SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
ENTOMOLOGY
TRANSCRIPTIONAL REGULATORY ELEMENTS
GENE EXPRESSION

Received
14 May 2013

Accepted
24 October 2013

Published
14 November 2013

Correspondence and requests for materials should be addressed to Y.-Z.D. (yzdu@yzu.edu.cn) or J.Y.L. (lij@vt.edu)

Diapause, signal and molecular characteristics of overwintering *Chilo suppressalis* (Insecta: Lepidoptera: Pyralidae)

Ming-Xing Lu[1,2], Shuang-Shuang Cao[1], Yu-Zhou Du[1], Zhong-Xian Liu[1], Pingyang Liu[2] & Jianyong Li[2]

[1]College of Horticulture and Plant Protection & Institute of Applied Entomology, Yangzhou University, Yangzhou, China,
[2]Department of Biochemistry, Virginia Tech, Blacksburg, VA, United States.

Diapause is a complex and dynamic process. *Chilo suppressalis*, an important rice pest in Asia enters facultative diapause as larvae. Our results demonstrated in Yangzhou, China, diapause was initiated between September 4 and 12, 2010. After diapause termination, *C. suppressalis* remained in quiescence in the field for as long as three months. The average time between collection of field larvae of *C. suppressalis* and their pupation decreased as the season progressed from fall to next spring. Unexpectedly, the pupated ratio of female to male in the initiation of diapause was 0.22. The abundance of *hsp90*, *hsp70*, *hsp60* and *Cs*AQP1 all peaked on January 8 or 15, 2011. Nitric oxide (NO) is a secondary messenger that is positively correlated with the diapause of *C. suppressalis.* Among several geographically separated populations of *C. suppressalis*, there are no significant differences in the mRNA levels of *hsp70*, *hsp60* or *Cs*AQP1.

In many insects, the ability to enter diapause has contributed greatly to their evolutionary success. Through diapause, insects are able to survive adverse climatic conditions[1]. Diapause as a process is divided into four eco-physiological phases: pre-diapause, diapause, post-diapause quiescence, and post-diapause development[2]. Insect diapause is centrally mediated at specific developmental stages, either in response to key stimuli from the environment (facultative diapause) or as a fixed component of ontogeny (obligatory diapause)[3,4]. The maintenance of diapause itself is physiologically dynamic and changes over time in response to environmental and internal stimuli[3,5–7]. Another source of variation in diapause characteristics in many insects occurs among geographically separated populations of the same species[8–12]. However, most work on this point has thus far focused on the differences in photoperiodic induction of diapause between populations from different latitudes and work is lacking on variation in up or down regulation of those genes.

It is known that genes characteristic of reproductive growth are down-regulated during diapause, while other genes, often termed diapause-specific, are up-regulated, while the expression of still others is either not affected or changes in an intermittent fashion[13,14]. In recent years, some studies have indicated that expression of heat shock protein (HSPs) is regulated as a function of insect diapause[3,15–17]. Heat shock genes are a subset of a larger group of genes coding for molecular chaperones whose expression levels often increase in response to environmental stress[18]. The heat shock proteins, present in all organisms and conserved through evolution, have been categorized into six major families: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (sHSPs)[19]. Another gene family, the aquaporins, has been suggested to possess some relation to dormancy in work with *Brachionus plicatilis* Müller (Ploimida: Brachionidae)[20,21]. Aquaporins (AQPs), often known as water channels, are integral membrane proteins that regulate the flow of water across cell membranes[22]. Various studies have found insect AQPs to play an important role in cold hardiness, as the freeze tolerance of an insect is related to its ability to remove water from cells using aquaporins[23–25]. Also, it is likely that gene expression and progress through diapause are influenced by signaling pathways. For example, nitric oxide (NO) was believed to regulate diapause-specific developmental pathways through secondary messenger production[7]. The NO involved is produced in the mitochondrial respiratory chain through the action of NO synthase[26,27].

The striped stem borer, *Chilo suppressalis* (Walker) (Insecta: Lepidoptera: Pyralidae), is an important rice pest widely distributed in China and other Asian countries, which has become a more serious threat to rice in recent years in China. The majority of *C. suppressalis* individuals undergo six larval instars, but a few have five or seven instars in rice fields in China[28]. In the district of Yangzhou (32.23°N, 119.26°E), Jiangsu province, China, striped stem borer has two complete and a partial third generation each year. Diapause of *C. suppressalis* has been studied in the suburb of Nanchang (28.77°N, 115.83°E), Jiangxi province, China, showed that most larvae entered facultative diapause in August, and *C. suppressalis* terminated diapause in the spring of next year[29]. However, this study did not further describe diapaused trait of *C. suppressalis* in the field. In Japan (31–43°N), *C. suppressalis* populations exist as at least two diapause-ecotypes, the Shonai ecotype (SN ecotype) and the Saigoku ecotype (SG ecotype). SN ecotype larvae enter diapause in early September and terminate it in November. However, larvae of the SG ecotype maintain diapause until February[30,31]. Such differences suggest that geographic variation in diapause may exist in other populations of *C. suppressalis*. Although cold hardiness may or may not depend on diapause, the two are often connected[32–34]. However, the relationship between cold tolerance and diapause in *C. suppressalis* is complex[35].

To date, the diapause characteristics of *C. suppressalis* under field ecology have not been assessed in detail. Our understanding of the molecular and signaling mechanisms of diapause in this species is also limited, while the importance of geographic variation in the distribution and evolution of *C. suppressalis* likewise needs further study. Therefore, to better understand the diapause mechanism of *C. suppressalis* and the other insects, we undertook following studies: 1. we described the diapause characteristics of *C. suppressalis* under field ecology in Yangzhou. These traits included diapause initiation, maintenance, termination, post-diapause quiescence and post-diapause development. Our results also provided a better understanding of the relationship between diapause and cold hardiness in *C. suppressalis*. 2. We characterized gene expression (*hsp90*, *hsp70*, *hsp60* and *Cs*AQP1) and NO variation in overwintering larvae of *C. suppressalis* collected during different phases of diapause and from five geographic populations of *C. suppressalis*. Our long-term goal is to identify biological and molecular pathways regulating diapause in insects. Ultimately, these studies may provide insights into the evolutionary mechanisms and the integrated pest management of *C. suppressalis* and other insects.

