Physiological Entomology

Physiological Entomology (2018) **43**, 169–179 DOI: 10.1111/phen.12239

Elevated CO_2 may alter pheromonal communication in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

KYUNG SAN CHOI[1,*], SEUNG-JOON AHN[2,*,†], SU BIN KIM[3], JEONG JOON AHN[1], BONG NAM JUNG[1], SANG WOOK GO[1] and DONG-SOON KIM[3]

[1]Research Institute of Climate Change and Agriculture, National Institute of Horticultural & Herbal Science, RDA, Jeju, Republic of Korea, [2]Horticultural and Herbal Crop Environment Division, National Institute of Horticultural & Herbal Science, RDA, Wanju, Republic of Korea and [3]Department of Plant Resources Science, College of Agriculture & Life Sciences, SARI, Jeju National University, Jeju, Republic of Korea

Abstract. Carbon dioxide (CO_2) as a greenhouse gas has been increasing in recent decades. Because an elevated atmospheric CO_2 influences insect physiology and behaviour, we hypothesize that pheromone–mediated communication in the moth is affected by an increased CO_2 level. We test the behavioural responses of male *Helicoverpa armigera* to sex pheromone in a wind tunnel, demonstrating a significant reduction of approaching behaviour to the odour source at a high CO_2 level (1000 ppm). Electroantennogram (EAG) responses of male to the pheromone component are also significantly suppressed in high CO_2 environments (600 and 1000 ppm), indicating that a high CO_2 level inhibits both behavioural and electrophysiological responses of male to the sex pheromone. Interestingly, the EAG response of the whole head preparation of males is influenced more by the elevated CO_2 level than that of the antenna-cut preparation. A sequential increase of CO_2 levels from an ambient CO_2 level also decreases the EAG response of the whole head but not of the labial palp-removed head, implying a potential mediation of labial palp in the head where the CO_2 receptor is located. By contrast, sex pheromone production in females reared under or shifted to an elevated CO_2 condition is increased, and the putative underlying mechanism for this is discussed. The present study provides an insight into the adaptive strategy of moth pheromone communication in a changing environment.

Key words. Carbon dioxide, electroantennogram, *Helicoverpa armigera*, pheromone, wind tunnel.

Introduction

Carbon dioxide (CO_2) is a greenhouse gas that plays a significant role in global warming. The atmospheric concentration of CO_2 has dramatically increased in recent decades and is predicted to reach 538–936 ppm by the end of this century (IPCC, 2013). Understanding how rising atmospheric CO_2 levels affect insect communication is important for predicting potential changes in the physiology and behaviour of insect pests and the damages that they cause. Several studies report the effects of elevated CO_2 on chemical communication in insects (Boullis *et al.*, 2016). For example, increased CO_2 levels reduce the ability of a moth (*Cactoblastis catorum*) to detect host plants (Stange, 1997) and the escape behaviour associated with alarm pheromone is diminished under elevated CO_2 in the aphids *Chaitophorus stevensis* (Mondor *et al.*, 2004), *Sitobion avenae* (Sun *et al.*, 2010) and *Amphorophora idaei* (Hentley *et al.*, 2014). Also, an increase in CO_2 concentration reduces the production and emission of alarm pheromone in the aphid species *Acyrthosiphon pisum* (Boullis *et al.*, 2017). Because pheromones play a crucial role in mating, aggregation and alarm signals in insects (Rutowski, 1982; Cardé

Correspondence: Dong-Soon Kim, Department of Plant Resources Science, College of Agriculture & Life Sciences, SARI, Jeju National University, Jeju 63243, Republic of Korea. Tel.: +82 10 3119 5685; e-mail: dongsoonkim@jejunu.ac.kr

∗These authors contributed equally to this study.
†Present address: Department of Crop and Soil Science, Oregon State University, Corvallis, Oregon, U.S.A.

& Baker, 1984), an alteration in their communication system as a result of an increased CO_2 concentration will affect both mating and reproduction. The CO_2 receptors reported in various lepidopteran insect species are located in labial palp pits (Bogner, 1990). They have a high sensitivity and can detect differences in CO_2 levels as low as 0.5 ppm (Stange, 1992). Although the biological function of the CO_2 receptor in Lepidoptera remains unclear, it might be associated with the integration of odour signals originating from the antenna because sensory axons in the CO_2 receptor project to the antennal lobe where the ofactory neurons arrive (Guerenstein & Hildebrand, 2008).

However, comprehensive effects of increasing atmospheric CO_2 concentrations on the sex pheromone communication system have yet to be reported in any lepidopteran species. In the present study, we investigate the effect of elevated CO_2 on the sex pheromone system in *Helicoverpa armigera* not only by measuring the amount of pheromone production in females, but also by determining the electrophysiological and behavioural responses of males to pheromones at increased CO_2 levels. In addition, the putative coordinating effects of CO_2 receptors placed in the labial palp on pheromone perception are investigated by comparing labial palp-removed males with normal males.

Materials and methods

Carbon dioxide treatment

The effects of increased CO_2 were examined in three chambers ($2 \times 2 \times 2$ m) at different CO_2 levels. All other conditions were kept constant: LD 16 : 8 h photocycle at 25 ± 0.1 °C and $52 \pm 2\%$ relative humidity, with continuous air ventilation. CO_2 gas from a compressed CO_2 bottle (Dongyang, Korea) was supplied to two chambers to obtain concentrations of 600 and 1000 ppm, respectively. The control chamber received no exogenous CO_2 gas input. The CO_2 concentration in the chambers was monitored with a CO_2 sensor (GMT222; Vaisala Oyj, Finland). Temperature and humidity were monitored with a temperature sensor (107 temperature probe; Campbell Scientific Inc., Logan, Utah) and a humidity sensor (HMP60; Vaisala Oyj), respectively. These sensors were linked to a data logger (CR1000; Campbell Scientific Inc.) and CR-BASIC software (Soldan, Korea), enabling the adjustment of CO_2 concentration, temperature and humidity in each chamber via a control device (SDM-CD16AC; Campbell Scientific Inc.). All environmental conditions were recorded at intervals of 1 h. CO_2 concentrations were 451 ± 16.0 ppm in the control chamber (control), 604 ± 9.6 ppm in the 600 ppm chamber (600 ppm) and 965 ± 13.5 ppm in the 1000 ppm chamber (1000 ppm). O_2 concentrations in all of the chambers were maintained so that they were consistent with natural conditions (approximately 21%) during the experiments.

Insects and chemicals

Helicoverpa armigera were reared under an LD 16 : 8 h photocycle at 25 ± 1 °C in the laboratory using a semi-artificial diet modified from that described previously by Choi *et al.* (2011).

Synthetic sex pheromone components (*Z*)-9-hexadecenal (*Z*9-16Al), (*Z*)-11-hexadecenal (*Z*11-16Al) and (*Z*)-11-hexadecenyl acetate (*Z*11-16Ac) were purchased from Pherobank (The Netherlands). Their purities (> 97%) were confirmed by gas chromatography (GC) before conducting the experiments.

