 hours as in the above mentioned experiment. In case of continuous dark, the source cards were placed into the jars in the incubator at the beginning of the putative light phase.

Investigation of color preference by female T. evanescens

Three experiments were carried out. In the first experiment, the parasitoids were allowed to choose between two colors. Six different combinations of colors were tested; green vs. red, green vs. yellow, green vs. blue, red vs. yellow, red vs. blue or yellow vs. blue. About 100 eggs of *S. cerealella* were glued to a circle in the center of 3×3 cm pieces of standard "cut and paste" paper of the above mentioned colors. The two colored pieces of each combination were used as target cards. They were placed as distant as possible within a 10 cm diameter petri dish and a small source card (2×2 cm, assumed to produce about 200 wasps) was placed at the center of the dish. The dishes were left for 1 hour during the light phase, after which the colored pieces were collected and the parasitized eggs were counted. Nine replicates were tested in each trial.

In the second experiment, the female parasitoids were allowed to choose between five different colors. *S. cerealella* eggs were glued to the center of 2×2 cm pieces of green, red, blue, yellow and white paper. The five pieces were arranged equidistantly in a circle beside the wall of a 15.8 cm diameter petri dish. A source card, with a potential load of about 800 wasps, was placed right in the center of the dish. Twelve replicates were prepared as mentioned above, and after 1 hour in the light phase the colored pieces were collected and the parasitized eggs on each were counted.

The third experiment was exactly as the second one, in terms of setup and number of replicates. However, the petri dishes were kept in dark for 1-hour period of parasitization.

Statistical analysis

Data obtained from color preference experiments in which combinations of two colors were used were subjected to two-tailed t-test. In case of color preference among five different colors, the results were analyzed using one-way ANOVA, followed by Tukey-test for multiple comparisons, when significant differences were observed. All of the tests were conducted according to Fowler *et al.* (1998), aided by Microsoft Excel software.

3. RESULTS

Parasitization activity under normal light-dark cycle:

The parasitoids exhibited cyclical parasitization activity along the three days of experiment, where the activity during photophase was generally

higher than during scotophase. The photophase of the first day was the period of highest parasitization activity, compared to the photophase of second or third days, while the activity was almost the same during scotophase on the three days (Fig. 1).

Parasitization activity under continuous light or continuous darkness:

When the insects were subjected to continuous light, they exhibited a rhythmic parasitization activity similar to that observed under the dark-light cycle. Parasitization activity was higher during subjective photophase than during subjective scotophase (Fig. 2). However, when the parasitoids were presented to continuous darkness, no obvious cycling in the parasitization activity was observed. In the subjective photophase of the first day the activity was only slightly higher than during the rest of the 72-hour experiment (Fig. 2).

Color preference

When the parasitoids were allowed to choose between eggs glued to cards of two different colors, they exhibited parasitizing preference for eggs on one color more than on the other. This was the case in five out of the six color combinations used in the experiment. Only in the case of "green vs. yellow" combination, the parasitoid exhibited a noticeable but not significant preference for yellow ($P = 0.063$). In the other combinations, the preference for one color over the other ranged between significant and highly significant (Fig. 3).

When the parasitoids were tested during the light phase, given the choice among five colors, yellow was chosen significantly more than green, blue, white, and red. Green was chosen significantly more than the remaining three colors. There was no significant difference in the preference for blue and white ($F = 17.57$, $P = 2.4 \times 10^{-9}$, Tukey test at $P < 0.01$). Red was chosen significantly less than the four colors (Fig. 4). When the test was done in darkness there were no significant differences between the numbers of parasitized eggs on the five colors ($F = 0.131$, $P = 0.969$). However, generally few numbers of eggs were parasitized on the five colors, compared to those parasitized under the light conditions (Fig. 4).

