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ORIGINAL ARTICLE

The effect of *Wolbachia* on diapause, fecundity, and clock gene expression in *Trichogramma brassicae* (Hymenoptera: Trichogrammatidae)

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Abstract
The short day lengths of late summer in moderate regions are used to induce diapause in various insects. Many studies have shown the maternal effect of photoperiod on diapause induction of *Trichogramma* wasps, but there is no study to show the relationship between photoperiodic regimes and clock genes in these useful biological control agents. Here, we investigated the role of photoperiods on diapause, fecundity, and clock gene expression (*clk*, *cyc*, *cry2*, *per*, and *timeout*) in asexual and sexual *Trichogramma brassicae* as a model insect to find any differences between two strains. Asexual strain was infected by *Wolbachia*, an endosymbiont bacterium. The diapause percentage was significantly higher under short days (8 h in sexual and 12 h in the asexual *T. brassicae*), although the diapause percentage of the sexual strain was significantly higher than the asexual one in all the photoperiods. The ANOVA revealed no significant changes between different photoperiods in the clock gene expression in the sexual strain but significant photoperiodic changes in *clk*, *cyc*, and *timeout* in the asexual strain. Our results showed that the mRNA levels of clock genes of asexual *T. brassicae* were significantly lower than those of sexual strain. The fecundity was significantly higher in the asexual strain. These results suggest that *Wolbachia* infection makes disturbance on the clock gene expression which consequently reduces the percentage of diapause but increases the fecundity in asexual *T. brassicae*.

Keywords Clock genes · Diapause · Fecundity · Photoperiod · *Trichogramma brassicae* · *Wolbachia*

Introduction

Environmental conditions affect all aspects of animal's life such as ecology, biology, physiology, and genetics. Among them, photoperiod is a much more reliable and stable seasonal signal since similar geographic locations will experience the same length of day on a specific day each year (Meuti and Denlinger 2013). Diapause is one of the most predominant photoperiodic responses in insects (Denlinger 2002; Saunders et al. 2002; Reznik et al. 2011).

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Several studies have shown the correlation between clock genes and photoperiodic diapause in insects (Ikeno et al. 2010; Bertossa et al. 2014). Rhythmic expression of clock genes regulates about a 24-h oscillation of the so-called circadian clock. In *Drosophila*, the major players in the clock are *cycle* (*cyc*), *clock* (*clk*), *period* (*per*), and *timeless* (*tim*) (Hardin 2005). The CYC/CLK heterodimer acts as a positive regulator of the transcription of *per*/*tim* during the day and early evening and other output genes, whereas the PER/TIM heterodimer acts as a negative regulator of CYC/CLK activity in the late night (Meyer et al. 2006). Unlike most insect orders, hymenopterans, including ants, bees, and wasps have only *timeout* instead of *timeless* which shows plastic expressions related to adaptations to ecology (Gu et al. 2014). *Cryptochrome* (*cry*), the other gene that plays a crucial role in circadian clocks in animals is divided to two types in insects: *Drosophila*-type *cry* (*cry1*) and mammalian-type *cry* (*cry2*) (Sandrelli et al. 2008). In *Drosophila*, *cry1* regulates the circadian clock in a light-dependent fashion whereas in other insects including the monarch butterfly that have both a mammal-like and a

Drosophila-like version of *cryptochrome*, regulates the circadian clock in a light-independent fashion, and acts as a powerful repressor of *clk* and *cyc* (Yuan et al. 2007).

In this study, we investigated the role of photoperiod on the percentage of progeny diapause, fecundity, and the expression of *clk*, *cyc*, *cry2*, *per*, and *timeout* mRNA in *Wolbachia*-infected (asexual) and *Wolbachia*-uninfected (sexual) *Trichogramma brassicae* strains to find the relationship of diapause, fecundity, and clock gene expression. In most studied *Trichogramma* species, photoperiodic conditions of pupal and adult development have maternal effects on diapause of their progeny (Ivanov and Reznik 2008). Facultative winter diapause in *Trichogramma* wasps occurs at the prepupae stage (Reznik 2011). Among members of this genus, *T. brassicae* is the dominant species in Iran that has been reared and released for the biological control of some Lepidopteran key pests such as *Chilo suppressalis* (Lep: Pyralidae), *Ostrinia nubilalis* (Lep: Crambidae), and *Pieris brassicae* (Lep: Pieridae) (Poorjavad 2011).

Trichogramma wasps have two reproductive modes, arrhenotoky and thelytoky. The second one is caused by *Wolbachia* (Stouthamer and Werren 1993), an endosymbiotic bacterium from family Rickettsiaceae (Pinto and Stouthamer 1994). *Wolbachia* is an obligate intracellular parasite transmitted maternally from infected females to their progeny which is present in many different organisms such as isopods, nematodes, acari, and about 17–76% of all insect species (Yue et al. 2009). They manipulate host reproductive systems through a variety of strategies including cytoplasmic incompatibility (O'Neill and Karr 1990; Werren 1997), male killing (Hurst et al. 2000), feminization (Rousset et al. 1992), and parthenogenesis in some parasitoid wasps such as *Trichogramma* spp. (Stouthamer and Werren 1993).