Wind tunnel

To determine the effects of CO_2 on male behavioural responses to sex pheromones, males (4–5 days old) with or without CO_2 receptors (i.e. normal or labial palp-removed) were subjected to different CO_2 treatments in a wind tunnel apparatus (40×40 cm; length 1 m; wind velocity, approximately 40 cm s^{-1}) after modifying the push and pull type wind tunnel (Kehat & Dunkelblum, 1990). The labial palp-removed males were prepared 30 min before scotophase by dissecting off the whole labial palps and a plastic cage ($15 \times 15 \times 15$ cm) containing 10–15 males was placed at the downwind end in the wind tunnel installed inside different CO_2 chambers. The wind tunnel experiment was conducted at 5 h after lights off in the same CO_2 environment where the insect had been reared. The odour source comprised a sex pheromone lure containing 1 mg of two sex pheromone components, *Z*11-16Al and *Z*9-16Al, at a ratio of 97.5 : 2.5 deposited in a rubber septum (outer diameter 11 mm; Wheaton, DWK Life Sciences, Millville, New Jersey) with diluted solution (5 μg μL^{-1}) in *n*-hexane (> 99%; Sigma-Aldrich, St Louis, Missouri). The lure was placed on a plate (diameter 10 cm, height 8 cm) located 80 cm upwind from the cage. After placing the odour source and opening the cage, the responses of males to the odour source were monitored for 10 min under a pale infrared lamp (Philips, The Netherlands). The behavioural responses were classified into three types: (i) upwind, males flew upwind and landed within 30 cm from the odour source; (ii) approach, males approached within 5 cm from the odour source; males showing the approach behaviour were isolated promptly so that they could not obstruct other males' response; and (iii) contact, males contacted the odour source with a direct flight. Approximately 100 normal males and 100 labial palp-removed males were tested for each CO_2 treatment in each generation. This experiment repeated for three generations. Males exposed to lure were not used again in this experiment because pre-exposure can affect their behavioural response to the lure (Mafra-Neto & Baker, 1996). Males that did not fly out of the cage during the test were excluded from the analysis.

Electroantennography (EAG)

EAG of males reared at different CO_2 levels. To determine the effects of CO_2 on the antennal response to sex pheromone, 3-day-old males that had been adapted to different CO_2 treatments for three generations were EAG tested with various concentrations of the major sex pheromone component of *H. armigera*, *Z*11-16Al. The synthetic compound was serially

diluted in *n*-hexane (> 99%; Sigma-Aldrich) at different dosages (0.05, 0.5, 5, 50 and 500 ng μL^{-1}) and 20 µL of each solution was loaded onto filter paper strips (0.3 × 6 cm; Whatman No. 1; Whatman, U.K.). Air and 20 µL of *n*-hexane were used as a control. After approximately 1 min of drying, the filter paper was inserted into a Pasteur pipette (length 15 cm) and used as an odour source.

The head was cut from an adult male and one antenna was removed. After the neck was mounted onto a recording electrode, the tip of the remaining antenna was cut and connected to the reference electrode of the EAG probe (PRG-2) using electroconductive gel (Parker Laboratories, Fairfield, New Jersey). The preparation was then placed close to the outlet of an air-flowing glass tube (length 120 mm, inner diameter 8 mm) where the odour stimulus was loaded. A charcoal-filtered and humidified air stream was released continuously (600 mL min^{-1}) over the preparation via a stainless steel pipe during the EAG test. The glass pipette connected to the stimulus air controller (model CS-05; Syntech, The Netherlands) was used for stimulation. Signals were amplified with an amplifier (UN-06; Syntech) and displayed on a computer via a software interface (EAGPRO, Syntech). As soon as the EAG test with the head was finished, we proceeded to the next test, which investigated an antenna sample detached from the head that had been used in the previous EAG test (see Supporting information, Fig. S1A). The antenna preparation procedure for the EAG test was same as that mentioned above. EAG tests were conducted in the same CO_2 chamber where the insects had been reared under different CO_2 concentrations. The EAG responses of the antenna separated from or attached to head were also measured at higher (control to 1000 ppm) or lower (1000 ppm to control) CO_2 levels. A series of odour sources (air, hexane and 0.001–10 µg of *Z*11-16Al in turn with intervals of at least 15 s) were tested with four cycles of repeat onto each preparation (head and antennae in order) of 10 males in total.

EAG by sequential changes of CO_2 concentration. Previous EAG tests were not fully sufficient for evaluating the role of CO_2 receptors in the labial palp and the effects of CO_2 on antenna under conditions of changing CO_2 levels because the adapted adult males at each CO_2 level were tested at the same CO_2 level. For the purpose of examining the role of CO_2 receptors, EAG responses were compared between different antennal samples: (i) detached from the head; (ii) attached to the head; and (iii) attached to the head without labial palps from males reared in the control CO_2 environment. The labial palp-removed head was prepared as soon as the EAG test on the head was finished (see Supporting information, Fig. S2). The synthetic compound of *Z*11-16Al was serially diluted in *n*-hexane at different dosages (0.5, 5, 50, 500 and 5000 ng μL^{-1}) and 20 µL of each solution was loaded onto filter paper strips. A series of odour sources (air, hexane and 0.01–100 µg of *Z*11-16Al in turn with intervals of at least 15 s) were repeated for three cycles at each CO_2 level (450, 600, 1000, 1500 and 2000 ppm in order) for each sample (antenna, head and head without labial palps in order). The antennal samples were kept at the specified CO_2 levels of each test for 30 min to acclimatize. The relative EAG responses of the head and the labial palp-removed head at higher CO_2 levels were calculated after normalizing the EAG response to that of the control. Average CO_2 concentrations during the tests were 449 ± 14.3 ppm for the control, 629 ± 68.4 ppm at 600 ppm, 1039 ± 84.5 ppm at 1000 ppm, 1539 ± 44.5 ppm at 1500 ppm and 2056 ± 60.9 ppm at 2000 ppm.

Pheromone quantification

The sex pheromone gland of virgin females (2 days old) reared under different CO_2 concentrations was dissected at 2, 4, 5, 6, 7, 8 and 10 h after lights off by temporarily anaesthetizing the female with excessive CO_2 gas. The gland was extracted in 20 µL of *n*-hexane containing 5 ng μL^{-1} *Z*11-16Ac as an internal standard. The sex pheromone extract was analyzed by GC (QP-2010; Shimadzu, Japan) using a capillary column (30 m × 0.32 mm inner diameter, thickness 0.25 µm; Rtx5). The injector was set at 250 °C. The oven temperature was set at 80 °C for 1 min, raised 15 °C min^{-1} to 140 °C, raised 10 °C min^{-1} to 200 °C, and then maintained at 200 °C for 7 min. One microlitre of the extract was injected in splitless mode. Helium, a carrier gas, was released into the column at 2 mL min^{-1}. Column flow was controlled by a pressure mode set at 74.5 kPa. Flame ionization detector was set at 280 °C. Quantitative analysis with GC was conducted on the major sex pheromone component (*Z*11-16Al) of *H. armigera*. Fifteen females were extracted each time from each CO_2 concentration treatment. To examine the role of CO_2 receptors in the pheromone production, the labial palp-removed females were prepared before scotophase by dissecting out the labial palps. Pheromone glands of the labial palp-removed females ($n = 15$) were extracted every 2 h in each CO_2 treatment until the end of scotophase and pheromone amounts were quantified as described above. Additionally, sex pheromone production was examined for normal females when they were moved to a higher (control to 1000 ppm) or lower (1000 ppm to control) CO_2 level immediately before the scotophase.

Mating

A pair of virgin female and male (< 1 day) was transferred to a cylinder-type plastic cage (diameter 15 cm, height 10 cm) covered over with a piece of gauze. Mating was considered to be successful when the egg laid by female was hatched. Thirty pairs were observed in each CO_2 treatment until the females died. This experiment was replicated for three generations.

Statistical analysis

All data sets were tested for normality and subjected to analysis of variance using SAS (SAS Institute, 2006). Means were separated by Tukey's range test at $P = 0.05$. Student's *t*-tests were also conducted to compare the results between two samples.