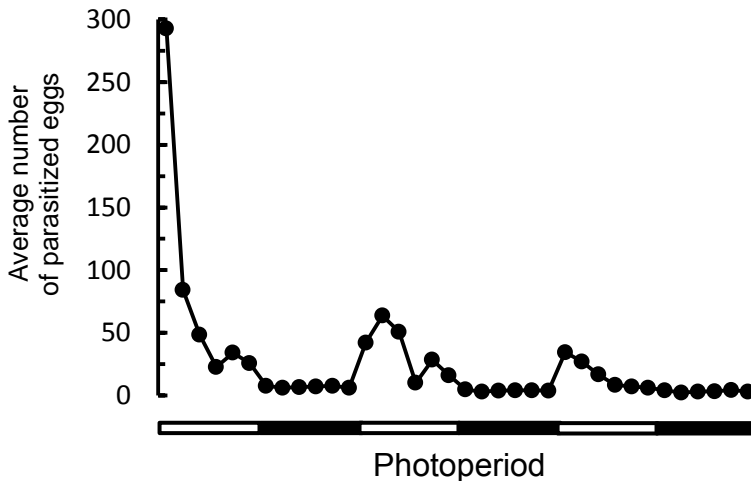


Fig. 1. Parasitization activity of female *T. evanescens* during the photophase (□) and scotophase (■) of three consecutive days after emergence. Three replicates, each comprising 200-250 wasps (males and females), were used for the experiment under 12L:12D cycle.

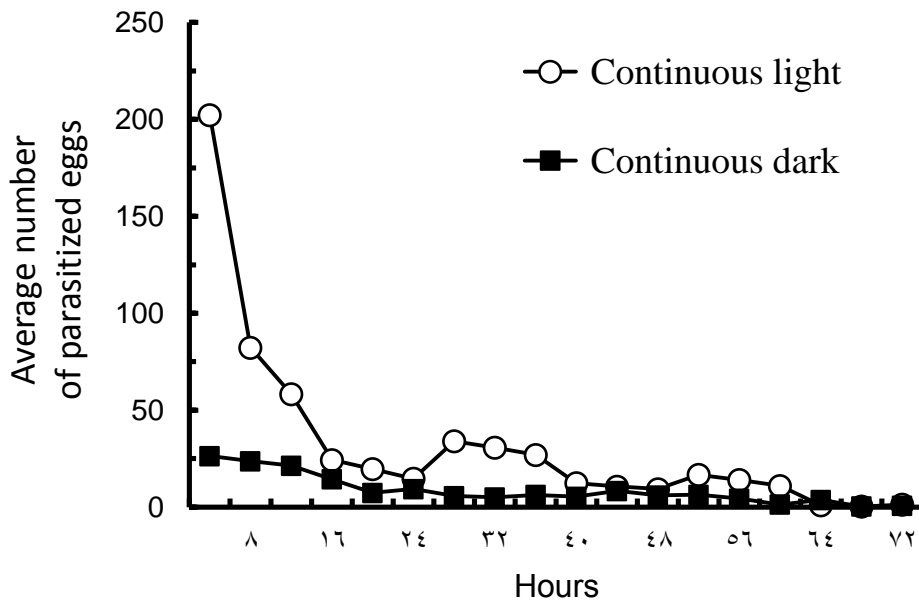


Fig. 2. Parasitization activity of female *T. evanescens* under continuous light or continuous dark conditions over three consecutive days after emergence. Three replicates, each comprising 200-250 wasps (males and females), were used for the experiment.