Results

Diapausing process of Yangzhou population of *C. suppressalis*. We observed that while 66% of *C. suppressalis* larvae collected on September 4, 2010 developed to pupae when taken into the laboratory (i.e., were not in diapause), only 39% of larvae collected on September 12 did so (i.e., 61% of the larvae had entered diapause by that date), demonstrating that in the Yangzhou district, diapause initiation occurred between September 4 and 12, in 2010 (Fig. 1A). However, this shift began even though the average temperature on September 12 was still high (25.8°C) (data from Yangzhou Bureau of Meteorology), and warm enough to support development of *C. suppressalis*. It was interesting that, with the exception of November 13, 2010, there were at least a few larvae in field samples on all collection dates that were still able to develop to pupae (i.e., were not in diapause). The termination of diapause for a population is considered to be the date when more than 50% of the diapausing stage (here, larvae) are able to resume development (if exposed to appropriated temperatures). However, in our study, the proportion of overwintering larvae able to resume development (given higher temperatures) fluctuated (dipping below 50% on some dates) and therefore we did not consider diapause to have been terminated until after January 27, after which the percentage of larvae able to proceed to pupation always exceeded 50% (Fig. 1A). For this same study site, other field research showed that the field population of larvae did not reach the 50% pupation point until May 16, 2011, indicating that in the field, *C. suppressalis* larvae remained in quiescence for as long as three months, presumably because field temperatures were too low to permit development, despite the termination of diapause.

Intensity of diapause and post-diapause development of overwintering *C. suppressalis*. Diapause intensity (DI) is a physiological trait that can be defined by the duration of diapause under given conditions of environment[38] DI of *C. suppressalis* larvae collected at different dates varied but was greatest on November 13, 2010 (Fig. 1B), when no larvae pupated after being taken to the laboratory and held at 28°C. Generally, DI of *C. suppressalis* decreased seasonally across samples (Fig. 1B). For larvae collected on September 4, 2010 (when 66% of field collected larvae were not in diapause) the average duration from field collection to pupation in the laboratory was 18.9 days. Until April 16, 2011 (when 83% of larvae pupated, indicating this percentage were out of diapause), the average duration was lower than for the September 4, 2010 collection (Fig. 1B).

Intriguingly, gender differences existed in some parameters, including the ratio of female and male larvae entering diapause in particular time periods. For example, from September 18 to October 30, 2010, when most larvae were in diapause and very few of the larvae taken into the laboratory continued on to pupate, most larvae that did pupate were male (Fig. 1C), indicating that a lower proportion of males than females had entered diapause, ratio only 0.22 on September 12, 2010. Also, DI of field samples of larvae differed between male and female larvae when held at 28°C (Fig. 1D).

The molecular basis of overwintering *C. suppressalis*. The expression levels of heat shock proteins or aquaporin genes didn't vary with field temperatures (Fig. 2). Three heat shock proteins of *C. suppressalis* exhibited different timing of expression during diapause. However, the mRNA levels of the three *hsps* all decreased between January 15 and February 12, 2011, the date by which all *C. suppressalis* larvae had terminated diapause. The abundance of *hsp90* peaked on January 8, 2011, while *hsp70* and *hsp60* levels peaked on January 15, 2011 (Fig. 3A–C). The expression level of *hsp90* remained stable until December 31, 2010, with the exception of October 2, 2010 (Fig. 3A). On January 15, 2011, the mRNA level of *hsp90* was significantly higher than on February 12 ($F_{11,\ 22} = 9.661$; $P < 0.001$), but it was not different from January 8 ($F_{11,\ 22} = 9.661$; $P < 0.001$) (Fig. 3A). The mRNA level of *hsp70* increased significantly between January 8 and 15, 2011, and then decreased on February 12, 2011 ($F_{11,\ 24} = 5.146$; $P < 0.001$) (Fig. 3B). Meanwhile, the level of *hsp60* remained even between January 15 and March 12, but was significantly different compared to February 12, 2011 ($F_{11,\ 24} = 5.146$; $P < 0.001$) (Fig. 3C). In order to clarify the relationship between water and diapause of *C. suppressalis*, the expression level of *Cs*AQP1 in this insect was determined during diapause. *Cs*AQP1 levels increased during diapause and peaked on January 15, 2011, at significantly higher levels than on December 31, 2010. By February 26, 2011, *Cs*AQP1 levels had decreased significantly ($F_{11,\ 22} = 5.431$; $P < 0.001$) (Fig. 3D). After the termination of diapause, although the temperature increased, the mRNA levels of *hsp90*, *hsp70*, *hsp60* and *Cs*AQP1 decreased (Fig. 1–3).

Signal characteristics of overwintering *C. suppressalis*. We examined a signal pathway in overwintering *C. suppressalis* by measuring NO levels at various stages of diapause. The trend of the NO levels was the opposite of that of heat shock proteins and aquaporin. The highest NO level was 28.78 μM on October 2, 2010, while its lowest level (2.82 μM) occurred on November 20, 2010, after which NO content increased again significantly ($F_{11,\ 24} = 7.475$; $P < 0.001$) (Fig. 4).