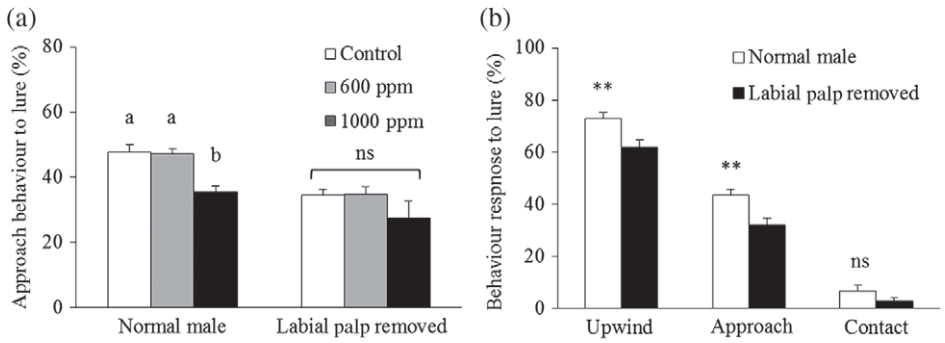

Fig. 1. Wind-tunnel behavioural response of *Helicoverpa armigera* male reared at different CO_2 levels. Data are the mean ± SE. (A) Approach behaviour of the male to 1 mg of sex pheromone lure containing *Z*11-16Al and *Z*9-16Al at a ratio of 97.5 : 2.5 (normal male: $n = 237$ in control; $n = 246$ in 600 ppm; $n = 228$ in 1000 ppm; $P < 0.05$, labial palp-removed: $n = 215$ in control; $n = 229$ in 600 ppm; $n = 221$ in 1000 ppm; $P > 0.05$). (B) Upwind, approach and contact behavioural responses between normal male (n = 711) and labial palp-removed male ($n = 665$) (**$P < 0.01$).

Results

Development and fecundity of H. armigera *at different CO_2 levels*

Through successive rearing for three generation at each of the different atmospheric CO_2 levels, no differences were apparently observed, except that the development time of the immature stage shortened slightly and female fecundity increased at higher CO_2 levels compared with the lower atmospheric CO_2 levels (data not shown).

Behavioural response of male at different CO_2 levels

A wind tunnel test was conducted with normal males and labial palp-removed males at different atmospheric CO_2 levels. In the normal group of males, the approach behaviour decreased significantly at 1000 ppm CO_2 (normal male: d.f. = 2,6, $F = 9.15$, $P = 0.0151$) (Fig. 1A). The approach behaviours of labial palp-removed males were not significantly different between the different CO_2 levels (labial palp-removed male: d.f. = 2,6, $F = 0.94$, $P = 0.4428$) (Fig. 1A). However, the labial palp-removed males showed a highly significant reduction in upwind and approach behaviours compared with normal males (upwind: d.f. = 16, $t = 2.92$, $P = 0.0099$; approach: d.f. = 16, $t = 3.34$, $P = 0.0042$; contact: d.f. = 16, $t = 1.62$, $P = 0.1249$) (Fig. 1B).

EAG

EAG of males reared at different CO_2 levels. The EAG responses of *H. armigera* males were affected largely by CO_2 concentrations and antenna preparation methods (Fig. 2). EAG responses from the whole head preparation were significantly lower at higher CO_2 levels (600 and 1000 ppm) compared with the normal CO_2 level (control) at different pheromone doses (head: $F = 2.79$, $P = 0.0791$ in air; $F = 4.11$, $P = 0.0276$ in hexane; $F = 4.66$, $P = 0.0183$ in 0.001 μg; $F = 4.83$, $P = 0.0161$ in 0.01 μg; $F = 5.89$, $P = 0.0075$ in 0.1 μg; $F = 6.93$, $P = 0.0037$ in 1 μg, $F = 4.72$, $P = 0.0175$ in 10 μg; with d.f. = 2,27 in all cases) (Fig. 2). However, EAG responses of antenna preparation were not significantly different between CO_2 levels at all sex pheromone doses except at 1 μg of *Z*11-16Al (antenna: $F = 1.37$, $P = 0.2719$ in air; $F = 2.4$, $P = 0.1099$ in hexane; $F = 1.8$, $P = 0.1848$ in 0.001 μg; $F = 1.45$, $P = 0.2516$ in 0.01 μg; $F = 2.66$, $P = 0.0881$ in 0.1 μg; $F = 3.76$, $P = 0.0362$ in 1 μg, $F = 0.89$, $P = 0.4227$ in 10 μg; with d.f. = 2,27 in all cases) (Fig. 2).

When males were transiently transferred from the rearing CO_2 condition to different CO_2 concentrations for the EAG test, the EAG responses were affected by the CO_2 concentration at which the EAG test was conducted (Table 1). In the present study, we measured EAG responses in four groups of males: constant CO_2 conditions (reared and tested in the control or 1000 ppm) and altered CO_2 conditions (reared in a control environment but tested in 1000 ppm or reared in 1000 ppm but tested in the control environment). Based on tests of antenna sections, EAGs decreased when males moved to high CO_2 (control → 1000 ppm) or increased when males moved to low CO_2 (1000 ppm → control). Based on the tests of whole head, however, the EAG responses of males moved to a high CO_2 environment were not readily fitted to those of the same constant CO_2 environment (Table 1).

EAG by sequential changes of CO_2 concentration.. EAG responses of *H. armigera* males reared under normal CO_2 conditions (control) and subjected to sequential increase of CO_2 concentration are shown in Fig. 3. EAG responses were gradually increased with increasing pheromone doses in the antenna section. They were significantly different between CO_2 levels at all pheromone doses tested (Fig. 3A; see also Supporting information, Table S1). The response of whole head showed a significant difference between CO_2 levels at 1 and 10 μg of *Z*11-16Al (Fig. 3A; see also Supporting information, Table S1). The EAG pattern was largely different between the whole head and a labial palp-removed head. The results obtained for the labial palp-removed head did not show a significant difference in EAG responses between different CO_2 levels

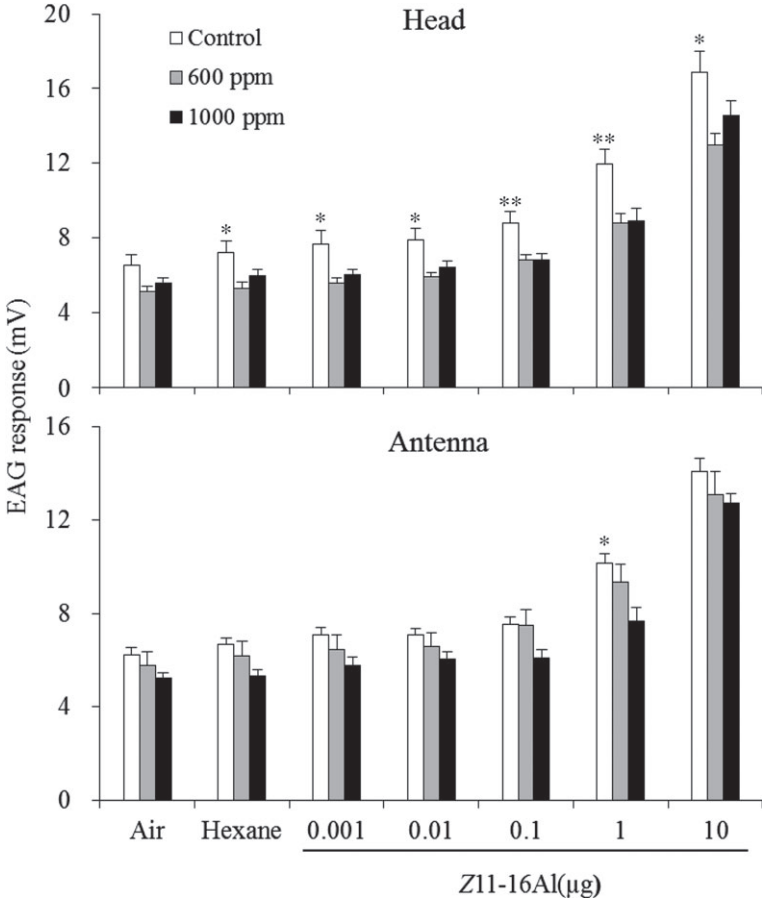

Fig. 2. Electroantennogram (EAG) response of *Helicoverpa armigera* males (3 days old) reared at different CO_2 levels to a series of concentrations of a synthetic sex pheromone component, *Z*11-16Al. EAG response of the head and antenna in turn ($n = 10$). Data are the mean ± SE. (*$P < 0.05$, **$P < 0.01$).

