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Temperature adaptability of two clades of *Aphelinus mali* (Hymenoptera: Aphelinidae) in China

Min Su[1†], Xiumei Tan[1†], Qinmin Yang[2], Fanghao Wan[1,3] and Hongxu Zhou[1*]

Abstract

Aphelinus mali (Haldeman) (Hymenoptera: Aphelinidae) is an effective natural enemy used in China to control the woolly apple aphid (*Eriosoma lanigerum* [Hausmann]) (WAA). Population of *A. mali* in China falls into two distinct genetic clades (Shandong clades and Liaoning clades). In the present results, the developmental threshold temperature of the Shandong clade (9.82 ± 1.44 °C) was lower than that of the Liaoning clade (10.72 ± 0.24 °C), while the effective accumulated temperature of the Shandong clade needed for development from oviposition to adult eclosion (126.45 ± 16.81 day-degree) was significantly higher than that of the Liaoning clade (107.99 ± 3.44 day-degree). The supercooling and freezing points of the Liaoning clade (− 27.66 °C, − 27.17 °C) were significantly lower than those of the Shandong clade (− 26.04 °C, − 25.54 °C).

Some other differences between the two clades as well were the content of fat, trehalose, and protein of overwintering larvae of the Liaoning clade (60.8%, 7.57 μg/one insect, 10.11 μg/one insect) as these were significantly higher than those of the Shandong clade (45.5%, 5.73 μg/one insect, 8.05 μg/one insect). The occurrence of the first adult emergence of the Shandong clade of *A. mali* was earlier in the year than that of the Liaoning clade, allowing this clade to better control WAA in early spring. Meanwhile, the developmental duration from oviposition to adult emergence of the Shandong clade was longer than that of the Liaoning clade, and the cold tolerance of one of these, the more northerly Liaoning clade, is greater than that of the other, the more southerly Shandong clade. All of these factors imply differences in the pest control ability of the two clades of *A. mali* in their respective regions.

Keywords: *Eriosoma lanigerum*, Effective accumulated temperature, Supercooling point, Cold tolerance

Background

Woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann), is a worldwide pest of apple, *Malus pumila* Miller (Jaume et al. 2015), and a quarantine pest in China (Zhang and Luo 2002). Since this aphid, which is native to North America, was introduced into China in Shandong province (Weihai) in 1914, in Liaoning province (Dalian) in 1929 from Japan, and in Yunnan province (Kunming) in 1930 through apple trees from America, it has had a serious impact on fruit production and acceptance of fruit for export. *Aphelinus mali* (Haldeman), a key parasitoid of this pest, was introduced several times into China during the period between 1940 and 1960 from the former Soviet Union and Japan and plays an important role in controlling WAA in Chinese orchards (Long et al. 1960).

Previous studies have found *A. mali* to be the most important parasitoid of WAA, making it a logical target for biological control by conservation (Gontijo et al. 2012). *A. mali* has been shown to provide a good control of WAA throughout the growing season in China, with field parasitism rates (50–90%) in apple orchards (Zhou et al. 2010).

However, in China, WAA continues to expand westward into new apple-growing regions (Lu et al. 2013). Woolly apple aphid was first found in Shanxi province in 1999 in one location (Linfen), but by 2007, it had spread across 360 km^2, infesting 6% of the area apple

* Correspondence: hxzhou@qau.edu.cn
†Equal contributors
[1]College of Plant Health and Medicine, Key Lab of Integrated Crop Pest Management of Shandong Province, Qingdao Agricultural University, Qingdao 266109, China
Full list of author information is available at the end of the article

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orchards (Wang et al. 2011). In Shandong province, surveys from 2000 to 2002 found WAA in about 8000 ha of orchards in Rizhao, Shandong area, with 10–20% of apple trees infested, resulting in an annual loss of 5×10^6 kg of apples (Wang et al. 2011). In Jiangsu province, WAA was found in 2005 in 4333 ha (Chu et al. 2008). Since 2007, WAA has spread to Hebei province, where it has caused great damage to orchards in several regions (Qinhuangdao, Tangshan, and Shijiazhuang) (Wu et al. 2009).