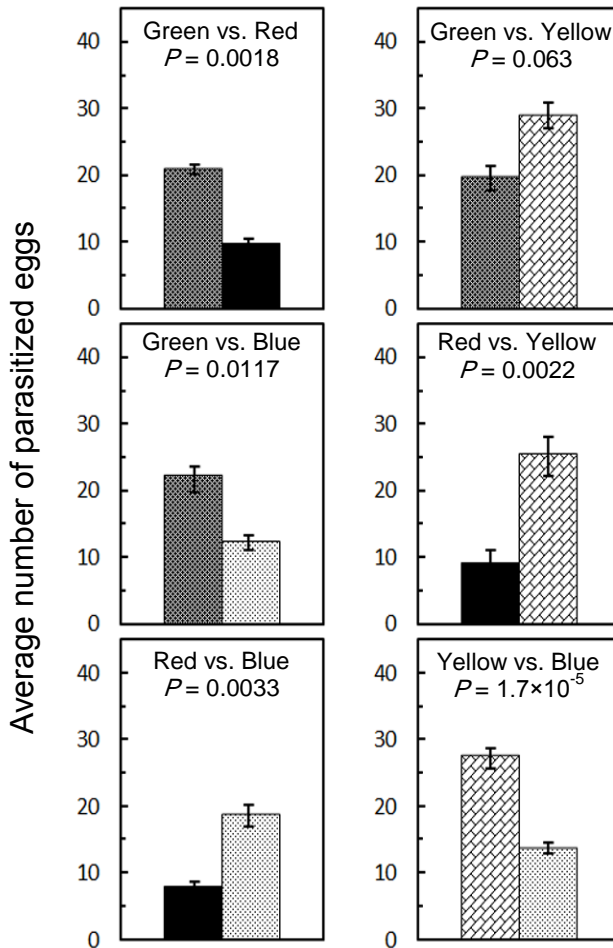


Fig. 3. Color preference by female *T. evanescens* when allowed to parasitize eggs mounted on two different colors. About 200 wasps were used in each test. Bars represent data from 9 replicates. The standard errors of mean are graphically shown and the P-values drawn from a tow-tailed *t*-test are also given.

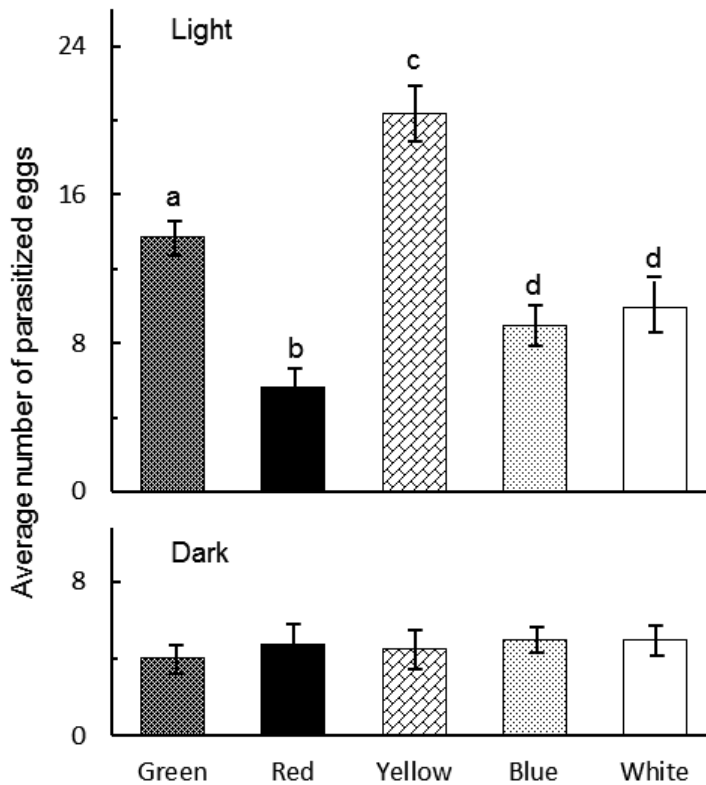


Fig. 4. Color preference by female *T. evanescens* when given access to eggs mounted on five different colors, either in light or dark conditions. About 800 wasps were used in each test. Bars represent data from 12 and 8 replicates in light and dark, respectively. The standard errors of mean are graphically shown. Bars denoted with different letters are significantly different at $P < 0.05$ (ANOVA followed by *Tukey*-test for multiple comparisons).

