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Does *Wolbachia* Change Diapause and Energy Reserves of *Trichogramma brassicae* in Response to Light Wavelengths?

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ABSTRACT

The present study examined the light wavelengths effect on the diapause percentage of progeny and energy reserves of maternal generation in sexual and asexual *Trichogramma brassicae* that had been reared under different light wavelengths before oviposition. Photoperiod has a maternal effect on the diapause induction in *Trichogramma* wasps; however, the light wavelengths effect on their diapause has not been studied. In this study, we reared the maternal generation of both strains under five light wavelengths including blue (455~475 nm), green (515~535 nm), orange (585~595 nm), red (620~630 nm), and white (5,000~10,000 K), and allowed 24 hours old females to oviposit in *Ephestia kuehniella* eggs. The diapausing generation was placed at 10°C and absolute darkness for two months. The results showed that *Wolbachia* infection and light wavelengths had significant effects on the diapause percentage and energy reserves of *T. brassicae*, excepting glycogen contents. The maximum and minimum diapause percentage was observed under green and white light in asexual, and under white and green light in sexual strain. The data showed that the sexual strain had lower lipid and protein levels than the asexual strain, except when exposed under white light. The diapause percentage in the sexual strain was higher than in the asexual strain under all light wavelengths, and the reaction of parasitoids toward light wavelengths was different in the two strains. Therefore, *Wolbachia* can cause a different reaction to light wavelengths in both diapause percentage and pattern of the parasitoid. These results should be considered to improve mass-rearing and long-term storage of this parasitoid.

Keywords: Egg parasitoid, Mass-rearing, Maternal effect.

INTRODUCTION

Diapause is a critical component of unfavorable conditions for most insects in temperate regions (Denlinger, 1991). It represents a complex process characterized by physiological and behavioral features (Kostal, 2006). It is known that most of the *Trichogramma* species show facultative diapause during the prepupal stage when they encounter low temperatures in nature or laboratory conditions.

Low temperatures are one of the most important factors that induce diapause in *Trichogramma* species (Zaslavski and Umarova, 1981; Keller, 1986; Sorokina and Malesnnikova, 1987; Garcia *et al*., 2002). While the maternal influence of photoperiod on the diapause induction of many *Trichogramma* wasps has been studied (Zaslavski and Umarova, 1990; Garcia *et al*., 2002; Ma and Chen, 2006; Ivanov and Reznik, 2008; Pizzol and Pintureau, 2008; Reznik *et al*., 2011; Voinovich *et al*., 2013), no studies have investigated the effect of light wavelengths on the diapause induction of *Trichogramma* wasps.

On the one hand, *Trichogramma* is positive light-dependent wasps (Costas, 1951) and, on the other hand, light wavelengths have an influence on insect

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behavior (Ripfel and Becker, 1982). Experimental works have shown that color perception exists in insects and they are sensitive to light color ranging from ultraviolet to red, being especially responsive to the shorter wavelengths of the visible spectrum and UV light (Hamdorf *et al*., 1971). When insects are exposed to light, they may show positive or negative phototaxis to the source of light, they may increase or decrease the rate of their general activity in response to the light wavelengths (Bertholf, 1940). In the case of diapause, insects showed a different response to the light wavelengths (Williams *et al*., 1965; Harris *et al*., 1967; Beck, 1980; Ismail *et al*., 2011) thus we assumed that the percentage of diapause in *Trichogramma* may also be altered by light wavelengths.

T. brassicae is known as the most abundant species that parasitize a wide range of economically important agricultural and forest Lepidopteran pests in Iran (Poorjavad, 2011). Similar to most *Trichogramma* species, *T. brassicae* has two reproductive modes: arrhenotoky, which produces sexual wasps, and thelytoky, which produces asexual wasps. Most of the thelytokous *Trichogramma* wasps are infected by an endosymbiont bacterium, which is called *Wolbachia* (Stouthamer and Werren, 1993).

The aim of this study was to check two hypotheses. The first one was that white lights might increase the percentage of diapause in *T. brassicae*, which is followed by more energy reserves in females reared under this light. The second hypothesis was that there should be a difference in response of sexual and asexual strains to various light wavelengths, which is followed by different energy reserves in those females. Since there was no information regarding energy reserves of the maternal generation of *Trichogramma* wasps who were ovipositing progenies for entering diapause, this research was carried out to compare energy reserves of newly emerged females reared on different light wavelengths.

