Lethal and Sublethal Effects of Long-term Cold Storage on Indoor-reared Harmonia Axyridis Adults

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Research Article

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Abstract

Indoor-reared natural enemy with high quality after long-term cold storage is crucial for sustainable pest management. However, besides survivals, the sublethal effects were not widely been reported. In this study, *Harmonia axyridis* (Pallas), an important biological control agent in Asia, was reared with β-carotene-amended artificial diet (Ha-Car) before storing at 6°C. After 30, 60, 90 and 120 days of storage, a series of biological parameters were measured to evaluate the fitness of *H. axyridis*.

We found that: (1) Survivals significantly decreased with prolonged storage, and more Ha-Car individuals survived at day 120 compared to Ha-CK (control); (2) The contents of glycogen and trehalose dramatically decreased following storage, and the weight losses gradually increased; (3) The average egg production and hatch rates within 15 days were not significantly different among treatments Ha-Car and Ha-CK following long-term storage (90 and 120 days), while the daily hatch rates gradually decreased from relatively high to zero at day 14 and 15; (4) The number of micropyles deposited on eggs also gradually decreased along with oviposition period. After re-mating with a new non-stored partner, the egg viability gradually increased again, while low egg viability was still detected in F1 generation. Moreover, reduced number of micropyles were detected on their eggs. In summary, Ha-Car can be cold stored for about 120 days with relatively high survivals and fecundity, but long-term storage produced remarkable intra- and trans-generational negative effects on fertility. Even though, the cold-stored *H. axyridis* had great potential being used in biological control program with inevitable promiscuity with field individuals.

Introduction

Long term cold storage of laboratory-reared natural enemy is very important for developing feasible biological control programs\(^1\), especially for those relying on large number releasing of natural enemies\(^2\). However, somatic damages causing by direct and/or indirect chilling injuries may accumulate during storage, e.g. accumulation of toxic metabolites and elimination of energy reserves\(^3\). In practice, some quantified parameters can be measured to evaluate the lethal and sublethal effects induced by cold storage. For most studies, significantly lowered survivals have been detected with prolonged storage (reviewed by Rathee and Ram\(^2\)), while the sublethal effects were not widely been reported\(^4\). Even so, the sublethal effects still needed to be closely monitored when make a comprehensive evaluation of a storage technique.

Specifically, the sublethal effects generally include delayed oviposition, lower fecundity and fertility and lower voracity\(^4\) or parasitism\(^5\). Until now, these effects were mostly conducted on parasitoids and rarely quantified in predators\(^4\). For example, the parasitism rate and fecundity of *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) significantly decreased after cold storage at 3°C or 5°C\(^6\); fecundity of *Trichogramma brassicae* Bezdenko (Hymenoptera, Trichogrammatidae) also decreased at a lower rate after storage at 10°C\(^7\). For the mymarid wasp, *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae), cold storage of immature wasps for 40 days even caused 44% female sterility, and tremendous
parasitism and fecundity reduction of 70% and 73%, respectively\(^8\). Moreover, studies showed that some sublethal effects can even be passed to their offsprings, called “transgenerational effects”\(^9\). For \(G.\ ashmeadi\), when cold storage of the adult parents was more than 20 d, delayed development, decreased fecundity, reduced longevity, and increased male production were observed in F1 generation\(^10\). For predators, significantly decreased fecundity was found in \(Hippodamia\ variegate\) (Goeze) (Coleoptera: Coccinellidae) when the adults were stored at 6°C (> 35 days) or at 12°C/0°C (> 45 days)\(^4\); similar reduced fecundity has also been detected in \(Cryptolaemus\ montrouzieri\) Mulsant (Coleoptera, Coccinellidae) when the adults were stored at 15°C for 20 days\(^11\) and in \(Rhyzobius\ lophantae\) Blaisdell (Coleoptera Coccinellidae) when the adults were stored at 12°C (10–40 days)\(^12\).