Asexual strain would increase the rearing productivity in commercial insectaries by producing only female offspring. Many studies have been conducted to compare asexual and sexual strains to understand which of them is useful as biological control agents. However, the results are controversial. Some studies show positive effects of *Wolbachia* infection on longevity, dispersal capacity, and parasitism rate of *Trichogramma* wasps (Girin and Bouletreau 1995; Pintureau et al. 2002; Stolk and Stouthamer 1996), while others show negative effects of *Wolbachia* infection on the number of progeny, emergence rate and lengthening of the life cycle of *Trichogramma* spp. (Hohmann et al. 2001; Huigens et al. 2004; Tagami et al. 2001). According to Huigens et al. (2004), asexual *Trichogramma kaykai* showed lower survival rates in comparison with sexual ones. However, some studies show no essential effect of *Wolbachia* infection on the proportion of emergence in diapausing individuals or on their fecundity (Pintureau et al. 2003). Some host species such as *Asobara tabida* cannot reproduce if *Wolbachia* are eliminated with antibiotics, and some other host species like filarial nematodes cannot even survive, without *Wolbachia* infection (Werren 2003).

There is a possibility that *Wolbachia* interfere diapause of *T. brassicae* through changing their behavior (Kishani Farahani et al. 2015), physiology (Stouthamer et al. 1999), or genetics (Huigens et al. 2004). Kishani Farahani et al. (2015) reported that asexual *T. brassicae* females have shown reduction in host discrimination capacity. This reduction of host discrimination in asexual females has been explained by reduced ability to perceive signals or poor integration of information at the neurological level.

The present study was thus aimed to investigate the effects of *Wolbachia* infection on the diapause, the expression pattern of clock genes, and the fecundity using *Wolbachia*-infected asexual and *Wolbachia*-uninfected sexual strains. We found that the *Wolbachia*-infected asexual strains showed significantly lower diapause incidences, lower clock gene expression, and higher fecundity. Although the pattern of photoperiodic variation of diapause incidence and fecundity was basically similar between the two strains, the clock gene expression pattern was different. The results are discussed in relation to the *Wolbachia* infection.

Materials and methods

Trichogramma culture

Two colonies of *Trichogramma brassicae*, *Wolbachia*-infected (asexual) and *Wolbachia*-uninfected (sexual) strains were used in this study. Both of which were collected from north of Iran and reared on the fresh eggs (less than 24 h old) of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lep: Pyralidae), for many generations (actually more than 100 generations). All experiments were conducted with wasps reared at 20 ± 1 °C, 70 ± 5% r.h., and L16:D8.

Detection of *Wolbachia* in asexual *T. brassicae*

To test whether *T. brassicae* strain was infected by *Wolbachia* or not, we used polymerase chain reaction with general primers for the *wsp* gene (Table 1). The total DNA from 50 frozen *T. brassicae* was extracted in a mixture of 30 ml of 5% Chelex solution and 2 ml of proteinase K (25 mg/ml), at 60 °C for 2 h followed by 15 min at 94 °C. Reaction cocktails for PCR amplification consisted of 2.5 μl of buffer 10× PCR, 2.5 U of Taq polymerase, 2.5 mM MgCl2, 0.75 mM dNTPs, 1.5 μl of *wsp* forward primer, 1.5 μl of *wsp* reverse primer, and 10 ng of DNA template in a final volume of 25 μl (Table 2). The PCR conditions were 94 °C for 3 min followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, and finally 72 °C for 10 min. The size of the PCR product was determined using standard agarose gel. The

Table 1 Oligonucleotide primers used in this study for sequencing

Primer	Nucleotide sequence (5′ to 3′)
F-*clk*	AGAAGAAGCGGCGGGAYCARTTYAA
R-*clk*	GGTCTGCAGCCAGATCCAYTGYTG
F-*cyc*	GACAGTGCTGCGAATGGCNGTNCARCA
R-*cyc*	CGCTGATCGACGAACAGGAAYTTNCCNTC
F-*cry2*	TGTTCGTGATCCGAGGACARCCNGCNGA
R-*cry2*	CACTGGGATGTACTTTCGGATGWARTCNCCRTT
F-*per*	GTCCAACGATCAGAAGGCCA
R-*per*	GGATTTTCGCGTGGAATCGG
F-*timeout*	CGGATAAACGCGAGCACAAG
R-*timeout*	CGATGATTTTGGACCGTCGC
F-*EF-1-alpha*	GCCCGAGCCGTTTAGGTTAT
R-*EF-1-alpha*	GCCTCAGAGTATGGTGGCTC

migration of the PCR product was realized using standard agarose gel and the expected size was consistent with that expected for *Wolbachia* (about 500 bp).

Diapause induction

For the first step, 24 cardboard paper strips (each with 200–300 *E. kuehniella* eggs) were submitted to 4 h for parasitization by 500 asexual and 1000 sexual *T. brassicae* (both 24 h old) in transparent, plastic cylinders (approximately 18 cm tall × 8 cm diameter) with an opening place that covered with a mesh in order to ventilation. The number of sexual *T. brassicae* was doubled in order to take into account males. High-quality eggs of *E. kuehniella* glued on separate cardboard paper strips (8 cm × 1 cm) using non-toxic and water-soluble glue (Canco) for each *T. brassicae* strains. We sprayed 20% honey water on the wall of cylindrical to feed *T. brassicae* adults. The cards with parasitized host eggs (the maternal generation) were individually placed in glass tubes and incubated at the same temperature conditions (20 ± 1 °C) and 70 ± 5% r.h. but under different 24-h light-dark (L:D) conditions (4:20, 8:16, 12:12, 16:8, 20:4, 24:0).