MATERIALS AND METHODS

Insects

Both sexual and asexual *T. brassicae* strains used in this study were identified by Dr. N. Pourjavad (Isfahan University of Technology, Isfahan, Iran). Both of these were collected from the North of Iran (south of the Caspian Sea) from eggs of *Ostrinia nubilalis* (Hubner) (Lep: Crambidae), and obtained from a culture maintained at the Ecology and Behavior Laboratory, University of Tehran. They were reared on eggs of *E. kuehniella* Zeller (Lep: Pyralidae) under constant Laboratory conditions (20±1°C, 16L:8D photoperiod and 70±5% RH) for many generations (more than 100 generations).

Experimental Design for Diapause Induction

Five cardboard paper strips (8×1 cm) with about 200-300 *E. kuehniella* eggs, which were less than 24 hours old, were used for each strain and light wavelength. The eggs of Mediterranean flour moth were subjected for 4 hours to parasitization by 100 asexual and 200 sexual *T. brassicae* in plastic cylinders (approximately 18 cm tall×8 cm in diameter) with an opening that was covered with a mesh in order to allow for ventilation. Then, these cards with parasitized host eggs were individually placed in glass tubes and incubated at 20±1°C, 70±5% RH and 10L: 14D photoperiod under five different light wavelengths, where white light (5,000~10,000 K) was the control and the other four treatments were as follows: blue (455~47 5nm), green (515~535 nm), orange (585~595 nm), and red (620~630 nm). Introduction of the best light wavelengths for each strain could improve diapause percentage of *T. brassicae*. The glass tubes were positioned in boxes with white background that had light barriers on both sides. All white boxes were placed in a bigger black box. At the day of mass emergence, 40 cardboard paper strips (5×1

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cm) with about 50 host eggs per card were offered to emerged females (24 hours old) of each strain and exposed to light wavelength for 2 hours in plastic cylinders, individually. In all treatments, oviposited females were removed after 2 hours, and the parasitized eggs were stored at 70±5% RH, in absolute darkness, and 10±1°C. Parasitized eggs were transferred to control temperature (20°C) after two months to speed up the development and to facilitate the emergence of living individuals. In the end, the emergence rate of both *T. brassicae* strains was measured by dividing the number of parasitized eggs with an emergence hole with the total number of parasitized eggs. We did not include cardboard paper strips with less than 10 parasitized *E. kuehniella* eggs in our experiments.

Sample Preparation to Determine Energy Reserves

About 100 newly emerged females (0.001±0.0001 g) were used to quantify the content of proteins, glycogen, carbohydrate and total lipids of maternal *T. brassicae* generation, which were reared at 20±1°C, 70±5% RH and 10L:14D under different light wavelengths, with three replications. Protein, glycogen, carbohydrate and lipid levels were determined using common biochemical analysis protocols (Foray *et al.*, 2012). First, *T. brassicae* were weighed and homogenized in 90 µL of aqueous lysis buffer solution [100 mm KH_2PO_4, 1 mm DiThioThreitol (DTT) and 1 mm EthyleneDiamineTetraacetic Acid (EDTA), pH 7.4]. Then, the samples were centrifuged at 180 g for 10 min at 4 ºC.

Protein Determination

The protein concentration was determined as described by Bradford (1976). At the beginning, 10 µL of supernatant was mixed with 500 µL Coomassie Brilliant Blue G-250. Then, protein concentration was measured at 595 nm after incubation at room temperature for 15–20 minutes, using a dilution-series of Bovine Serum Albumin (BSA) as a standard. Each measurement was performed in triplicates.

Glycogen Determination

To determine the glycogen, carbohydrate, and lipid contents, 10 µL of lysis buffer solutions, 10 µL of 2% Na_2SO_4 and 750 µL of chloroform: methanol (1:2) were added to the first supernatant. Then, the samples were centrifuged for 15 minutes at 180×g and 4°C to remove glycogen from the supernatant, which was transferred into a new tube to determine the carbohydrate and lipid contents. The pellet was used to determine the glycogen content. After vigorous vortexing and centrifuging for 5 min at 16,000×g and 4°C, 500 µL of anthrone reagent was added to the pellet, followed by 15 min of incubation at 90°C in a water bath. The glycogen content was then determined by measuring the absorbance of the sample at 625 nm with glycogen as the standard. Each measurement was performed in triplicates.