Table 1. Electroantennogram (EAG) response of male *Helicoverpa armigera* to sex pheromone component *Z*11-16Al at different CO_2 levels.

CO_2 level[a] (ppm)		Dose of *Z*11-16Al (μg)[b]						
Reared	Tested	Air	Hexane	0.001	0.01	0.1	1	10
Antenna section								
Control	Control	6.2 ± 0.32 ab[c]	6.7 ± 0.27 ab	7.1 ± 0.35 a	7.1 ± 0.27 a	7.5 ± 0.33 a	10.2 ± 0.42 a	14.1 ± 0.55 a
Control	1000	5.5 ± 0.36 ab	5.9 ± 0.30 b	6.4 ± 0.31 a	6.5 ± 0.29 a	7.0 ± 0.38 ab	8.1 ± 0.40 bc	13.7 ± 0.63 a
1000	Control	6.7 ± 0.41 a	6.5 ± 0.38 ab	6.9 ± 0.39 a	7.1 ± 0.36 a	7.7 ± 0.34 a	9.9 ± 0.33 ab	14.4 ± 0.41 a
1000	1000	5.2 ± 0.21 b	5.3 ± 0.28 ab	5.8 ± 0.35 a	6.0 ± 0.31 a	6.1 ± 0.36 b	7.7 ± 0.58 c	12.7 ± 0.41 a
Whole head								
Control	Control	6.5 ± 0.58 a	7.2 ± 0.62 a	7.7 ± 0.72 a	7.9 ± 0.62 a	8.8 ± 0.64 a	12.0 ± 0.77 a	16.9 ± 1.12 a
Control	1000	7.1 ± 1.15 a	7.9 ± 1.31 a	7.7 ± 1.04 a	8.2 ± 1.33 a	8.8 ± 1.2 a	11.4 ± 1.67 a	18.1 ± 2.14 a
1000	Control	7.6 ± 0.57 a	7.6 ± 0.55 a	8.0 ± 0.60 a	8.1 ± 0.53 a	9.0 ± 0.46 a	11.8 ± 0.51 a	17.4 ± 0.76 a
1000	1000	5.6 ± 0.29 a	6.0 ± 0.36 a	6.0 ± 0.31 a	6.4 ± 0.37 a	6.8 ± 0.33 a	8.9 ± 0.66 a	14.6 ± 0.74 a

[a]Moths were reared and/or tested under the specific CO_2 environment for three generations (control = 451 ± 16.0 ppm; 600 = 604 ± 9.6 ppm; 1000 = 965 ± 13.5 ppm).

[b]EAG tests were conducted with 3-day-old males ($n = 10$). Each stimulus was treated four times in turn.

[c]Means followed by same letters in a column are not different by the honestly significant difference test at $P = 0.05$ (Antenna section: $F = 3.73$, $P = 0.0196$ in air; $F = 3.6$, $P = 0.0227$ in hexane; $F = 2.5$, $P = 0.0752$ in 0.001 μg; $F = 2.3$, $P = 0.0937$ in 0.01 μg; $F = 3.69$, $P = 0.0206$ in 0.1 μg; $F = 7.07$, $P = 0.0007$ in 1 μg; $F = 1.82$, $P = 0.1612$ in 10 μg, whole head: $F = 1.28$, $P = 0.2963$ in air; $F = 1.01$, $P = 0.4008$ in hexane; $F = 1.45$, $P = 0.2438$ in 0.001 μg; $F = 0.98$, $P = 0.4114$ in 0.01 μg; $F = 1.79$, $P = 0.1665$ in 0.1 μg; $F = 1.81$, $P = 0.1623$ in 1 μg; $F = 1.18$, $P = 0.3301$ in 10 μg; with d.f. = 3, 36 in all cases).

at all pheromone doses tested (Fig. 3A; see also Supporting information, Table S1). The scaled EAG pattern to that of normal CO_2 (control) clearly revealed a difference in EAG between heads with and without CO_2 receptors (Fig. 3B). The scaled EAG responses of those with a CO_2 receptor were decreased in a CO_2 concentration-dependent manner. However, the scaled EAG responses were unchanged for those without labial palps. The relative proportion of the EAG of a whole head compared with the labial palp-removed head decreased nonlinearly with increasing CO_2 (Fig. 3C).