The multicolored Asian ladybird beetle, \(Harmonia\ axyridis\) (Pallas) (Coleoptera: Coccinellidae), native to Asia, has established populations worldwide and has also been considered as an important invasive insect species\(^13\)–\(^15\). At present, this predatory ladybird beetle has been commercially reared and widely used as a biocontrol agent in many agricultural fields\(^16,17\) due to its excellent voracity, dispersal capacity and multivoltine life-cycle\(^18,19\). However, \(H.\ axyridis\) is a “chill intolerant” predator\(^20\), and the logistics of low-temperature storage has been shown to be one of the most important limitations in using it in biological control programs\(^21,22\). Abundant studies have been done to improve the cold storage techniques, but most of them only focused on the field collected pre-overwintering individuals\(^1\). For example, the quality of pre-storage diet has been confirmed to have notable effect on the post-storage fitness of \(H.\ axyridis\), and injured wine grape berries or sugar showed to be more optimal than water in decreasing mortality of overwintering beetles\(^23\). The indoor-reared individuals have been reported to be much more chilling intolerant\(^24,25\). For these populations, we recently found that the meridic artificial diet is fruitful pre-storage nutrition for long-term cold storage (almost 60 days)\(^1\). As is well known, carotenoids are ubiquitous in organisms and have many important biological and physiological roles, e.g. light detection, photo-protection, coloration, hormone precursors and antioxidants\(^26,27\). In our previous study, we have confirmed that supplementation of \(\beta\)-carotene in artificial diet can greatly increase the larval survival of \(H.\ axyridis\)\(^28\). Here, we speculated that feeding \(H.\ axyridis\) adults with carotenoid-amended artificial diet before storage might contribute to improving their cold tolerance.

As for the post-storage fitness of \(H.\ axyridis\) adults, previous studies reported that cold storage generally duration dependent, had obvious lethal effects on survival rate\(^1,29\)–\(^31\). For sublethal effects, the persistence of high egg hatch rate (75–85% during two months)\(^30\) and shortened pre-oviposition duration\(^29\) has been reported by a few studies. Besides, no adverse effects on fecundity has also been reported before\(^1,29,30,32\).

In this study, we still focused on the effect of cold storage on indoor-reared \(H.\ axyridis\) adults, and the adopted storage techniques were similar to those used in our previous studies with some modifications\(^1\). Briefly, \(\beta\)-carotene-amended artificial diet was supplemented as pre-storage nutrition, and the rearing period was prolonged to 10 days due to the deduction that rearing on artificial diet for longer periods
might result in higher post-storage survivals\textsuperscript{1,33}. After that, the adults were stored at 6°C for different periods (30, 60, 90, or 120 days), and their post-storage fitness as well as offspring’s reproductive capabilities were measured to evaluate the lethal and sublethal effects. In addition, the contents of trehalose and glycogen that play important roles in cold hardiness of insects\textsuperscript{22} were measured to evaluate their dynamic changes during storage. Besides, the number of micropyles located on eggshell, which play important roles in egg fertilization\textsuperscript{34}, was determined to reveal the potential mechanisms of cold storage on fertility.

**Results**

**Post-storage survivals**

After storage, significantly different survivals were detected between Ha-Car and Ha-CK (female: df = 1, $\chi^2 = 4.545$, $p = 0.03302$; male: df = 1, $\chi^2 = 4.769$, $p = 0.02898$), and decreased survivals were detected following prolonged storage duration (female: df = 3, $\chi^2 = 98.155$, $p < 0.001$; male: df = 3, $\chi^2 = 50.075$, $p < 0.001$). However, no significant diet × duration interaction was detected (female: df = 3, $\chi^2 = 1.395$, $p = 0.70672$; male: df = 3, $\chi^2 = 2.941$, $p = 0.40075$). In a further analysis of one compared to another, we found that the post-storage survivals of both Ha-Car and Ha-CK females were displayed as following order: 30 DIS > 60 DIS > 90 DIS and 120 DIS (Ha-Car: df = 3, $\chi^2 = 41.570$, $p < 0.001$; Ha-CK: df = 3, $\chi^2 = 57.980$, $p < 0.001$) (Fig. 1A). Similar variations were also detected in males (Ha-CK: df = 3, $\chi^2 = 30.623$, $p < 0.001$; Ha-Car: df = 3, $\chi^2 = 22.394$, $p < 0.001$) (Fig. 1B). For both females and males, a significant difference between Ha-Car and Ha-CK was only detected at 120 DIS (female: df = 1, $\chi^2 = 4.004$, $p = 0.04540$; male: df = 1, $\chi^2 = 6.896$, $p = 0.00864$) (Fig. 1A and B).