Molecular traits of geographic populations of overwintering *C. suppressalis*. Among the five geographic populations of *C. suppressalis* sampled (Fig. 5), the expression level of *hsp90* was significantly higher in the Yangzhou population ($F_{4,\ 9} = 4.149$; $P = 0.036$). However, there were no significant differences among the Chengdu, Yichang, Hanzhong and Changchun populations ($F_{4,\ 9} =$

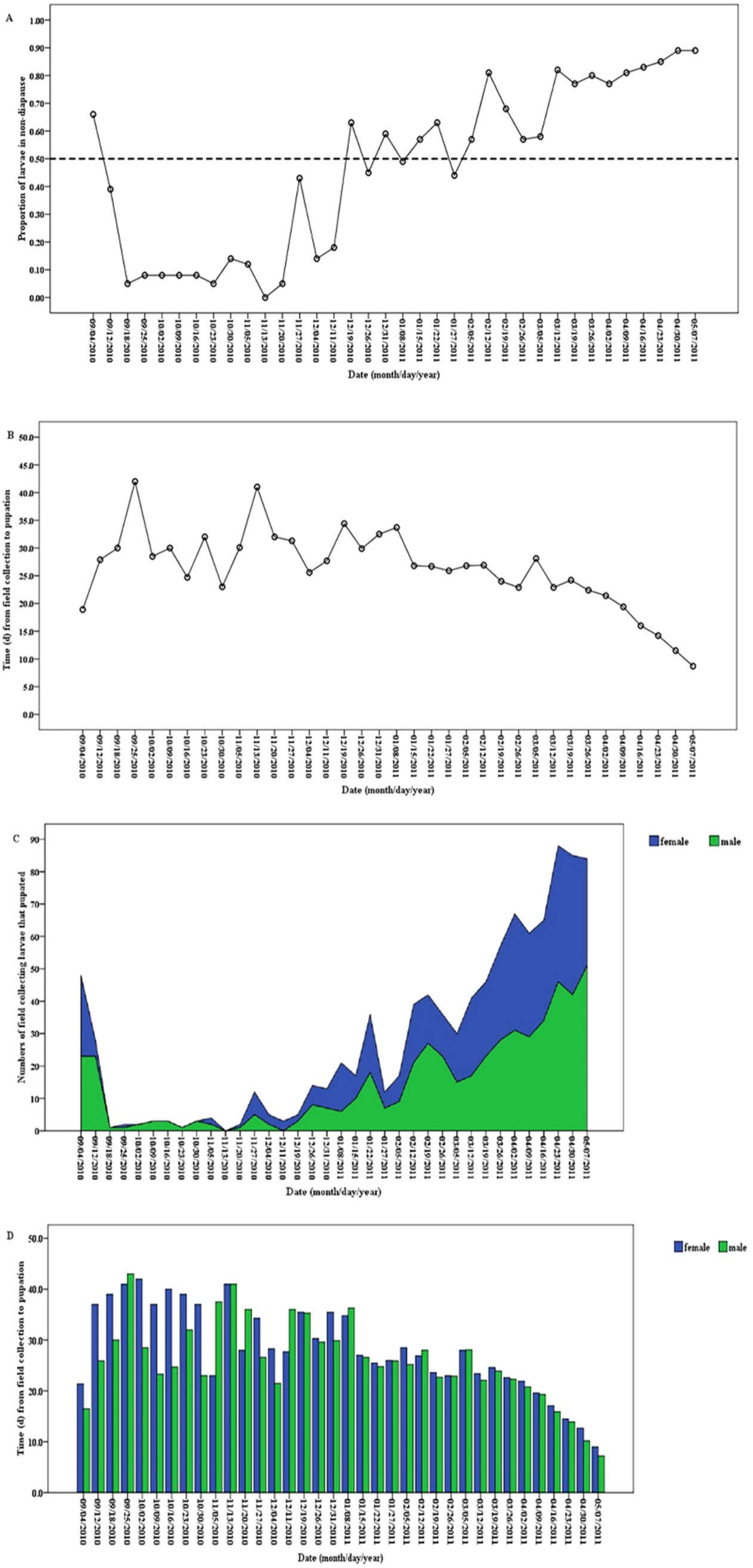

Figure 1 | **Diapausing characteristics of *C. suppressalis* in the field.** (A) Pupation rate in the laboratory of larvae collected from the field from September, 2010 to May, 2011 and then held at 28°C (pupation indicates a larva was not in diapause). (B) The average number of days between collection and pupation for those larvae that did pupate after being taken from the field into the laboratory and held at 28°C. (C) Number of larvae that pupated after collection that were female or male. (D) Average number of days between field collection and pupation in the laboratory for female versus male larvae, for those that did pupate.