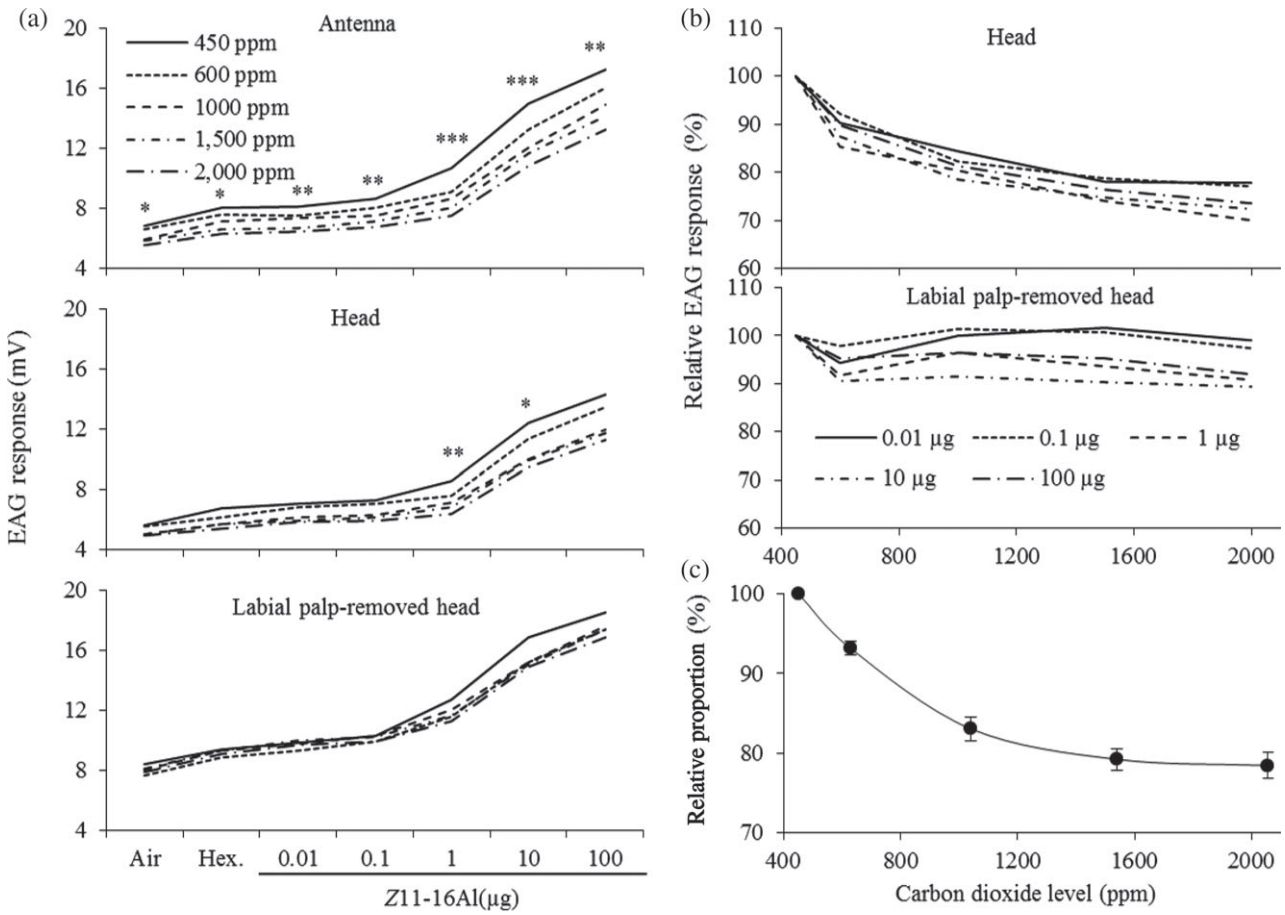

Fig. 3. Electroantennogram (EAG) response of *Helicoverpa armigera* male (3 days old) at increasing CO_2 levels to a series of concentrations of a synthetic sex pheromone component, *Z*11-16Al. Data are the mean ± SE. (A) The EAG test was conducted with the opposite antenna, the head and the labial palp-removed head in turn ($n = 9$; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (B) Relative EAG response of the head and that of the labial palp-removed head, respectively, normalized to the response with control CO_2. (C) Relative portion of the head response to the response of labial palp-removed head (d.f. = 2,220, $F = 24.48$, $P < 0.0001$).

Sex pheromone perception of male at different CO_2 levels

The intensity of EAG signals was diminished in higher CO_2 environments both in the antennal and head preparations (Fig. 2). Interestingly, the EAG patterns were largely different between the antennal and head preparations, showing lower responses in the head preparation as the CO_2 levels increased. In the wind-tunnel experiment, the approach behaviour of normal males was different from that of males without CO_2 receptors (Fig. 1A). Furthermore, the EAG response of labial palp-removed head did not show a significant difference among different CO_2 levels for all pheromone doses tested and appeared to be no different from each other at higher CO_2 levels (Fig. 3A). The scaled EAG pattern compared with that of a normal CO_2 concentration (control) showed a clear difference in EAGs between heads with and without labial palps (Fig. 3B). EAGs for heads with labial palps were decreased in a CO_2 concentration-dependent manner. However, EAGs for heads without labial palps were almost unchanged. The relative proportion of the EAG of the whole head compared with that of labial palp-removed head decreased nonlinearly with increasing CO_2 (Fig. 3C). The lessening effect of EAG may be mediated by CO_2 receptors in labial palps.

Pheromone quantification

Elevated CO_2 significantly increased pheromone biosynthesis of *H. armigera* in females ($n = 103$ in control; $n = 105$ in 600 ppm; $n = 105$ in 1000 ppm) (Fig. 4B). Females reared at 600 and 1000 ppm CO_2 showed a general increase in sex pheromone production compared with those reared under control conditions of CO_2 (d.f. = 2,40 in 2 h, d.f. = 4,42 in others; 2 h: $F = 3.85$, $P = 0.0296$; 4 h: $F = 9.57$, $F = 0.0004$; 5 h: $F = 5.88$, $P = 0.0056$; 6 h: $F = 1.54$, $P = 0.2266$; 7 h: $F = 12.31$, $P < 0.0001$; 8 h: $F = 3.57$, $P = 0.037$; 10 h: $F = 2.49$, $P = 0.0953$) (Fig. 4B). The labial palp-removed female showed a significantly lowered sex pheromone production than normal females, particularly under control conditions and at 600 ppm ($n = 105$ in control and 600 ppm; $n = 104$ in 1000 ppm; control: d.f. = 26,

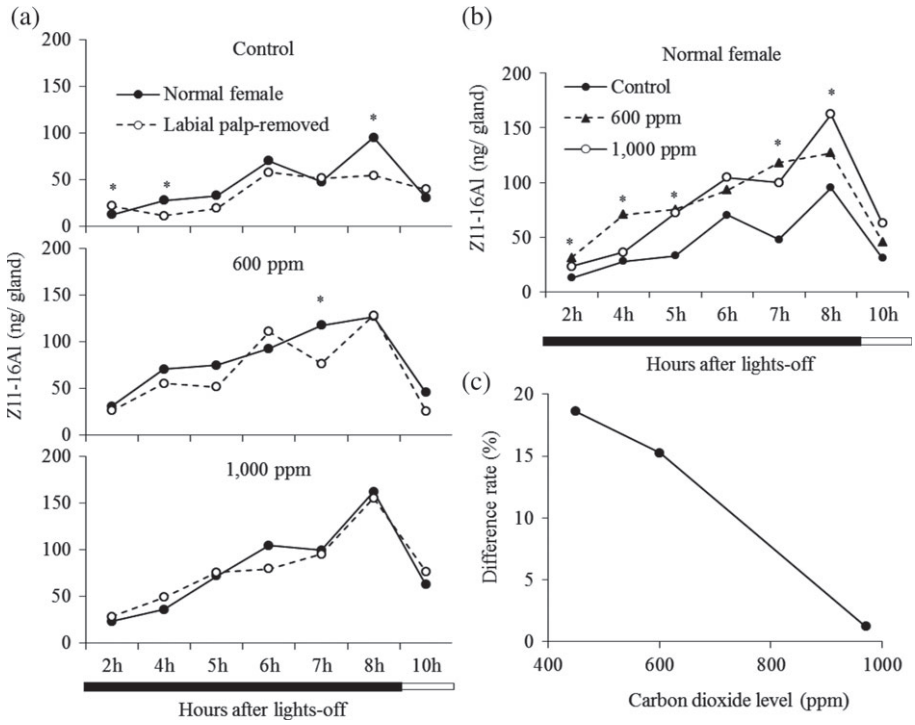

Fig. 4. Sex pheromone production of *Helicoverpa armigera* females at different CO_2 levels. Data are the mean ± SE (■, scotophase; □, photophase) (*$P < 0.05$). (A) Comparison of the average amount of a major sex pheromone component *Z*11-16Al produced per female reared at different CO_2 levels with a series of time after lights off between normal female and labial palp-removed female (normal female: $n = 103$ in control; labial palp-removed female: $n = 104$ in 1000 ppm; others: $n = 105$). (B) Changes in the *Z*11-16Al production of normal females at different CO_2 levels. (C) Changes in the difference rate in the total amount of the sex pheromone production of the labial palp-removed female compared with that of the normal female at different CO_2 levels.

$|t| = 3.5$, $P = 0.0023$ in 2 h; d.f. = 28, $|t| = 3.8$, $P = 0.001$ in 4 h; d.f. = 28, $|t| = 2.31$, $P = 0.0285$ in 8 h; 600 ppm: d.f. = 27, $|t| = 2.42$, $P = 0.0222$ in 7 h; others: $P > 0.05$) (Fig. 4A). The difference in total amounts of sex pheromone between the labial palp-removed females and normal females showed a decreasing tendency as the CO_2 levels increased (Fig. 4C). In addition, sex pheromone production was changed when females were transferred to higher or lower CO_2 levels before socotophase (4 h: d.f. = 3,56, $F = 6.46$, $P = 0.0008$; 5 h: d.f. = 3,56, $F = 3.13$, $P = 0.0326$; 5 h: d.f. = 3, 55, $F = 2.4$, $P = 0.0781$; 6 h: d.f. = 3,56, $F = 10.37$, $P < 0.0001$; 8 h: d.f. = 3,54, $F = 5.17$, $P = 0.0033$) (Table 2). When transferred to higher CO_2 levels (control to 1000 ppm), females produced a high amount of sex pheromone, which was sometimes as much as that of females reared at 1000 ppm CO_2, especially 5 and 6 h after lights off. In addition, females moved to lower CO_2 levels (1000 ppm to control) showed a reduction in sex pheromone production, which was sometimes as much as that of females reared under control conditions of CO_2 at 6 and 8 h after lights off.

