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Diurnal rhythm of volatile emissions from damaged *Brachypodium distachyon* affects the temporal preferences of tritrophic interactions

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ABSTRACT

Diurnal rhythm of volatile emissions from grasses in response to herbivory may temporally affect the oviposition behaviors of the conspecific herbivores and prey searching of natural enemies of the herbivores in a diurnal cycle of the ecosystem. We assessed volatiles emitted from the temperate grass *Brachypodium distachyon* L. (Poaceae) damaged by *Mythimna separata* Walker (Noctuidae), a specialist herbivore of Poaceae, in light/dark cycle conditions. Volatiles were preferentially emitted from the damaged plants in both light and dark phases, but their quantitative compositions were different. The generalist predator *Nesidiocoris tenuis* Reuter (Miridae) was attracted to the damaged plants in both light and dark conditions. However, adult females of *M. separata* preferred to oviposit on undamaged plants mostly in the dark owing to the cue of undamaged plant volatiles, partly in a circadian rhythm-dependent manner. The findings suggested that volatiles released in the dark contribute to both plant-herbivore and plant-herbivore enemy communications.

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KEYWORDS
Diurnal cycle; oviposition behavior; tritrophic interaction; volatile organic compound

Introduction

Various plant species can be induced to emit a specific blend of volatile organic compounds (VOCs) in response to herbivores' attack as infochemicals for attraction of natural enemies of herbivores (Takabayashi and Dicke 1996; Arimura et al. 2005; Aljbory and Chen 2018). Herbivore attacks are in many cases known to induce substantially higher levels of VOCs in the light phase (day) than the dark phase (night), because a mono- and hemi-terpenes, a major group of VOCs, are emitted in a light-dependent manner or according to circadian rhythms, as representatively shown in cases of constitutive release of floral scents or light-dependent isoprene emissions (Loivamäki et al. 2007; Roeder et al. 2007). Bee-pollinated snapdragon flowers have been shown to emit methyl benzoate mostly during the day, to coincide with the activity of bumble bees (Kolosova et al. 2001). In some cases, however, VOCs are released substantially at night, as shown, for instance, for benzenoids in the floral scents of *Petunia axillaris* that act as a cue for a nocturnal pollinator, *Manduca sexta* (Hoballah et al. 2005).

The release of herbivore-induced plant volatiles (HIPVs) is a phenotypically plastic response of plants to diurnal or nocturnal timing of herbivores' attack (Arimura et al. 2008), and the temporally different cues may modulate the activity of herbivores and their natural enemies. The evasion or attraction behaviors of herbivorous arthropods and their natural enemies have been shown to depend on temporally different HIPV blends that are repellent against adult female moths for their oviposition sites (De Moraes et al. 2001) and attractive to their natural enemies (Turlings et al. 1995; Zhang et al. 2010; Signoretti et al. 2012; Joo et al. 2017; Naranjo-Guevara et al. 2017).

Although diurnal rhythms of HIPV emissions have frequently been observed in a variety of plant taxa (Loughrin et al. 1997; Halitschke et al. 2000; Maeda et al. 2000; Zhang et al. 2010, 2013; Cai et al. 2012; Clavijo McCormick et al. 2014; Joo et al. 2017), little is known about the ecological implications of nocturnal volatiles. In the current study, we therefore highlighted the ecological function of HIPV cues for the interactions between *Brachypodium distachyon* L. (Poaceae) plants and the nocturnal herbivore *Mythimna separata* Walker (Noctuidae) (Tanaka 1976). We used *B. distachyon* because this temperate grass has not been selected artificially, so it has several advantages as an experimental model organism for genetic and ecological studies (Kellogg 2015). Artificially selected plants frequently have different morphology and physiology from their wild ancestors, and consequently cause an altered balance of the tritrophic interactions of the plants with herbivores and carnivores (Chen et al. 2015). It has been shown, for instance, that wild cabbage (*Brassica oleracea* L.) plants damaged by *Pieris rapae* L. emit HIPVs that are strongly attractive to the specialist parasitoid *Cotesia rubecula* Marshall, due to the different composition of HIPVs compared to those from artificially selected cultivars (Gols et al. 2011).

To understand the nature of tritrophic interactions of *B. distachyon*, we spotlighted *M. separata*, a specialist herbivore of Poaceae, because it may respond to plant volatiles, since its larvae use plant volatiles as cues for day versus night (Shiojiri et al. 2006). *M. separata* is known as a nocturnal herbivore that feeds on host plants during the night and, in turn, conceals itself during the day (Sato et al. 1983). Such activities with diurnal rhythm may possibly affect tritrophic interactions, as plants emit lower levels of HIPVs after feeding

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ceases (Mandour et al. 2011). Therefore, nocturnally damaged plants would benefit by nocturnal emission of HIPVs to attract nocturnally active herbivore enemies. As a natural enemy species, *Nesidiocoris tenuis* Reuter (Miridae) was used because it preys on various herbivorous arthropods, including lepidopteran, whitefly, mite, thrips, and aphid eggs or larvae (Torreno 1994; Li et al. 2008; Sanchez 2008; Calvo et al. 2009). This predator has been shown to consume the eggs of *M. separata* (personal observation).

