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

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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 CsAQP1 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 CsAQP1.

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. Diapause as a process is divided into four eco-physiological phases: pre-diapause, diapause, post-diapause quiescence, and post-diapause development. 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). The maintenance of diapause itself is physiologically dynamic and changes over time in response to environmental and internal stimuli. Another source of variation in diapause characteristics in many insects occurs among geographically separated populations of the same species. 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. In recent years, some studies have indicated that expression of heat shock protein (HSPs) is regulated as a function of insect diapause. 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. 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). Another gene family, the aquaporins, has been suggested to possess some relation to dormancy in work with Brachionus plicatilis Müller (Ploimida: Brachionidae). Aquaporins (AQPs), often known as water channels, are integral membrane proteins that regulate the flow of water across cell membranes. 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. 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. The NO involved is produced in the mitochondrial respiratory chain through the action of NO synthase.

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. 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. However, this study did not further describe diapauased trait of *C. suppressalis* in the field. In Japan (31–43°N), *C. suppressalis* populations exist at as 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.

### 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. 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 hsp genes 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 5, 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 CsAQ1 in this insect was determined during diapause. CsAQ1 levels increased during diapause and peaked on January 15, 2011, at significantly higher levels than on December 31, 2010. By February 26, 2011, CsAQ1 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 CsAQ1 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(4, 9) = 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 Zhongzhou population (F(4, 9) = 4.149; P = 0.036). However, there were no significant differences among the Chengdu, Yichang, Hanzhong and Changshun populations.
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.
4.149; \( P > 0.050 \)). The mRNA levels of \( hsp70 \) and \( hsp60 \) for the five geographic locations likewise showed no significant differences (\( F_{3,29} = 0.465; P > 0.050, F_{3,9} = 2.507; P > 0.050 \)) (Fig. 6A). Although the highest value of \( CsAQP1 \) was found in the Changchun population, the levels of \( CsAQP1 \) 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\(^3\). 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\(^\circ\)N, 119.26\(^\circ\)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\(^4\). *C. suppressalis* enters this facultative state in response to short-day conditions during the autumn\(^5\). 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\(^6\). Some studies, however, have found that cold hardiness increased in the post-diapausing stage\(^7,8\). 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 \(-18^\circ\)C, 2 h upon being observed in the laboratory for larvae of this population collected on November 14, 2010\(^9\), 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\(^10\). 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)\(^10\).

Recently, studies have found that heat shock proteins contributed to successful diapause\(^11-15\). In this report, we demonstrate the linkage between diapause and three heat shock proteins (\( hs p 90, h s p 70 \) and \( h s p 60 \)) in *C. suppressalis*, which each had a different expression pattern during diapause. However, the abundance of \( h s p 90, h s p 70 \) and \( h s p 60 \) 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 \( h s p 90, h s p 70 \) and \( h s p 60 \) 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* Lelebvre (Lepidoptera: Noctuidae), in which *SnoHsc70* was induced during diapause\(^15\). In contrast, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) was found to have its *hs p 90* transcripts down regulated during its pupal diapause\(^12\). It is noteworthy that in our study, three *h s p s* of *C. suppressalis* were synchronously elevated.

![Figure 2](https://www.nature.com/scientificreports/) 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.
Figure 3 | Relative expression levels of hsp90, hsp70, hsp60, and CsAQP1 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.
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\(^4\), 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)\(^\text{3}\), 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\(^4\). 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*\(^5\). 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 allow for the subsequent development of *C. suppressalis*.

**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.

**Figure 5** | Location of *C. suppressalis* populations used in this study (solid circles). The map was constructed by ArcView GIS3.3.
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. The trend of CsAQP1 in overwintering C. suppressalis was similar to that of the three HSPs, but the mRNA level of CsAQP1 was elevated significantly in early diapause (Fig. 3D), suggesting that CsAQP1 played the important role in the cold hardiness and diapause initiation of C. suppressalis. In B. plicatilis, aquaporins seem related to dormancy. 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. 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. 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. 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. Southern populations have shorter, while northern populations have longer critical photoperiods. 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 CsAQP1, 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,

![Figure 6](https://www.nature.com/scientificreports)

Relative expression levels of hsp70 and CsAQP1 in C. suppressalis sampled from different locations. (A) Relative expression levels of hsp90, hsp70 and hsp60. (B) Relative expression levels of CsAQP1. 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.

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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 hardness in C. suppressalis encountering adverse environmental conditions would further elevate the levels of the hsps and CsaQPI.

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 population of C. suppressalis. Subsequent pupation of overwintering larvae were 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 diapausind C. suppressalis, we analyzed the mRNA expression of hsp90, hsp70, hsp60, and CsaQPI 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 geo
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