In China, *A. mali* is comprised of two distinct genetic clades (Zhang et al. 2014; Zhou et al. 2014). We hypothesized that the expansion of WAA in recent years is related to differences in low-temperature adaptability between these clades. Earlier studies outside of China have found that low-temperature adaptability differs among geographic populations of *A. mali* (Mols and Boers 2001). For example, in the Annapolis River valley of Nova Scotia (Canada), the parasitoid population appears earlier in the year than a strain of *A. mali* found in the Netherlands, thus providing better control in Nova Scotia than Holland. By the time, the Dutch strain of *A. mali* becomes active and its population was grown to levels that make its control by *A. mali* difficult (Mols and Boers 2001). The Nova Scotian and the Dutch strains of *A. mali* also differ in their low-temperature threshold (8.6 and 9.4 °C, Nova Scotia vs Holland, respectively) and their effective accumulated temperature requirements, which were lower for the Canadian strain than for the Dutch strain (123.5 and 136.4 day-degree, respectively) (Mols and Boers 2001).

The present study aimed to determine the differences in the cold tolerance of different clades of *A. mali* in China to improve our understanding of the mechanism of WAA outbreaks and to provide insights into how to better use *A. mali* to control this economically important pest.

Materials and methods

Target insects

Woolly apple aphid was collected from Qingdao (36° 20′N) in Shandong province. *A. mali* used in this study was collected from 4 to 5 apple orchards in the two locations: (1) Shandong province (Qingdao 36° 20′N, 120° 12′E) and (2) Hebei province (Qinhuangdao 119° 20′E, 39° 25′N), which is 889 km apart, representing the Shandong and the Liaoning clades, respectively. To obtain parasitoids from the field, in May and June 2015, apple branches, infested with woolly apple aphids were collected. The black-colored aphids (indicating parasitization) were isolated, and parasitized aphids were held in 1.5-mL centrifuge tubes until adult parasitoid's eclosion. *A. mali* and WAA were held at 25 °C, 70% RH, and a 16:8-h L:D photoperiod.

Measurement of effective accumulated temperature and developmental threshold temperature

Fifteen males and 15 females of *A. mali* from each clade were placed together with an excess of hosts (ca 200 aphids) in Petri dishes (13.5 cm dia). The parasitoids were removed after 24 h, and the exposed WAA allowed to develop under one of five temperatures (18, 20, 23, 25, and 28 °C), noting the day of adult parasitoid emergence from each parasitized aphid. This process was replicated five times for each temperature, with an average of 0.073 ± 0.006, 0.087 ± 0.018, 0.120 ± 0.031, 0.160 ± 0.035, and 0.173 ± 0.024 parasitism rates within each replicate for each clade, respectively. All groups of potentially parasitized aphids were held at 70% RH and a 16:8-h L:D photoperiod and observed daily to record when aphids turned black and adult parasitoid eclosion began. Newly emerged adults of *A. mali* were held separately under the same environmental conditions provided with 10% honey water. The sex and date of death of *A. mali* individuals were determined as well as the number of Day-Degree (DD) for each stage of development. Longevity of adults of each clade at each temperature was also determined.

The effective accumulated temperature (K) and the developmental threshold temperature (C) were calculated according to Ma (2009)

$$K = \frac{n\sum VT - \sum V \sum T}{n\sum V^2 - (\sum V)^2}$$

$$C = \frac{\sum V^2 \sum T - \sum V \sum VT}{n\sum V^2 - (\sum V)^2}$$

where n is the number of groups in every experiment, T the constant temperature, and V is the average development rate.

Supercooling point and freezing temperature of overwintering parasitoid larvae

Overwintering larvae of *A. mali* were obtained by dissecting parasitized (black) woolly apple aphids collected in Changli (4~16 °C) for the Liaoning clade on October 26, 2015, and in Qingdao (7~13 °C) for the Shandong clade on November 17, 2015, using an anatomical lens.

Supercooling point (SCP) and freezing point (FP) of *A. mali* larvae were measured using a SUN-V type intelligent instrument in combination with a − 80 °C ultra-low temperature refrigerator. When the instrument was connected with the temperature probe, one larva was placed on the probe and then inserted into the 200-μL gun head of transfer liquid gun (to avoid contact with the pipe wall). The gun head was tightly wrapped with absorbent cotton and placed in the testing box, where absorbent cotton prevented rapid changes in the cooling rate. The whole probe and associated device were then

placed into an ultra-low temperature refrigerator, where software of the measuring instrument system of the supercooling point -V1.3 was used to record temperature each second. Once below freezing, the SCP was found by reference to a sudden rise of temperature as energy is released upon larval freezing. The peak of this rise in temperature is the freezing point. This process was repeated 30 times for each clade.