DISCUSSION

Results of the present study showed that female *T. evanescens* parasitize their factitious host eggs almost entirely during the light phase. Under light-dark conditions, an obvious rhythmic parasitization activity was observed, in which high parasitization rates were observed during the light phase and low rates were observed during the dark phase. These results are consistent with those reported on a *Trichogramma* species (Reznik *et al.*, 2009). Under

continuous light, the rhythmicity was largely kept the same. The persistence of rhythmic parasitization activity for 3 days under continuous light suggests that the activity is regulated by an endogenous circadian clock mechanism similar to various behavioral activities in insects (Giebultowicz, 2000). The presence of daily rhythms based on the endogenous circadian oscillations that are synchronized with the external photoperiod has also been demonstrated in *Trichogramma* species (Karpova, 2006). However, under continuous darkness the rhythm in the present study appeared to be completely damped out. Such direct effects of environmental factors on circadian rhythm are not unusual in insects. It is known that the circadian clock system consists chiefly of three elements, input pathways which are mainly photoreceptors, an internal pacemaker or oscillator, and effector pathways that manifest the overt rhythm (Page, 2003). The phase of endogenous oscillation can be shifted by the influence of environmental factors, such as temperature or light (Karpova and Reznik, 2002). Therefore, the inhibitory effect of darkness on parasitization activity observed in the present study indicates that the manifested rhythm involves a mixture of endogenous and input pathways (Saunders, 1982).

It is established that *Trichogramma* species are diurnal parasitoids, with adult emergence occurring in the early morning (Pompanon *et al.*, 1995; Karpova and Reznik, 2002), quickly followed by locomotor, dispersal and mating activities (Martel, 2007). This may interpret why the activity of the female parasitoids was inhibited under dark conditions. The great majority of parasitization was also observed on the first day of contact with host eggs. It has been demonstrated that it takes *Trichogramma* wasps 6 days to disperse up to 180 meters, and 21 days are required for a dispersal distance of 230 meters, from the release site (Wright *et al.*, 2001). *T. evanescens*, despite being a warm climate species, is also more sensitive than other *Trichogramma* species to environmental factors, such as wind and temperature (Fournier and Boivin, 2000). Long dispersal is thus time and energy consuming, and may expose the parasitoids to deleterious factors. The higher activity in the early female's life observed in the present study may therefore be of adaptive value since it enables females to parasitize as many host eggs as possible while being young, and reduces both the energy expenditure required for dispersal and the risk of dying before reproduction.

The results suggest also that host finding in *T. evanescens* is affected by visual cues. The observed rhythmic parasitization behavior, with peak activity during light, appears therefore to be highly adaptive as it allows the parasitoids to gain and utilize visual information. However, behavioral

plasticity is common in parasitic wasps. Various oviposition behaviors aiming to optimize the clutch size have already been reported in *Trichogramma* (Honda and Luck 2001; Nisani and Honda, 2010). It is thus obvious that the temporal pattern of parasitization activity of female *T. evanescence* observed in the present study, and its vulnerability to environmental factors, particularly darkness, cannot be characterized by a simple model, and the behavioral plasticity of the wasps should rather be considered.

The color preference experiments demonstrated that visual information may be critical for host finding. The female parasitoids were able to discriminate between eggs on cards of different colors, as manifested in the striking preferences for certain colors. At present color preference by *Trichogramma* species other than *T. evanescens* has been demonstrated almost only in three studies (Kadlubowski, 1970; Romeis *et al.*, 1998; Lobdel *et al.*, 2005). The present work may be the first to show that *T. evanescens* can utilize color stimuli. This finding contradicts what has been reported more than 80 years ago, in a classic and heavily cited study by Salt (1935) who concluded that *T. evanescens* does not select hosts based on color. Several reasons may account for this disagreement. For instance, the conclusion of Salt (1935) was based on a no-choice test. The female parasitoids were also allowed to parasitize a wide range of suitable and unsuitable host-like objects that happened to vary in color. It is thus assumed that the valuable study of Salt (1935) did not involve controlled experiments specifically designed to test for egg color preferences. The present work may, therefore, represent a significant contribution to the limited information regarding the use of visual stimuli by *T. evanescens*. This should not overshadow the fact that *Trichogramma* wasps can also find and parasitize their host eggs in darkness (Calvin *et al.*, 1997). However, the rate of host finding seem to increase strikingly in light.