Overall, our data support a significant role of the diurnal rhythm of HIPV emissions from *B. distachyon* plants damaged by the nocturnal caterpillars in repelling the herbivore's adult females for oviposition as well as recruiting natural enemies of herbivores.

Materials and methods

Plants

Brachypodium distachyon (Bd21) seeds were obtained from the Riken Bio Resource Center (Tsukuba, Japan). After seeds were washed with a mixture of 1% (v/v) sodium hypochlorite and 0.02% (v/v) Tween-20, they were placed on a wet filter paper in a plastic dish (ø 9 cm × 1.8 cm) at 4°C in the dark. After 1 week, the plastic dish was transferred to a climate-controlled room at 24 ± 1°C with a photoperiod of 16 h (6:00–22:00, 80 μE m^{-2} s^{-1}, fluorescent light, FL40SSW/37, NEC lighting, Ltd., Tokyo, Japan) until seedlings emerged. The seedlings were transplanted to soil in a plastic pot (ø 7 cm × 7.5 cm) and grown in the above conditions for 5 weeks, at which time plants were around 30 cm in height with 30–40 leaves.

For the insect oviposition assay under the inverse light/dark cycle (see below), plants grown in the condition in which light and dark cycles were inversely set during the day and nighttime (i.e. dark period between 10:00 and 18:00; and light period between 18:00 and 10:00) were used. The plants were kept in the inverse light condition immediately after transplantation to soil in a plastic pot. Except for these light/dark regimes, plants were grown in the same conditions described above.

Insects

All the insects used in this study were incubated in climate-controlled rooms at 24 ± 1°C with a photoperiod of 16 h (6:00–22:00). The larvae of *M. separata* were reared on artificial diet (Insecta-LF; Nihon Nousan Kougyou Ltd., Tokyo, Japan) in a plastic container (19 cm × 12 cm × 7 cm) with a mesh-covered lid (6 cm × 8 cm). Three times a week, feces in the plastic case were removed and a piece of artificial diet was added. The pupae were moved to a plastic case (27 cm × 18 cm × 15 cm) for rearing adults. The emerged adults mated and oviposited onto accordion-folded paper hung from the lid. Cotton wool soaked with sugar water was provided as food for adults. Twice a week, the cotton wool was replaced with a new one. Once a day, the oviposition substrates were replaced with new ones. About 100 eggs were obtained and transferred to a plastic container, and the newly hatched larvae were incubated with artificial diet (see above).

N. tenuis was obtained from Mitsuki Shimomoto and Kazuhide Nakaishi (Kochi Prefectural Agricultural Technology Research Center, Nangoku, Japan) in 2012. *N. tenuis* adults were kept in a lidded plastic case (12 cm × 17 cm × 5 cm) and eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were provided as food. *Sedum rubrotinctum* (Rosales: Crassulaceae) leaves were also provided as an oviposition substrate and a water source for *N. tenuis*. The plastic case had a screened opening (1 cm × 1 cm) in the lid and on the side for air ventilation. Twice a week, *E. kuehniella* eggs were provided and the *S. rubrotinctum* leaves were replaced. Leaves with eggs were placed into a new plastic case.

Determination of headspace volatiles of B. distachyon *plants*

We collected headspace volatiles from an undamaged *B. distachyon* plant in a pot and from *B. distachyon* plants damaged by *M. separata* larvae. Undamaged plants or plants challenged with five 3rd-instar *M. separata* larvae were placed in an open glass container (2 L) covered with mesh on the top at 10:00 of the 1st day. Plants in the container were incubated at 24 ± 1°C in a 16 h light/8 h dark cycle (photoperiod during 6:00–22:00, with 80 μE m^{-2} s^{-1}, fluorescent light, FL40SSW/37, NEC lighting). VOCs emitted from the potted plants were collected by using the volatile collection system of GERSTEL-Twister (polydimethylsiloxane [PDMS] coated stir bar, 0.5 mm film thickness, 10 mm length, GERSTEL GmbH & Co. KG, Mülheim an der Ruhr, Germany). The glass bottle (2 L) containing the individual plant with Twister and *n*-tridecane (0.1 μg) as internal standard was sealed completely with a glass lid during static headspace volatile collection. The Twister bar was positioned at the middle (around 20 cm height) of the glass bottle with a magnet. We collected VOCs for 2 h with 2-h intervals between collections at 5 time points. The VOC collections were carried out at 34–36 h, 38–40 h, 42–44 h, 46–48 h, and 50–52 h after the onset of incubation (from 20:00 on the 2nd day to 14:00 on the 3rd day for all).