Table 2 Oligonucleotide primers used in this study for PCR

Primer	Nucleotide sequence (5′ to 3′)
F-*wsp*	TGGTCCAATAAGTGATGAAGAAAC
R-*wsp*	AAAAATTAAACGCTACTCCA
F-*clk*	GACAAATCGACGGTCCTGAA
R-*clk*	TCGAGGGCCTCTAAAGTCAA
F-*cyc*	CAGTGCACGGGCTATCTGAA
R-*cyc*	TGTTGGTGCTGACGGTGG
F-*cry2*	CGTGCCGACTTTGAGGAGAA
R-*cry2*	CGGCTGTCTTGTAACTCGCT
F-*per*	ATCAATGCTTCGCCAATGCCAA
R-*per*	CCGGCATGTAGTAAACCGGA
F-*timeout*	CCTGGTGGAGCAGATGAGAA
R-*timeout*	TGCGCAAGTGTTTCGGATCA
F-*EF-1-alpha*	CGACAAGCGTACCATTGAGA
R-*EF-1-alpha*	ACGCTCAGCCTTCAGTTTGT

At the day of mass emergence of the maternal generation, 120 cardboard paper strips, with *E. kuehniella* eggs (ca. 50 eggs per card) were offered to 2 h for parasitization in a plastic cylinders with emerged wasps (24 h old) from each photoperiod. For all photoperiods, parasitization was conducted at the same time of the day: between 10:00 and 12:00, (3–5 h after the light-on). In all replicates, females were removed after 2 h oviposition and the parasitized eggs were kept at 14 ± 1 °C, 70 ± 5% r.h., and absolute darkness.

Adults emerged after about 45–50 days of parasitization at 14 ± 1 °C. After the mass emergence, all the parasitized host eggs were dissected, and the number of living diapausing prepupae and non-diapausing individuals (emerged adults, few dead adults, dead or alive pupae, and dead prepupae inside the host chorion) were counted. Each alive prepupa was assumed to be diapausing individual at this temperature. The percentage of diapausing individuals was separately calculated for each card.

The effect of photoperiod on parasitization by *T. brassicae*

In order to evaluate the effect of *Wolbachia* infection and photoperiod on parasitization of *E. kuehniella* eggs by *T. brassicae*, we measured the fecundity of 20 females of asexual and sexual *T. brassicae* under laboratory conditions (20 ± 1 °C, 70 ± 5% r.h. and photoperiods of L:D = 4:20, 8:16, 12:12, 16:8, 20:4, and 24:0 h). For each replicate, one paper card (5 cm × 1 cm) with about 50 *E. kuehniella* eggs was exposed to one 24 h old *T. brassicae* female which developed and were kept under different photoperiod regimes with a small droplet of 20% honey water. After 48 h, all the females were separated from *E. kuehniella* eggs. This experiment was carried out under laboratory conditions at 20 ± 1 °C, L16:D8, and 70 ± 5% r.h. One week after parasitization, the number of parasitized (black) eggs per card were counted.

Specimen collection and light conditions

There were four replicates in each photoperiod for each strain. Four cardboard paper strips (8 cm × 1 cm) each containing 200–300 Mediterranean flour moth eggs glued onto each card were exposed for 4 h to parasitization by about 500 asexual and 1000 sexual *T. brassicae* (both 24 h old and ready to oviposit) in plastic cylinders. Then ovipositing females were separated of host eggs

and each paper card placed in a test tube separately and incubated at the same temperature conditions (20 ± 1 °C), but under different photoperiods (L:D = 4:20, 8:16, 12:12, 16:8, 20:4, and constant light). The glass vials were checked daily to remove newly emerged *E. kuehniella* larvae because of their cannibalism behavior. Both asexual and sexual adults emerged about 17–18 days after parasitization. Adults of *T. brassicae* were kept for 1 day at the same photoperiods and collected 2 h after light-on in the second day. Finally, they were quickly frozen in liquid nitrogen after sampling and stored at − 80 °C until use (Fig. 1).

Sequencing and alignments

To identify *clk*, *cyc*, *cry2*, *per*, *timeout*, and *elongation factor 1-alpha* (*Egf1*) sequences of *T. brassicae*, total RNA was extracted from the whole body of *T. brassicae* with TRIzol (Invitrogen, Carlsbad, CA). Total RNA was treated with DNase I to remove potentially contaminating DNA. First-strand cDNA synthesis was performed from 500 ng of total RNA with random 6 mers using Primescript RT reagent kit (Takara Bio, Ohtsu, Japan). We designed primers for *Egf1*, *per*, and *timeout* based on sequences of *Trichogramma pretiosum* (*Egf1*: XM_014381510.1, *per*: XM_014378036.1, and *timeout*: XM_014379200.1). Also, we used degenerate primers for amplification of *clk*, *cyc*, and *cry2* genes of *T. brassicae* (Table 1). Using these primers, we performed PCR with a condition of 30 s for denaturation at 95 °C, 60 s for primer annealing at 58 °C, and 1 min for extension at 72 °C for 35 cycles with EmeraldAmp® GT PCR Master Mix (Takara Bio, Ohtsu, Japan). PCR conditions were optimized until a single PCR product was yielded. The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN) and sequenced with Applied Biosystems 3500/3500xL Genetic Analyzer (Hitachi Corp, Tokyo, Japan). Sequences were analyzed by the GENtle V1.9.4. software and have been deposited in the GenBank database (Table 3).

