



Article

Molecular Basis of CO₂ Sensing in *Hyphantria cunea*

Jian Zhang ^{1,†}, Shiwen Duan ^{2,†}, Wenlong Wang ¹, Duo Liu ^{1,*} and Yinliang Wang ^{2,*}

¹ School of Life Sciences, Changchun Normal University, Changchun 130033, China

² School of Life Sciences, Northeast Normal University, Changchun 130024, China

* Correspondence: liuduocsfu@163.com (D.L.); wangyl392@nenu.edu.cn (Y.W.)

[†] These authors contributed equally to this work.

Abstract: Carbon dioxide (CO₂) released by plants can serve as a cue for regulating insect behaviors. *Hyphantria cunea* is a widely distributed forestry pest that may use CO₂ as a cue for foraging and oviposition. However, the molecular mechanism underlying its ability to sense CO₂ has not been elucidated. Our initial study showed that CO₂ is significantly attractive to *H. cunea* adults. Subsequently, 44 *H. cunea* gustatory receptors (GRs) were identified using transcriptome data, and 3 candidate CO₂ receptors that are specifically expressed in the labial palps were identified. In vivo electrophysiological assays revealed that the labial palp is the primary organ for CO₂ perception in *H. cunea*, which is similar to findings in other lepidopteran species. By using the *Xenopus* oocyte expression system, we showed that the *HcunGR1* and *HcunGR3* co-expressions produced a robust response to CO₂, but *HcunGR2* had an inhibitory effect on CO₂ perception. Finally, immunohistochemical staining revealed sexual dimorphism in the CO₂-sensitive labial pit organ glomerulus (LPOG). Taken together, our results clarified the mechanism by which *H. cunea* sense CO₂, laying the foundation for further investigations into the role of CO₂ in the rapid spread of *H. cunea*.

Keywords: CO₂ sensing; fall webworm; oviposition; foraging; sexual dimorphism; gustatory receptor



Citation: Zhang, J.; Duan, S.; Wang, W.; Liu, D.; Wang, Y. Molecular Basis of CO₂ Sensing in *Hyphantria cunea*. *Int. J. Mol. Sci.* **2024**, *25*, 5987. <https://doi.org/10.3390/ijms25115987>

Academic Editor: Guy Smagghe

Received: 23 January 2024

Revised: 25 May 2024

Accepted: 26 May 2024

Published: 30 May 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

CO₂, as a ubiquitous gas in the natural atmosphere, is produced by almost all organisms when they obtain energy via respiration. Insects possess the ability to detect subtle changes in the concentration of carbon dioxide (CO₂) in their environment [1]. CO₂ not only serves as a danger signal for insects, but in social insects such as honeybees, CO₂ could also cause an imminent increase in the nest temperature; thus, they expel CO₂ by flapping their wings to lower the temperature of their nest [2]. CO₂ is also important in host-seeking and oviposition in insects; e.g., blood-sucking mosquitoes use CO₂ as a cue to locate their vertebrate hosts [3,4]; in phytophagous insects, evidence has shown that CO₂ can guide *Elasmopalpus lignosellus* larvae to locate the freshest parts of the plant [5], while *Manduca sexta* and *Cactoblastis cactorum* use CO₂ to locate suitable places to lay eggs [6,7]. However, the CO₂-sensing mechanism of most other lepidopteran pests, such as the major forestry pest *Hyphantria cunea*, remains unclear. *H. cunea* is a highly polyphagous and fertile pest; one female can lay up to 500 eggs with a hatch rate of over 95% [8], and can forage on more than 400 plant species, resulting in great damage to forest ecosystems, which is known as “smokeless fires” locally [9]. One of the predominant reasons for their rapid spread is their ability to choose suitable foraging and oviposition sites [10], suggesting that CO₂ cues might contribute to their adaptation to the environment.

The CO₂ sensing pathway in insects varies among different taxa. Two CO₂ receptor genes were found in *Drosophila melanogaster*, and only *DmelGr21a* and *DmelGr63a* were co-expressed in response to CO₂ [11,12], suggesting that a heterodimer is needed in *D. melanogaster* to carry out CO₂ sensing. However, CO₂ receptor homologs have not been identified in honeybees, ants, or blacklegged ticks [13], suggesting that these species utilize distinct mechanisms for CO₂ sensing [14,15]. Three CO₂ receptors have been found in

lepidopterans, such as *Danaus Plexippus*, *Bombyx mori*, *Heliconius Melpomene*, and *Helicoverpa armigera* [16,17]. A functional study of *H. armigera* showed that although *HarmGr1*, *HarmGr3*, and *HarmGr2* were expressed in the same neuron of the labial palps, only the co-expression of *HarmGr1+HarmGr3* or *HarmGr1+HarmGr2+HarmGr3* resulted in a robust response to Sodium bicarbonate (NaHCO_3) [18], indicating that *HarmGr1* and *HarmGr3* were necessary for CO_2 perception in *H. armigera*, whereas the role of *HarmGr2* is still unclear. To date, the molecular CO_2 sensing pathway in *H. cunea* is unclear, and whether the CO_2 sensing mechanism in *H. cunea* is conserved among lepidopteran species remains unknown.

At the central nervous system (CNS) level, the location and projection modes of CO_2 -sensing glomeruli also vary among different insects. In *D. melanogaster*, CO_2 -sensitive neurons in the antennae project to a ventrally distributed glomerulus called the *V glomerulus* [19,20]; in *Aedes aegypti* and in several other mosquitoes, CO_2 -sensitive neurons in the maxillary palps are linked to the dorsomedial glomerulus in the antennal lobe (AL) [21–23]. These two species both have an ipsilateral projection; i.e., CO_2 neurons project to only one side of the AL, which is also called the “single side projection mode”. In Lepidoptera, the well-accepted hypothesis is that labial palp pit organs (LPO) are used for CO_2 sensing [24], and LPO neurons project to a specific glomerulus located on the most ventral side of the antennal lobe; this type of glomerulus is referred to as the “labial pit organ glomerulus” (LPOG) [24,25]. The LPO neurons in lepidopterans project to both the ipsilateral and contralateral antennal lobes via bilateral projection. LPOG is considered a specific CO_2 glomerulus, and no other olfactory neurons project to this glomerulus; therefore, an assessment of its projection mechanisms and olfactory glomerulus volume could provide further motivation to assess the importance of CO_2 sensing in *H. cunea* through higher-level mechanisms. In conclusion, although the rapid spread of *H. cunea* may be related to herbivore-induced plant volatiles (HIPVs) such as pinene [26], its ability to sense CO_2 also plays a major role. But to our knowledge, no studies have investigated the role of CO_2 in the spread of *H. cunea* using molecular-level approaches. Our study will contribute to a better understanding of the important role that CO_2 plays in the dispersal of *H. cunea*, which will provide a theoretical basis for pest management.

2. Results

2.1. Effect of CO_2 on *H. cunea* Behavior

To verify the effect of CO_2 on the behavior of female *H. cunea*, Y-tube olfactometer tests were conducted with 60 *H. cunea* at different CO_2 concentrations. Compared with those of the negative control (pure air without CO_2 , Figure 1), 32 moths preferred 1% CO_2 . As the CO_2 concentration increased, 34 moths were attracted to the side with CO_2 at a 3% concentration, 37 moths were attracted to 5% CO_2 , 38 moths were attracted to 8% CO_2 , and 41 moths were attracted to 10% CO_2 . These results showed that CO_2 concentrations ranging from 8%–10% had an attractive effect on *H. cunea* adults; within this range, the attractive effect of CO_2 increased with increasing CO_2 concentration.

2.2. Identification and Homology Analysis of *HcunGRs*

To identify CO_2 receptors in *H. cunea*, 44 candidate gustatory receptors (GRs) were identified from *H. cunea* transcriptome data; 24 GRs were full-length sequences, and 20 were truncated sequences. Blast results revealed that the similarity between *HcunGRs* and those of other species ranged from 35% to 91% (Table S1). *HcunGR16* was the homolog of the fructose receptors of *D. melanogaster*, *B. mori*, *H. armigera*, and *H. melpomene* and was also hypothesized to be a candidate fructose receptor in *H. cunea* (Figure 2) [16]. *HcunGR4-10* and *HcunGR12-14* clustered together with the conserved sugar receptor *DmelGR64* [27], suggesting that these GRs have a sugar receptor (non-fructose) sensing role. *HcunGR44* is a candidate for bitter receptors in *H. cunea*. Most importantly, we found that *HcunGR1*, *DmelGR21a*, and *HarmGR1*; *HcunGR2* and *HarmGR2*; and *HcunGR3*, *DmelGR63a*, and *HarmGR3* were grouped together, indicating that these three *HcunGRs* might play a role in CO_2 sensing in *H. cunea*.

