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Optimization of *Orius majusculus* release: photoperiodic sensitivity at different temperatures and storage of diapausing adults

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Abstract: The photoperiodic sensitivity and storage of *Orius majusculus* (Reuter) from Antalya was investigated to obtain data for the optimization of its release. In the current study, it was not possible to induce a high (up to 100%) incidence of diapause unless all the nymphal instars and adults were successively exposed (i.e. full exposure) to a short day length (11.5 h) at constant temperatures of 18 and 26 °C, and fluctuating temperatures of 10/26 °C. Hence an intermittent exposure to long day lengths, even for short periods of time (only during instars IV–V or adult stage), would avoid a high incidence of diapause at 10/26 °C and 26 °C. The response of *O. majusculus* nymphal instars and adults to a short day length with a low incidence of diapause under fluctuating temperatures suggests that greenhouses, where such temperatures prevail, provide an environment that is conducive to diapause prevention. The release of adults is a better approach than the release of nymphs, since the adults and their nymphs, until the eclosion of new adults, are expected to respond to short day lengths with a low incidence of diapause in greenhouses. When nymphs are released, on the other hand, a high incidence of diapause would be inevitable. Increasing the constant temperature to 30 °C at full exposure did not prevent diapause and consequently did not change the incidence of diapause. Storage of diapausing *O. majusculus* adults was possible for 1 month with a higher survival rate and fecundity than in a study based on the storage of nondiapausing adults at a lower temperature.

Key words: Biological control, diapause, *Frankliniella occidentalis*, *Orius majusculus*, photoperiodic sensitivity, storage

1. Introduction

Minute pirate bugs, *Orius* spp., are used in greenhouses worldwide in the augmentative biological control of the western flower thrips, *Frankliniella occidentalis* (Pergande) (van Lenteren, 2012). Because of the risks associated with the introduction of nonindigenous natural enemies, priority has been given in recent years to the use of indigenous species in the biological control of pests (van Lenteren et al., 2003; De Clercq et al., 2011). Three species of *Orius* (*O. laevigatus* (Fieber), *O. majusculus* (Reuter), and *O. niger* (Wolff)) are common in the Palearctic region, including Turkey (Péricart, 1972; Önder, 1982). Among them, *O. laevigatus* is the most widely used predator against *F. occidentalis* in vegetable greenhouses in Europe (van Lenteren, 2012), including Turkey. However, the rate of successful biological control programs that use *Orius* spp. against *F. occidentalis* may be increased, and a search for alternatives is warranted. It has been demonstrated that a strain of *O. niger* from Antalya (Başı and Tunç, 2008), similar to a strain of *O. laevigatus* from Sicily (Tommasini and van Lanteren, 2003), does not enter reproductive

diapause, which is a major impediment for the use of *Orius* under the short day length conditions of winter. Despite the ability of the Antalya strain of *O. niger* to avert diapause, the low fecundity and survival rates during mass rearing reduce its attractiveness as a candidate for use in biological control programs (Başı and Tunç, 2008).

Another alternative, *O. majusculus*, appears to be a less demanding and easy-to-handle species. In addition, it appears to have high fecundity and survival rates, which are the most desired traits for successful mass rearing of a biological control agent (Alauzet et al., 1992; Blümel, 1996; Başı and Tunç, 2012). Several reports have indicated that *O. majusculus* possesses biological characteristics and predation activities comparable to those of the major commercial species, *O. laevigatus* (Tommasini and Nicoli, 1993, 1994; Riudavets et al., 1995; Arno et al., 2008). The main drawback of the use of *O. majusculus* (its reproductive diapause during short day lengths) can be averted with supplementary lighting (Jacobson, 1993; Başı and Tunç, 2012). A southern strain of *O. majusculus* from Antalya was shown to have a considerably shorter critical day

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length than the northern strains (van den Meiracker, 1994), which would require a shorter supplementary lighting period. A short day length supplemented with artificial light as low as 10 lx would be sufficient to avoid diapause (Bahşi and Tunç, 2012).