Determination levels of selected cryoprotectants in overwintering larvae

Overwintering larvae of *A. mali* were collected all from the same single site and date. Thirty black aphids were taken at each time, the larvae were dissected out, and each treatment was repeated five times.

Free-water and fat content in overwintering A. mali larvae

The fresh weight (FW) of 30 overwintered larvae as a group was determined using a microbalance (Sartorius BSA224S-CW, Beijing Co., Ltd.), then larvae were dried together at 60 °C for 48 h. The dry weight (DW) of the samples was then obtained using the same microbalance, and the water content of the individual sample determined as (DW − FW)/FW × 100 (Folch et al. 1957).

After measuring the DW, the sample of 30 dried larvae was placed in a 1.5-ml centrifuge tube and homogenized in 20 μL of a chloroform mixture (2:1) then 0.6 ml of the chloroform and methanol mixture was added. The sample was then centrifuged at 2600 rpm for 10 min, and this process was repeated three times, each time removing the resulting supernatant. The residue was then baked at 60 °C in an oven for 72 h to determine the lean dry weight (LDW). The fat content of the insect was determined by the following formula: [(DW − LDW)/DW] × 100 (Folch et al. 1957).

Trehalose and glycogen content in overwintering A. mali larvae

Thirty overwintering larvae were added to 40 μL of 10% trichloroacetic acid solution and homogenized by grinding. The material was then rinsed by 0.2 ml of 10% trichloroacetic acid solution and centrifuged three times at 5000 rpm for 10 min. Between bouts of centrifugation, the precipitate was dissolved by 0.15 ml of 10% trichloroacetic acid solution. Final supernatant was mixed with 0.5 ml of anhydrous ethanol and then placed in a refrigerator at 4 °C for 24 h. 0.4 ml of the supernatant was centrifuged at 10,000 rpm for 15 min. The resulting supernatant was added to 0.4 ml of 0.15 mol/l H_2SO_4 solution that placed in a boiling water bath for 15 min and afterwards allowed to return to room temperature. When cooled, 0.4 ml of a 30% KOH solution was slowly added while stirring, and the solution was returned to the boiling water bath for 15 min before the trehalose determination.

Table 1 Oviposition to eclosion durations of the non-overwintering generation of *Aphelinus mali* for two genetic clades in China

Oviposition to eclosion		Shandong clade		Liaoning clade	
		Duration (*D*)	Velocity (*V*)	Duration (*D*)	Velocity (*V*)
18 °C	♀	15.83 ± 1.73a	0.0632	15.26 ± 0.43a	0.0655
	♂	14.83 ± 1.52ab	0.0674	15.22 ± 0.40a	0.0657
20 °C	♀	12.75 ± 0.79b	0.0784	11.58 ± 0.23b	0.0864
	♂	12.94 ± 0.66b	0.0773	11.94 ± 0.06b	0.0838
23 °C	♀	9.69 ± 0.61c	0.1033	9.67 ± 0.69c	0.1034
	♂	9.25 ± 0.83cd	0.1206	6.83 ± 0.60de	0.1464
25 °C	♀	8.43 ± 0.85cd	0.1186	7.84 ± 0.09d	0.1275
	♂	8.29 ± 0.85cde	0.1206	7.33 ± 0.33d	0.1364
28 °C	♀	6.50 ± 1.51e	0.1538	5.80 ± 0.79e	0.1724
	♂	7.17 ± 1.44de	0.1395	7.06 ± 0.53de	0.1416

Values in the table are mean ± SD; different lowercase letters indicate significant differences in the same column

Thirty microliters of the liquid, with 300 μL of anthrone, was placed in a boiling water bath for 15 min and then held in the dark for 20~30 min. The reflectance of the sample at 620 nm was then used to determine the level of trehalose by comparison to a known standard.

To determine the level of glycogen, the remaining precipitate was mixed with 0.5 ml of distilled water, and when the precipitate was fully dissolved, it was used to measure glycogen levels (using the same method as above for trehalose). After the treatment with 300 μL of anthrone, the reflectance of the sample at 620 nm was used to determine the level of glycogen by comparison to a known standard. These processes were repeated for five samples (each from the same time and place) for each parasitoid clade.