Measurement of mRNA levels

Quantitative real-time PCR was performed to measure mRNA levels using CFX96 Connect™ Real-Time System (BIO RAD) using KAPA SYBR® FAST qPCR Master Mix (2X), (KAPA BIOSYSTEMS, Tokyo, Japan) with specific primers that are shown in Table 2 with a condition of 10 min at 95 °C, 39 cycles of 15 s at 95 °C, 60 s at 60 °C, and 60 s at 95 °C. In order to calculate mRNA content of respective genes, standard curves were plotted using seven dilutions (10^2–10^8 copies) of amplified cDNAs and included in each PCR run. A melting curve was recorded at the end of the PCR amplification to confirm that a unique transcript product had been amplified. Candidate gene expression was normalized by the geometric mean of the expression level of housekeeping gene (*Egf1*) as described previously (Moriyama et al. 2008). Four independent experiments were used to calculate the mean ± SEM.

Statistical analyses

All data were checked for normality using the Minitab 16. statistical software and variances were checked for equality. The effect of photoperiod on the percentage of progeny diapause, fecundity, and gene expression were checked by a one-way ANOVA and the Tukey honestly significant difference (HSD) test for each sexual and asexual strain, separately. The effect of *Wolbachia* infection on the percentage of progeny diapause, fecundity, and gene expression were checked by *t* test. All calculations were performed using the SAS software (Version 9.4.).

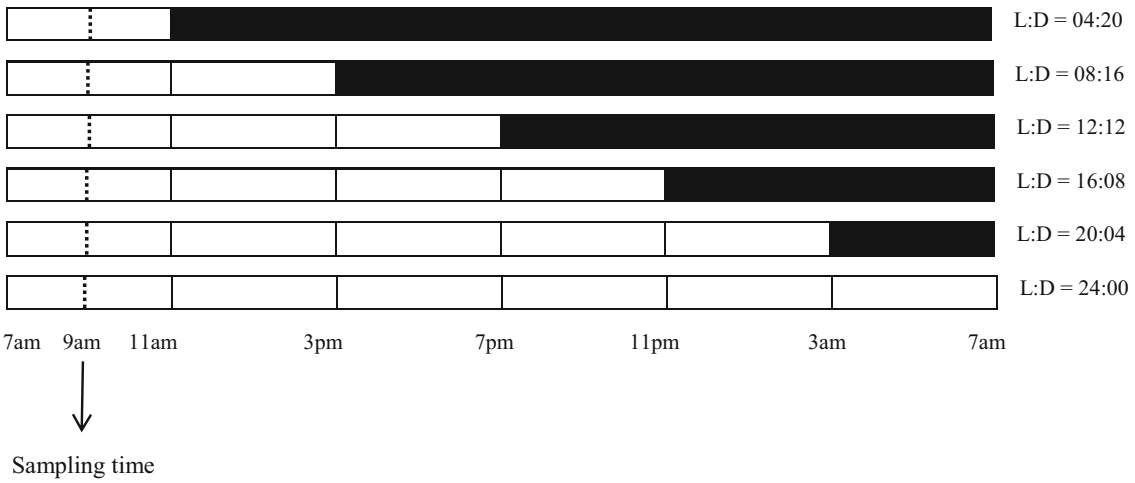

Fig. 1 The experimental conditions in which both asexual and sexual *T. brassicae* reared under different number of daylight hours of L:D = 4:20, 8:16, 12:12, 16:8, 20:4, and 24:0 h at 20 ± 1 °C with r.h. 70 ± 5%

Table 3 GenBank accession no. for the cDNA fragment of the clock genes deposited in the GenBank database and used to design the species specific primers

Gene	Accession number
clk	KU748130
clk	KY064170
cyc	KU748129
cry2	KY064171
per	KU748131
timeout	KU748132
EF-1-alpha	KY064169

Results

Photoperiodic induction of diapause

We examined the effect of photoperiods on diapause induction in sexual and asexual strains of laboratory *T. brassicae*. Our results showed that the percentage of diapausing progeny significantly depended on photoperiod in both sexual ($F = 10.91$; d.f. = 5, 714; $P = < 0.0001$) and asexual ($F = 10.74$; d.f. = 5, 714; $P = < 0.0001$) strains. The results are shown in Fig. 2. In the sexual strain, diapause induction was highest in 8 L:16D followed by L24:D0 and lowest in L4:D20: day lengths of 8 and 24 induced diapause in 17.4 and 15.8% of progeny, respectively, whereas in 4 h day length, diapause individuals were 5.8%. The asexual strain showed very low diapause incidence in comparison to the sexual strain ($t = 15.97$; $P = < 0.001$), with the highest in L12:D12 followed by L24:D0 and L8:D16: the diapause percentage of asexual strain was 3.9, 3.2, and 2.9% at a day length of 12, 24, and 8 h, respectively, and around zero for other day lengths.