Orius majusculus plays an important role in biological control systems where multiple pests are targeted by multiple natural enemies. This minute pirate bug has the ability to control multiple pests and to become established in greenhouse crops when the target pest is absent or present at low densities because it can feed on alternative types of prey. The combined use of *O. majusculus* with aphid predators and parasitoids resulted in control of both aphids and thrips (Messelink et al., 2013). The bug was also reported to have the potential to serve as an important biological control agent of whitefly *Bemisia tabaci* (Gennadius) and *F. occidentalis*, and it is known to survive by feeding on either of these 2 pests in greenhouse crops where they occur together (Arno et al., 2008). Furthermore, it can be sustained on plant material as an alternative food source during times of prey scarcity (Pumariño and Alomar, 2012).

Proper timing and follow-up for greenhouse releases of *Orius* that undergo diapause necessitate the determination of the life stages that are sensitive to diapause-inducing photoperiods (Ruberson et al., 2000). Available data on the photoperiodic sensitivity of different life stages in *Orius* were obtained using single temperature regimes only (van den Meiracker, 1994; Ruberson et al., 2000; Cho et al., 2005). Since temperature was demonstrated to have an impact on the photoperiodic response of *Orius* species (Musolin et al., 2004; Musolin and Ito, 2008), photoperiodic sensitivity of different life stages was detected using both constant and fluctuating temperature regimes in the present study.

There is a need to store mass-reared *O. majusculus* during periods of low demand or short days when diapause is likely to occur. Storing such predators in a state of diapause is assumed to enable storage for longer periods and to have less of a negative impact than storing nondiapausing predators at low temperatures (Tauber et al., 1993; Ruberson et al., 2000). To determine the extent to which this assumption is true, the survival, fecundity, and termination of diapause (preoviposition period) of adults were examined in this study after storage under diapause-inducing conditions for varying periods of time.

2. Materials and methods

2.1. *Orius majusculus* rearing

A strain of *O. majusculus* collected from Antalya (37.0°N, 30.5°E), Turkey, was used for this study. Adults and nymphs were reared separately in ventilated, 500-mL, clear plastic containers with a supply of frozen *Ephestia kuehniella* Zeller eggs as food and bean pods as the egg-

laying substrate and moisture source. Moth eggs were obtained from a culture maintained at the Department of Plant Protection, Akdeniz University, Antalya, and the green beans were purchased from a local market. The floor of the containers was lined with dried bean pod fragments to avoid cannibalism. The stock culture was kept in a walk-in growth chamber at 26 ± 1 °C, $60 \pm 10\%$ RH, 16:8 (L:D) h, and 2400 lx.

2.2. Photoperiodic sensitivity

Four temperature- and light-controlled incubators were used for the experiments. Light of 2400 lx was provided by 15-W or 18-W fluorescent lamps inside the incubators.

A day length shorter than 11.5 h induced reproductive diapause in all females of the investigated *O. majusculus* strain (Bahşi and Tunç, 2012). Photoperiods of 11.5:12.5 (L:D) and 12.5:11.5 (L:D) were used for exposing the insects to short and long day lengths, respectively. To determine the photoperiodic sensitivity of *O. majusculus* nymphal instars and life stages, 8 different exposure regimes were designed by transferring the given sequence of nymphal instars and life stages to a short day length as follows: (1) only eggs, (2) only instars I–III, (3) only instars IV and V, (4) all instars I–V, (5) only adults, (6) instars IV and V and the adults, (7) eggs and all instars, and (8) all instars and adults (full exposure). The test insects were exposed to the long day length at the same temperature during the remaining instars and/or stages (Table 1).

The effect of temperature on the photoperiodic sensitivity of the nymphal instars and life stages was investigated under constant temperatures of 18 ± 1 and 26 ± 1 °C, and fluctuating temperatures of $10 \pm 1/26 \pm 1$ °C (each applied for 12 h, the dark period was synchronized with 10 °C, transition between the 2 temperatures lasted about 30 min) at $60 \pm 10\%$ RH and 2400 lx. The possibility of the aversion of diapause and change in the diapause incidence by increasing temperature under the short day length conditions were investigated by exposing all the nymphal instars and adults (full exposure) at 30 ± 1 °C as well (not included in Table 1).