**Weight loss during cold storage**

For females, the weight loss during storage was significantly affected both by pre-storage diet ($F_{1,216} = 8.863$, $p = 0.00324$) and storage duration ($F_{3,216} = 96.822$, $p < 0.001$). There was also a significant diet × duration interaction ($F_{3,216} = 3.998$, $p = 0.00849$). Specifically, the variation order among different storage periods in Ha-Car was 30 DIS < 60 DIS < 90 DIS and 120 DIS ($F_{3,119} = 59.010$, $p < 0.001$), while in Ha-CK was 30 DIS < 60 DIS and 90 DIS < 120 DIS ($F_{3,97} = 41.500$, $p < 0.001$). However, the significant difference between Ha-Car and Ha-CK was only detected at 30 and 60 DIS (30 DIS: $F_{1,83} = 7.612$, $p = 0.00713$; 60 DIS: $F_{1,62} = 15.700$, $p < 0.001$) (Fig. 2A).

For males, the weight loss during cold storage was not affected by pre-storage diet ($F_{1,234} = 0.135$, $p = 0.7140$), but was significantly affected by storage duration ($F_{3,234} = 46.374$, $p < 0.001$). There was no significant diet × duration interaction ($F_{3,234} = 0.277$, $p = 0.8420$). As for the variations among storage periods, the weight loss in both Ha-Car and Ha-CK was 30 DIS < 60 DIS and 90 DIS < 120 DIS (Ha-Car: $F_{3,129} = 23.590$, $p < 0.001$; Ha-CK: $F_{3,105} = 23.820$, $p < 0.001$) (Fig. 2B).
Determination of glycogen and trehalose content

Among the four storage durations, the content of glycogen was not significantly different, while the content of trehalose was significantly lower in Ha-Car-120 than in Ha-Car-30. However, compared to non-stored control (Ha-Car-0), both the glycogen and trehalose contents were significantly lower in individuals that had cold storage experiences (trehalose: Kruskal-Wallis $\chi^2 = 28.355, p < 0.001$; glycogen: Kruskal-Wallis $\chi^2 = 19.277, p < 0.001$) (Fig. 3).

Post-storage reproductive capabilities

F0 generation. After removing from refrigerator, the adults began to lay eggs at day 6–7, which were similar among the four treatments (Kruskal-Wallis $\chi^2 = 2.914, p = 0.4051$) (Fig. 4A). Daily egg production was quite stable within 15 days and showed to be fluctuated around 30 (Fig. 4B). However, the daily egg hatch rates were relatively high only at the first 5 days (above 50% in Ha-Car-120, Ha-CK-90, and Ha-CK-120), and dramatically decreased since then. Especially at day 12 and 13, the hatch rates were close to 0 (In fact, at day 11, 12 and 13, only small proportion of hatchable eggs can be detected from one pair of adults), and all eggs absolutely lost viability at day 14 and 15 (Fig. 4C). Within the whole oviposition period of 15 days, the average number of eggs and hatch rates both were not significantly different among the four treatments (average number of eggs: $F_{3,22} = 0.876, p = 0.5065$; average egg hatch rate: $F_{3,22} = 0.284, p = 0.836$) (Fig. 4D).