The volatiles adsorbed on the Twister were analyzed using a 6890/5972 GC-MS system (Agilent Technologies, Santa Clara, CA, USA), equipped with a thermal desorption system (TDS), a cooled injection system (CIS), and a cold trap system (CTS) (GERSTEL GmbH & Co. KG). An HP-5MS capillary column (30 m long, 0.25 mm I.D. and 0.25 μm film thickness; Agilent Technologies) was used with He (1 mL min^{-1}) as carrier gas. Headspace volatiles collected on a Twister were released from the PDMS by heating in the TDS at 200°C for 4 min, within a He flow. The desorbed compounds were collected in the CIS at −130°C, and then the collected compounds were released from the CIS by heating. The desorbed compounds were collected again in the CTS at −50°C, and then flash heating of the CTS provided sharp injection of the compounds into the capillary column of the gas chromatograph to which the CTS was connected. The temperature of the GC was programed to rise from 40°C (9 min hold) to 280°C at 5°C min^{-1}. Mass spectra in the electron impact mode were generated at 70 eV. Limonene (98%, Wako Pure Chemical Industrials, Ltd., Osaka, Japan), geranylacetone (Wako), (*Z*)-3-hexenyl acetate (Wako), and (*E*)-β-caryophyllene (Wako) were identified by comparing their mass spectra and retention times with those of authentic compounds. Sesquiterpenoids were tentatively identified by comparison with mass spectra in the Wiley 7 N database. The relative amounts of individual compounds were calculated from the peak areas of the total ion chromatogram,

based on the internal standard. Replicate analyses were conducted with four to six independent plant individuals.

Note that volatiles released from *M. separata* larvae alone, without the host plant, did not show any particular VOC detected (not shown).

Y-tube olfactometer olfactory assay

Olfactory assays towards HIPVs and VOCs from *B. distachyon* plants were performed as previously described by Rim et al. (2015) with slight modifications. *B. distachyon* plants were exposed to five 3rd-instar *M. separata* larvae from 10:00 on the 1st day. The olfactory preference of *N. tenuis* adults between the undamaged plants versus the damaged plants (*M. separata* larvae were removed just before the onset of assays) was tested in the light condition between 8:00 and 10:00 (daytime on the 3rd day) and dark condition between 24:00 and 02:00 (nighttime on the 2nd day). *N. tenuis* adults used for the bioassays were naïve to volatiles from the potted plants. Each *N. tenuis* was gently placed at the starting point (2 cm from the downwind end of the main tube) in the Y-tube using an insect aspirator made for handling of these predators. When a tested *N. tenuis* arrived at the end of either side of the Y-tube, we judged that it made a choice. When *N. tenuis* did not choose either of the branches within 5 min, it was counted as a no-choice predator and excluded from data analysis. Twenty males and 20 females of *N. tenuis* (5–10 days old after emergence) were tested each time. Each experiment was conducted on three different experimental days with new sets of odor sources. All the insects were used once and then discarded. Note that we performed all the assays that needed to be undertaken in darkness under a red LED flashlight (Hugsby LE-T11).

Ovipositional preference of M. separata adult females

Plants were grown in two sets of light cycle program: either with a normal light cycle program (photoperiod between 6:00 and 22:00) or an inverse light cycle program (photoperiod between 18:00 and 10:00). For two-choice oviposition preference assays using plants grown in normal light–dark condition, a pair of undamaged plants and a pair of the potted *B. distachyon* plants exposed to five *M. separata* larvae from 10:00 on the 1st day (see above) in the respective light cycle programs were set alternately at each of the corners in a plastic cage (34 cm × 34 cm × 25.5 cm) (Figure 4(a)). The larvae were removed prior to the assays. Three mated *M. separata* adult females (4–5 days old after emergence) grown in the normal light program were released in the center of the cage at 10:00 on the 3rd day or 22:00 on the 2nd day, and all the plants were taken out after 8 h to count the number of eggs.

Otherwise, when plants grown in the inverse light–dark condition were used, the assays were performed in the inverse light dark condition. The damaged plants were exposed to five *M. separata* larvae from 22:00 on the 1st day in these assays. Three mated *M. separata* adult females grown in the normal light condition were released in the center of the cage at 10:00 on the 3rd day (start of dark period in the inverse light–dark condition) or 22:00 on the 3rd day (4 h after start of light period in the inverse light–dark condition), and all the plants were taken out after 8 h to count the number of eggs.

Four sets of assays (daytime in the normal light/dark cycle), nine sets of assays (nighttime in the normal light/dark cycle), six sets of assays (daytime in the inverse light/dark cycle), and four sets of assays (nighttime in the inverse light/dark cycle) were conducted at 24 ± 1°C. The positioning of the four individual plants was changed clockwise among the four corners every single assay for the same set.

Statistical analysis

All the statistical analyses were performed with R version 3.4.2 (R Core Team 2017).