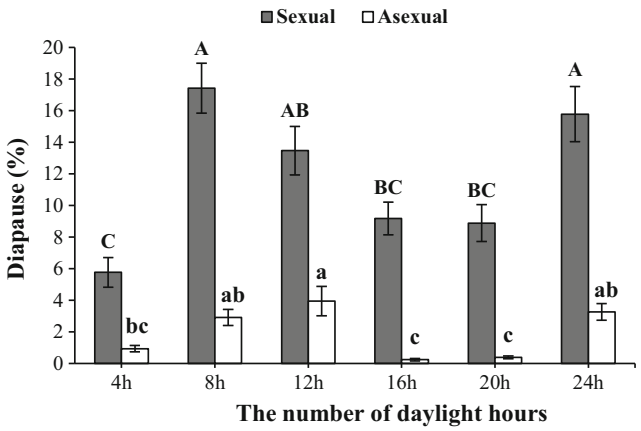

Fig. 2 The influence of different number of daylight hours on the diapause percentage of *T. brassicae* in relation to *Wolbachia* infection at 14 °C. The different numbers of daylight hours include 4, 8, 12, 16, 20, and 24 h of light phase, respectively. Capital and lowercase letters are used to indicate statistically significant differences between different numbers of daylight hours in sexual and asexual *T. brassicae*, respectively

Photoperiodic effects on clock gene expression

We have obtained cDNAs of five clock genes, i.e., *clk*, *cyc*, *cry2*, *per*, *timeout*, and *Egf1* as a house keeping gene: the length of cDNA fragments was 781, 661, 486, 459, 564, and 474 bp, respectively. To examine whether transcript levels of clock genes varied with maternal photoperiods and differed between asexual and sexual strains, the mRNA levels of *clk*, *cyc*, *cry2*, *per*, and *timeout* were measured by qPCR in asexual and sexual *T. brassicae* (Figs. 3 and 4). The samples were collected 2 h after light-on in six different photoperiods. The ANOVA indicated no significant changes between different photoperiods for all clock genes examined in sexual *T. brassicae* strains, while in the asexual strain, *clk* ($F = 5.45$; d.f. = 5, 12; $P = 0.007$), *cyc* ($F = 8.38$; d.f. = 5, 12; $P = 0.001$), and *timeout* ($F = 3.54$; d.f. = 5, 12; $P = 0.03$) showed significant photoperiodic variation (Fig. 4).

Effects of *Wolbachia* infection on gene expression

The mRNA levels of clock genes of asexual *T. brassicae* were found to be significantly lower than those of sexual strain in all the clock genes, *clk* ($t = -15.73$; $P = < 0.001$), *cyc* ($t = -10.09$; $P = < 0.001$), *per* ($t = -12.82$; $P = < 0.001$), *timeout* ($t = -3.4$; $P = 0.0018$), and *cry2* ($t = -9.39$; $P = < 0.001$) (Fig. 3).

Fecundity in sexual (uninfected) and asexual (infected) strains

The fecundity of *T. brassicae* females in various photoperiodic conditions is summarized in Fig. 5. The fecundity was dependent on photoperiod in both sexual ($F = 2.97$; d.f. = 5, 114; $P = < 0.0001$) and asexual ($F = 4.87$; d.f. = 5, 114; $P = 0.0004$) *T. brassicae*. The fecundity of females which developed and had been kept under L12:D12 (average eggs per a female were 30.75 ± 2.18 and 20.25 ± 1.63 for asexual and sexual strains, respectively) was significantly lower than for those which developed and had been kept under long day conditions of L16:D8, L20:D4, and continuous light in the sexual strain or under long day of L20:D4 and ultra-short day of L4:D20 in the asexual strain. It is notable that the fecundity of asexual *T. brassicae* was significantly higher than sexual one under all photoperiod regimes ($t = 11.19$; $P = < 0.001$) (Fig. 5).

Discussion

The results indicate that the sexual strain is more successful in diapause in comparison with asexual strain (Fig. 2),