At day 16, each Ha-Car-120 female or male was paired with a refreshed new partner (neither mated nor being exposed to cold storage before). After pairing, the daily egg productions in treatments ReM- were generally higher than those in ReM- within 7 days, but the average number per day was not significantly different between these two treatments ($F_{1,9} = 0.556, p = 0.4750$) (Fig. 5A, B). The average egg hatch rate per day was also similar in treatments ReM- and ReM- ($F_{1,9} = 0.111, p = 0.7460$) (Fig. 5C), while the daily egg hatch rates in treatments ReM- were also generally higher than those in ReM- within 7 days, and they all gradually increased with prolonged oviposition period and finally reached to about 50% (Fig. 5D).

F1 generation.

Female adults in F1 generation began to lay eggs after almost 7 days of post-emergence development, and thereafter produced an average of 34.5 eggs within 10 days, which were similar to those in control (pre-oviposition period: $F_{1,18} = 0.395, p = 0.538$; egg number: $F_{1,18} = 0.137, p = 0.716$). It is worth noting the corresponding egg hatch rate (19.6%) was still significantly lower than that in control (57.8%) ($F_{1,18} = 27.090, p < 0.001$) (Table 2).

Number of micropyles

The average number of micropyles in Ovi-5 (21.0) was significantly higher than that in Ovi-14 (19.0), but was similar to that in Ovi-8 (19.4) or Ovi-11 (19.2). In addition, eggs from ReM-8 or F1-11 also had
significantly lower number of micropyles (18.5 and 18.1, respectively) compared to that in Ovi-5 ($F_{5,474} = 5.396, p < 0.001$) (Fig. 6).

**Discussion**

Tauber *et al.* stated that an effective storage technique must fulfill the following fitness requirements after storage: high survival, synchronous and predictable initiation of reproduction, and sustained high fecundity and fertility. In this study, the indoor-reared *H. axyridis* adults were stored at 6°C with the longest period of 120 days, which was closed to that used for field-collected pre-overwintering individuals. After storage, the adults exhibited appreciable post-storage fitness, such as relatively high survival and a great power in reproduction with stable pre-oviposition period. However, there were also some noticeable negative effects that had rarely been reported before, which would encourage more studies to reveal the mechanisms and develop more effective storage techniques to evade these negative effects.

More than 90 percent individuals survived after 30 days of storage, but the survivals gradually decreased with the increasing of storage duration. Although those at 90 or 120 DIS were significantly lower, there were still more than 30 percent. Chilling injury during storage was an important factor causing mortality. In addition, other indirect effects including accumulation of toxic metabolites and elimination of energy reserves have also been suggested as probable reasons for death. Here, with prolonged storage, the weight loss gradually increased and, especially, the glycogen and trehalose contents sharply decreased even at 30 DIS. These results indicated that energy reserves, especially those directly related to cold tolerance, were rapidly consumed during storage. Specifically, at 60 DIS, the survivals of Ha-CK were about 70% which were similar to those reported in our previous studies that using the same pre-storage diet. However, Ha-CK still had a survival rate of 31.3% (female) and 35.3% (male) at 120 DIS, which was superior to that reported before (all adults died at 90 DIS under similar conditions). These results confirmed our speculation that feeding *H. axyridis* with artificial diet for prolonged periods (10 versus 2 days) can improve the cold tolerance of indoor-reared *H. axyridis* adults. Moreover, compared to control diet treatment (Ha-CK), we found that feeding *H. axyridis* with β-carotene-amended artificial diet before storage (Ha-Car) can further improve the post-storage survivals when storage duration was prolonged to 120 days (> 50%). However, the weight losses of Ha-Car and Ha-CK were not significantly different at 120 DIS. As we know, carotenoids are powerful non-enzymatic antioxidants and supplementation of β-carotene can thus reduce the costs of innate immune response. In addition, other physiological functions of carotenoids might also be benefit for survivals.