Carbohydrate Determination

To determine the carbohydrate contents, 150 µL of supernatant was transferred to a microtube and was evaporated for approximately 30 minutes at room temperature. Then, it was mixed with 240 µL of anthrone reagent and was heated for 15 minutes at 90°C in a water bath. The carbohydrate contents were then determined by measuring the absorbance of the sample at 625 nm with glucose as the standard (van Handel, 1965; 1985a). Each measurement was performed in triplicates.

Lipid Determination

The cholesterol was used as the standard to determine the total amount of lipids of each

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treatment according to the vanillin assay procedure (van Handel, 1985b). For the assay, 100 µL of the supernatant was transferred into a borosilicate microplate well and heated at 90°C until complete solvent evaporation. Then, 10 µL of 98% sulfuric acid was added and the microplate was incubated at 90°C for 2 minutes in a water bath. After cooling the microplate on ice, 190 µL of vanillin reagent was added. The plate was homogenized, incubated at room temperature for 15 minutes, and its absorbance was measured spectrophotometrically at 525 nm. Each measurement was performed in triplicates.

Statistical Analysis

The data was transformed by Johnson transformation *[(-2.06231+1.33263)×Ln (X+39.2659)/(100.991-X)]* and the effects of strain (*Wolbachia* infection) and light wavelengths on the percentage of diapause of *T. brassicae* and energy compartments were compared using two-way ANOVA. Data was subjected to two-way Analyses Of Variance (ANOVA), followed by Tukey test to separate means when differences were significant at $P< 0.05$. All analyses were performed with SAS 9.1.3. Version.

RESULTS AND DISCUSSION

The results revealed that strain (F= 339.42; df= 1, 390; P< 0.0001), light wavelengths (F = 7.20; df= 4, 390; P< 0.0001) and interaction between strain and light wavelengths (F= 24.06; df= 4, 390; P< 0.0001) had statistically significant influence on the diapause percentage of *T. brassicae*.

White light caused the highest percentage of diapause (85.88±1.07%), followed by blue (83.74±0.95%), red (82.35±1.13%), orange (82.00±1.79%), and green (79.66±1.46%) lights in the sexual strain, whereas the highest percentage of diapause occurred under green light (73.02±1.46%), followed by blue (69.01±2.16%), red (66.99±1.44%), orange (63.44±1.51%), and white (41.19±2.16%) lights in the asexual strain (Figure 1). According to the results, sexual and asexual *T. brassicae* strains responded differently when they were exposed to different light wavelengths.

Thus, we concluded that diapause was greatly influenced by light wavelengths in both strains, although more diapause occurred in sexual *T. brassicae* compared to

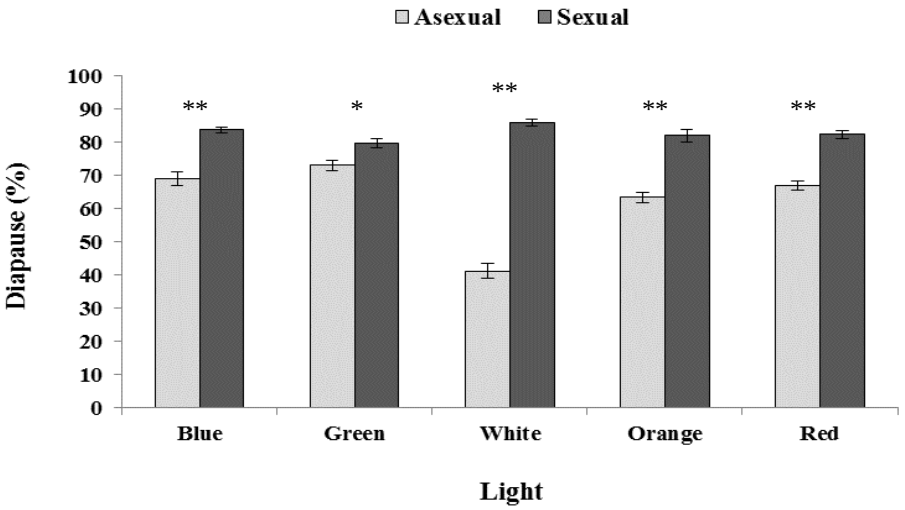

Figure 1. Effects of strain (*Wolbachia* infection) and different light wavelengths on the diapause percentage of *T. brassicae*. The different light wavelengths include blue (455~475 nm), green (515~535 nm), cool white (5,000~10,000 K), orange (585~595 nm) and red (620~630 nm). There is a statistically significant difference (P< 0.0001) according to the two-way ANOVA.