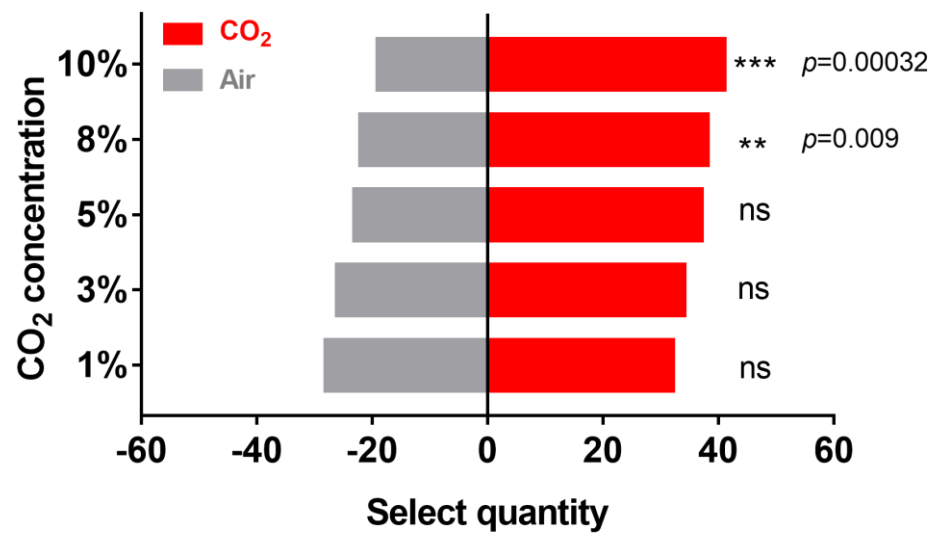


Figure 1. The number of female *Hyphantria cunea* attracted to different concentrations of CO₂. Statistical differences were evaluated via the Chi-square test. ** $p < 0.01$, *** $p < 0.001$, ns: no significance.

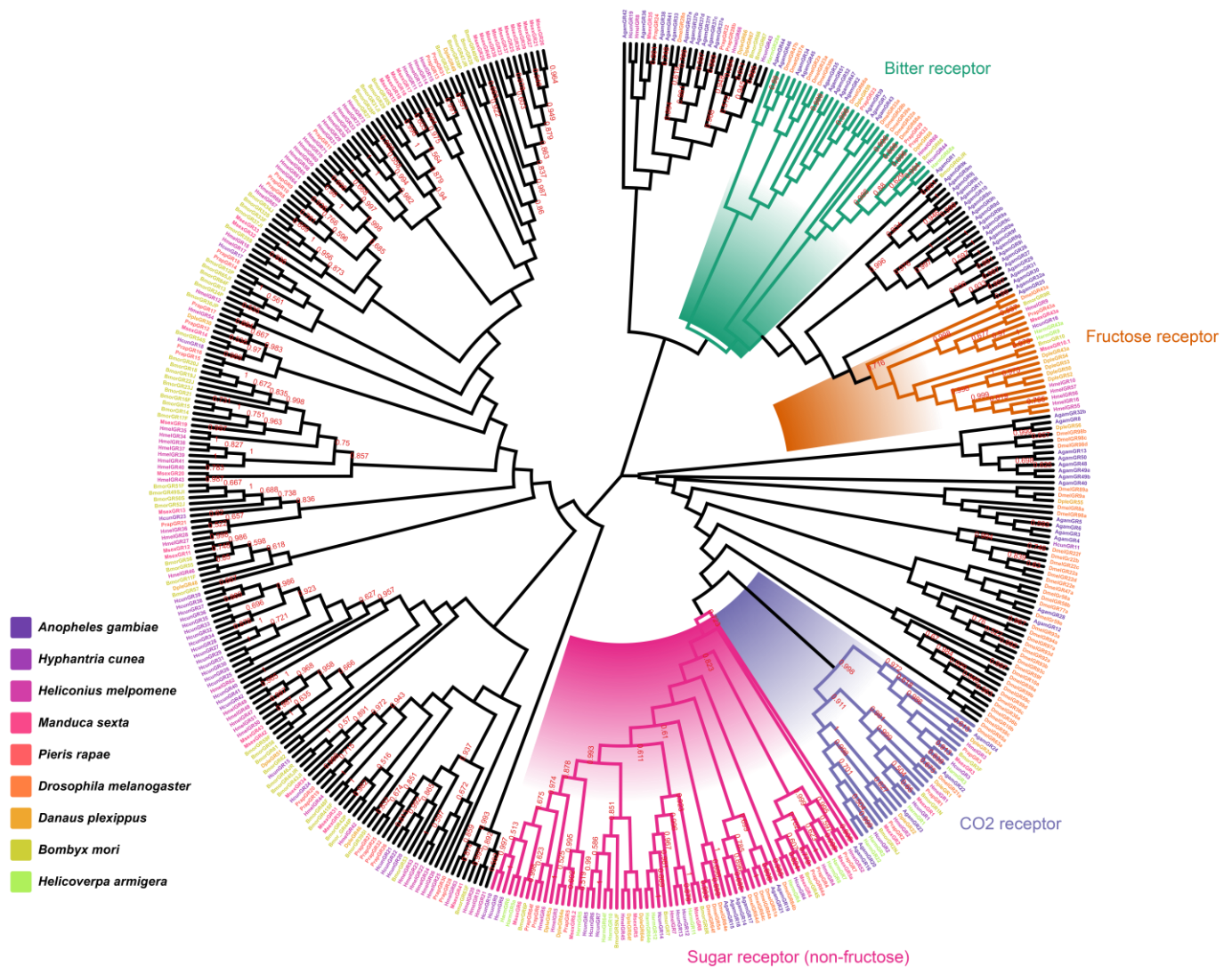


Figure 2. The maximum likelihood tree of candidate gustatory receptors (GRs). Bootstrap replications up to 1000.

Notably, the expression levels of the GRs showed that *HcunGR1*, *HcunGR2*, and *HcunGR3* were highly abundant in the labial palps (Figure 3a). The expression patterns of *HcunGR1*, *HcunGR2*, and *HcunGR3* were further verified by q-PCR (Figure 3b), and the qPCR results were consistent with the heatmap. These results indicate that the main organ responsible for CO₂ sensing in *H. cunea* is the labial palp, which is similar to that in other lepidopteran species.