Table 2 Longevity of adults of the non-overwintering generation of *Aphelinus mali* for two genetic clades in China

Temperatures (°C)		Shandong clade		Liaoning clade	
		Duration (*D*)	Velocity (*V*)	Duration (*D*)	Velocity (*V*)
18	♀	25.00 ± 2.34a	0.0400	22.30 ± 1.52b	0.0448
	♂	23.00 ± 5.29a	0.0435	19.72 ± 1.67bcd	0.0507
20	♀	16.94 ± 3.49b	0.0590	34.47 ± 1.32a	0.0290
	♂	15.06 ± 3.69bc	0.0664	31.94 ± 0.97a	0.0313
23	♀	15.63 ± 3.00bc	0.0639	21.03 ± 1.29bc	0.0475
	♂	13.94 ± 3.56bcd	0.0717	17.41 ± 1.65cde	0.0574
25	♀	13.64 ± 7.26cde	0.0733	14.84 ± 1.42ef	0.0674
	♂	10.93 ± 3.24def	0.0915	10.94 ± 0.78f	0.0914
28	♀	10.10 ± 7.38ef	0.0990	16.39 ± 1.54de	0.0610
	♂	8.33 ± 3.14f	0.1200	13.89 ± 1.06ef	0.0719

Values in the table are mean ± SD; different lowercase letters indicate significant differences in the same column

Table 3 The lower developmental thresholds and total day-degree of non-overwintering generation of *Aphelinus mali* for two genetic clades in China

Parasitoid clade tested	Lower developmental threshold (°C)		Total DD (day-degree) per life stage (oviposition to adult eclosion)	
	Shandong clade	Liaoning clade	Shandong clade	Liaoning clade
♀	9.32 ± 1.62a	12.96 ± 1.56b	134.61 ± 20.77a	88.42 ± 16.65b
♂	10.61 ± 1.58a	13.33 ± 0.79b	116.12 ± 16.60a	82.02 ± 7.36b
♀ + ♂	9.82 ± 1.44	10.72 ± 0.24	126.45 ± 16.81	107.99 ± 3.44

Values in the table are mean ± SD

Protein content of overwintering larvae

Thirty overwintering *A. mali* larvae were grouped and placed in a 1.5-ml centrifuge tube to which 0.1 ml of 0.04 mol/l phosphate buffer (pH = 7.0) was added. The samples were ground to homogenize the larvae and washed with 1.1 ml of 0.04 mol/l of phosphate buffer solution (pH = 7.0). The homogenized sample was then fully extracted with 0.04 mol/l phosphate buffer at 20~25 °C for 4 h, and the solution was centrifuged at 6000 rpm for 10 min. A sample of 20 μL of the resulting supernatant was collected and added to 80 μL of 0.04 mol/l phosphate buffer (pH = 7. 0) and 200 μL of Coomassie brilliant blue (Shanghai Kayon Biological Technology Co., Ltd.). The sample was mixed by shaking and allowed to stand for 2 min before colorimetric analysis at 595 nm to measure the absorbance value, from which the free protein content was calculated by reference to a standard. This was done five times (from one sample date and place) for each parasitoid clade.

Statistical analyses

The average data of development at respective temperature conditions were calculated as the mean ± standard deviation (SD) by SPSS 20.0. Significant differences in duration times were tested using one-way analysis of variance (ANOVA) corrected by SPSS 20.0. Independent sample *t* tests were used to analyze the developmental duration of each clade by SPSS 20.0.

Results and discussion

Effective accumulated temperature and developmental threshold temperature

Developmental duration

Under the same temperature regime, there was no significant difference in development duration (egg to adult emergence) between males and females of the same clade of *A. mali*. Development duration significantly decreased with increasing temperature in each clade of *A. mali* (Table 1).

For females, development duration (from oviposition to adult emergence) of the Shandong clade was significantly longer than that of the Liaoning clade ($F = 0.390$, df = 4, $P = 0.048$) at 20 °C (Table 1).

Adult longevity

Under the same temperature regime, female adults survived longer than males of the same clade, but this difference was not significant. Significant differences were found for longevity at different temperatures within the same clade (Table 2). Adult longevity decreased significantly (from 25.00 ± 2.34 to 10.10 ± 7.38 for female, 23.00 ± 5.29 to 8.33 ± 3.14 for male) with increasing temperature 18 to 28 °C in the Shandong clade, while the longest duration in the Liaoning clade was at 20 °C (34.47 ± 1.32 for female, 31.94 ± 0.97 for male) and the shortest was at 25 °C (14.84 ± 1.42 for female, 10.94 ± 0.78 for male).