We used ANOVA to compare the amounts of each volatile compound from undamaged plants and *M. separata*-damaged plants at every time point. Then, to compare diurnal and nocturnal volatiles from undamaged plants or damaged plants, we conducted principle coordinates analysis (PCoA) for the volatiles data of 24:00–02:00 vs. 08:00–10:00 and 04:00–06:00 vs. 12:00–14:00 on the Bray–Curtis dissimilarities. The relative peak areas of all the volatile compounds from undamaged plants or damaged plants collected during the sampling times indicated above were compared with a permutational multivariate analysis of variance (PERMANOVA; no. of permutations performed = 9999) based on Bray–Curtis dissimilarities of the quantity of the volatile compounds. PCoA and PERMANOVA were calculated using the capscale function and adonis function of the vegan package (Oksanen et al. 2017) in R version 3.4.2 (R Core Team 2017). Before PERMANOVA was conducted, we confirmed that there was no significant difference in the dispersion of volatile compounds between diurnal and nocturnal volatiles.

We analyzed the data of oviposition experiments using a generalized linear model (GLM) with Poisson distribution, because the type of data of this experiment was count data (number of oviposited eggs). First, we compared the number of eggs on the undamaged plants and the damaged plants at daytime or nighttime in normal or inverse light–dark cycle, respectively. Then we analyzed the combined number of eggs on the undamaged and the damaged plants of the above conditions to examine the effects of time and light condition on the ovipositional behavior of *M. separata*.

For the Y-tube olfactometer experiments, we analyzed data with a repeated *G*-tests of goodness-of-fit using the RVAideMemoire package (Herve 2017).

Results

VOC emissions from B. distachyon plants according to light/dark rhythms

Sixteen major VOCs, consisting of 13 sesquiterpenoids, 2 monoterpenoids (limonene and geranylacetone), and a green leaf volatile ((*Z*)-3-hexenyl acetate), were observed in the headspace of undamaged and *M. separata*-damaged *B. distachyon* plants (Figure 1). Overall, some of the VOCs, as representatively observed in the case of (*E*)-β-caryophyllene, were more strongly emitted from the damaged plants than undamaged plants in both the light and dark phases. Nevertheless, it appeared that both undamaged and damaged plants emitted higher levels of total VOCs in the dark phase than in the light phase according to principal coordinates analyses (PCoA) for the comparison between time-points of the light phase (08:00–10:00, 2 h after light on) and dark phase