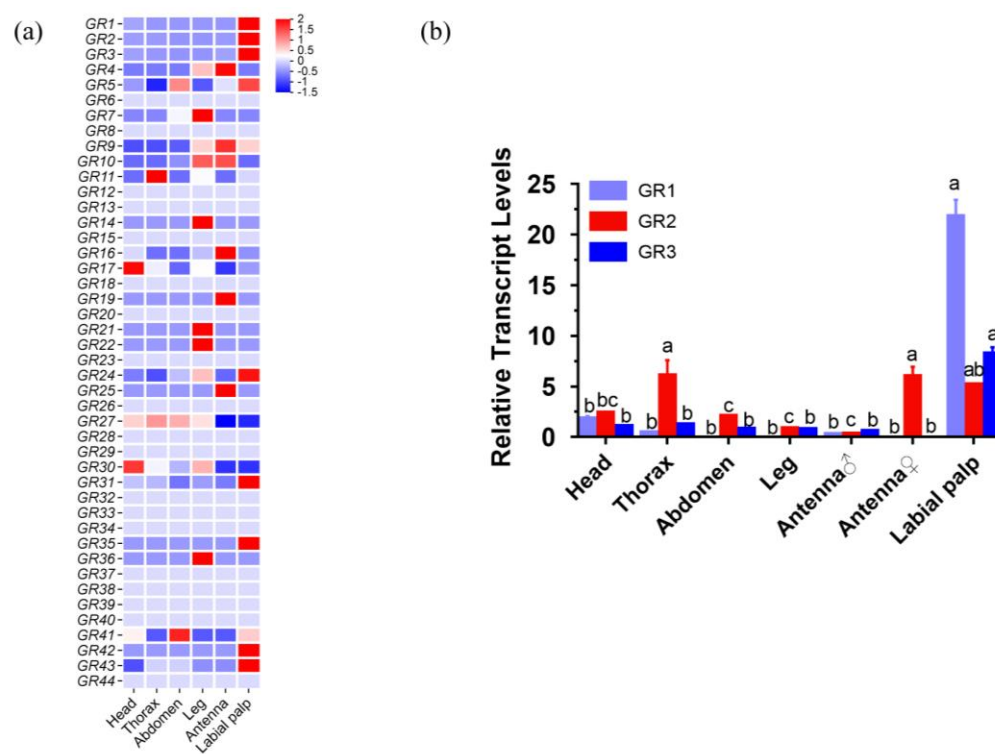


Figure 3. Expression profiles of *HcunGRs* in different body parts of adult *H. cunea*. (a) Characteristic expression patterns of 44 *HcunGRs* in different body parts based on FPKM (normalization by row). FPKM: Fragments per kilobase of transcript per million fragments mapped. (b) Expression patterns of three candidate CO₂ GRs in different body parts of adult *H. cunea*. A different lowercase indicates a significant difference based on one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). The data are presented as the means $\pm$ standard errors of the means (SEMs), $N = 3$.

2.3. Electrophysiological Response of the Antennae and Labial Palp to CO₂

The results of electrolabialpalpography (ELPG) and electroantennogram (EAG) showed that the response to CO₂ was significantly higher in the labial palp of female *H. cunea* than in males (Figure 4a) ($p = 0.0313$), but there was no significant difference in the antennae response to CO₂ (Figure 4b). In addition, the response of the labial palp to CO₂ was consistently higher than that of the antennae at 1%–10% concentrations (Figure 4). These findings suggest that the labial palp is the main organ involved in CO₂ sensing in *H. cunea* and that female moths have stronger CO₂-sensing abilities than males.

2.4. Two-Electrode Voltage Clamp (TEVC) Response of *HcunGr1*, *HcunGr2*, *HcunGr3*, and Their Combinations

By analyzing the dissolved CO₂ concentration in NaHCO₃ solution, a direct correlation between the concentration of NaHCO₃ and the dissolved CO₂ concentration was identified (Figure S1). Moreover, we found that Na⁺ in NaCl solution also elicits a channel current (Figures 5a–g and S2). Therefore, eliminating the Na⁺ effect from the NaCl solution is necessary when calculating the “real response”; we used full response minus NaCl response to look at the real response of CO₂ [28]. Oocytes expressing *HcunGR1*, *HcunGR2*, or *HcunGR3* alone or with co-expressions of *HcunGR1*+*HcunGR2* or *HcunGR2*+*HcunGR3*

did not respond to CO₂ after excluding the effect of Na⁺ (Figure 5c–g). However, the *HcunGR1+HcunGR3* and *HcunGR1+HcunGR2+HcunGR3* expression sets significantly responded to CO₂ beginning at a concentration of 100 mM (equivalent to 51 ± 3 ppm CO₂) (Figure S2), and the response increased gradually with increasing CO₂ concentration (Figure 5a,b). In the range of 100–300 mM, the response of *HcunGR1+HcunGR3* ranged from 257 ± 87 nA to 1387.67 ± 162.7 nA, and the response of *HcunGR1+HcunGR2+HcunGR3* ranged from 245.67 ± 114.33 nA to 575.3 ± 89.3 nA (Figure 5h). The response of the *HcunGR1+HcunGR2+HcunGR3* set was significantly lower than that of the *HcunGR1+HcunGR3* set from a concentration of 200 mM or greater (Figure 5i).

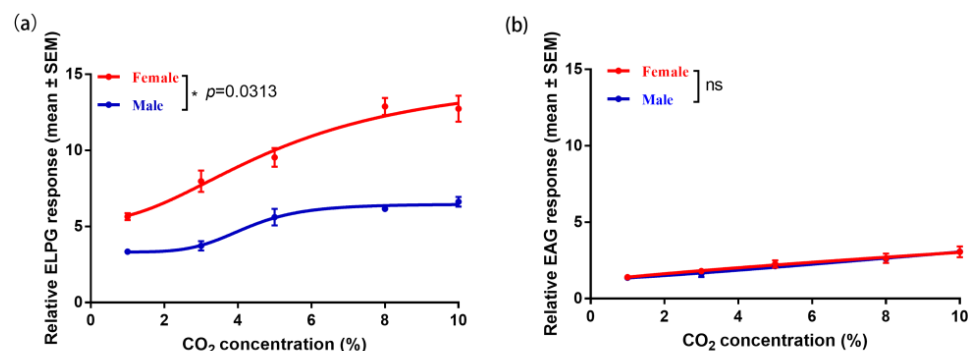


Figure 4. Electrolabialpalpography (ELPG) and electroantennogram (EAG) nonlinear regression curve for CO₂. (a) Response of the labial palps of the *H. cunea* to CO₂; (b) response of the antennae of the *H. cunea* to CO₂. Statistical differences were evaluated by the Wilcoxon signed-rank test. * $p < 0.05$, ns: no significance. The data are presented as the means $\pm$ SEMs, $N = 3$ biological replicates (Tables S4 and S5).

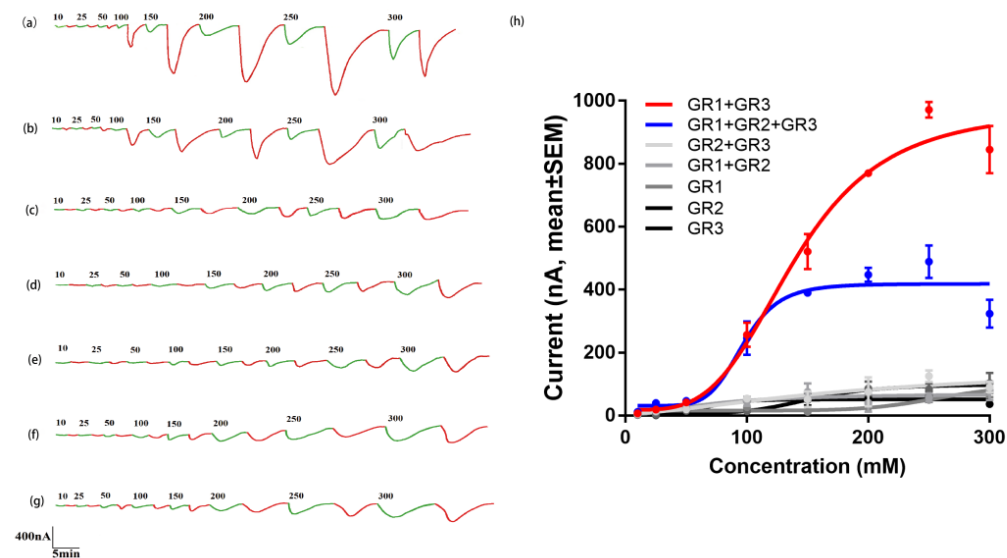


Figure 5. Two-electrode voltage clamp recording (TEVC) responses of *HcunGR1*, *HcunGR2*, and *HcunGR3* alone and in combination with different concentrations of NaCl and NaHCO₃. (a) Response of *HcunGR1+HcunGR3*; (b) response of *HcunGR1+HcunGR2+HcunGR3*; (c) response of *HcunGR1*; (d) response of *HcunGR2*; (e) response of *HcunGR3*; (f) response of *HcunGR1+HcunGR2*; (g) response of *HcunGR2+HcunGR3*; green traces represent the response in NaCl solution; red traces represent the response in NaHCO₃ solution; the number involved in (a–g) indicates concentration NaCl and NaHCO₃; (h) nonlinear regression curve of *HcunGR1*, *HcunGR2*, and *HcunGR3* alone and in combination after excluding the influence of Na⁺. $N = 3$ biological replicates (Table S6); The data are presented as the mean $\pm$ SEM.