Mating

The successful mating of *H. armigera* was increased by approximately two-fold at 600 ppm CO_2 compared with the control, although it was not significantly increased at 1000 ppm (d.f. = 2, 6, $F = 7.66$, $P = 0.0223$) (Fig. 5).

Discussion

Sex pheromone-mediated communication is crucial for mating and reproduction, particularly in moths (Rutowski, 1982; Cardé & Baker, 1984). However, the effects of the atmospheric CO_2 concentration on the pheromone system are not well understood. In the present study, we report the modulatory effects of an elevated CO_2 level on the pheromone-mediated communication system of *H. armigera*. The elevated CO_2 levels significantly decrease and disrupt behavioural (Fig. 1) and electrophysiological (Figs 2 and 3 and Table 1) responses of *H. armigera* males to the sex pheromone. The higher CO_2 environment also prolongs the sex pheromone production of females (Fig. 4B). Our results indicate that pheromone perception in *H. armigera* might be disrupted by elevated CO_2 levels, which could be complemented by increased sex pheromone production (Fig. 4B). We anticipate that our results will not only provide new insights into the interaction between insects and CO_2, but also a new direction for pest management in the future in relation to expected climate changes.

Sex pheromone receptors in antenna are positioned in porous olfactory sensilla (Steinbrecht, 1997) where olfactory neurons

Table 2. Changes in amount of *Z*11-16Al biosynthesized in the sex pheromone gland of female *Helicoverpa armigera* at different CO_2 levels.

CO_2 level[a] (ppm)		Amount (ng) of *Z*11-16Al produced hours after lights off[b]				
Reared	Tested	4 h	5 h	6 h	7 h	8 h
Control	Control	27.7 ± 3.68 b[c]	32.8 ± 6.00 b	70.2 ± 8.76 a	47.6 ± 7.65 c	95.2 ± 12.52 b
Control	1000	26.9 ± 3.68 b	64.7 ± 6.00 ab	109.3 ± 8.76 a	63.3 ± 7.65 bc	83.4 ± 12.52 b
1000	Control	65.0 ± 10.77 a	59.0 ± 10.64 ab	70.6 ± 14.41 a	119.2 ± 15.61 a	105.7 ± 13.23 ab
1000	1000	36.1 ± 6.63 b	72.1 ± 11.01 a	104.7 ± 17.00 a	99.7 ± 8.16 ab	162.3 ± 19.64 a

[a]Moths were reared and/or tested in the CO_2 environment (control: 451 ± 16.0 ppm; 600: 604 ± 9.6 ppm; 1000: 965 ± 13.5 ppm).
[b]Quantification on the sex pheromone component was conducted with the sex pheromone gland detached from 2 day-old females ($n = 15$) at each hour.
[c]Means followed by same letters in column are not different by the honestly significant difference test at $P = 0.05$ (4 h: d.f. = 3,56, $F = 6.46$, $F = 0.0008$; 5 h: d.f. = 3,56, $F = 3.13$, $P = 0.0326$; 6 h: d.f. = 3,55, $F = 2.4$, $P = 0.0781$; 7 h: d.f. = 3,56, $F = 10.37$, $P < 0.0001$; 8 h: d.f. = 3,54, $F = 5.17$, $P = 0.0033$).

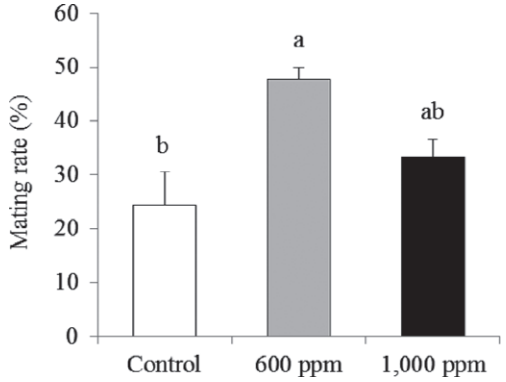

Fig. 5. Mating rate of *Helicoverpa armigera* adult pair reared at different CO_2 levels. Data are the mean ± SE ($n = 90$; d.f. = 2,26, $F = 7.66$, $P = 0.0233$).

are embedded in the receptor lymph. The gaseous CO_2 can be easily infiltrated into the inner part of olfactory sensilla via the pore. It is then rapidly converted into carbonic acid (H_2CO_3), probably by carbonic anhydrase present in the receptor cells (Brown *et al.*, 1984), resulting in an acidification of sensilum-lymph same as the mechanism in haemolymph whose pH decreases when exposed to carbon dioxide gas (Badre *et al.*, 2005). Acidification of sensilum-lymph can cause conformational changes of pheromone-binding proteins (PBPs) and alter their binding affinities to pheromone molecules. A great conformational change in PBP occurs at pH 5 and 6 in *Antheraea polyphemus* (Mohantyl *et al.*, 2004). The PBP of *Bombyx mori* is reported to release pheromone compound as a result of a lower binding affinity at lower pH near the dendrite membrane surface (Damberger *et al.*, 2013). In addition, the ion concentration in sensilum-lymph can be affected by an increased CO_2 level. Transepithelial potential (TEP) represents the voltage source of a receptor neurone. It is generated by H^+-dependent K^+ ion transport energized by H^+-pumping V-ATPase in the membrane of tormogen and trichogen (Thurm & Wessel, 1979; Kaissling, 1986; Klein, 1992). K^+ ion transport is linked to pH gradient and V-ATPase activity (Dow, 1992). This could be inhibited by acidification in the extra- or intracellular micro-environment (Zeiske *et al.*, 2002). Consequently, reduced K^+ ion transport activity causes a reduced voltage and increased resistance in the membrane (Küppers & Bunse, 1996). Furthermore, CO_2 can affect the neuronal signal by decreasing the firing rate and hyperpolarization (Dean *et al.*, 1989; Williams *et al.*, 2007). Based on these mechanisms, an increased CO_2 concentration can decrease sensitivity to odour molecules and/or suppress signal transduction in the olfactory system.

The hypothetical neural network of pheromone production and CO_2 receptor in insect central nervous system is shown in Fig. 6(A). The sex pheromone signal from male antennae is encoded in the antennal lobe (AL) and is then transmitted to the protocerebrum (PC) (De Belle & Kanzaki, 1999; Hansson & Christensen, 1999). The CO_2 sensory axons of *H. armigera* sensilla extend to the labial pit organ glomerulus (LPOG) in each AL, to the suboesophageal ganglion (SOG) and to the ventral nerve cord (VNC) (Zhao *et al.*, 2013). Consequently, the CO_2 concentration-dependent reduction of the EAG response measured on male heads with CO_2 receptors can be explained partially by the role of the CO_2 receptor with respect to modulating the signal of the pheromone receptor in the PC in accordance with the signal induced by the CO_2 receptor, probably as a relative rate (Fig. 3C). For example, a radio has a distinctive procedure for sieving the sound from a noise. The filtrated signals are amplified and noise is removed. Similarly, the AL might be an organ for such a function, although it only deals with the signal produced from the antenna. We show that the signal intensity produced from the antenna could change at different CO_2 levels. The pheromone signal encoded by the AL could have some false information. To date, there are no reports available about any organ mediating or correcting the differences between outside and inside information. However, this type of organ should exist in insects because they pivotally depend on chemical communication and chemoreception. Therefore, CO_2 receptors might act as probes for the outside environment and help interpret the pheromone signal by providing a CO_2 induced signal to correct signal errors or variations of the receptor that are easily affected by the surrounding CO_2 concentration. Consequently, an elevated CO_2 level can directly affect pheromone receptor in the antenna and decrease the perception ability of males with respect to the female sex pheromone. In addition, CO_2 receptor in the labial palp might be involved in a process of pheromone-mediated perception in this moth.