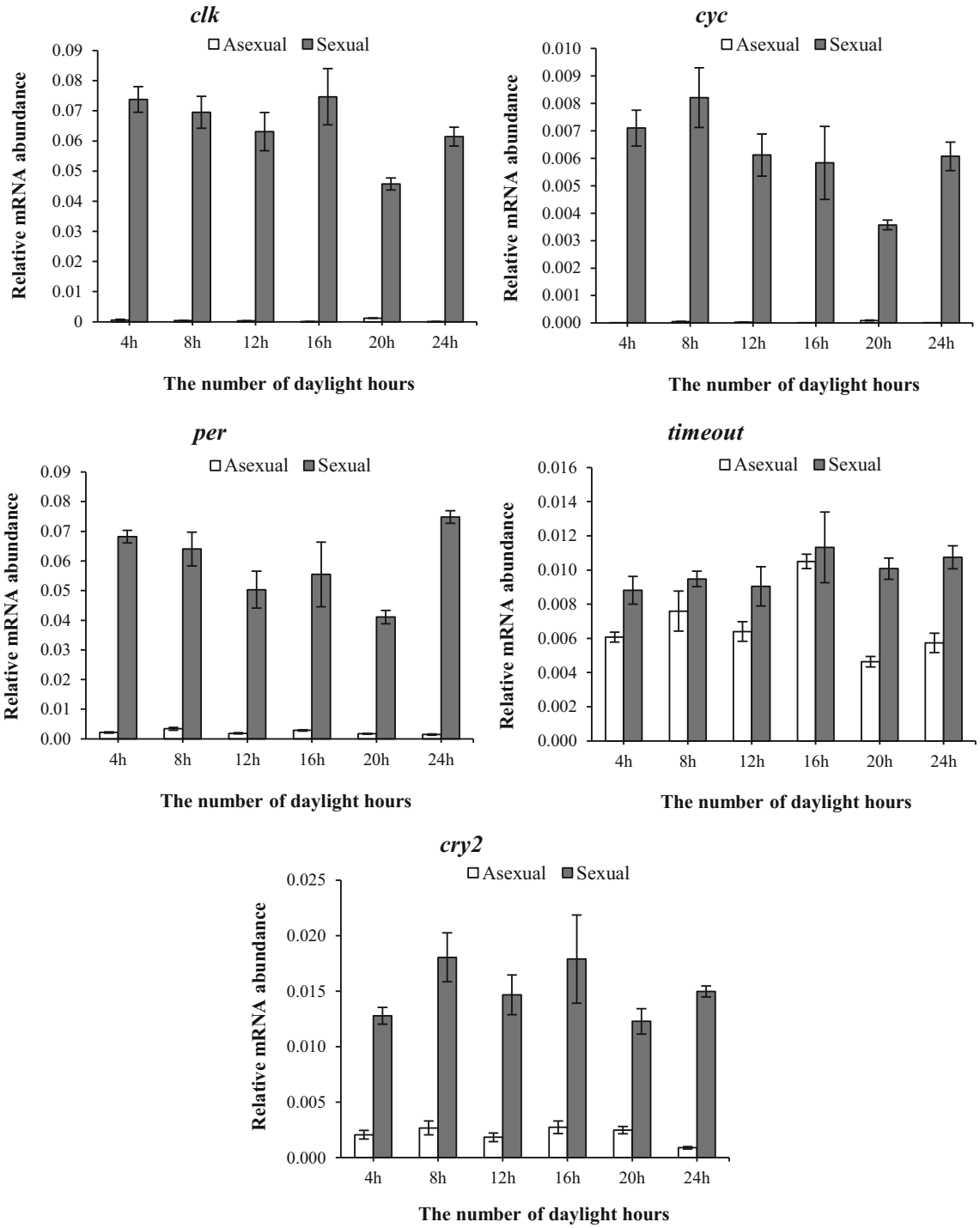

Fig. 3 Relative mRNA abundance (*Tb'clk*; *Tb'cyc*; *Tb'cry2*; *Tb'timeout*; *Tb'per*) of sexual (gray column) and asexual (white column) *T. brassicae* strains under 4, 8, 12, 16, 20, and 24 h light-on, 20 °C. The abundance of all genes mRNA was measured by quantitative real-time RT-PCR with total RNA extracted from the whole body of *T. brassicae* that were collected at 6 photoperiods starting at 2 h after light-on. The value shown is relative to the amount of EF-1α mRNA. Vertical bars indicate SEM. Black and white bars indicate darkness and light, respectively

suggesting that *Wolbachia* endosymbiosis prevents successful diapause. Similar results were reported in *Nasonia vitripennis* (Walker) (Hym: Pteromalidae) (Bordenstein and Werren 2000) and *Trichogramma oleae* (Voegelé & Pointel) (Pintureau et al. 2002) as sexual individuals were better able to enter diapause in comparison with asexual ones.

Diapause was induced by the short days and inhibited by the long days in most studied *Trichogramma* species, particularly in *T. pintoi* Voegele (Zaslavski and Umarova 1990),