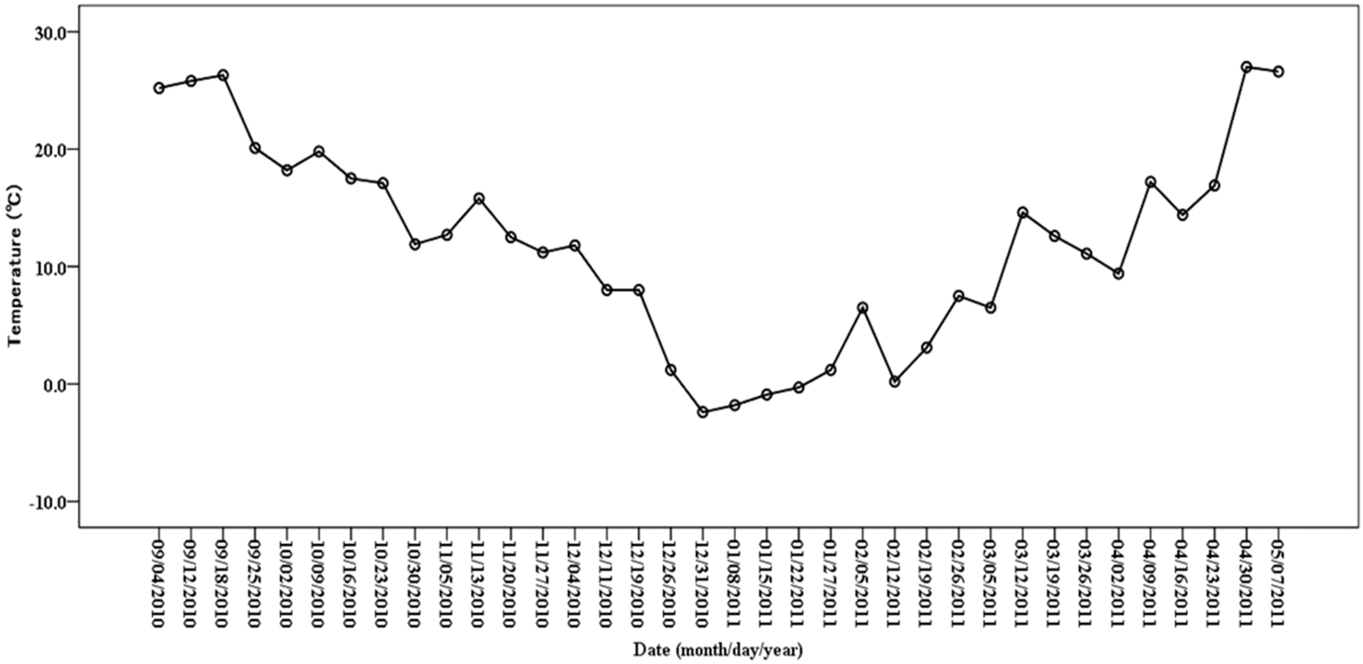

Figure 2 | **Daily average temperature was used in this study from September 2010 to May 2011 in the field of Yangzhou.** Data were recorded from Yangzhou Bureau of Meteorology.

4.149; $P > 0.050$). The mRNA levels of *hsp70* and *hsp60* for the five geographic locations likewise showed no significant differences ($F_{4,9} = 0.465$; $P > 0.050$, $F_{4,\,9} = 2.507$; $P > 0.050$) (Fig. 6A). Although the highest value of *Cs*AQP1 was found in the Changchun population, the levels of *Cs*AQP1 from the five geographic locations were not significantly different ($F_{4,\,9} = 3.024$; $P > 0.050$) (Fig. 6B).

Discussion

Most multivoltine insects have evolved facultative diapause, a strategy that allows them to survive under adverse conditions such as winter[39]. Diapause is undoubtedly a complex and dynamic process. *Chilo suppressalis*, one of the most economically damaging insect pests in China, enters facultative diapause as a larva, but this process is not fully understood, and control of diapause, quiescence, and post-diapause development require further study. Most previous experiments have focused on the photoperiod induced diapause of *C. suppressalis* in the laboratory, but such laboratory conditions might not be relevant to field conditions. In this study, we therefore described the diapause, signal and molecular characteristics of overwintering *C. suppressalis* larvae in the field.

We found that in the district of Yangzhou (32.23°N, 119.26°E) *C. suppressalis* entered diapause in early September, and terminated diapause in late January or early February of the following year. This borer, therefore, maintains diapause for almost five months and remains in quiescence for as long as three more months in the field, waiting for favorable conditions for development in May. Similarly, in the suburbs of Nanchang, Jiangxi province, China, full-grown larvae entered diapause in September[40]. *C. suppressalis* enters this facultative state in response to short-day conditions during the autumn[29,41]. For the Yangzhou *C. suppressalis* population, 61% entered the diapause on or before September 12 (Fig. 1A), at a time when rice was ripe and would soon be harvested, removing the borer's primary food source. Diapause in most cases enhances tolerance to cold stress[3]. Some studies, however, have found that cold hardiness increased in the post-diapausing stage[31,42]. In previous studies, we observed a maximum cold tolerance of *C. suppressalis* on January 15, 2011, in contrast to a lack of such tolerance (98% larval mortality at -18°C, 2 h upon being observed in the laboratory for larvae of this population collected on November 14, 2010)[43], which led us to conclude that at least in our population of *C. suppressalis*, cold hardiness was somewhat independent of diapause.

Generally, the average time between collection of field larvae and their pupation in the laboratory when held at 28°C (diapause intensity) decreased as the season progressed from fall to spring. Although diapause of *C. suppressalis* terminated in late January or early February, average pre-pupation time was still long on February 5 (26.8 days). However, on May 7, pre-pupation time had decreased to only 8.7 days (Fig. 1B). During the post-diapause quiescence, lingering physiological effects of diapause of *C. suppressalis* gradually dissipated, preparing larvae to initiate development as soon as favorable conditions returned. Differences in the intensity of diapause effects due to the sex of the insect are still poorly understood[44]. Our experiment indicated that in *C. suppressalis* there is a sexual difference in the intensity of diapause effects, especially for larvae just entering diapause. In general, intensity of diapause was stronger for females than for males (Fig. 1C and D). A similar sex-related difference in the timing of post-diapause development under natural conditions was found for *Leptocorisa chinensis* Dallas (Hemiptera: Alydidae)[45].

Recently, studies have found that heat shock proteins contributed to successful diapause[46–50]. In this report, we demonstrate the linkage between diapause and three heat shock proteins (*hsp90*, *hsp70* and *hsp60*) in *C. suppressalis*, which each had a different expression pattern during diapause. However, the abundance of *hsp90*, *hsp70* and *hsp60* all unexpectedly peaked on January 8 or 15, 2011, and decreased between January 15 and February 12, 2011 when *C. suppressalis* terminated the diapause (Fig. 3A–C). The presence of mRNAs encoding *hsp90*, *hsp70* and *hsp60* was tightly linked to the entire diapause period, with levels increasing as diapause got underway, and then decreasing during the post-diapause period (Fig. 3A–C). This finding is consistent with a similar result for *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae), in which *SnoHsc70* was induced during diapause[51]. In contrast, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) was found to have its *hsp90* transcripts down regulated during its pupal diapause[53]. It is noteworthy that in our study, three *hsps* of *C. suppressalis* were synchronously elevated.