The significant color preference in the multiple choice situation was in the order of yellow > green > blue or white > red. The disappearance of this preference when the same set of five colors was presented to the female wasps in darkness indicates that the preference was unlikely to be the result of tactile, olfactory or gustatory cues, and that the preference was almost exclusively based on color vision. In dual choice tests yellow was remarkably but not significantly preferred over green, whereas preference in other combinations largely matched the preference in the multiple choice test. The lack of significant difference between the parasitoids' preferences for yellow and green in the dual test may be due to that both colors are

generally more attractive to the parasitoids than other colors, and hence may be mutually targeted when found together. In terms of discrimination between different colors, the results are largely consistent with studies on other *Trichogramma* species (Bhattacharya and Basit, 1997; Singh *et al.*, 2001; Baitha and Sinha, 2002). However, regarding the preference for specific colors, some differences are found between results of the present study and those reported by other investigators. For instance, green was more preferred than yellow in *T. chilonis* (Baitha and Sinha, 2002) and white was preferred more than pink in *T. carverae* (Begum *et al.*, 2004). Yellow was more preferred than green in *T. ostriniae*, a finding that is consistent with the present study, but white was more preferred than green (Lobdell *et al.*, 2005). These differences may be attributed not only to the difference in species studied, but also to the differences in settings. For instance, Baitha and Sinha (2002) have not controlled for light, and conducted their tests at a different temperature and used different food for the parasitoids. Begum *et al.* (2004) have tested the choice between of alyssum flowers of different colors and Lobdell *et al.* (2005) have tested the choice of egg-mimicking colored beads, whereas the present study used colored cards.

It has been demonstrated that the compound eyes of *T. evanescens* are of the apposition type, and the calculations derived from the eye structure suggest that it possesses trichromatic color vision (Fischer *et al.*, 2011). The majority of Hymenoptera investigated so far have trichromatic vision with maximal sensitivity values near ultraviolet, blue, and green (Peitsch *et al.*, 1992). This profile of sensitivity may explain why red was least preferred in the present study. It is worth noting that the present study tested the behavior of *T. evanescens* toward the background color, not the color of host eggs themselves. It may be experimentally difficult to completely segregate the response of *Trichogramma* to egg color from their response to background color. Investigations that focus on host egg color (e.g., Lobdell *et al.*, 2005) and those focusing on the background color are both necessary for gaining better understanding of the parasitization behavior of *Trichogramma*. Host-seeking behavior in parasitic wasps has been divided into four stages, habitat location, host location, host recognition, and host acceptance (Vinson, 1998). It can then be concluded from the present study that color cues are utilized by *T. evanescens* at least in the stage of habitat location. This corroborates other studies in which the background color has been found to influence the success of *Trichogramma* in foraging and realized parasitism (Begum *et al.*, 2004).