2.5. Anterograde Dye Filling of Labial Palps in *H. cunea*

After we concluded that the labial palp is the main organ for sensing CO₂, an anterograde dye-filling experiment on the labial palps was conducted to further explore the transmission of CO₂ signals to the central nervous system. We observed the dye's entry from the labial palp nerves, passing through the gnathal ganglion (GNG), then dividing into two bundles, and finally arriving at the LPOG (Figure 6a–f). The LPOG is located in the ventral region of the ALs, where it is similar to the DP region in *D. melanogaster*; we named this region DP1. The projections displayed a clear boundary, and the neurons exhibited a bilateral projection pattern.

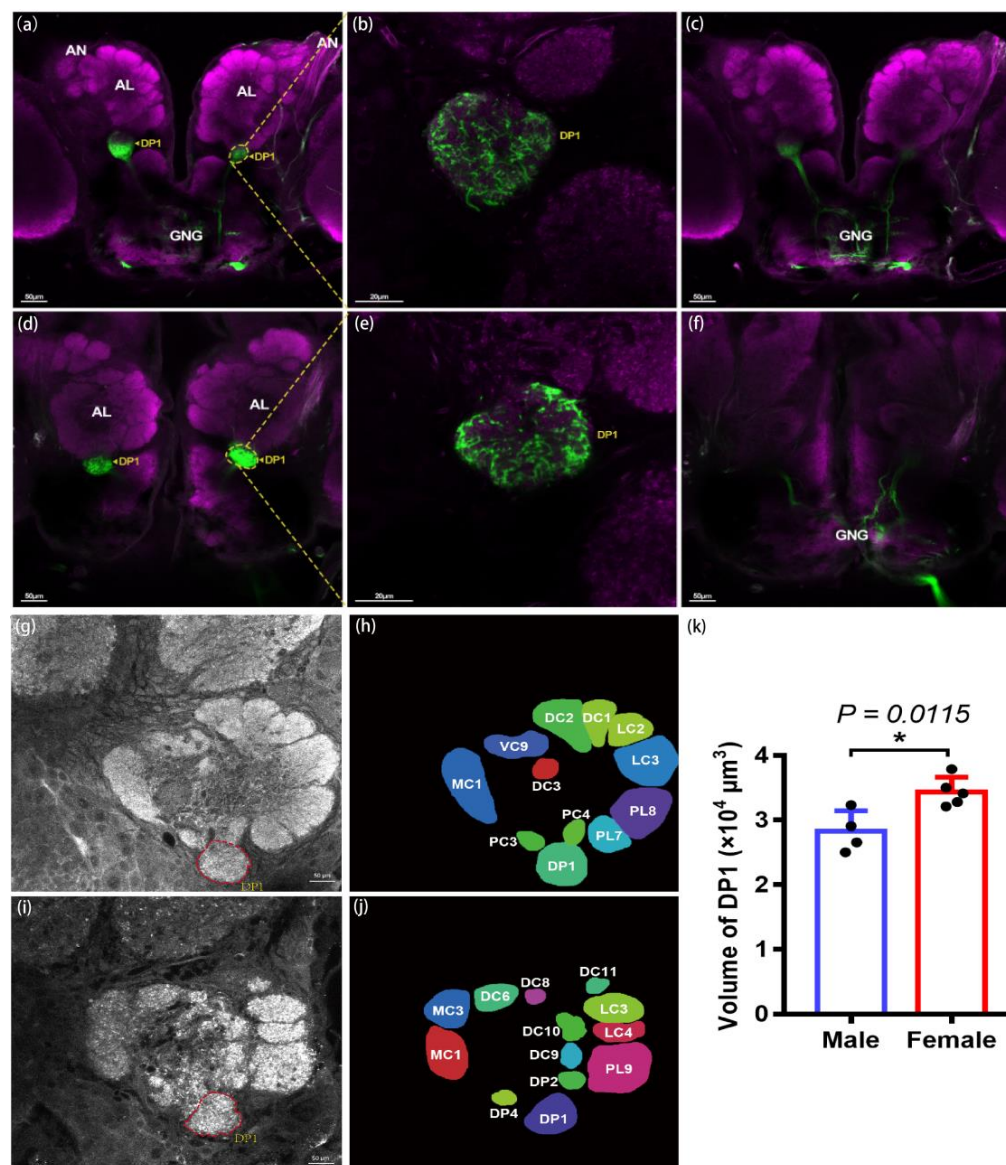


Figure 6. Anterograde dye-filling of labial palps and two-dimensional reconstructions of the antennal lobe (AL) in the DP1 region of *H. cunea*. (a–c) The central projections of female labial pit organ sensory neurons passing through the gnathal ganglion (GNG) to DP1; (d–f) the central projections of male labial pit organ sensory neurons passing through the gnathal ganglion (GNG) to DP1; (g,h) confocal images of the male *H. cunea* AL glomeruli seen from the ventral view. The sections are from anterior to posterior at a depth of 156 μm . Scale bar = 50 μm ; (i,j) confocal images of female *H. cunea* AL glomeruli taken from the ventral view. The sections are from anterior to posterior at a depth of 142 μm . Scale bar = 50 μm ; (k) the volume compares male and female DP1s. The data are presented as the mean $\pm$ SEM, $N \geq 4$. Statistical differences were evaluated by unpaired *t* tests. * $p < 0.05$.

To precisely locate and calculate the volume of the LPOG in the AL, we, based on the nine individuals (five females and four males), performed two-dimensional and three-dimensional reconstructions of all the glomeruli (Figure 6g–j) (Figure S3). It was found that female *H. cunea* possess 81 glomeruli, whereas male moths have only 74; most of the glomeruli were roughly spherical, while a few were irregularly shaped, forming a central fiber nucleus. After computing the surface area and volume of DP1 in both male and female *H. cunea*, we found that the total surface area of DP1 in females ($5741.25 \mu\text{m}^2$) was nearly the same as that in males ($5728.94 \mu\text{m}^2$); however, the average volume of DP1 in females ($34,388.3 \mu\text{m}^3$) was significantly ($p = 0.0115$) greater than that in males ($28,852.4 \mu\text{m}^3$) (Figure 6k), suggesting that sexual dimorphism existed in the DP1 glomerulus.

3. Discussion

Initially, we discovered that *H. cunea* adults exhibit a preference for CO_2 concentrations ranging from 1% to 10%; this preference might be linked to the foraging and oviposition behavior of *H. cunea*. Studies have shown that many plants, such as *Nepenthes*, release in excess of 5% concentration of CO_2 [29], which is consistent with the CO_2 concentration in our behavioral test. In addition, researchers have found that adults *H. cunea* prefer to forage and oviposition at night [30], which is the peak time of CO_2 release, which partially explains why the high concentration of CO_2 could also attract *H. cunea* females. Previous studies have shown that the level of respiratory metabolism in plants is a main indicator reflecting plant quality. A high respiration rate indicates the presence of more nutrients, such as carbohydrates [7]; therefore, *H. cunea* can choose strong nectar as food by sensing the CO_2 released by plants [31]. On the other hand, it has been shown that insect spawning increases with increasing CO_2 concentration [6], suggesting that *H. cunea* can also choose oviposition sites by CO_2 cues to maximize the survival of their offspring. Field observations have also shown that *H. cunea* prefer to lay eggs on the dorsal sides of leaves [32], which may be because the undersides of terrestrial plant leaves have more stomata and can generate higher CO_2 concentrations [33]. *H. cunea* can use this CO_2 cue to lay eggs on the dorsal side of the leaf to prevent damage from direct sunlight. In summary, our behavioral results showed that CO_2 can attract adult *H. cunea*, which may be related to their oviposition behavior and its adaptation. And the possibility of further study about CO_2 baited traps such as mosquitos will be an exciting topic in the future [34].

Homology analysis revealed that three CO_2 receptor homologs exist in *H. cunea*, which is consistent with findings in other lepidopteran insects [16,17], indicating that the CO_2 sensing pathway might be relatively conserved in Lepidoptera. The expression levels of the three candidate CO_2 receptors demonstrated that they were all specifically expressed in labial palps; however, their expression was low in the antennae. And the response of the labial palp is significantly greater than that of the antennae, suggesting that the labial palp is the primary organ for sensing CO_2 in *H. cunea*, as is the response of other lepidopteran species, such as *H. armigera* and *M. sexta* [18,25]. The fact that females produce stronger action potentials than males in the labial palp also suggests that CO_2 may play an important role in female-specific behaviors such as spawn selection in *H. cunea*. In *D. melanogaster*, the molecular mechanisms for CO_2 sensing in taste and olfaction are mutually independent [35], and ionotropic receptors (IRs) are involved in the detection of CO_2 [36–38]. In addition, the response of *D. melanogaster* to CO_2 is regulated by two different neural pathways, one for CO_2 attraction and the other for avoidance [39]. Therefore, it is necessary to further investigate whether there are other CO_2 -sensing pathways in *H. cunea*.