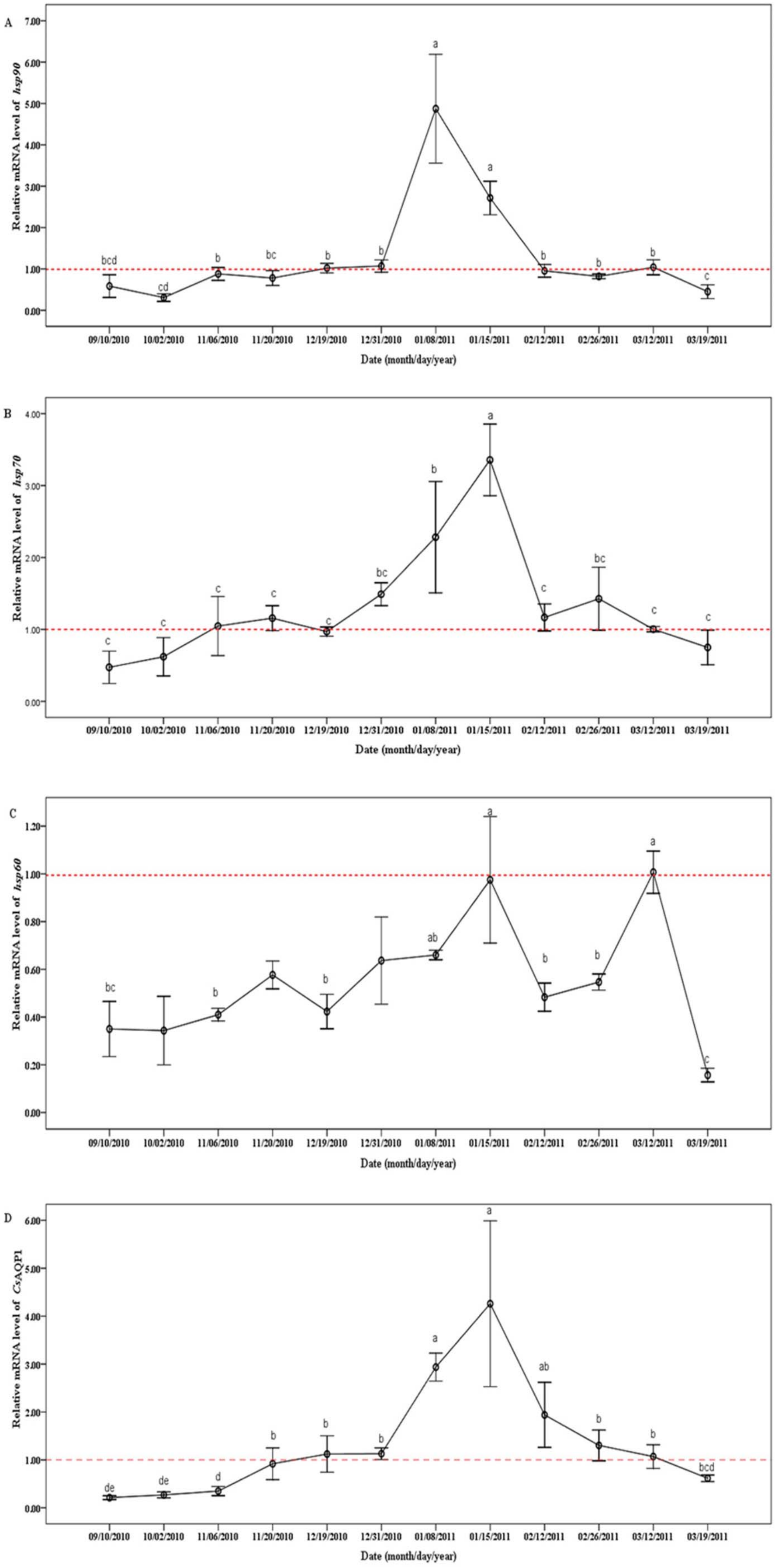

Figure 3 | Relative expression levels of *hsp90*, *hsp70*, *hsp60*, and *Cs*AQP1 in overwintering *C. suppressalis*. The quantity of each gene mRNA is normalized to the abundance of 18SrRNA. Significant differences are indicated as $P < 0.05$. Values are denoted as the mean ± SE.

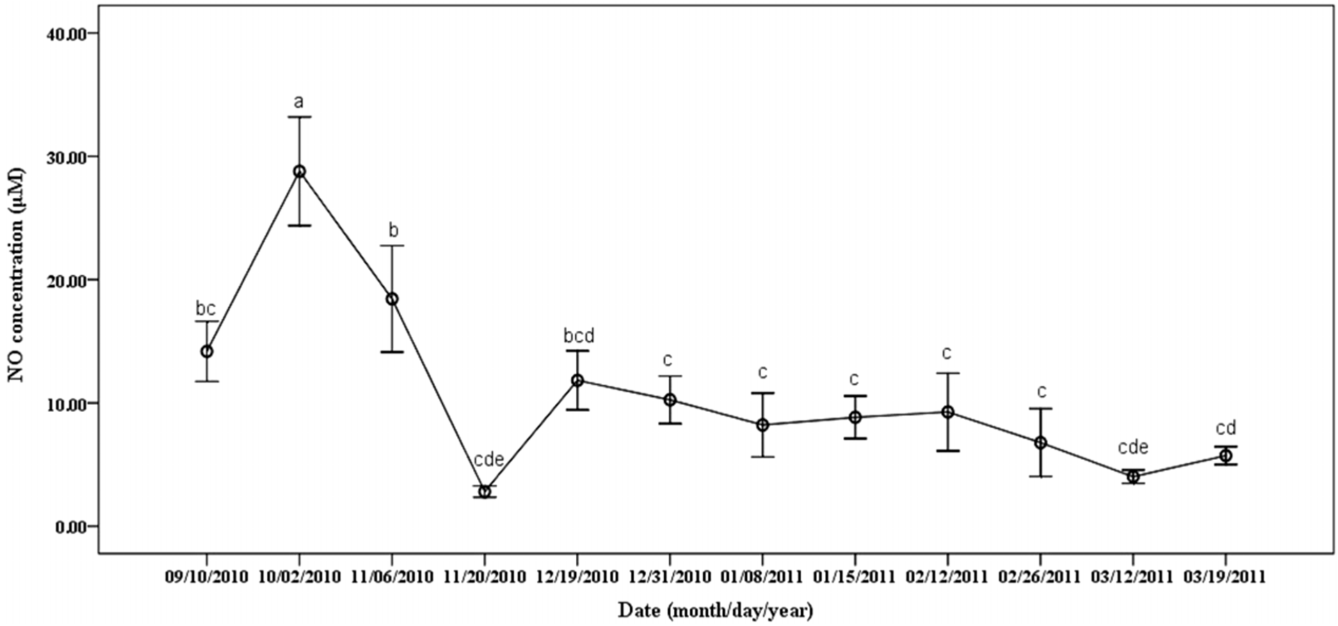

Figure 4 | Nitric oxide (NO) content in overwintering *C. suppressalis*. Significant differences are indicated as $P < 0.05$. Values are denoted as the mean ± SE.