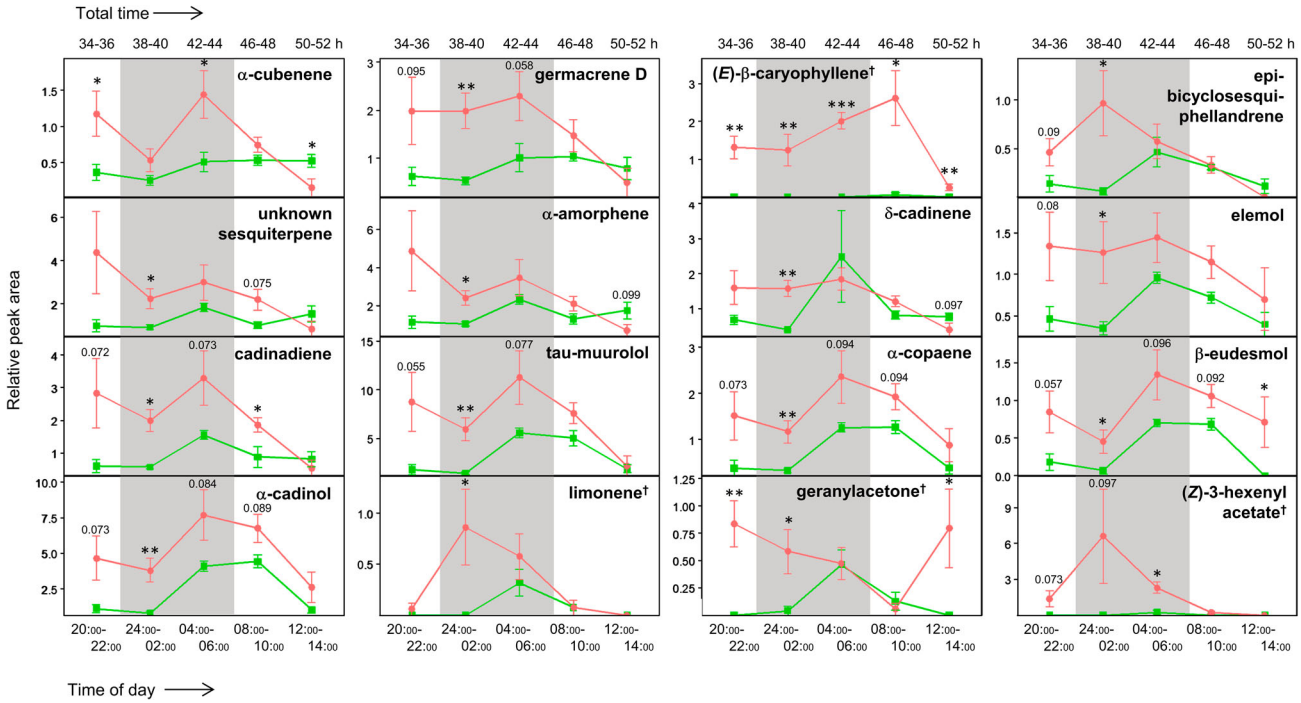

Figure 1. Volatile organic compounds emitted from undamaged *Brachypodium distachyon* plants and plants exposed to *Mythimna separata* larvae in a light/dark cycle. Red lines represent emission from *M. separate*-damaged plants while green lines correspond to emission from undamaged plants. Two-hour collection periods are shown across the bottom of the *x*-axis, while total elapsed time after the onset of insect challenge is shown on the top of the *x*-axis. Alternating light and shaded sections along the graph correspond to 16 h light and 8 h dark periods. Data represent the means and standard errors ($n = 4$–6). Data marked with an asterisk are significantly different from those of undamaged plants, based on an ANOVA (***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$). Otherwise, the means followed by a *P*-value are marginally different. The compound name with a cross (†) was identified by comparing their mass spectra and retention times with those of authentic compounds. The other compounds were tentatively identified with database alone.

(24:00–02:00, 2 h after light off) (Figure 2(a,b)) (PERDISP: $P = 0.6274$, PERMANOVA: $P = 0.0048$ for undamaged plants; PERDISP: $P = 0.2757$, PERMANOVA: $P = 0.061$ for damaged plants, see Table S1). Likewise, PCoA for comparison between other time-points of the light phase (12:00–14:00, 6 h after light on) and dark phase (04:00–06:00, 6 h after light off) also showed higher levels of emission of total VOCs in the dark phase (Figure 2(a,b)) (PERDISP: $P = 0.2104$, PERMANOVA: $P = 0.0049$ for undamaged plants; PERDISP: $P = 0.9519$, PERMANOVA: $P = 0.022$ for damaged plants, see Table S1).

Olfactory responses of generalist predator to VOCs emitted from B. distachyon *in light and dark conditions*

Based on the above findings on rhythmic emission of VOCs from *B. distachyon* plants, we assessed olfactory responses of the generalist predator *N. tenuis* to undamaged-plant VOCs vs. damaged-plant VOCs in the light and dark conditions. *N. tenuis* is an omnivorous mirid bug with diverse prey, including lepidopteran larvae/eggs such as those of *S. litura* (Wei et al. 1997; Rim et al. 2015). Both females and males of *N. tenuis* preferred VOCs from damaged plants over undamaged plants in both light and dark conditions (Figure 3(a,b)) (male: light phase, $P < 0.001$ and dark phase, $P < 0.01$; female, light phase: $P < 0.05$, dark phase: $P < 0.001$, *G*-test).

Ovipositional preference of M. separata *adult females*

Next, ovipositional preference of *M. separata* adult females was assessed using *B. distachyon* plants that had been grown in the normal or inverse light/dark cycle. *M. separata* preferred to lay eggs on the undamaged plants over damaged plants during the dark phase (22:00–06:00). They laid eggs on neither undamaged plants nor damaged plants in the light phase (10:00–18:00) (Figure 4(b)). Moreover, when adult females were released to *B. distachyon* plants that had been grown in the inverse light/dark cycle condition, they still preferred to lay eggs on the undamaged plants over damaged plants in the dark phase during the daytime (10:00–18:00) (Figure 4(b)). They laid only a few eggs in the light phase during the nighttime (22:00–06:00), indicating that the dark condition is an essential factor for their oviposition performance. A comparison of the total numbers of eggs laid on both undamaged and damaged plants in the whole dataset showed that the adult females dominantly preferred the dark phase to oviposit (Figure 4(c)). Yet, the number of eggs laid during nighttime on *B. distachyon* plants that had been grown in the inverse light/dark cycle condition was lower than the number laid on the plants that had been grown in the normal light/dark cycle condition, indicating that ovipositional performance of *M. separata* was even controlled by a circadian clock. There were significant effects of different factors, i.e. daytime or nighttime and light or dark, on the ovipositional preference of *M. separata* (Table S2).

Discussion

In response to attack by *M. separata*, *B. distachyon* plants released HIPVs (Figure 1). Of great interest is the fact that the total quantity of HIPVs released was larger in the dark phase than in the light phase (Figure 2). These findings do not accord with many cases in previous studies in which the diurnal pattern of HIPV release showed that the release generally peaked in the daytime, irrespective of whether diurnal or