Results of the present work may have profound ecological and practical implications. In addition to demonstrating that *T. evanescens* possesses color vision, the present study showed that the parasitoid follow an obvious circadian rhythmic pattern in their parasitization behavior and the background color may affect host finding. In nature the potential host eggs of *Trichogramma* are generally white, yellowish, or greenish white, and are mostly oviposited on a green background, i.e., leaves or green stems. The extremely high preference for yellow and green in the present study may, therefore, be of an adaptive significance to these parasitoids. Moreover, many phytophagous moths lay their eggs on plant parts that are not necessarily green, e.g., flowers (Bopp and Gottsberger, 2004) and fruits (Blackmer *et al.*, 2001). The corn earworm for instance sometimes lays eggs on corn silk, a pale yellow substratum (Oatman, 1966). The preference for yellow may therefore be important for finding potential host eggs of generally yellowish color or eggs deposited on yellowish plant parts. In essence, the present study showed that the highest activity of *T. evanescens* is attained during daytime and the females can be visually attracted towards potential host eggs or host habitats as part of their normal behavior. Therefore, the efficacy of *T. evanescens* as a biocontrol agent can be enhanced by adopting certain measures. Such measures may include rearing the parasitoids under light-dark regimes in phase with the ambient light-dark conditions in the target field, and thereafter release them shortly before sunrise. This may ensure preparing the parasitoids to be ready for attacking their hosts once they are in the field, i.e., when they are still young and vigorous. Because *T. evanescens* in the present study hardly parasitized host eggs in darkness, the species may also not be the most suitable parasitoid for use against insect pests that live in dark habitats such as stored product insects.

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تفضيل الألوان ونمط التوزيع الزمني لتطفل إناث *Trichogramma evanescens* على عائلها

أسماء جمال ثابت عبد الساتر* ، عبد العليم جابر محمد* ، حمدي حسين محمود* ،
دعاء شحاتة محمد* ، مدحت مريد صادق**
* معهد بحوث وقاية النبات، مركز البحوث الزراعية، أسيوط.
** قسم علم الحيوان، كلية العلوم، جامعة أسيوط.

تم فحص الإيقاع اليومي لنشاط طفيل *Trichogramma evanescens* في تطفله على بيض فراشة أنجوموا للحبوب، *Sitotroga cerealella* في المعمل. وجد أن إناث الطفيل أبدت إيقاعا يوميا واضحا في نشاطها التطفلي عندما اختبرت تحت ظروف من الإضاءة والإظلام (١٢ ساعة إضاءة : ١٢ ساعة إظلام) على مدار ثلاثة أيام متتالية، حيث كان النشاط مرتفعا في فترة الضوء ومنخفضا في فترة الظلام. وقد لوحظ أيضا أن هناك إيقاعا مماثلا عندما اختبرت إناث نفس الطفيل لمدة ثلاثة أيام متتالية في ظروف من الضوء المستمر، وهو ما يشير إلى أن إيقاع النشاط التطفلي تنظمه ساعة بيولوجية داخلية. ولكن عندما أجري الاختبار في ظروف الظلام المستمر لوحظ أن الإيقاع يتلاشى، حيث انخفض معدل التطفل انخفاضا كبيرا طوال الأيام الثلاثة للتجربة. يشير اضمحلال الإيقاع في حالة الإظلام إلى أن الضوء ضروري لعمل الآلية الداخلية لتنظيم الإيقاع، وأن الظلام يؤثر في هذه الآلية على نحو مباشر، مما يدل على مرونة ملحوظة في سلوك الطفيل استجابة للعوامل البيئية. وفي تجربة أخرى أتيح لإناث الطفيل أن تتطفل على مجموعتين من بيض عائلها موضوعتين على مربعين ورقبيين مختلفي اللون، حيث أظهر الطفيل تفضيلا ملحوظا للون الأصفر على اللون الأخضر أو الأحمر أو الأزرق، كما فضل الطفيل أيضا الأخضر على الأحمر أو الأزرق، وفضل الأزرق على الأحمر. وعندما أتيح للطفيليات أن تتطفل على خمس مجموعات من البيض موضوعة على خمسة مربعات ورقية مختلفة الألوان، أظهرت الطفيليات تدرجا في التفضيل من الأصفر إلى الأخضر، ثم الأبيض والأزرق، ثم الأحمر. وعندما أجريت التجربة في الظلام لم تفضل الطفيليات أيا من الألوان الخمسة، وهو ما يشير إلى ضعف احتمال أن يكون سبب التفضيل مرتبطا بالشم أو التدوق أو اللمس، وأن رؤية الألوان كانت سبب التفضيل.