Each exposure regime began with the eggs laid on bean pods exposed to the females for 24 h under rearing conditions. The bean pods containing eggs were transferred to the given exposure regime condition at the given temperature. Exposure of the given stage or stages and/or nymphal instars to short day lengths took place according to exposure regimes detailed above. The stages or instars not exposed to short day lengths were kept at long day lengths at the same temperature. The number of eggs used for each exposure regime at a given temperature was 100–200. The hatched nymphs were confined to bean leaf discs in clear plastic petri dishes and supplied with *E. kuehniella* eggs. Each dish accommodated 10 nymphs, which hatched on the same day, and were counted as 1

replicate. Ten dishes, or 10 replicates, were used for each exposure regime at a given temperature. The numbers of female and male nymphs used for each exposure regime at a given temperature are provided in the footnotes in Table 1. The dishes were examined daily and emerged adults were removed.

One newly eclosed female and 1 newly eclosed male were placed into ventilated 40-mL glass jars and supplied with *E. kuehniella* eggs and bean pod pieces approximately 10 mm in length. *Ephestia kuehniella* eggs were replaced every 2 days, but the pod pieces were replaced daily. Fifteen pairs were used in each exposure regime at a given temperature. The preoviposition period was determined by examining bean pods daily for the eggs laid. Females that failed to oviposit within a period at least 2 times longer than the typical preoviposition period of nondiapausing females, which was 5, 6, and 10 days at 26 °C, 10/26 °C, and 18 °C, respectively, were considered to be in reproductive diapause (Şerife Ünal Bahşı, unpublished data).

2.3. Storage of diapausing adults

The possibility of storing diapausing adults was investigated for a short day length of 11.5 h at 18 ± 1 °C, $60 \pm 10\%$ RH, and 2400 lx. The insects were exposed to these conditions from the egg stage to the end of the given storage period, which was 30, 60, or 90 days. Bean pods containing eggs up to 24 h in age were placed in 250-mL clear plastic containers. The hatched nymphs were provided with *E. kuehniella* eggs 3 times a week and bean pods as a moisture source. The eclosed adults were transferred to new containers and provided with *E. kuehniella* eggs and bean pods at similar intervals. About 200 adults were maintained in 5 containers. No eggs were laid during the storage period. Mortality was recorded at the end of each storage period. The effect of storage on the preoviposition, oviposition, and postoviposition periods and fecundity were determined by subjecting 25 females to long day lengths of 12.5 h at 26 ± 1 °C after 30 or 60 days of storage. The females were individually confined in ventilated, 40-mL glass jars and supplied with *E. kuehniella* eggs and bean pod pieces. The indicated parameters were determined by examining the bean pods daily for the number of eggs laid, and the females that did not oviposit were recorded. These parameters could not be applied to individuals stored for 90 days because the mortality rate was 90% and exhaustion of the surviving individuals was high.

2.4. Data analysis

A chi-square test was used to analyze data on the incidence of diapause and survival (Minitab, 1998). Data on the preoviposition, oviposition, and postoviposition periods and fecundity were subjected to one-way general linear model analysis of variance (ANOVA) using normal error distribution to determine if the differences were significant, and the Tukey test was used to separate the means (SAS, 1998).

3. Results

3.1. Photoperiodic sensitivity

The incidence of reproductive diapause in *O. majusculus* exposed to a short day length was affected ($P < 0.001$) by the exposure and temperature regimes (Table 1). The responses of the life stages and/or nymphal instars differed significantly between exposure regimes at a given temperature and between temperatures for a given exposure regime, except for the exposure of all nymphal instars and adults together (full exposure). When considering the exposure regimes, other than full exposure, diapause incidence was low with minimal variation (between 7% and 20% overall at 10/26 °C and 26 °C), with the exceptions of the exposure of nymphal instars I–III alone at 10/26 °C and the exposure of all nymphal instars I–V at 26 °C. Diapause incidence was higher (80%–93%) for the majority of exposure regimes at 18 °C compared to 10/26 °C and 26 °C, except for the exposure options that included nymphal instars I–III alone and I–V alone, respectively. The incidence of diapause during exposure of the egg stage alone did not exceed 33% at any given temperature.

The highest incidence of diapause at all temperatures was recorded when all nymphal instars and adults, in succession, were exposed to a short day length. A diapause incidence of 100% was attained at 18 °C and 26 °C, but only 84% at 10/26 °C; these data were not significantly different ($P > 0.05$). Shorter exposure (e.g., exposure of nymphal instars I–III, IV and V, I–V, or adults separately, or even instars IV and V plus adults) did not induce 100% diapause at any temperature regime.