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the asexual strain. Similar results were reported in *Nasonia vitripennis* (Walker) (Hym: Pteromalidae) (Bordenstein and Werren, 2000) and *T. oleae* Voegele and Pointel (Pintureau *et al*., 2002).

Similar to the sexual strain observed in this research, Ismail *et al*. (2011) showed that the highest diapause percentage occurred under white colors, which was followed by green in *Tetranychus urticae* Koch (Acari: Tetranychidae), but none of them entered diapause under red color. Bunning and Joerrens (1960) indicated that blue light induced diapause in *Pieris brassicae* L. (Lep: Pieridae) during early photophase, while diapause occurred later under red light. Beck (1980) indicated that the most effective wavelengths for diapause induction are between 400-550 nm for most species. According to his experiment, red light works very much like absolute darkness in diapause induction, whereas when the pupae of *Antheraea pernyi* Guer (Lep: Attacidae) were exposed to wavelengths shorter than 560 nm, it caused complete breaking of diapause (Williams *et al*., 1965). Harris *et al*. (1967) indicated the same results: *Anthonomus grandis* Boh (Col: Curculionidae) could not enter diapause when it was exposed to wavelengths between 485 and 560 nm.

Lipid, Protein, Carbohydrate, and Glycogen Concentration

Most energy reserves in the maternal generation of *T. brassicae* were in the form of lipids (Figure 2). The light wavelengths, strain and their interaction had a significant effect on the lipid concentration of *T. brassicae* (Table 1). Lipids are usually the main energy reserve for overwintering of insects (Yaginuma and Yamashita, 1978; Beenakkers *et al*., 1981; Storey and Storey, 1983). It seems that accumulation of lipids in insects is to support their energy demands during harsh conditions (Bashan *et al*. 2002). Amiri and Bandani (2013) reported that lipids were accumulated by prediapause Sunn pest bugs for their metabolic needs during diapause. Data showed that the sexual strain had lower lipids than the asexual strain, with the exception of white light (Figure 2-a).

Similarly, the light wavelengths, strain, and their interaction had a significant effect on the protein concentration of *T. brassicae* (Table 1). According to the results, asexual *T. brassicae* had higher protein levels than sexual *T. brassicae*, with the exception of white light (Figure 2-b).

Sexual *T. brassicae* had higher carbohydrate than asexual *T. brassicae* in all the light wavelengths (Figure 2-c). Light wavelengths and strain had significant effects on the carbohydrate concentration (Table 1), whereas the interaction of light wavelengths and strain did not have a significant effect on the carbohydrate concentration of the parasitoid.

Glycogen content was the lowest energy of reserves in *T. brassicae*. According to the data analysis, sexual *T. brassicae* had higher glycogen than asexual *T. brassicae*, with the exception of blue and orange lights (Figure 2-d). Light wavelengths, strain, and their interaction did not show significant effect on the glycogen concentration of *T. brassicae* (Table 1), which indicates that this parasitoid may not use it as an energy reserve for diapause. However, previous studies have recognized the influence of accumulation of low molecular weight sugars and polyols at the beginning of diapause in many species of insects (Han *et al*., 2005; Khani *et al*., 2007; Han *et al*., 2008).

The results demonstrated that carbohydrates and glycogen may convert to lipids in the maternal generation of both sexual and asexual *T. brassicae*. Numerous studies have stated that carbohydrates were converted into lipids within the insects' fat body (Hines and Smith, 1963; Bailey, 1975; Venkatesh and Morrison, 1980; Inagaki and Yamashita, 1986; Briegel, 1990). Zhou *et al*. (2004) have reported that the fat body of *Aedes aegypti* (L.) (Dip: Culicidae) females have a high capacity for the synthesis of