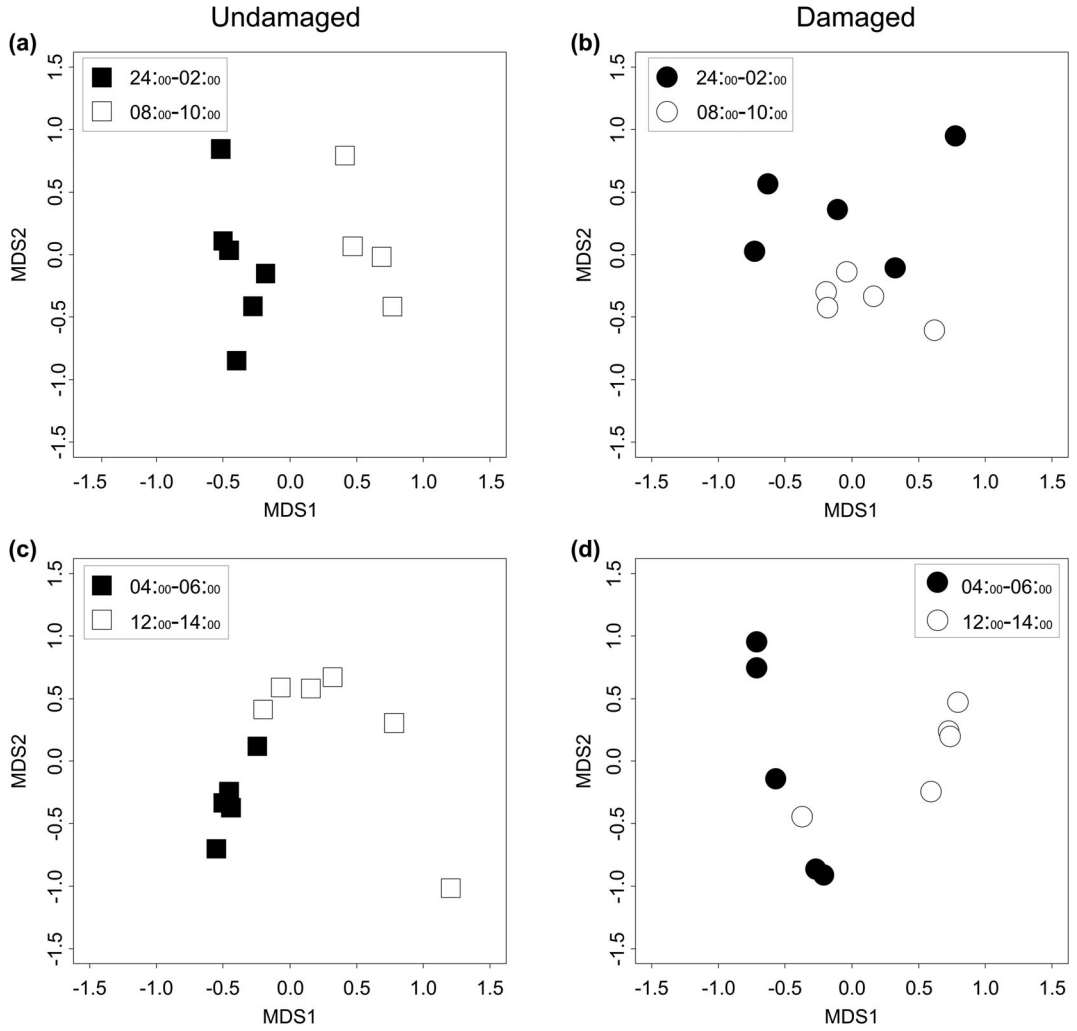

Figure 2. Multidimensional scaling (MDS) plot on volatile composition data. A MDS plot (for the first two axes) was made using principal coordinates analysis (PCoA) of a Bray-Curtis distance matrix on volatile composition data from (a) undamaged plants and (b) damaged plants during 24:00–02:00 (scotoperiod) versus 08:00–10:00 (photoperiod), and (c) undamaged plants and (d) damaged plants during 04:00–06:00 (scotoperiod) versus 12:00–14:00 (photoperiod). The black-filled squares or circles represent samples during scotoperiods while the empty squares or circles represent samples during photoperiods. The percentages of variance in the data explained by the first and second axes of the PCoA are (a) 79.02 and 7.91%, (b) 50.48 and 31.76%, (c) 68.94 and 17.09% and (d) 80.11 and 12.95%, respectively.

nocturnal herbivores explored (Loughrin et al. 1994, 1997; Halitschke et al. 2000; Maeda et al. 2000; De Moraes et al. 2001; Gouinguené and Turlings 2002; Arimura et al. 2004, 2008; Zhang et al. 2010, 2013; Cai et al. 2012; Clavijo McCormick et al. 2014; Joo et al. 2017). However, (*Z*)-3 hexenyl acetate, one of the green leaf volatiles (GLVs), appeared to be emitted predominatly in the dark phase, and this is certainly agreed with pevious studies (Arimura et al. 2008). Unlike GLVs, however, the dark-dependent emission of limonene (monoterpenoid) was not in accord with the common knowledge that monoterpenoids are exclusively synthesized via the 2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP) pathway in a light-dependent manner (Arimura et al. 2008). In the case of *B. distachyon*, however, de novo synthesis of monoterpenoids may rather rely on the alternative cytosolic mevalonate pathway or the cross-talk with the MEP pathway, in which the terpenoid precusor(s) (e.g. isopentenyl diphosphate) is exclusively exchanged, in a manner similar to that in sesquiterpenoid biosynthesis. In accord with this, teas have been shown to emit some monoterpenids ((*E*)-β-ocimene and 1,3,8-*p*-menthatriene) nocturnally in response to attack by the weevil *Myllocerinus aurolineatus* (Cai et al. 2012).