By contrast to the decreasing receptivity of males to the pheromone component, elevated CO_2 significantly increases pheromone biosynthesis in females of *H. armigera* (Fig. 4B). Changes in CO_2 levels cause alterations in sex pheromone

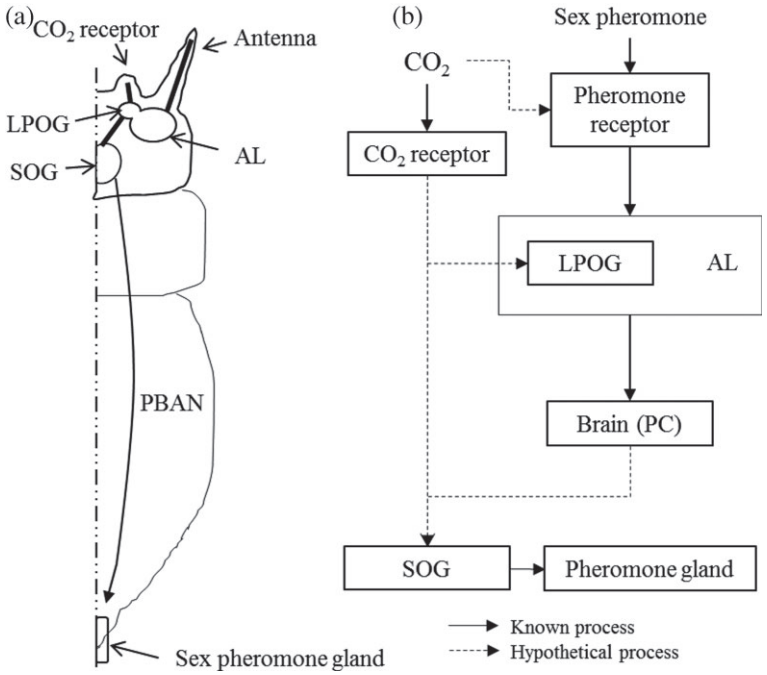

Fig. 6. Hypothetic process of carbon dioxide effect on the sex pheromone perception of male and its biosynthesis in female moth. (A) Illustration of organs related with sex pheromone perception and biosynthesis in a moth: AL, antennal lobe; LPOG, labial pit organ glomerulus; SOG, suboesophageal ganglion; PC, protocerebrum; PBAN, pheromone biosynthesis activating neuropeptide. (B) Hypothetic networks for CO_2 receptor-mediated control of pheromone production in females and pheromone perception in males.

production (Table 2). Furthermore, sex pheromone production of the labial palp-removed female is lower than that of the normal female and the difference becomes smaller as CO_2 levels increase (Fig. 4A,C). The synthesis of sex pheromone in insect species is controlled by pheromone biosynthesis activating neuropeptide (PBAN) (Rafaeli *et al.*, 1991; Rafaeli, 2009). The neuropeptide is first synthesized in the brain and then released into haemolymph through SOG, resulting in the activation of PBAN receptors on the pheromone gland, which triggers a pheromone biosynthesis cascade (Fig. 6A). Coincidently, the neuron of the CO_2 receptors projects to the SOG, where PBAN is produced and released, indicating that CO_2 receptors might be involved in pheromone production, probably by affecting SOG in the central nervous system (Fig. 6B).

In the present study, we find that a high CO_2 concentration negatively affects the detection ability of *H. armigera* males to sex pheromone, with CO_2 receptors being involved. By contrast, sex pheromone production of females is increased at elevated CO_2 levels. The discriminative response between males and females to the CO_2 environment may be an adaptive strategy enabling their survival in a variable CO_2 environment because a low detection ability of males to sex pheromone can be compensated for by increased pheromone production in females. This strategy could be effective for increasing the mating rate between males and females within a short distance. The mating rate of *H. armigera* increases significantly at 600 ppm, although this is not the case at a higher level (1000 ppm) of CO_2 (Fig. 5). However, it is difficult to expect such successful mating in the natural environment because males and females are generally located far away in complex structural habitats.

Some previous studies report the negative effect of CO_2 on the behaviour of animals, including numerous arthropods. Increased CO_2 levels reduce the detectability of the cactus moth (*C. catorum*) to host signals (Stange, 1997) and diminish escaping behaviour in several aphid species *Chaitophorus stevensis* (Mondor *et al.*, 2004), *S. avenae* (Sun *et al.*, 2010) and *A. idaei* (Hentley *et al.*, 2014). In addition, ocean acidification as a result of elevated CO_2 levels is reported to have reduced the olfactory response in a marine fish and disrupted its discriminatory ability (Munday *et al.*, 2009). Also, the production and emission of alarm pheromone in an aphid, *A. pisum*, is reduced under elevated CO_2 (Boullis *et al.*, 2017). Such results are suggested to be a result of the elevated CO_2 condition affecting the central nervous system (Mondor *et al.*, 2004; Sun *et al.*, 2010). Furthermore, the results of the present study provide a possible reason explaining how chemical communication in a moth can be disrupted under elevated CO_2 levels. Over the evolutionary time scale, the atmospheric CO_2 concentration had increased up to approximately 2000 ppm by the end of the Permian and up to 2500 ppm in the Jurassic (Royer, 2006). Insects belonging to eight different orders became extinct at the end of Permian and also one order became extinct in the Jurassic (Carpenter & Burnham, 1985). To our knowledge, the direct relationship between the extinction and the CO_2 level has not been reported previously. The results of the present study indicate that the increased CO_2 level in the past might have diminished perception ability and disrupted chemical communication in the extinct insect species. Most current lepidopteran species evolved in the Cenozoic (Labandeira & Sepkoski, 1993) when the CO_2 level was below 500 ppm (Royer, 2006). Therefore, it is most likely that lepidopteran species cannot live in a high CO_2 environment, although some social insects such as bees or ants can thrive in a hive or underground where CO_2 gas is densely accumulated (Jones, 2013). Taking together with the results of the present study and previous reports, it is expected that moth populations will largely decline in the future as a result of disrupted mating

putatively caused by elevated CO_2 levels. In addition, the present results not only provide an insight into the interaction between insects and CO_2, but also a new perspective for future pest management in the challenging era of climate change.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Electroantennogram (EAG) response of male *Helicoverpa armigera* to sex pheromone component *Z*11-16Al at sequentially increased atmospheric CO_2 levels (see Materials and methods).

Figure S1. Electroantennogram (EAG) experiment on male antenna and head samples. (A) EAG process for population reared at CO_2 levels of 450 (control), 600 and 1000 ppm, (B) Representative EAG response and intensity measurement and (C) representative EAG responses for an antenna at 450 ppm CO_2.

Figure S2. EAG experiment on male antenna, head, and head with the labial palps removed at changing CO_2 levels. (A) Experimental procedure for increasing CO_2 levels (450–2000 ppm) and (B) removal of the labial palps from the head.

Acknowledgements

The present study was conducted with the support of the Cooperative Research Program (Project Title: Short-term assessment of increased CO_2 concentration to major lepidopteran insect pests of horticulture; Project Number: PJ009264), RDA, Republic of Korea.