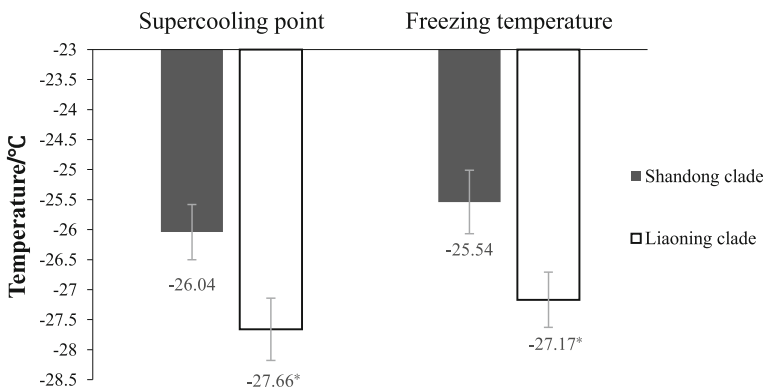

Fig. 1 Supercooling and freezing points of overwintering *Aphelinus mali* larvae for two genetic clades found in China. Asterisk indicates a significant difference at the 0.05 level

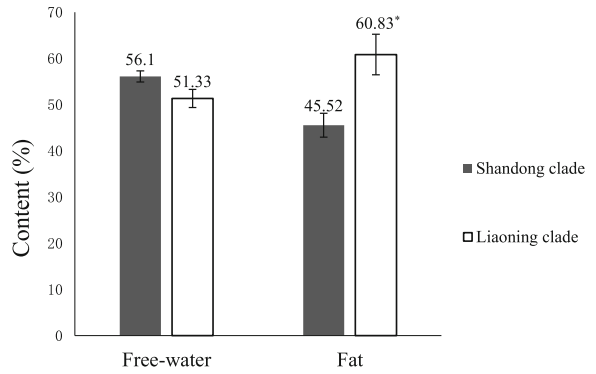

Fig. 2 Free-water and fat content of *Aphelinus mali* for two genetic clades found in China. Asterisk indicates a significant difference at the 0.05 level

Longevity of adult females was significantly greater for the Liaoning clade than for the Shandong clade at 20 °C ($F = 0.394$, df = 4, $P = 0.000$) and 23 °C ($F = 1.302$, df = 4, $P = 0.021$). There were no significant differences in female longevity between the clades at the other temperatures (Table 2).

For males, adult longevity was significantly longer for the Liaoning clade than for the Shandong clade at 20 °C ($F = 1.811$, df = 4, $P = 0.000$) and 28 °C ($F = 0.080$, df = 4, $P = 0.028$), but not significantly different for the other temperatures (Table 2).

Lower developmental thresholds and total day-degree for stage completion

Results represented in Table 3 showed that the lower developmental thresholds of both males and females of the Shandong clade were lower ($F = 3.350$, df = 3, $P = 0.026$; $F = 0.150$, df = 3, $P = 0.012$) than those of the Liaoning clade.

Meanwhile, the total day-degree for stage completion of the Shandong clade was greater than that of the Liaoning clade ($F = 5.907$, df = 3, $P = 0.028$) (Table 3).

In this study, we found that the larval and pupal stages of Shandong clade females (9.32 ± 1.62 °C) and males (10.61 ± 1.58 °C) both had lower developmental threshold temperatures than that of the Liaoning clade (12.96 ± 1.56 °C, 13.33 ± 0.79 °C, respectively). The total day-degree for stage completion of Shandong clade females (134.61 ± 20.77 DD) and males (116.12 ± 16.60 DD) were both higher than that of the Liaoning clade (88.42 ± 16.65 DD, 82.02 ± 7.36 DD) from oviposition to adult eclosion. In Mols and Boers' study, the low-temperature threshold of the Nova Scotian (Canada) strain (8.6 °C) was lower than that of the Dutch (Nederland) strain (9.4 °C) (Mols and Boers 2001), allowing it to appear earlier in spring, thus providing better control. The developmental threshold temperature of the Shandong clade was lower than that of the Liaoning clade in the present study, suggesting that *A. mali* of Shandong clade can occur earlier in spring and may therefore provide better control of woolly apple aphid at a lower population level of the pest.

Supercooling points and freezing temperatures of overwintering larvae of the two clades

The supercooling (– 26.04 °C) and freezing (– 25.54 °C) points of the Shandong clade were significantly higher than corresponding values for the Liaoning clade (– 27.66 °C and – 27.17 °C, respectively) ($F = 0.167$, df = 58, $P = 0.024$; $F = 0.088$, df = 58, $P = 0.023$, respectively) (Fig. 1).