In vitro expression of *HcunGR1*, *HcunGR2*, and *HcunGR3* revealed that CO_2 responses occurred only when *HcunGR1* was combined with *HcunGR3* or when *HcunGR1* was combined with *HcunGR2*+*HcunGR3*. These results support the crucial role of the *HcunGR1*+*HcunGR3* co-expression in CO_2 sensing in *H. cunea*, which is consistent with the findings in *H. armigera* [18]. Notably, the response of the *HcunGR1*+*HcunGR2*+*HcunGR3* ternary complex was significantly lower than that of the co-expression at concentrations greater than 200 mm. The role of *HcunGR2* in CO_2 sensing remains unknown. Our results could be due to two

possible explanations. One possibility is that the limited number of ion channels on the oocyte surface results in a reduction in the expression ratio of the *HcunGR1+HcunGR3* co-expression when *HcunGR2* is expressed [40], therefore suppressing the TEVC response of *HcunGR1+HcunGR3*; another possibility is that *HcunGR2* may serve as a modulator, playing an inhibitory role in the CO₂ sensing process in *H. cunea*. In conclusion, we have shown that the co-expression formed by *HcunGR1+HcunGR3* plays a primary role in CO₂ sensing, but the function of *HcunGR2* requires further exploration.

At the CNS level, a bilateral projection pattern of labial palps was observed in *H. cunea*; this result is consistent with what has been observed in *H. armigera* and *Anopheles gambiae* (*A. gambiae*) but differs from the unilateral projection pattern observed in *D. melanogaster* and *A. aegypti*. Our heatmap result showed that *GR1*, *GR2*, and *GR3* were the top three highest expressing receptors in labial palps, which were responsible for CO₂ sensing; thus, the projection from the labial palp into the brain was mainly for CO₂ sensing. But we also found that there are other GRs also expressed in the labial palp, which may be related to wider sensing of different cues [41]. And previous research has suggested that bilateral projections enable olfactory receptor neurons (ORNs) to release an asymmetric amount of neurotransmitters on both sides of ALs [42], which leads to a stronger signal in one projection neuron (PN) than in the other, enhancing the contrast of odor concentration gradients between the two brain hemispheres [43]. This asymmetrical projection pattern may help *H. cunea* detect the differences in CO₂ concentrations between the two labial palps and rapidly locate the CO₂ source. Moreover, the average volume of DP1 in females was significantly higher than that of males; however, the total surface area of DP1 was nearly the same in both genders. This indicates that the shape of DP1 is different between males and females, which is consistent with our observations that the male's DP1 had an irregularly shaped (Figure S3). And the sexual dimorphism was observed in the morphology of LPOGs; the average volume of LPOGs was significantly greater in females than in males. It is well accepted that a larger glomerulus indicates greater odor sensitivity due to the greater number of synaptic connections [44,45]. An enlarged LPOG may reflect the crucial role of CO₂ sensing in *H. cunea* females; in contrast, no significant sexual dimorphism was observed on the LPOG in *M. sexta* [46]. In summary, the identified sexual dimorphism of labial palp projections in *H. cunea* may somewhat explain the differences in CO₂ function between males and females, which might be linked to female-specific behavior such as oviposition.

4. Materials and Methods

4.1. Insect

A single fall webworm egg mass was collected from a Manchurian ash (*Fraxinus mandshurica*) tree in Animal and Plant Park, Jinlin Province, China (43°86'96.71" N, 125°33'29.82" E), and was reared in an artificial incubator (BIC-300 artificial incubator, Boxun, Shanghai, China) at 26 °C, 80% RH and a 19:5 light: dark cycle beginning in 2019. After each 12 generations, the wild population was collected again from the same place and crossed with the laboratory colony for rejuvenation for more than 30 generations. The larvae were fed on the leaves of mulberry trees (*Morus alba*), and the adults were given a 10% sucrose solution for energy supplementation.

4.2. Binary Choice Assay

The airflow of CO₂ (Juyang Company, Changchun, China) was controlled by a flow meter and mixed with Zero Air (21% O₂ and 79% N₂, CO₂ free, Juyang Company, China) to ensure the delivery of 1%, 3%, 5%, 8%, and 10% CO₂. A Y-tube olfactometer (1.6 cm in diameter, 7.2 cm in base and arm length) was used for the binary choice assay, and pure air without any CO₂ was used as a negative control. At 7 p.m. (peak spawning), day 3 after emergence, females were selected. A moth was first placed at the entrance of the main arm, and a "choice" was recorded when it entered an arm and stayed for more than 30 s. If no choice was made within 5 min, the data were recorded as "no choice". A total of 60 *H. cunea* were tested. The Y-tube was cleaned with hexane, and the position of the stimulus was

exchanged before and after each test. All the assays were performed in a dark room with red light (Intelligent LED solutions, Berkshire, UK) to avoid light interference.

4.3. Homology Analysis of Gustatory Receptors (GRs)

The GR sequence of *H. cunea* used in this study was obtained from our previous transcriptome studies (Table S1) [47], and the genome sequences of six lepidopteran species, *B. mori* (GCA_026075555.1), *M. sexta* (GCA_014839805.1), *H. melpomene* (GCA_900068175.1), *Pieris rapae* (GCA_905147795.1), *H. armigera* (GCA_026262555.1), and *D. plexippus* (GCA_009731565.1) were used for homology analysis, and *D. melanogaster* (GCA_000001215.4) and *A. gambiae* (GCA_000005575.1) were used as outgroups. The amino acid sequences of the GRs were first aligned by MUSCLE, after which Molecular Evolutionary Genetics Analysis (MEGA; State College, PA, USA) version 6 was used to construct a maximum likelihood (ML) tree with the Jones–Taylor–Thornton (JTT) model [48]. Bootstrap support values were based on 1000 replicates. The resulting homological tree was visualized with FigTree 1.42 (<http://tree.bio.ed.ac.uk/software/figtree/>, accessed 29 May 2023). And the fragments per kilobase of transcript per million fragments mapped (FPKM) were used to measure gene expression [49]. R (R Foundation for Statistical Computing, Vienna, Austria) version 4.1.3 was used to construct the heatmaps.

4.4. Electrophysiological Recording

EAG and ELPG were used to detect the electrophysiological responses of the antennae and labial palp to CO₂ in *H. cunea*. Three days after emergence, females and males were selected at night (oviposition peak time). Glass electrodes were pulled by using a micropipette PC-10 (Narishige, Tokyo, Japan) and then filled with 1 M potassium chloride containing 1% polyvinylpyrrolidone. The reference electrode was subsequently inserted into one eye of the insects, while the recording electrode was placed in contact with the tips of the antennae and labial palp using the micromanipulator MP-12 (Syntech, Kirchzarten, Germany). The obtained signals were amplified by a high-impedance ac/dc preamplifier (Syntech, Kirchzarten, Germany). CO₂ stimuli ranging from 1% to 10% were injected into a carbon-filtered and humidified airflow for 0.2 s to deliver the stimulus to the antenna and labial palp at 500 mL/min generated by an air stimulus controller CS-55 (Syntech, Kirchzarten, Germany). A minimum of 3 individuals were tested, and 3 puffs were performed for each antenna or labial palps. The EAG and ELPG data were acquired with EAG Pro version 2.0 software (Syntech, Kirchzarten, Germany) and normalized by the response of negative control (21% O₂ and 79% N₂, CO₂ free) (Juyang Company, China) by the equation “relative EAG response = EAG response of CO₂ / EAG response of negative control”. The data were subsequently analyzed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

4.5. RNA Extraction, Expression Pattern Analysis, Quantitative PCR, and Cloning

Previous studies have shown that some GRs are differently expressed in the olfactory sensing organ (antennae) between males and females, but in non-olfactory organs (such as the head, chest, abdomen, and legs), no sex-based different expressions were detected; thus, we use mix samples of these body parts as a negative control [50]. The head, thorax, abdomen, leg, and labial palp carefully dissected from 15 individuals (female: male = 1:1) with DEPC-treated forceps under a stereomicroscope (Motic, Hong Kong, China). Then, female and male antennae were dissected from 15 individuals in the same way. Total RNA was isolated from homogenized body parts with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. After extraction, the total RNA concentration was assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gel (Sangon Biotech, Shanghai, China) electrophoresis.