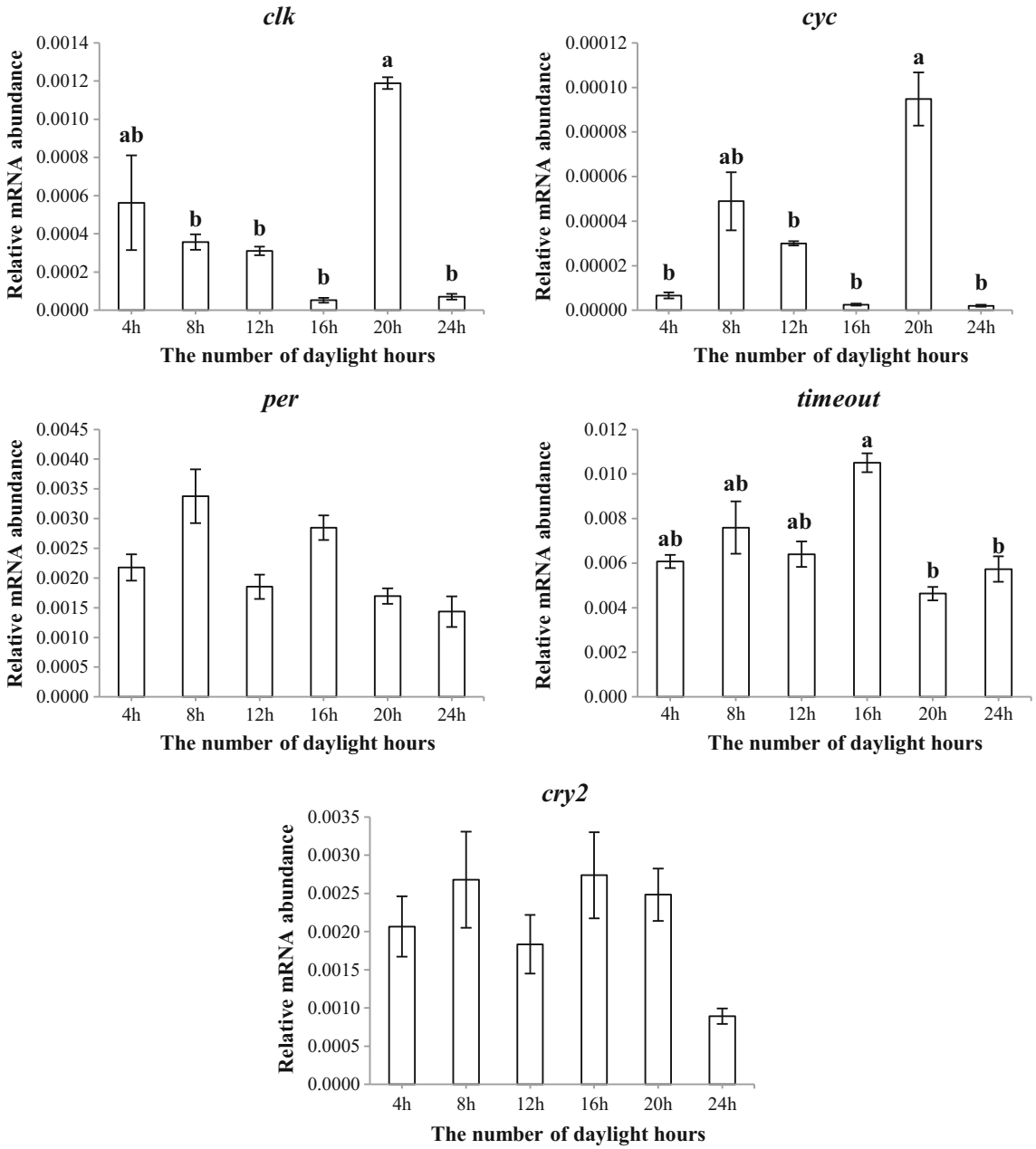

Fig. 4 Relative mRNA abundance (*Tb'clk*; *Tb'cyc*; *Tb'cry2*; *Tb'timeout*; *Tb'per*) of asexual *T. brassicae* strain depicted from Fig. 4 with expanded ordinate under 4, 8, 12, 16, 20, and 24 h light-on at 20 °C. For further explanations, see Fig. 4. Lowercase letters are used to indicate statistically significant differences between different numbers of daylight hours in asexual *T. brassicae*

T. embryophagum Hartig (Voinovich et al. 2003), *T. principium* Sug & Sor (Reznik and Kats 2004), and *T. piceum* Djur (Reznik et al. 2013). However, in *T. brasicae*, the percentage of diapause induction showed a significantly higher value at continuous light (L24:D0) and a lower value in ultra-short photoperiod (L4:D20) both in sexual and asexual strains, suggesting that diapause suppressed in a restricted range of long photoperiods and of ultra-short photoperiods (Boivin 1994). These values at L24:D0 and L4:D20 may not have ecological significance because the wasps never encounter these extreme conditions, but have physiological significance which still remains to be explained (Danilevski 1965).

Many studies have shown that maternal influence of photoperiod is better observed at temperatures above threshold. The thermal threshold of development for *T. brassicae* is about 10 °C (Babendreier et al. 2003). For example, Bonnemaison (1972) demonstrated that most of the prepupae of *Trichogramma evanescence* went to diapause at 10 °C regardless of photoperiodic conditions whereas at 15 °C the wasp showed a clear photoperiodic responses with most individuals entered diapause in short photoperiods. Similarly,

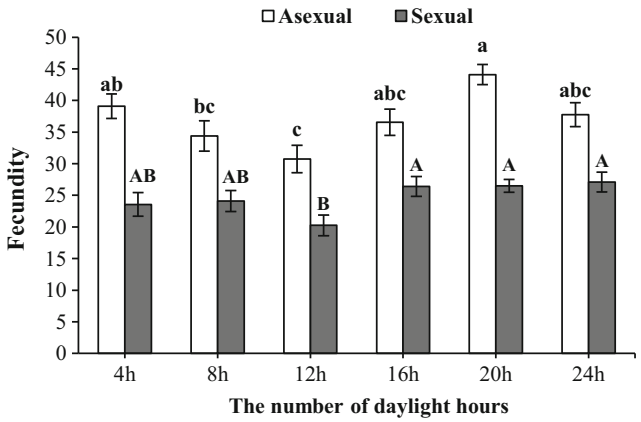

Fig. 5 The fecundity of asexual and sexual *T. brassicae* under 20 ± 1 °C, r.h. = 70 ± 5% and different numbers of daylight hours L:D = 4:20, 8:16, 12:12, 16:8, 20:4, and 24:0 h. Capital and lowercase letters are used to indicate statistically significant differences between different numbers of daylight hours in sexual and asexual *T. brassicae*, respectively

Pizzol and Pintureau (2008) have shown that photoperiod experienced by the maternal generation had effect on diapause of *Trichogramma cacoeciae* at 13 °C but not at 10 °C. In the present study, we transferred the progeny to 14 °C and absolute darkness to examine diapausing rate in sexual and asexual *T. brassicae*. The yielded clear photoperiodic responses suggest that the temperature is in a right range for maintenance of maternal photoperiodic responses. Actually, the temperature range was equivalent to that observed during autumn in Karaj, Alborz Province, Iran. The meteorological data (Meteorology Organization of Alborz Province) have announced that average monthly temperatures were 16.4 and 15.4 °C in October 2013 and 2014, respectively.