Freeze tolerance and freeze avoidance are two alternative strategies for survival at sub-zero temperatures, with freeze avoidance being thought to predominate in moderately cold and predictable thermal environments (Sean et al. 2015). Freeze tolerance, on the other hand, is the ability of certain insects to enhance their cold resistance by regulating the body's supercooling state (Zhang and Ma 2013) so that at temperatures below freezing, the insect's body fluid remains liquid. The supercooling points of the Shandong clade of overwintering larvae and freezing point were both

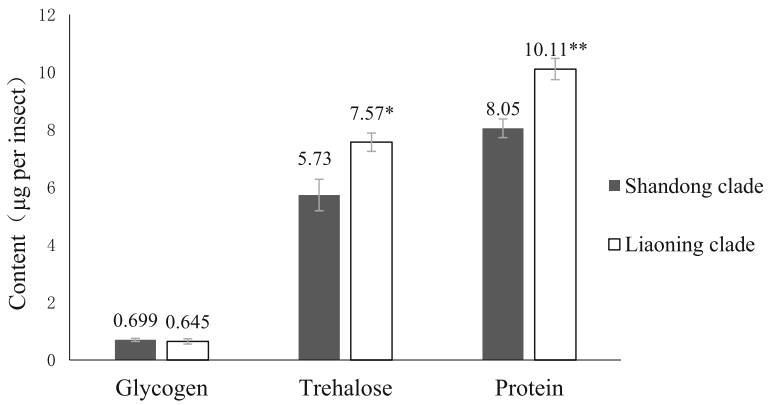

Fig. 3 Glycogen, trehalose, and protein levels of *Aphelinus mali* for two genetic clades found in China

higher than that of the Liaoning clade, suggesting that the cold resistance of the Liaoning clade is stronger than that of Shandong clade.

Levels of cryoprotectants in overwintering parasitoid larvae of each clade

While there were differences in sample means for the free-water content of the two clades, they were not significant ($F = 0.625$, df = 8, $P = 0.072$). In contrast, the fat content of the Shandong clade (45.5%) was significantly different from that of the Liaoning clade (60.8%) ($F = 2.836$, df = 8, $P = 0.017$) (Fig. 2).

There were no significant differences in glycogen content between the two clades ($F = 0.216$, df = 8, $P = 0.613$). Trehalose levels, however, were significantly lower in the Shandong clade (5.73 g/larvae, 25.37 g/mg) than in the Liaoning clade (7.57 g/larvae, 36.12 g/mg) ($P = 0.020$, $P = 0.008$, respectively). Protein content was also significantly lower in the Shandong clade (8.05 g/larvae, 35.68 g/mg) than in the Liaoning clade (10.11 g/larvae, 48.20 g/mg) ($P = 0.003$, $P = 0.001$, respectively) (Fig. 3).

In cold environments, the free-water content in insect bodies is greatly reduced, leading to an increase in the concentration of body solutes and thus reducing its freezing point (Feng et al. 2014). Cold-resistant substances of insects include two types, small molecules and antifreezing proteins (Chen et al. 2010). Cold-resistant small molecules include glycerol, sorbitol, mannitol, five carbon polyol (probably Arabia sugar alcohol or nucleic acid alcohol), trehalose, glucose, fructose, and some amino and fatty acids (Chen et al. 2010).

Conclusions

Recent study of Zhang and Ma (2013) reported that insects in low-latitude areas had higher cooling points than insects in high-latitude areas. By comparing the supercooling point and the levels in the insect's body of cold-resistant materials for the two clades of *A. mali*, we found that the cold resistance of the Liaoning clade was higher than that of Shandong clade, possibly because of increasing latitude or genetic differentiation. Future studies should compare the cold resistance ability of the two clades from populations at the same latitude. A better understanding of the life table parameters of this parasitoid, as well as the genetic variation within populations, will allow for more accurate use of this insect to control WAA in China.

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Authors' contributions

MS carried out the whole experiments at room condition and drafted the manuscript. XT corrected the English manuscript. QY collected A. mali from Qingdao and Qinhuangdao. FW participated in the design of the study HZ conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Author details

[1]College of Plant Health and Medicine, Key Lab of Integrated Crop Pest Management of Shandong Province, Qingdao Agricultural University, Qingdao 266109, China. [2]General Station of Plant Protection of Shandong Province, Jinan 250100, China. [3]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100081, China.

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