Cold hardiness and winter diapause are essential for survival in most overwintering insects. The relations between cold hardiness and winter diapause are physiologically complicated. Previous studies have shown the greatest cold hardiness of *C. suppressalis* arising in January[43], when all three heat shock proteins exhibited their greatest abundance. As molecular chaperones, these heat shock proteins could be involved in maintaining the integrity of key metabolic enzymes or structural proteins during the low temperatures of winter[3]. *Hsp90*, *hsp70* and *hsp60* protect *C. suppressalis* from cold injury. Unlike in *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae)[3], cold hardiness is not firmly linked to diapause in *C. suppressalis*. Diapausing larvae of *C. suppressalis* were initially not cold hardy, but cold hardiness peaked four months after initiating diapause[43]. Interestingly, their attainment of cold hardiness coincided with their acquisition of the ability to synthesize heat shock proteins. *C. suppressalis* terminated diapause in late January or early February. By February 5, 2011, the mRNA levels of *hsp90*, *hsp70*, and *hsp60* had all decreased significantly from their peak (Fig. 3A–C). A similar pattern was found for *S. crassipalpis*[52]. From these results, we concluded that all three heat shock proteins play important dual roles in the cold hardiness and diapause of *C. suppressalis*. Indeed, HSPs could form the bridge between cold hardiness and diapause in *C. suppressalis*. In most cases, expression of *hsps* is incompatible with insect development, so down-regulation of these three *hsps* is required to all for the subsequent development of *C. suppressalis*.

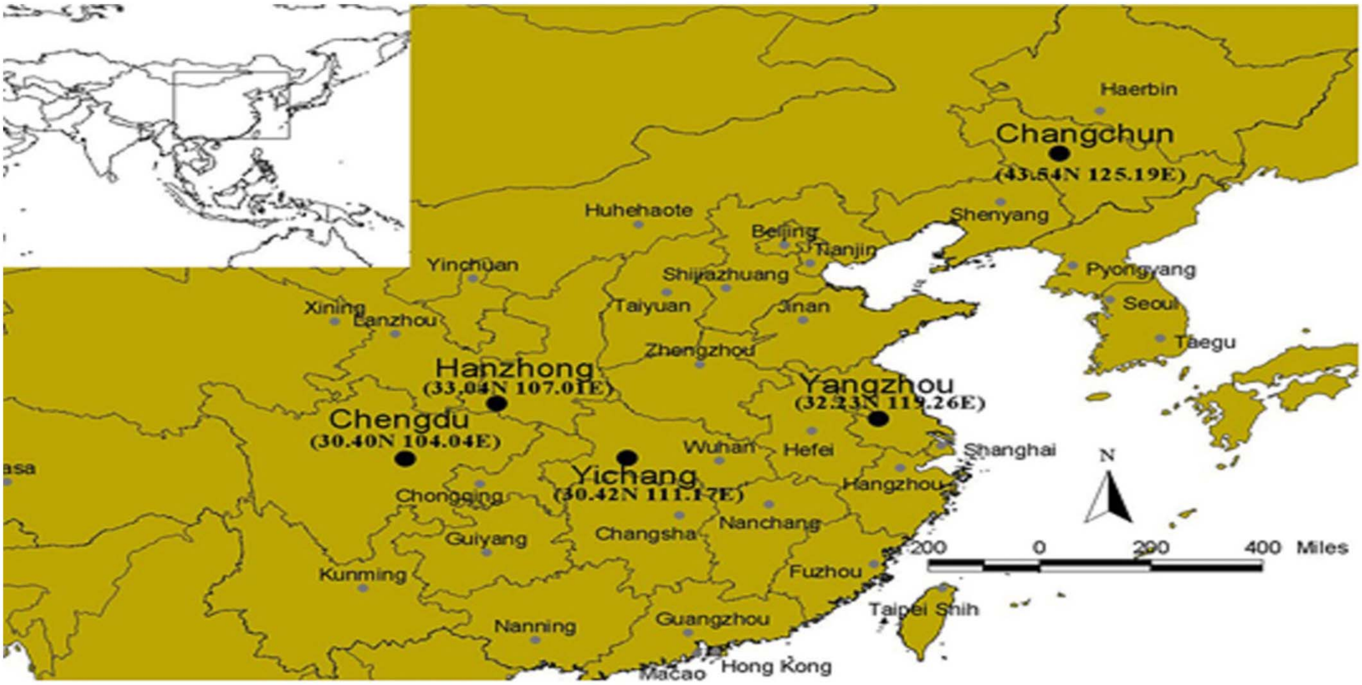

Figure 5 | Location of *C. suppressalis* populations used in this study (solid circles). The map was constructed by ArcView GIS3.3.