Interestingly, *clk* and *cyc* showed a similar tendency of photoperiod dependent changes in their expression levels with greatest expression in L20:D4 in asexual strain, suggesting that they were likely regulated in a common mechanism. *Timeout* gene showed a different pattern from *clk* and *cyc* with a peak at L16:D8 and a small non-significant peak at L8:D16. A similar pattern was also seen in *per* and *cry2*, although the variation was not statistically significant, suggesting that they were affected by a similar mechanism. These data also suggest that the positive clock elements, *clk* and *cyc*, and the negative elements, *per* and *cry2*, are regulated by separate mechanisms. This is reminiscent that the circadian clock plays an important role in photoperiodic responses (Sakamoto et al. 2009) and that the positive and the negative elements plays different role in determination of photoperiodic phenotype (Ikeno et al. 2011). In both sexual and asexual strains, the amount of *clk* gene expression was ten times higher than that of *cyc* gene.

Wolbachia infection suppresses clock gene expression in *T. brassicae*. The *Wolbachia* infection has been shown to affect the level of some genes such as immune genes (Moreira et al. 2009; Bian et al. 2010; Kambris et al. 2010), metalloprotease gene (Hussain et al. 2011), genes involved in oogenesis and programmed cell death (Kremer et al. 2012). Kremer et al. (2012) suggested that *Wolbachia* might have an effect on expression of genes involved in development, PCD, and immunity, especially through the regulation of oxidative stress.

Turley (2013) have examined the role of *Wolbachia* on circadian rhythms and age-related neurodegeneration in *Aedes aegypti*. Their results showed no significant differences between infected and cured *Ae. aegypti* in expression of genes (*sws*, *vap*, *tau*, *kh*, and *nrxI*) related to neurodegeneration and only minimal effects on expression of *per* gene. One possible explanation for their results is due to the elimination of *Wolbachia* using antibiotics. Certainly, removal of obligate bacteria like *Wolbachia* (parasite or symbiont) by antibiotics hardly eliminates those mechanisms evolutionary acquired in association with endosymbiosis. Thus lack of significant difference in *Ae. aegypti* between *Wolbachia*-infected and *Wolbachia*-cured strains can be quite predictable.

Ikeno et al. (2010) have shown that the *cyc* gene plays an important role in ovarian development or diapause in *Riptortus pedestris*, when continuous *cyc* expression leads to an increase in ovarian development (nondiapause) compared to when *cyc* is knocked down by RNAi, regardless of day length. However, the hypothesis in *Riptortus* cannot explain the difference in fecundity between the sexual and asexual *T. brassicae*, because the asexual *T. brassicae* showed less *cyc* expression than sexual strain which has less fecundity than asexual females.

As shown in Fig. 5, in both asexual and sexual of *T. brassicae*, the fecundity of females of which maternal generation was kept in the short photoperiods (L8:D16 and L12:D12 for the asexual strain; L12:D12 for the sexual strain) was less than those of which maternal generation were kept in the long photoperiods (L20:D4 for the asexual strain; L16:D8; L24:D0 for the sexual strain). Therefore, we expected that higher *cyc* gene expression in those long photoperiods than short photoperiods: the high *cyc* expression in L20:D4 well correlated with high fecundity in asexual strain, but other conditions were not the case. Thus, the fecundity might be controlled by factors other than the clock genes, which were affected by *Wolbachia*, although the involvement of the clock genes cannot be ruled out.

In the present study, we measured clock gene levels only at a single time point in each of the photoperiodic conditions. Most of the clock genes are known to be expressed in a cyclic manner. An expression pattern of some genes changes in a photoperiod-dependent manner (Majercak et al. 1999). For example, *per* gene is rhythmically expressed but the peak phase changed with photoperiods while for *tim*, the amplitude of its mRNA expression changes in *Sarcophaga carssipalpis* (Goto and Denlinger 2002). Thus, the daily expression profiles of the clock genes should be examined in our future study to clarify the functional role in photoperiodic control of fecundity as well as diapause.

However, the differences in these traits may be due to the divergence of host traits and genes after the *Wolbachia*-infected thelytokous strain have evolved from an uninfected ancestral strain. There is also a possibility that these traits had already diverged between the ancestral populations of the two strains before *Wolbachia* infection occurred. Hence, elimination of *Wolbachia* by antibiotics or high-temperature treatments is suggested. Although, the application of antibiotics or high temperatures may directly affect the expression of these traits. Antibiotic treatments might have negative effects on oocyte production of *T. brassicae* female as Dedeine et al. (2001) have reported for the parasitic wasp, *Asobara tabida* Nees (Hymenoptera: Braconidae), and undoubtedly this destructive change will reduce the productivity of the biological control agent.

Conclusion

In this study, two strains differed in diapause incidence, fecundity, and the expression patterns and levels of circadian clock genes. It seems that the differences in these traits between the infected and uninfected strains are attributable to *Wolbachia* infection, and *Wolbachia* may interfere the expression of circadian clock genes, reducing the success rate in entering diapause in the asexual *T. brassicae*. According to our results, higher clock gene expression in the sexual *T. brassicae* strain results in higher rate of entering diapause state. Our results suggest that there is a molecular link between the expression level of clock genes and the occurrence of diapause. The mechanism through which *Wolbachia* controls the clock gene expression is a challenging issue in our future study.

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