Our findings about HIPV emission from *B. distachyon* in the dark raised the question of the ecological implication(s) of

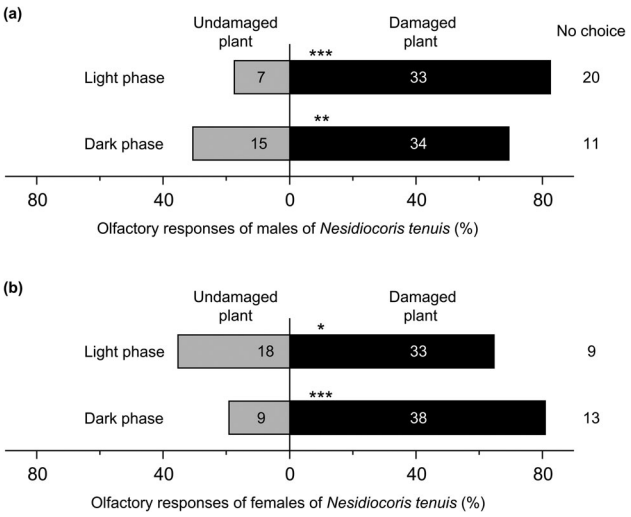

Figure 3. Olfactory response of (a) males and (b) females of *Nesidiocoris tenuis* performed during daytime and nighttime for comparison between undamaged *Brachypodium distachyon* plants vs. *B. distachyon* plants that had been damaged by five 3rd-instar *Mythimna separata* larvae. The plants exposed to *M. separata* larvae from 10:00 on the 1st day were incubated until the onset of bioassays (08:00, light condition on the 3rd day and 24:00 dark condition on the 2nd day). A replicated G-test was conducted to evaluate the significance of attraction in each experiment (***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$).

nocturnal HIPVs. Regarding this, it appeared that the response of adult females of the nocturnal herbivore *M. separata* to HIPVs induced by conspecific larvae was to avoid performing oviposition on the host plants in the dark (Figure 4). Such avoidance of HIPV-emitting host plants would be advantageous for the herbivores' offspring to avoid potential competition within the same species and accidental encounters with natural enemies. It should also be noted that *M. separata* adult females oviposited eggs in the dark irrespective of whether it occurred during daytime or nighttime, but they showed a preference to oviposit during nighttime rather than daytime (Figure 4). Accordingly, their oviposition is likely to rely primarily on light/dark conditions and partly on circadian rhythm.

In turn, the generalist predator *N. tenuis* was attracted to HIPVs irrespective of whether they were in light or dark conditions (Figure 3). This is probably because *N. tenuis* is active in both daytime and nighttime and is able to respond to VOCs from various plant taxa due to its wide range of prey and host plant dependencies (Lins et al. 2014; Rim et al. 2015, 2017). In some cases, light-dependent HIPV emissions may be beneficial for attracting day-active *Cotesia* parasitoids (Sato et al. 1983; Fukushima et al. 2002; Sharma et al. 2002). However, since *B. distachyon* is actually invaded by both diurnal and nocturnal herbivores, VOCs that are emitted during both day and night should also serve as specific infochemicals that attract herbivore enemies during both day and night and repel nocturnal herbivores at night. As described above, *N. tenuis* consumes only the eggs but not larvae of *M. separata*. This suggests the possibility that in the natural situation, the eggs of the herbivore are predated by *N. tenuis* attracted when young stages of *M. separate* larvae (~3rd instar used in the current study) that developed earlier induce the release of HIVPs from the damaged *B. distachyon* host plants.

The present study demonstrated that (1) HIPVs are emitted from *B. distachyon* plants at the highest concentrations by damaged *B. distachyon* plants in the dark, (2) predators are attracted to damaged plants in both the light and dark, and (3) herbivores oviposit on undamaged plants in the dark. Together, these findings indicate the significance