A 100% diapause was induced at full exposure to a short day length at constant temperatures of 18, 26, and 30 °C (the latter not included in Table 1).

3.2. Storage of diapausing adults

The preoviposition period (i.e. the period of diapause termination), survival, and the percentage of the individuals that remained in diapause were affected ($P < 0.05$) by the storage periods (Table 2). The preoviposition period was significantly longer (Tukey, $P < 0.05$) in females stored for 30 days (13.5 versus 8.6) than in those stored for 60 days. Survival decreased significantly from 83% to 55% to 10% as the period of storage increased from 30 to 60 to 90 days. A significant increase, from 7% to 18%, in the rate of females that remained in diapause was found with an increase in the storage period from 30 to 60 days. Fecundity was not affected significantly by the storage periods, although it decreased somewhat in the females stored for 60 days. Individuals stored for 90 days had a high mortality rate and parameters other than survival could therefore not be assessed.

Table 1. The incidence of reproductive diapause in *Orius majusculus* exposed to short day lengths (11.5 h) during varying stages and/or instars at different temperatures.

Stages exposed to short day	Incidence of diapause (%)			Chi-square test within rows
	Temperature (°C)			
	18	10/26	26	
E ^a	33	20	7	X ² = 14.2 df = 2 P < 0.001
I-III ^b	21	79	10	X ² = 50.4 df = 2 P < 0.001
IV, V	80	13	0	X ² = 83.2 df = 2 P < 0.001
A ^c	93	13	10	X ² = 73.9 df = 2 P < 0.001
I-V	27	7	84	X ² = 55.4 df = 2 P < 0.001
IV, V + A	80	7	20	X ² = 57.8 df = 2 P < 0.001
E + I-V	80	20	46	X ² = 25.3 df = 2 P < 0.001
I-V + A	100	84	100	X ² = 1.0 df = 3 P = 0.785
Chi-square Test within columns	X ² = 75.5 df = 7 P < 0.001	X ² = 152.0 df = 7 P < 0.001	X ² = 194.7 df = 7 P < 0.001	

^a Egg; the number of eggs used in each exposure regime at a given temperature was 100–200.

^b Nymphal instar; the number of nymphs used in each exposure regime at a given temperature was 29–49 ♀ and 26–47 ♂.

^c Adult; the number of adult pairs for each exposure regime at a given temperature was 15.

4. Discussion

4.1. Photoperiodic sensitivity

The photoperiodic response of life stages and nymphal instars of *Orius* species in terms of diapause incidence may not always be straightforward or consistent under controlled conditions (Ruberson et al., 1991; Bahşı and Tunç, 2012). In nature, they are exposed to regular photoperiods, whereas in an experimental setting they are exposed to intermittent photoperiods at any given life stage or sequence of nymphal instars. In addition, they were

exposed to a combination of constant photoperiods and constant temperatures that do not exist in nature. Even the constant short and long day lengths that are accompanied by fluctuating temperatures do not sufficiently simulate the natural environment. The sensitivity of life stages and/or nymphal instars to diapause-inducing photoperiods has been investigated in only a few *Orius* species. Furthermore, data on their sensitivity under different temperature regimes are lacking. The present data suggest that a given sequence of nymphal instars or life stages of *O. majusculus*

Table 2. The effect of storage under diapause-inducing conditions (L:D = 11.5:12.5 h, 18 °C) on the biological traits of *Orius majusculus* females (values are mean ± standard error).

Period of storage (days)	Preoviposition ^a (days)	Oviposition period (days)	Postoviposition period (days)	Fecundity Eggs per female	Survival ^b (%)	Percentage remained in diapause %
30 ^c	13.5 ± 1.5 A ^d	18.9 ± 1.9 A	7.3 ± 0.8 A	108.6 ± 12.1 A	83	7
60	8.6 ± 1.2 B	17.3 ± 2.0 A	3.3 ± 1.0 B	88.6 ± 14.2 A	55	18
90	NA ^e	NA	NA	NA	10	NA
	Chi-square test				X ² = 21.2 df = 2 P < 0.001	X ² = 4.3 df = 1 P = 0.038

^aThe preoviposition period after transfer to long day lengths.