References

Badre, N.H., Martin, M.E. & Cooper, R.L. (2005) The physiological and behavioral effects of carbon dioxide on *Drosophila melanogaster* larvae. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **140**, 363–376.

De Belle, J.S. & Kanzaki, R. (1999) Protocerebral olfactory processing. *Insect Olfaction* (ed. by B.S. Hassoon), pp. 243–281. Springer, Germany.

Bogner, F. (1990) Sensory physiological investigation of carbon dioxide receptors in Lepidoptera. *Journal of Insect Physiology*, **36**, 951–957.

Boullis, A., Detrain, C., Francis, F. & Verheggen, F.J. (2016) Will climate change affect insect pheromonal communication? *Current Opinion in Insect Science*, **17**, 87–91.

Boullis, A., Fassotte, B.S., Sarles, L. *et al.* (2017) Elevated carbon dioxide concentration reduces alarm signaling in aphid. *Journal of Chemical Ecology*, **43**, 164–171.

Brown, D., Garcia-Segura, L.M. & Orci, L. (1984) Carbonic anhydrase is present in olfactory receptor cells. *Histochemisty*, **80**, 307–309.

Cardé, R.T. & Baker, T.C. (1984) Sexual communication with pheromones. *Chemical Ecology of Insects* (ed. by W.J. Bell and R.T. Cardé), pp. 355–383. Springer, New York, New York.

Carpenter, F.M. & Burnham, L. (1985) The geological record of insects. *Annual Review of Earth and Planetary Sciences*, **13**, 297–314.

Choi, K.S., Park, Y.M. & Kim, D.S. (2011) Rearing method for *Ascotis selenaria* (Lepidoptera: Geometridae) using an artificial diet. *Korean Journal of Applied Entomology*, **50**, 55–63.

Damberger, F.F., Michel, E., Ishida, Y. *et al.* (2013) Pheromone discrimination by a pH-tuned polymorphism of the *Bombyx mori* pheromone-binding protein. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 18680–18685.

Dean, J.B., Lawing, W.L. & Millhorn, D.E. (1989) CO_2 decreases membrane conductance and depolarizes neurons in the nucleus tractus solitarii. *Experimental Brain Research*, **76**, 656–661.

Dow, J.A.T. (1992) pH gradients in lepidopteran midgut. *Journal of Experimental Biology*, **172**, 355–375.

Guerenstein, P.G. & Hildebrand, J.G. (2008) Roles and effects of environmental carbon dioxide in insect life. *Annual Review of Entomology*, **53**, 161–178.

Hansson, B.S. & Christensen, T.A. (1999) Functional characteristics of the antennal lobe. *Insect olfaction* (ed. by B.S. Hassoon), pp. 125–161. Springer, Germany.

Hentley, W.T., Vanbergen, A.J., Hails, R.S. *et al.* (2014) Elevated atmospheric CO_2 impairs aphid escape response to predators and conspecific alarm signals. *Journal of Chemical Ecology*, **40**, 1110–1114.

IPCC (2013) Annex II: Climate system scenario tables. *Climate Change 2013: The physical science basis. Contribution of working group I to the fifth assessment report of the Intergovernmental panel on climate change* (ed. by T.F. Stocker, D. Qin, G.-K. Plattner *et al.*), pp. 1395–1445. Cambridge University Press, U.K.

Jones, W. (2013) Olfactory carbon dioxide detection by insects and other animals. *Molecules and Cells*, **35**, 87–92.

Kaissling, K.E. (1986) Chemo-electrical transduction in insect olfactory receptors. *Annual Review of Neuroscience*, **9**, 121–145.

Kehat, M. & Dunkelblum, E. (1990) Behavioral response of male *Heliothis armigera* (Lepidoptera: Noctuidea) moths in a flight tunnel to combinations of components identified from female sex pheromone glands. *Journal of Insect Behavior*, **3**, 75–83.

Klein, U. (1992) The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport:immunological evidence for occurrence of a V-ATPase in insect ion transporting epithelia. *Journal of Experimental Biology*, **172**, 345–354.

Küppers, J. & Bunse, I. (1996) A primary cation transport by a V-type ATPase of low specificity. *Journal of Experimental Biology*, **199**, 1327–1334.

Labandeira, C.C. & Sepkoski, J.J. (1993) Insect diversity in the fossil record. *Science*, **261**, 310–315.

Mafra-Neto, A. & Baker, T.C. (1996) Elevation of pheromone response threshold in almond moth males pre-exposed to pheromone spray. *Physiological Entomology*, **21**, 217–222.

Mohantyl, S., Zubkov, S. & Gronenborn, A.M. (2004) The solution NMR Structure of *Antheraea polyphemus* PBP provides new insight into pheromone recognition by pheromone-binding proteins. *Journal of Molecular Biology*, **337**, 443–451.

Mondor, E.B., Tremblay, M.N., Awmack, C.S. & Lindroth, R.L. (2004) Divergent pheromone-mediated insect behaviour under global atmospheric change. *Global Change Biology*, **10**, 1820–1824.

Munday, P.L., Dixson, D.L., Donelson, J.M. *et al.* (2009) Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 1848–1852.

Rafaeli, A. (2009) Pheromone biosynthesis activating neuropeptide (PBAN): regulatory role and mode of action. *General and Comparative Endocrinology*, **162**, 69–78.

Rafaeli, A., Hirsch, J., Soroker, V. *et al.* (1991) Spatial and temporal distribution of pheromone biosynthesis-activating neuropeptide in *Helicoverpa (Heliothis) armigera* using RIA and in vitro bioassay. *Archives of Insect Biochemistry and Physiology*, **18**, 119–129.

Royer, D.L. (2006) CO_2-forced climate thresholds during the phanerozoic. *Geochimica et Cosmochimica Acta*, **70**, 6556–5675.

Rutowski, R.L. (1982) Mate choice and lepidopteran mating behavior. *Florida Entomologist*, **65**, 72–82.

SAS Institute (2006) *SAS Enterprise Guide*. SAS Institute Inc., Cary, North Carolina.

Stange, G. (1992) High resolution measurement of atmospheric carbon dioxide concentration changes by the labial palp organ of the moth *Heliothis armigera* (Lepidoptera: Noctuidae). *Journal of Comparative Physiology A*, **171**, 317–324.

Stange, G. (1997) Effects of changes in atmospheric carbon dioxide on the location of host by the moth, *Cactoblastis cactorum*. *Oecologia*, **110**, 539–545.

Steinbrecht, R.A. (1997) Pore structures in insect olfactory sensilla: a review of data and concepts. *International Journal of Insect Morphology and Embryology*, **26**, 229–245.

Sun, Y.C., Su, J.W. & Ge, F. (2010) Elevated CO_2 reduces the response of *Sitobion avenae* (Homoptera: Aphididae) to alarm pheromone. *Agriculture, Ecosystems & Environment*, **135**, 140–147.

Thurm, U. & Wessel, G. (1979) Metabolism-dependent transepithelial potential differences at epidermal receptors of arthropods. *Journal of Comparative Physiology*, **134**, 119–130.

Williams, R.H., Jensen, L.T., Verkhratsky, A. *et al.* (2007) Control of hypothalamic orexin neurons by acid and CO_2. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 10685–10690.

Zeiske, W., Meyer, H. & Wieczorek, H. (2002) Insect midgut K^+secretion: concerted run-down of apical/basolateral transporters with extra-/intracellular acidity. *Journal of Experimental Biology*, **205**, 463–474.

Zhao, X.C., Tang, Q.B., Berg, B.G. *et al.* (2013) Fine structure and primary sensory projections of sensilla located in the labial-palp pit organ of *Helicoverpa armigera* (Insecta). *Cell and Tissue Research*, **353**, 399–408.

Accepted 5 March 2018
First published online 16 April 2018