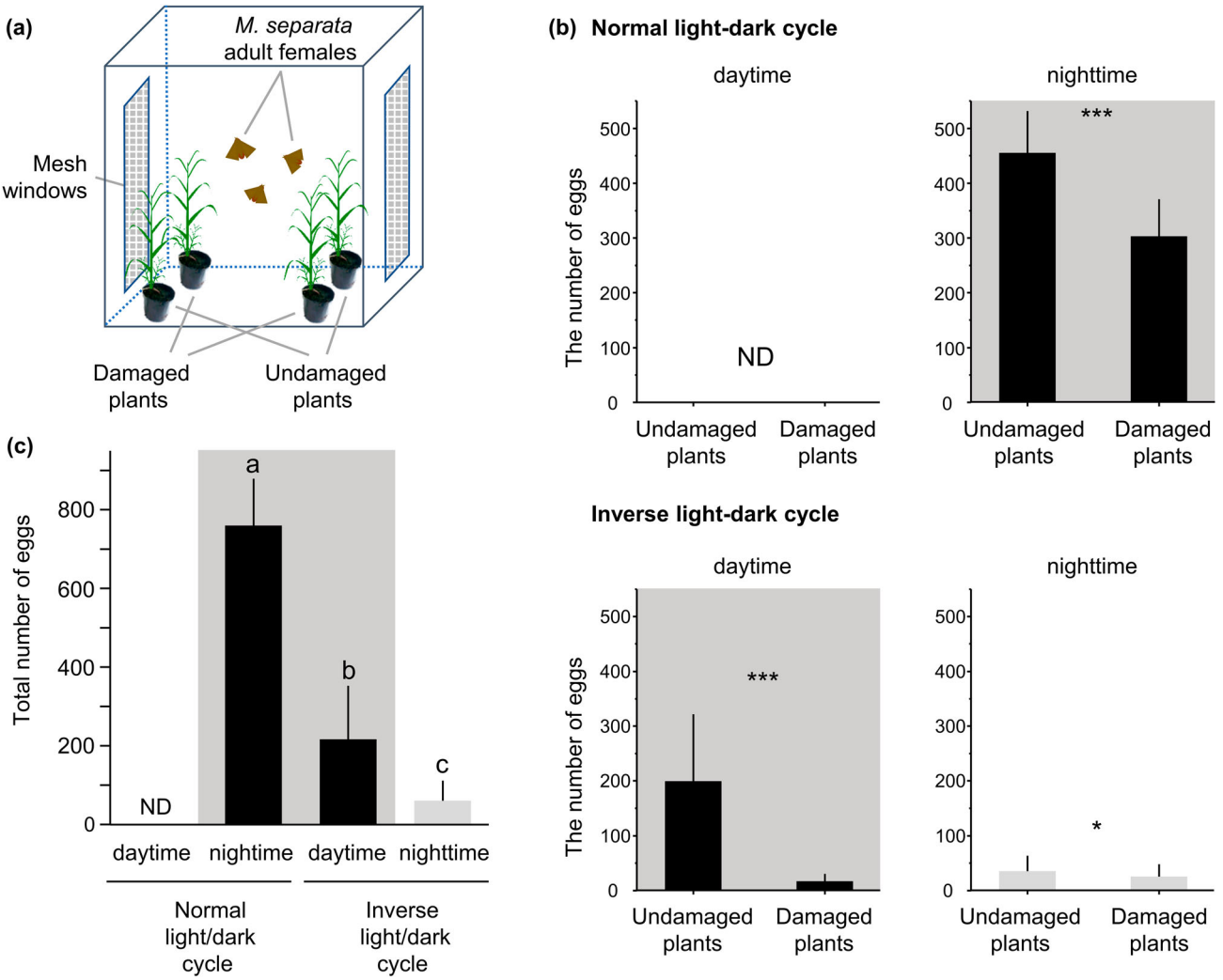

Figure 4. Ovipositional performance of *Mythimna separata* adult females in different light/dark cycle programs. A pair of undamaged *Brachypodium distachyon* plants and a pair of plants that had been damaged with *M. separata* larvae as illustrated in (a). The plants exposed to *M. separate* larvae from 10:00 (for normal light condition) or 22:00 (for inverse light condition) on the 1st day were incubated until the onset of bioassays. (b) Assays were performed during daytime (10:00–18:00 on the 3rd day for normal light condition or 22:00–06:00 on the 3rd–4th day for inverse light condition) and nighttime (22:00–06:00 on the 2nd–3rd day for normal light condition or 10:00–18:00 on the 3rd day for inverse light condition) for comparisons between undamaged and damaged plants that had been grown in the normal or inverse light/dark cycle program. Asterisks indicate a significant difference between the number of eggs on damaged and undamaged plants (***, $P < 0.001$; *, $0.01 \leq P < 0.05$, generalized linear model with Poisson distribution). (c) Total numbers of eggs laid on both undamaged and damaged plants in the different light/dark regimes in (b) and (c) were reanalyzed. Means indicated by different small letters are significantly different, based on a generalized linear model with Poisson distribution ($P < 0.05$). ND, no eggs were detected. Light and shaded sections along the graph correspond to assays performed during the photoperiod and dark period.

of the nocturnal blend of HIPVs for reducing the loss of fitness by herbivore damage, owing to not only its ability to attract predators but also to repel the adult females of the herbivores. However, it remains to be determined what volatile cue(s) is primarily working, and this issue should be addressed in future studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Notes on contributors

H.R., S.S., Y.K., and G.A. conceived and designed the experiments. H.R., S.S., and R.O. performed the experiments. H.R. and S.S. analyzed the data. H.R. and G.A. wrote the manuscript.

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