For the qPCR assay, 1 µg of total RNA was transcribed into cDNA by using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). qPCR was performed with a LightCycler 480 II Detection System (Roche, Shanghai,

China) and TransStar Tip Top Green qPCR Supermix (TransGen Biotech, Beijing, China) under the following conditions: 94 °C for 30 s; 45 cycles of 94 °C for 5 s, 55 °C for 15 s, and 72 °C for 10 s; the β -actin gene was used as an internal control. The primers used in this study are listed in Table S2. The qPCR results were analyzed via the $2^{-\Delta\Delta CT}$ method [51]. The data were subsequently analyzed with GraphPad Prism 6.0 (GraphPad Software, CA, USA).

The primer was designed by Primer 3 (<https://bioinfo.ut.ee/primer3-0.4.0>, accessed on 29 June 2023) (Table S3), the full ORF of GRs containing 5'UTR and 3'UTR were obtained by PCR and ligated to pUCm-T Vector (Sangon Biotech, Shanghai, China) for sequencing. Subsequently, the ORF of GRs were amplified by a specific primer and subcloned to pGEMHE using pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China) with BamHI and HindIII restriction sites (New England Biolabs, Ipswich, MA, USA). The recombinant plasmids were transformed in DH5 α (TransGen Biotech, Beijing, China) competent cell, and then plasmids were extracted with a SanPrep Column Plasmid Mini-Preps Kit (Sangon Bio, Shanghai, China). After transformation, the inserts were verified via DNA sequencing (Sangon Biotech, Shanghai, China).

4.6. cRNA Synthesis and Oocyte Microinjection

The full-length open reading frames (ORFs) of *HcunGr1*, *HcunGr2*, and *HcunGr3* were expressed in *Xenopus laevis* oocytes individually or in combination; thus, seven sets of oocytes were obtained expressing the following: *HcunGr1*, *HcunGr2*, *HcunGr3*, *HcunGr1+HcunGr2*, *HcunGr1+HcunGr3*, *HcunGr2+HcunGr3*, and *HcunGr1+HcunGr2+HarmGr3*. cRNAs of *HcunGr1*, *HcunGr2*, and *HcunGr3* containing the 3' (126bp) and 5' (43bp) *Xenopus* globin UTR from the pGEMHE vector were synthesized using the mMACHINE T7 Transcription Kit (Ambion, Austin, USA) according to the manufacturer's instructions. RNA concentration and purity were analyzed by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and 1% agarose gel (Sangon Biotech, Shanghai, China) electrophoresis. A total of 46 nL (36.8 ng) of relevant cRNA was microinjected into each oocyte at a 1:1 or 1:1:1 ratio by a NanoLitter 2000 injector (World Precision Instruments, Sarasota, FL, USA). Afterward, the injected oocytes were incubated at 18 °C for 2 to 8 days in Barth's solution (96 mM NaCl, 2 mM KCL, 5 mM MgCl₂, 0.8 mM CaCl₂ and 5 mM HEPES; pH adjusted to 7.6 by NaOH) supplemented with 10 μ g/ mL gentamycin, 50 μ g/ mL tetracycline, 100 μ g/ mL streptomycin and 500 μ g/ mL sodium pyruvate.

4.7. CO₂ Quantification and TEVC

Since a TEVC requires a liquid environment, it is not possible to directly measure the response of GRs to CO₂ in air; we chose to quantify the function of GRs by their response to dissolved CO₂ in NaHCO₃ solution, which was previously described by Xu et al. [28]. In brief, the concentration of dissolved CO₂ in NaHCO₃ solution can be calculated by the following equation; thus, we can obtain different concentrations of dissolved CO₂ by controlling the pH and the concentration of bicarbonate.

$$[CO_2(aq)] = \frac{10^{pK_{overall} - PH} \times [HCO_3^-]_{nominal}}{1 + 10^{pK_{overall} - PH}}$$

$K_{overall}$, which is sometimes referred to as K_a , is a constant that incorporates the CO₂ hydrolysis constant (K_h) and the first dissociation constant of carbonic acid (K_{a1}), i.e., $K_{overall} = K_h \times K_{a1}$. The pKa value is 6.3 (<https://pubchem.ncbi.nlm.nih.gov/compound/sodium-bicarbonate#section=pKa>, accessed on 6 August 2023).

The TEVC technique was used to record the channel currents in *Xenopus* oocytes at a holding potential of −80 Mv [52]. Signals were amplified with an Axonclamp 900A amplifier (Molecular Devices, San Jose, CA, USA) and 50-Hz low-pass filters and digitized at 1 kHz. Data acquisition and analysis were performed using Axon Digi 1550B and pCLAMP10 software (Molecular Devices, San Jose, CA, USA). We used the same concentration of Na⁺ in NaCl solution as negative control and then used full response

minus NaCl response to look at the real response of CO₂. The data were subsequently analyzed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

4.8. Anterograde Dye Filling and Immunohistochemical Staining of the Labial Palps

In order to furthermore explore labial palps projection on AL, the anterograde dye filling was performed on labial palp with both genders. According to previous studies [53], the adult insects were fixed in a plastic tube with dental wax so that their heads were exposed. The base of the labial palp was then cut off, and the fluorescent dye, tetramethyl-rhodamine dextran (MicroRuby, Molecular Probes; Invitrogen, Eugene, OR, USA), was applied at the cutting surface by using a needle. After staining, the insects were placed in a refrigerator with moist filter paper overnight, allowing transportation of the dye to the sensory axons. The next day, the brain and ventral nerve cord were dissected in Ringer's saline, fixed in 4% paraformaldehyde (PFA) for 1 h, dehydrated via an EtOH gradient, cleared with methyl salicylate, and mounted in Permount on perforated aluminum slides with coverslips.

For immunohistochemical staining, the brains of *H. cunea* were carefully removed with forceps, fixed with 4% PFA at 4 °C overnight, and then rinsed with PBST three times for 45 min. The fixed brain body part was then transferred to 5% normal goat serum (NGS; Thermo Fisher Scientific, Waltham, MA, USA) and preincubated at 4 °C for 15 h. After preincubation, the primary antibody 3C11 (anti-SYNORF1, 1:100 dilution with 5% NGS and PBST) (DSHB, University of Iowa, Johnson County, IA, USA) was applied and incubated at 4 °C for 5 days. Afterward, the brain was rinsed with PBST again and treated with the secondary antibody Cy2 coupled to an Alexa Fluor™ 488 (1:300 dilution with 1% NGS and PBST) (Invitrogen, Eugene, OR, USA) at 4 °C for 3 days. Finally, the brain was dehydrated using an alcohol gradient, cleared with methyl salicylate, stored at 4 °C, and mounted with 1 mm aluminum slides for confocal laser microscopy imaging.

The slides were observed under a laser scanning confocal microscope (LSM880, Carl Zeiss, Jena, Germany) with an excitation wavelength of 488 nm and collected between 490 nm and 560 nm. A clear image was captured with ZEN v2.6. The identified neuropils within the brain and ventral nerve cords were reconstructed by using the 3D reconstruction software Amira 5.4.3 (FEI, Hillsboro, OR, USA) [54].

4.9. Statistical Analysis

One-way ANOVA followed by Tukey's test or Dunnett's test was used for multigroup comparisons (Figure 3b). Wilcoxon signed-rank test was used as a comparison between two curves (Figure 4). When two sets of data were compared, the data were first evaluated by the Shapiro–Wilk normality test. If $p \leq 0.05$, the data sets will be applied for the Mann–Whitney test. If $p > 0.05$, the data will subsequently apply to the F-test to check the equal variances. If the data pass the F-test, it will apply to unpaired *t*-tests (Figures 6k and S2). If they do not pass the F-test, the data will apply to the unpaired *t*-tests with Welch's correction. The data were analyzed with SPSS 27.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). And for electrophysiological and TEVC experiments, the value of each biological replicate is the average of its three technical replicates, and three technical replicates mean that when conducting an antenna and labial palp or oocyte preparation, the same stimulus was applied three times.