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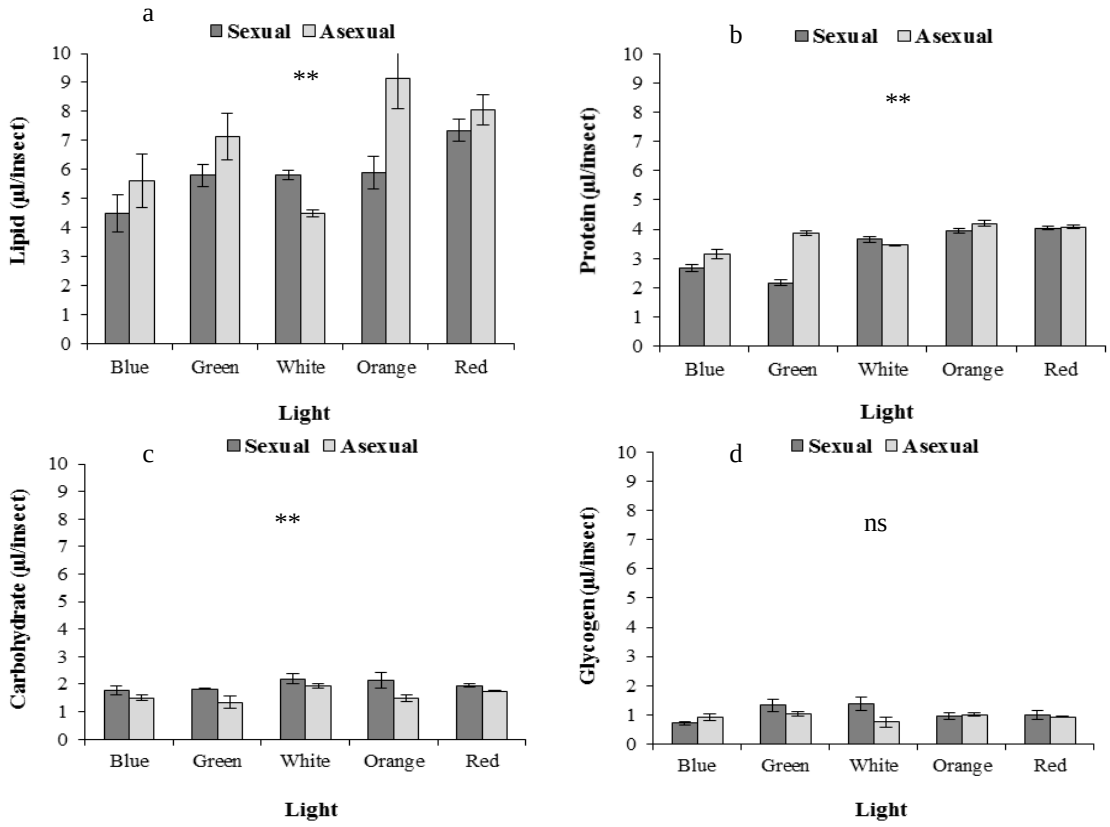

Figure 2. Effects of strain (*Wolbachia* infection) and different light wavelengths on the contents of (a) Lipid, (b) Protein, (c) Carbohydrate and (d) Glycogen (µg insect^{-1}) of *T. brassicae* females. The light wavelengths are defined under Figure 1 and text. There is a statistically significant difference (P< 0.0001) according to the two-way ANOVA.

Table 1. Effects of the strain (*Wolbachia* infection), light wavelengths and the interaction of strain and light wavelengths on lipid, protein, carbohydrate and glycogen contents (µg insect^{-1}) of *T. brassicae*.

Energy reserves	Source of variation	*df*	Mean square	*F*
Lipid	Strain	1	7.70	6.73*
	Wavelength	4	9.56	8.36**
	Strain×Wavelength	4	3.93	3.44*
	Error	20	1.14	
Protein	Strain	1	1.48	50.65**
	Wavelength	4	1.82	62.31**
	Strain×Wavelength	4	0.81	27.55**
	Error	20	0.03	
Carbohydrate	Strain	1	1.06	16.46**
	Wavelength	4	0.21	3.33*
	Strain×Wavelength	4	0.06	0.96^{ns}
	Error	20	0.06	
Glycogen	Strain	1	0.17	3.01^{ns}
	Wavelength	4	0.11	1.99^{ns}
	Strain×Wavelength	4	0.16	2.74^{ns}
	Error	20	0.06	

*, **: Significantly difference at 5 and 1% probability level, respectively.

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lipids than glycogen, and this may explain the higher content of lipids compared to glycogen in the maternal generation of both sexual and asexual *T. brassicae*. As glycogen is rapidly catabolized (Rambabu and Rao, 1994; Sancho *et al*., 1998), the reduction in glycogen contents may be a result of energy generation for flight. It seems that the high amount of proteins in comparison with carbohydrate and glycogen is due to using protein as an energy source, which has been reported in some insects such as *Glossina* spp. (Dip: Glossinidae) and *Leptinotarsa decemlineata* (Col: Chrysomelidae) (Niaqi *et al*., 1992).