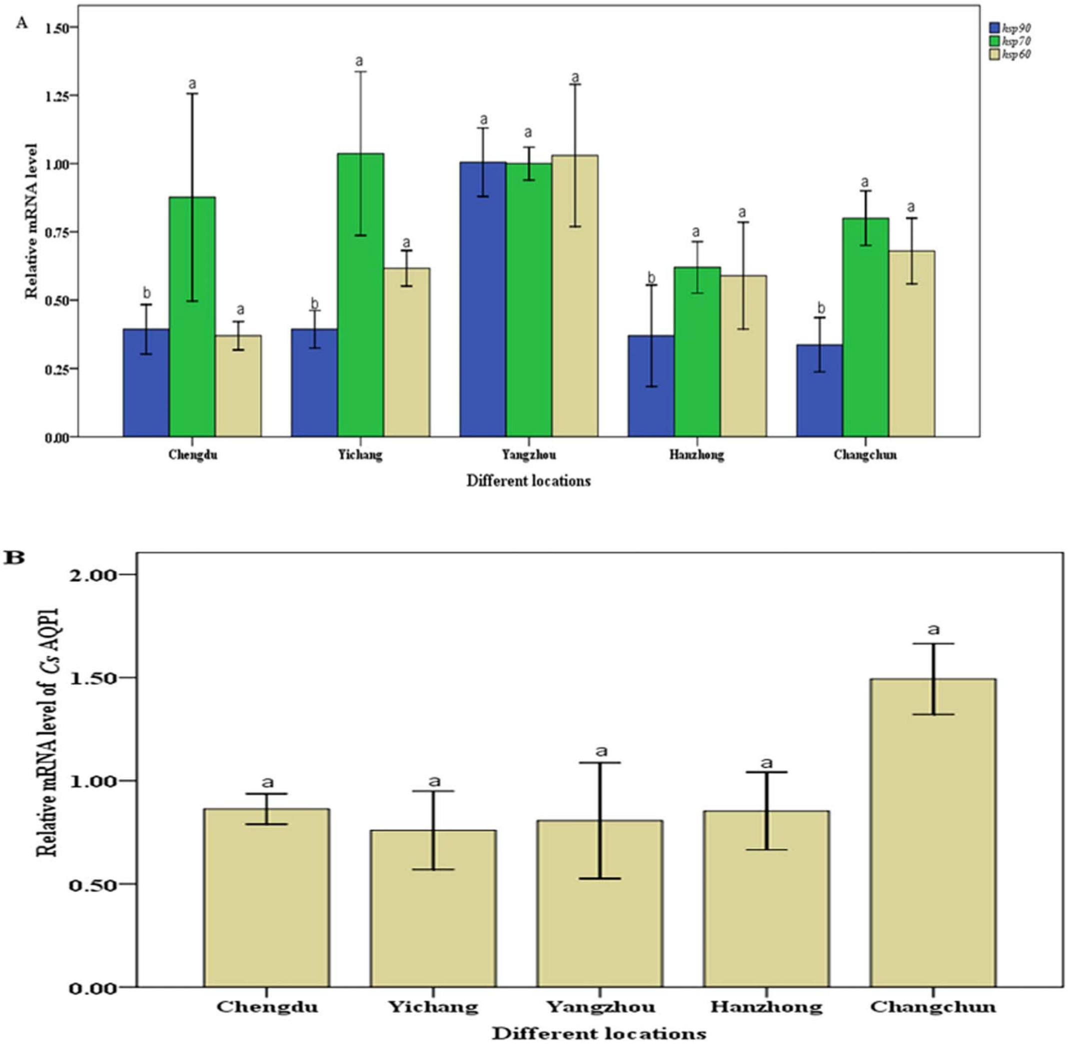

Figure 6 | Relative expression levels of *hsps* and *Cs*AQP1 in *C. suppressalis* sampled from different locations. (A) Relative expression levels of *hsp90*, *hsp70* and *hsp60*. (B) Relative expression levels of *Cs*AQP1. The quantity of genes mRNA is normalized to the abundance of 18SrRNA. Significant differences are indicated as $P < 0.05$. Values are denoted as the mean ± SE.

The role of water in the diapause of insects is often overlooked, despite it being one of the most fundamental molecules for all living organisms. Aquaporins are integral membrane proteins belonging to a large family of water channel proteins that assist in rapid movement of water across cellular membranes. It has been suggested that these proteins also play a significant role in the movement of low molecular weight solutes[24,35]. The trend of *Cs*AQP1 in overwintering *C. suppressalis* was similar to that of the three HSPs, but the mRNA level of *Cs*AQP1 was elevated significantly in early diapause (Fig. 3D), suggesting that *Cs*AQP1 played the important role in the cold hardiness and diapause initiation of *C. suppressalis*. In *B. plicatilis*, aquaporins seem related to dormancy[20,21]. To our knowledge, our study is the first to examine the connections between aquaporin, cold hardiness and diapause in insects.

Gene expression and progress of an insect through diapause are likely to be influenced by signaling pathways. NO, acting as a secondary messenger, may regulate diapause by influencing specific developmental pathways[7,27]. The larvae collected in mid-November exhibited the greatest level of diapause intensity (Fig. 1B). The highest content of NO in a field-collected larvae, was 28.78 μM in early October, which was a marked increase from early September (Fig. 4), and coincided with the period when *C. suppressalis* initially entered diapause (Fig. 1A). This increase suggests that NO may play an important role during the initiation of diapause of *C. suppressalis*. Because once insect enters the diapause, metabolism is reduced and development is arrested. The action of NO on the cell cycle has been proposed to be responsible for the effective arrest of its progression[27,54]. However, the lowest NO content found in sampled insects in our study was 2.82 μM on mid-November, 2010 (Fig. 4), while many *C. suppressalis* larvae overwintering in the field pupated after mid-November (Fig. 1). It is possible that the low concentrations of NO might enhance cell proliferation[55]. The *C. suppressalis* could actively modulate NO to prepare for development when favorable conditions arrive.

Geographic differences in the photoperiodic induction of diapause between populations from different latitudes have been well studied in many insects[5,9,56]. Southern populations have shorter, while northern populations have longer critical photoperiods[5]. Geographic variation, however, in the molecular basis of diapause has rarely been discussed. We investigated diapausing *C. suppressalis* larvae from five different locations: Chengdu (30.40°N, 104.04°E), Yichang (30.42°N, 111.17°E), Yangzhou (32.23°N, 119.26°E), Hanzhong (33.04°N, 107.01°E) and Changchun (43.54°N, 125.19°E). Regardless of location, we found no significant differences in the expression of *hsp70*, *hsp60*, or *Cs*AQP1, while the expression level of *hsp90* in the Yangzhou population was significantly higher than in the other populations (Fig. 6A and B). While up regulation of heat shock proteins during diapause is essential[54],