5. Conclusions

Although the detailed biological functions of CO₂ in foraging and oviposition in *H. cunea* could not be fully clarified, some conclusions were reached at this stage. First, CO₂ (ranging from 1% to 10%) strongly affects *H. cunea* adults; second, the main organ involved in CO₂ sensing in *H. cunea* is the labial palp, and female moths have a more sensitive ability to perceive CO₂ than male moths, whereas *HcunGR1* and *HcunGR3* are indispensable elements in the CO₂ sensing process; and third, sexual dimorphism is observed in the volume of the LPOG, the main CO₂ projection region in the antennal lobe. In summary,

these results showed that CO₂ has an attractive effect on *H. cunea*, which may be related to their oviposition behaviors; by identifying the CO₂ receptors in *H. cunea*, the olfactory sensing pathway was further clarified. These results would benefit the development of a CO₂-based trap for the monitoring and control of *H. cunea*, meanwhile providing more evidence on the biological function of CO₂ among varied insects, especially those pests that take serious damage to the agriculture and forest.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms25115987/s1>.

Author Contributions: Conceptualization, Y.W. and D.L.; methodology, Y.W. and S.D.; software, J.Z.; validation, J.Z. and S.D. formal analysis, J.Z., S.D. and W.W.; writing—original draft preparation, S.D. and Y.W.; writing—review and editing, Y.W., D.L., J.Z. and S.D.; funding acquisition, Y.W. and D.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Key R&D Program of China (2023FYE0113600) and the Natural Science Foundation of Jilin Province (grant 20230101252JC).

Institutional Review Board Statement: *Xenopus laevis* approved by the Ethics Committee of Northeast Normal University and used for this study were collected, cared for, and treated under strict compliance with all ethical practices and laws.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data sets analyzed in the current study are available from the corresponding author upon reasonable request.

Acknowledgments: We thank our colleagues for their assistance in the specimen collection of the insects. The authors would like to thank all the reviewers who participated in the review.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Guerenstein, P.G.; Hildebrand, J.G. Roles and Effects of Environmental Carbon Dioxide in Insect Life. *Annu. Rev. Entomol.* **2008**, *53*, 161–178. [\[CrossRef\]](#)
2. Seeley, T.D. Atmospheric carbon dioxide regulation in honey-bee (*Apis mellifera*) colonies. *J. Insect Physiol.* **1974**, *20*, 2301–2305. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Bowen, M.F. The Sensory Physiology of Host-Seeking Behavior in Mosquitoes. *Annu. Rev. Entomol.* **1991**, *36*, 139–158. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Gillies, M.T. The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culicidae): A review. *Bull. Entomol. Res.* **1980**, *70*, 525–532. [\[CrossRef\]](#)
5. Huang, X.; Mack, T.P. Artificial Carbon Dioxide Source to Attract Lesser Cornstalk Borer (Lepidoptera: Pyralidae) Larvae. *J. Econ. Entomol.* **2001**, *94*, 860–867. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Abrell, L.; Guerenstein, P.G.; Mechaber, W.L.; Stange, G.; Christensen, T.A.; Nakanishi, K.; Hildebrand, J.G. Effect of elevated atmospheric CO₂ on oviposition behavior in *Manduca sexta* moths. *Glob. Chang. Biol.* **2005**, *11*, 1272–1282. [\[CrossRef\]](#)
7. Stange, G.; Monro, J.; Stowe, S.; Osmond, C.B. The CO₂ sense of the moth *Cactoblastis cactorum* and its probable role in the biological control of the CAM plant *Opuntia stricta*. *Oecologia* **1995**, *102*, 341–352. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Loewy, K.J.; Flansburg, A.L.; Grenis, K.; Kjeldgaard, M.K.; Mccarty, J.; Montesano, L.; Vernick, J.; Murphy, S.M. Life History Traits and Rearing Techniques for Fall Webworms (*Hyphantria cunea* Drury) in Colorado. *J. Lepid. Soc.* **2013**, *67*, 196–205. [\[CrossRef\]](#)
9. Schowalter, T.D.; Ring, D.R. Biology and Management of the Fall Webworm, *Hyphantria cunea* (Lepidoptera: Erebidae). *J. Integr. Pest Manag.* **2017**, *8*. [\[CrossRef\]](#)
10. Wu, N.; Zhang, S.; Li, X.; Cao, Y.; Liu, X.; Wang, Q.; Liu, Q.; Liu, H.; Hu, X.; Zhou, X.J.; et al. Fall webworm genomes yield insights into rapid adaptation of invasive species. *Nat. Ecol. Evol.* **2019**, *3*, 105–115. [\[CrossRef\]](#)
11. Jones, W.D.; Cayirlioglu, P.; Kadow, I.G.; Vossell, L.B. Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*. *Nature* **2006**, *445*, 86–90. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Kwon, J.Y.; Dahanukar, A.; Weiss, L.A.; Carlson, J.R. The molecular basis of CO₂ reception in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 3574–3578. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Robertson, H.M.; Kent, L.B. Evolution of the Gene Lineage Encoding the Carbon Dioxide Receptor in Insects. *J. Insect Sci.* **2009**, *9*, 1–14. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Hu, J.; Zhong, C.; Ding, C.; Chi, Q.; Walz, A.; Mombaerts, P.; Matsunami, H.; Luo, M. Detection of Near-Atmospheric Concentrations of CO₂ by an Olfactory Subsystem in the Mouse. *Science* **2007**, *317*, 953–957. [\[CrossRef\]](#) [\[PubMed\]](#)