^bSurvival during storage.

^cThe number of pairs used in each treatment was 25.

^dMeans followed by the same capital letter in columns are not significantly different (Tukey P < 0.05).

^e NA: parameter could not be assessed due to the high death rate of females.

would respond differently in terms of diapause incidence at changing temperatures.

Strain and/or latitudinal differences may also lead to the differences in the response of life stages of *O. majusculus* to the short day length at a given temperature. The data on response of the adult stage of *O. majusculus* at 18 °C do not match those of the strains from the Netherlands and Antalya. The diapause incidence at 18 °C was not significantly high at the adult stage of *O. majusculus* from the Netherlands (van den Meiracker, 1994), whereas the adults were found to respond with a high incidence of diapause at 18 °C in the present study. However, the 2 studies agree that exposure of all nymphal instars and the adults, in succession, to a short day length is required for a 100% diapause induction at 18 °C.

For a high incidence of diapause, exposure of a certain sequence of instars plus the adult stage to short day length is required for the *Orius* species studied so far (van den Meiracker, 1994; Ruberson et al., 2000; Cho et al., 2005). The exposure of adults is indispensable for all species, but the number and sequence of nymphal instars that should be exposed differs from one species to another. The exposure of the longest sequence of nymphal instars, I–V, is required in *O. majusculus* (van den Meiracker, 1994; present study) among the *Orius* species studied so far. Thus, under short day conditions it may be possible to avoid a high incidence of diapause by intermittent exposure to the long day lengths during certain stages of the life cycle in *O. majusculus*. Indeed, the pattern of photoperiodic sensitivity of *O. majusculus* life stages in the

present study suggests that an exposure to long day lengths only during nymphal instars IV and V or adult stage would avoid a high diapause incidence at 10/26 °C and 26 °C.

The response of nymphal instars and adults of *O. majusculus* from Antalya to the short day length with a low incidence of diapause under fluctuating temperatures suggests that greenhouses, where such temperatures prevail, provide an environment that is conducive to low rates of diapause. However, this phenomenon needs to be verified by using more combinations of fluctuating temperatures.

The results of the present study suggest that the release of *O. majusculus* adults is a better approach than the release of *O. majusculus* nymphs. A sequence of exposure that consisted of the released adults and their progeny, until eclosion of new adults, is expected to decrease diapause under fluctuating greenhouse temperatures. On the other hand, if nymphs are released, an exposure of nymphs plus adults would inevitably lead to a high incidence of diapause at all temperatures.

The aversion of diapause and changing the incidence of diapause at full exposure to short day length was not possible by increasing temperature for *O. majusculus* from Antalya. Unlike some other *Orius* species (van den Meiracker, 1994; Musolin et al., 2004; Musolin and Ito, 2008), its response remained the same in terms of diapause incidence, which was 100% at full exposure, under a wide range of constant temperatures (18–30 °C) in the present study. It is obvious that increasing the day length is the only way to avoid diapause in *O. majusculus* from Antalya.

4.2. Storage of diapausing adults

Storage of diapausing *O. majusculus* females at 18 °C led to changes in their biological traits. The preoviposition period was longer and fecundity was lower under 30-day storage compared to those exposed continuously, from egg to death, to the long day length at 26 °C in a study that used the same strain (Bahşi and Tunç, 2012).

It was demonstrated that with provisions of food and plant material the storage of diapausing adults of *O. majusculus* from Antalya for 1 month was possible with a higher survival rate and a higher fecundity than in the study (Rudolf et al., 1993) based on the storage of nondiapausing adults.

Diapause termination after switching to long day lengths took longer in *O. majusculus* (van den Meiracker, 1994 and present study) than in some other *Orius* species

(van den Meiracker, 1994; Cho et al., 2005). A long period of diapause termination might contribute to success of storage.

For the storage of *O. majusculus* under diapause, exposure to the short day lengths throughout the preimaginal and adult stages is necessary to induce diapause in all stored females. Therefore, the process of diapause induction should begin at the egg stage in order to ensure exposure of all nymphs from the time of hatching. Future research should aim to study the storage of diapausing adults with provisions of food at varying low temperatures in conjunction with complete darkness.

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