Table 1 | Primer sequences used in the real-time quantitative PCR

Gene	Genbank number	Primer sequence (5′–3′)	Fragment length (bp)
Hsp90	AB206477	TCGGTCAGTTTGGTGTTGGTT TTCATCGTCGTTGTGCTTGG	83
Hsp70	GU726137	TGCTGGTGACACACATTTGGG GACAGGGTTCGCTTCGCTCT	159
Hsp60	GQ265913	TGGCATTGACGGCTCTGTG TGGCATCTGTAAGTGCTGTTCGGGATG	149
*Cs*AQP1	JQ011314 JQ011315	AGATGCAGAAAAGGACG TTGATGGCGATACAGGCGG	144
18SrRNA	GQ265912	GTGATGGGACGAGTGCTTTTATT GCTGCCTTCCTTGGATGTGG	258

these proteins seem to play the same role in the diapause of different populations of *C. suppressalis*. Together, these three heat shock proteins and aquaporin could form the molecular basis of diapause in *C. suppressalis*. However, the genetic characteristics of diapause are sometimes concealed in natural populations, and a particular individual may require particular external conditions for its expression. For instance, the requirement of strengthening cold hardiness in *C. suppressalis* encountering adverse environmental conditions would further elevate the levels of the *hsps* and *Cs*AQP1.

In conclusion, our studies exhibited comprehensively diapause, signal and molecular characteristics of overwintering *C. suppressalis*. These works are useful to better understand the mechanisms of diapause of *C. suppressalis* and the other insects. Such an understanding could allow us to construct a more accurate model to predict seasonal outbreaks and reveal new approaches to improve the integrated management of this insect pest.

Methods

Insect material and study design. *Chilo suppressalis* used in the main study were collected from experimental rice fields in a suburb of Yangzhou. It is an important rice pest, and becomes serious in recent years in China. We declared that no specific permissions were required for these activities. Also the field studies did not involve endangered or protected species. To determine the diapause characteristics of a population of *C. suppressalis* from a rice field in Yangzhou, China, including initiation, maintenance, termination and quiescence, we made daily observations on subsequent pupation of overwintering *C. suppressalis* larvae removed at various dates from the field. Overwintering larvae (88–170) were regularly collected from rice stubble between September 2010 and May 2011 (Fig. 1). Larvae were placed individually in glass tubes, 20 cm long 18 cm diameter along with fresh rice seedling. Tubes were placed in climate-controlled chambers (RXZ-288A, Jiangnan; with ±0.5°C error) maintained at 28°C, 70 ± 5% relative humidity and a 16 : 8 L:D photoperiod. Larvae were checked daily, and both mortality and pupation (female and male) were recorded. All insects were observed for at least 30 days, and the pupation of larvae was used as a criterion for non-diapause. We also noted the number of days between collection and pupation as a measure of the intensity of diapause at the time of collection of larvae in the field.

In order to study the molecular characteristic of diapausing *C. suppressalis*, we analyzed the mRNA expression of *hsp90*, *hsp70*, *hsp60*, and *Cs*AQP1 in overwintering *C. suppressalis* larvae collected from the field on 12 sample dates, from September 10, 2010 to March 19, 2011. As a separate experiment, to determine what effect geographical differences had on the diapause in *C. suppressalis*, we collected overwintering larvae from five different locations including Chengdu (30.40°N, 104.04°E), Sichuan province, Yichang (30. 42°N, 111.17°E), Hubei province, Yangzhou (32. 23°N, 119.26°E), Jiangsu province, Hanzhong (33.04°N, 107.01°E), Shaanxi province and Changchun (43.54°N, 125.19°E), Jilin province between October 15 and 28, 2010 (Fig. 5). Ten overwintering larvae were randomly chosen from each location and cooled to −70°C with liquid nitrogen for subsequent use in real-time quantitative PCR.

Quantitative real-time PCR (qPCR) analysis. Total RNA was extracted by the SV Total RNA Isolation System (Promega Z3100) followed by DNase treatment to eliminate DNA contamination. The reaction volume was 20 µl. Each reaction contained 10 µl of 2 × SYBR®Premix EXTaqTM (TaKaRa, Dalian, China) master mix, 0.8 µl of each of gene specific primers (Table 1), 0.4 µl of Rox reference Dye, and 2 µl of cDNA templates. Reactions were carried out on a CFX-96 real-time PCR system (Bio-Rad). The efficiencies of the target and reference genes were similar. The quantity of gene mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method[36], and normalized to the abundance of the 18SrRNA gene. Following qPCR, the homogeneity of the PCR products was confirmed by melting curve analysis.

Total NO analysis. Total NO content was measured for three replicates of overwintering larvae. For each replicate, one larva was crushed in a 0.9% sodium chloride solution. After centrifugation, the supernatant was transferred to three clean tubes, which were used for the assay. The experiment followed the Griess Reagent System (G2930, Promega). Briefly, the experimental sample was added to sulfanilamide solution and incubated for 10 minutes at room temperature. The reaction mixture needed to be protected from light. After incubation, the NED Solution was distributed and the reaction mixture solution was measured at 535 nm by microplate spectrophotometer (MQX200R2, Bio Tek).

Statistical analysis. Homogeneity of variances among different groups was evaluated by the Levene test. Significance differences between treatments were identified with the LSD test for multiple comparisons. The data were analyzed using SPSS16.0 software[37] and denoted as means ± SE (standard error).

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Acknowledgments

We would like to thank the members of Institute of Applied Entomology, Yangzhou University for giving the help of sample collection. We also appreciate Prof. Roy Van Driesche from University of Massachusetts for polishing the manuscript and giving helpful advice. This work was supported by the National Basic Research and Development Program of China (2012CB114100), the National Natural Science Foundation of China (31371937) and the Ph.D. Programs Foundation of the Ministry of Education of China (20113250110008).

Author contributions

Y.Z.D. and J.Y.L. conceived and designed the experiments. M.X.L., S.S.C., Z.X.L., and P.Y.L. carried out the experiments and analyzed the data. M.X.L. wrote the main manuscript text. All authors reviewed the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Lu, M.-X. *et al.* Diapause, signal and molecular characteristics of overwintering *Chilo suppressalis* (Insecta: Lepidoptera: Pyralidae). *Sci. Rep.* **3**, 3211; DOI:10.1038/srep03211 (2013).