15. Fischler, W.; Kong, P.; Marella, S.; Scott, K. The detection of carbonation by the *Drosophila* gustatory system. *Nature* **2007**, *448*, 1054–1057. [[CrossRef](#)] [[PubMed](#)]
16. Engsontia, P.; Sangket, U.; Chotigeat, W.; Satsook, C. Molecular Evolution of the Odorant and Gustatory Receptor Genes in Lepidopteran Insects: Implications for Their Adaptation and Speciation. *J. Mol. Evol.* **2014**, *79*, 21–39. [[CrossRef](#)] [[PubMed](#)]
17. Xu, W.; Anderson, A. Carbon dioxide receptor genes in cotton bollworm *Helicoverpa armigera*. *Sci. Nat.* **2015**, *102*, 1–9. [[CrossRef](#)] [[PubMed](#)]
18. Ning, C.; Yang, K.; Xu, M.; Huang, L.-Q.; Wang, C.-Z. Functional validation of the carbon dioxide receptor in labial palps of *Helicoverpa armigera* moths. *Insect Biochem. Mol. Biol.* **2016**, *73*, 12–19. [[CrossRef](#)] [[PubMed](#)]
19. Stocker, R.F.; Singh, R.N.; Schorderet, M.; Siddiqi, O. Projection patterns of different types of antennal sensilla in the antennal glomeruli of *Drosophila melanogaster*. *Cell Tissue Res.* **1983**, *232*, 237–248. [[CrossRef](#)]
20. Suh, G.S.B.; Wong, A.M.; Hergarden, A.C.; Wang, J.W.; Simon, A.F.; Benzer, S.; Axel, R.; Anderson, D.J. A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Nature* **2004**, *431*, 854–859. [[CrossRef](#)]
21. Distler, P.G.; Boeckh, J. Central Projections of the Maxillary and Antennal Nerves in the Mosquito *Aedes Aegypti*. *J. Exp. Biol.* **1997**, *200*, 1873–1879. [[CrossRef](#)]
22. Anton, S.; van Loon, J.J.; Meijerink, J.; Smid, H.M.; Takken, W.; Rospars, J.-P. Central projections of olfactory receptor neurons from single antennal and palpal sensilla in mosquitoes. *Arthropod Struct. Dev.* **2003**, *32*, 319–327. [[CrossRef](#)]
23. Anton, S.; Rospars, J. Quantitative analysis of olfactory receptor neuron projections in the antennal lobe of the malaria mosquito, *Anopheles gambiae*. *J. Comp. Neurol.* **2004**, *475*, 315–326. [[CrossRef](#)]
24. Zhao, X.-C.; Tang, Q.-B.; Berg, B.G.; Liu, Y.; Wang, Y.-R.; Yan, F.-M.; Wang, G.-R. Fine structure and primary sensory projections of sensilla located in the labial-palp pit organ of *Helicoverpa armigera* (Insecta). *Cell Tissue Res.* **2013**, *353*, 399–408. [[CrossRef](#)]
25. Guerenstein, P.G.; Christensen, T.A.; Hildebrand, J.G. Sensory processing of ambient CO₂ information in the brain of the moth *Manduca sexta*. *J. Comp. Physiol. A* **2004**, *190*, 707–725. [[CrossRef](#)]
26. Yu, H.; Khashaveh, A.; Li, Y.; Li, X.; Zhang, Y. Field Trapping of Predaceous Insects With Synthetic Herbivore-Induced Plant Volatiles in Cotton Fields. *Environ. Entomol.* **2017**, *47*, 114–120. [[CrossRef](#)]
27. Slone, J.; Daniels, J.; Amrein, H. Sugar Receptors in *Drosophila*. *Curr. Biol.* **2007**, *17*, 1809–1816. [[CrossRef](#)]
28. Xu, P.; Wen, X.; Leal, W.S. CO₂ per se activates carbon dioxide receptors. *Insect Biochem. Mol. Biol.* **2019**, *117*, 103284. [[CrossRef](#)]
29. Baby, S.; Johnson, A.J.; Zachariah, E.J.; Hussain, A.A. Nepenthes pitchers are CO₂-enriched cavities, emit CO₂ to attract preys. *Sci. Rep.* **2017**, *7*, 11281. [[CrossRef](#)]
30. Edosa, T.T.; Jo, Y.H.; Keshavarz, M.; Anh, Y.S.; Noh, M.Y.; Han, Y.S. Current status of the management of fall webworm, *Hyphantria cunea*: Towards the integrated pest management development. *J. Appl. Entomol.* **2019**, *143*, 1–10. [[CrossRef](#)]
31. Krenn, H.W. Feeding Mechanisms of Adult Lepidoptera: Structure, Function, and Evolution of the Mouthparts. *Annu. Rev. Entomol.* **2010**, *55*, 307–327. [[CrossRef](#)]
32. Morris, R.F. Fecundity and colony size in natural populations of *hyphantria cunea*. *Can. Entomol.* **1972**, *104*, 399–409. [[CrossRef](#)]
33. Clark, D. Images of Leaf Stomata: Little Things that Matter. *Microsc. Today* **2019**, *27*, 12–17. [[CrossRef](#)]
34. Eshun, O.; Gerry, A.; Hayes, W.K. Mosquito Capture Rate Using CO₂-Baited Traps in Relation to Distance From Water and Height: Implications for Avian Disease Transmission. *J. Med Entomol.* **2016**, *53*, 1378–1384. [[CrossRef](#)]
35. Chen, Y.-C.D.; Dahanukar, A. Recent advances in the genetic basis of taste detection in *Drosophila*. *Cell. Mol. Life Sci.* **2019**, *77*, 1087–1101. [[CrossRef](#)]
36. Benton, R.; Vannice, K.S.; Gomez-Diaz, C.; Vossell, L.B. Variant Ionotropic Glutamate Receptors as Chemosensory Receptors in *Drosophila*. *Cell* **2009**, *136*, 149–162. [[CrossRef](#)]
37. Sánchez-Alcañiz, J.A.; Silbering, A.F.; Croset, V.; Zappia, G.; Sivasubramanian, A.K.; Abuin, L.; Sahai, S.Y.; Münch, D.; Steck, K.; Auer, T.O.; et al. An expression atlas of variant ionotropic glutamate receptors identifies a molecular basis of carbonation sensing. *Nat. Commun.* **2018**, *9*, 4252, Correction in *Nat. Commun.* **2020**, *11*, 1131. [[CrossRef](#)]
38. Ai, M.; Min, S.; Grosjean, Y.; Leblanc, C.; Bell, R.; Benton, R.; Suh, G.S.B. Acid sensing by the *Drosophila* olfactory system. *Nature* **2010**, *468*, 691–695. [[CrossRef](#)]
39. van Breugel, F.; Huda, A.; Dickinson, M.H. Distinct activity-gated pathways mediate attraction and aversion to CO₂ in *Drosophila*. *Nature* **2018**, *564*, 420–424. [[CrossRef](#)]
40. Terhag, J.; Cavara, N.A.; Hollmann, M. Cave Canalem: How endogenous ion channels may interfere with heterologous expression in *Xenopus oocytes*. *Methods* **2010**, *51*, 66–74. [[CrossRef](#)]
41. Amat, C.; Marion-Poll, F.; Navarro-Roldán, M.A.; Gemenio, C. Gustatory function of sensilla chaetica on the labial palps and antennae of three tortricid moths (Lepidoptera: Tortricidae). *Sci. Rep.* **2022**, *12*, 1–11. [[CrossRef](#)]
42. Gaudry, Q.; Hong, E.J.; Kain, J.; de Bivort, B.L.; Wilson, R.I. Asymmetric neurotransmitter release enables rapid odour lateralization in *Drosophila*. *Nature* **2012**, *493*, 424–428. [[CrossRef](#)]
43. Mohamed, A.A.M.; Hansson, B.S.; Sachse, S. Third-Order Neurons in the Lateral Horn Enhance Bilateral Contrast of Odor Inputs Through Contralateral Inhibition in *Drosophila*. *Front. Physiol.* **2019**, *10*, 851. [[CrossRef](#)]
44. Mysore, K.; Subramanian, K.; Sarasij, R.; Suresh, A.; Shyamala, B.V.; VijayRaghavan, K.; Rodrigues, V. Caste and sex specific olfactory glomerular organization and brain architecture in two sympatric ant species *Camponotus sericeus* and *Camponotus compressus* (Fabricius, 1798). *Arthropod Struct. Dev.* **2009**, *38*, 485–497. [[CrossRef](#)]

45. Varela, N.; Avilla, J.; Gemenio, C.; Anton, S. Ordinary glomeruli in the antennal lobe of male and female tortricid moth *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) process sex pheromone and host-plant volatiles. *J. Exp. Biol.* **2011**, *214*, 637–645. [\[CrossRef\]](#)
46. Kent, K.S.; Harrow, I.D.; Quartararo, P.; Hildebrand, J.G. An accessory olfactory pathway in Lepidoptera: The labial pit organ and its central projections in *Manduca sexta* and certain other sphinx moths and silk moths. *Cell Tissue Res.* **1986**, *245*, 237–245. [\[CrossRef\]](#)
47. Chen, Q.; Zhao, H.; Wen, M.; Li, J.; Zhou, H.; Wang, J.; Zhou, Y.; Liu, Y.; Du, L.; Kang, H.; et al. Genome of the webworm *Hyphantria cunea* unveils genetic adaptations supporting its rapid invasion and spread. *BMC Genom.* **2020**, *21*, 1–22. [\[CrossRef\]](#)
48. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [\[CrossRef\]](#)
49. Trapnell, C.; Williams, B.A.; Pertea, G.; Mortazavi, A.; Kwan, G.; Van Baren, M.J.; Salzberg, S.L.; Wold, B.J.; Pachter, L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **2010**, *28*, 511–515. [\[CrossRef\]](#)
50. Zhao, Y.; Wang, F.; Zhang, X.; Zhang, S.; Guo, S.; Zhu, G.; Liu, Q.; Li, M. Transcriptome and Expression Patterns of Chemosensory Genes in Antennae of the Parasitoid Wasp *Chouioia cunea*. *PLoS ONE* **2016**, *11*, e0148159. [\[CrossRef\]](#)
51. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [\[CrossRef\]](#)
52. Zhou, Y.; Han, Q.; Feng, K.; Wang, J.; Zhou, H.; Wen, M.; Duan, H.; Wang, Y.; Ren, B. New binding sites of nicotinic acetylcholine receptors from *Myzus persicae*. *Entomol. Gen.* **2023**, *43*, 461–470. [\[CrossRef\]](#)
53. Ignell, R.; Anton, S.; Hansson, B.S. The maxillary palp sensory pathway of Orthoptera. *Arthropod Struct. Dev.* **2000**, *29*, 295–305. [\[CrossRef\]](#)
54. Ye, Z.; Liu, F.; Liu, N. Three-dimensional structure of the antennal lobe in the Southern house mosquito *Culex quinquefasciatus*. *Insect Sci.* **2021**, *28*, 93–102. [\[CrossRef\]](#)

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.