In conclusion, our results documented some behavioral and biochemical adaptations for maternal generation of sexual and asexual *T. brassicae*. A large amount of lipids and proteins accumulated prior to oviposition with overwintering abilities. For the first time a study showed how the form of energy reserves changes under the influence of different light wavelengths in the maternal generation of *T. brassicae,* which were ovipositing progenies for entering diapause. According to the results, lipids were the most abundant (about 50%) energy reserves in all the treatments. It seems that mothers accumulate lipids to protect their progenies from cold and adverse effects.

Light wavelengths had a significant effect on the diapause percentage of the parasitoids that were affected by *Wolbachia* infection. Furthermore, there was a relation between the diapause percentage of progenies of females developed under different light wavelengths and the amount of energy reserves of ovipositing females. On the one hand, the lowest lipid content under white light in comparison with other lights was followed by a reduction in the diapause percentage in the asexual strain. On the other hand, the lowest protein content under green light in sexual females was followed by the lowest diapause percentage in these wasps in comparison with other wavelengths. The maternal generation accumulated more lipid as they developed under the short photoperiod and 20°C, which induced diapause in their progenies (the same condition as that of late summer or onset of autumn). We assume that they probably transfer the lipids to the eggs of their diapausing generation. Finally, we can introduce the white light as a suitable wavelength for rearing sexual *T. brassicae*, and green light for asexual *T. brassicae*.

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آیا آلودگی به ولباکیا، درصد دیاپوز و میزان ذخایر انرژی زنبور پارازیتوییید *Trichogramma brassicae* را در پاسخ به طیف نوری تغییر می‌دهد؟

س. رحیمی کلده، ع. بندانی، و ا. عاشوری

چکیده

در این پژوهش، اثر قرارگیری در طیف‌های مختلف نوری بر تغییرات ذخایر انرژی پارازیتوییدهای ماده آلوده به ولباکیا و غیرآلوده *Trichogramma brassicae* و نیز درصد دیاپوز نتاج آن‌ها مورد مطالعه قرار گرفت. طول دوره نوری دارای اثر مادری در القای دیاپوز در زنبورهای پارازیتوییید جنس *Trichogramma* است اما تاکنون اثر طیف‌های نوری بر دیاپوز آن‌ها مطالعه نشده است. نسل مادری تحت طیف‌های نوری: آبی (۴۷۵-۴۵۵ نانومتر)، سبز (۵۳۵-۵۱۵ نانومتر)، نارنجی (۵۹۵-۵۸۵ نانومتر)، قرمز (۶۳۰-۶۲۰ نانومتر) و سفید (۵۰۰۰ تا ۱۰۰۰۰ کلوین) پرورش یافت و به ماده‌های ۲۴ ساعته اجازه داده شد تا در تخم‌های *Ephestia kuehniella* تخم‌گذاری کنند. نسل دیاپوزگذران در تاریکی مطلق و دمای ۱۰ درجه سلسیوس به مدت دو ماه نگهداری شد. بر اساس نتایج به دست آمده، آلودگی به ولباکیا و قرارگیری نسل مادری تحت طیف‌های مختلف نوری، بطور معنی‌داری در القای دیاپوز و نیز میزان ذخایر انرژی *T. brassicae* به استثنا میزان گلیکوژن اثرگذار است. حداکثر و حداقل درصد دیاپوز در جمعیت آلوده در طیف‌های سبز و سفید و در جمعیت غیرآلوده در طیف‌های سفید و سبز مشاهده شد. ماده‌های غیرآلوده در تمامی طیف‌های نوری به استثنا طیف نوری سفید، لیپید و پروتیین کمتری نسبت به ماده‌های آلوده داشتند. درصد دیاپوز در جمعیت غیرآلوده بیش از آلوده در تمامی طیف‌های نوری بود. دو جمعیت واکنش کاملاً متفاوتی را در برابر طیف‌های نوری نشان دادند. ولباکیا سبب بروز پاسخ متفاوت دو جمعیت از لحاظ درصد و الگوی دیاپوز شد. نتایج حاصل از این پژوهش می‌تواند نقش کلیدی در بهبود روند تولید انبوه و ذخیره بلندمدت این عامل کنترل بیولوژیک داشته باشد.

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