In addition, after 90 and 120 days of storage, the females began to lay eggs following 6–7 days’ development and maintained a quite stable and high daily fecundity (around 30 eggs) within 15 days. In previous studies, persistence of high post-storage fecundity has been widely reported in field collected...
pre-wintering *H. axyridis* adults. For example, after 6 months of storage at 6°C, the adults had a daily oviposition rate of 21 eggs during the first month\(^3\), storage at 3°C for 120–150 days had no adverse effect on reproductive capacity\(^2\,3\). For indoor-reared individuals, our previous research also found a high fecundity following 60 days of storage (39.9 eggs per day)\(^1\). Moreover, in this study, no significant difference was detected among the alive individuals of Ha-CK-90, Ha-CK-120, Ha-Car-90, and Ha-Car-120 feeding on *M. persicae*. Studies showed that aphid feeding during post-storage periods can cue ovarian development within several days which might reduce the differences originally existed between treatments\(^4\). These results also indicated that the fecundity of *H. axyridis* seems to be less sensitive to low temperature experiences. Actually, for many biocontrol agents, ability to survive after long-term cold storage with retaining reproductive capacity is their important property\(^4\).

Even so, obvious sublethal effects of long-term cold storage on fertility were detected in this study. In the four treatments, the daily egg hatchability dramatically decreased from day 6 and thereafter, and totally lost at day 14 and 15, which resulted in a relatively low average hatch rate (26–35%). Actually, our previous study had detected a relative lower egg hatch rate following 60 days of storage (28.1% vs 46.5% in non-stored control)\(^1\). Surprisingly, for field collected pre-overwintering adults, a very high hatch rate (85%) can be detected even after twofold durations of storage (up to eight months) at 6°C\(^3\). These results showed that the fertility of indoor-reared *H. axyridis* was much more sensitive to long-term cold storage, which means much more indirect chilling injuries were accumulated during storage\(^3\). Even so, the egg viability can be quickly recovered (day 3 post re-mating) and gradually increased by re-mating with a normal non-stored partner. To our knowledge, this study might be the first to report such mysterious negative effects of cold storage in coccinellids, while the possible reasons were still hard to deduce.

Some studies reported that heat can cause negative effects on fertility through compromised spermatogenesis, dropped viability (or less competitive of sperm), or compromised motility of sperm (Reviewed by McAfee *et al.*\(^4\)). Studies showed that the number of micropyles as well as their structures had crucial roles in the fertilization of eggs\(^3\), and eggs equipped with multiple micropyles offer several benefits\(^4\). In *H. axyridis*, the number of micropyles has been showed to be plastic but relatively stable (range from 18 to 21)\(^3\,3\,4\). Here, significantly reduced number of micropyles was detected on eggs in treatment Ovi-14 (19.0, egg hatch rate was 0) or ReM-8 (18.5, egg hatch rate was recovered to almost 50%) compared to that in Ovi-5 (21.0, egg hatch rate was almost 57%). These results further confirmed that the number of micropyles was plastic following long-term cold storage, but not directly related to the change of fertility.

More importantly, we found that the negative effects of cold storage on fertility can be transferred to the next generation. Eggs produced by the F1 offspring of Ha-Car still had significantly lower hatch rate compared to control treatment (19.6% versus 57.8%), but similar oviposition number was detected in these two treatments. However, Zhao *et al.* reported that cold acclimation (5°C) of *H. axyridis* parents for 5 days can cause negative effects on the fecundity of F1 offspring\(^4\). In addition, the number of micropyles in F1-11 (18.1) was still significantly lower than that in Ovi-5. The mechanism for how
passing maternal effects of chilling damage to the F1 generation has not been fully revealed, and DNA methylation was supposed to be an important approach\textsuperscript{45}. In future, the capability of recovering normal egg viability in F1 and following generations and how to realize still need to be revealed.

In summary, the indoor-reared \textit{H. axyridis} (Ha-Car) can be stored for about 120 days at 6°C with the survivals of above 50%, and the alive adults had very high egg production capability on \textit{M. persicae}. However, long-term cold storage had intra- and trans-generational negative effects on fertility, but it can be recovered by mating with a new non-stored male. Therefore, the stored \textit{H. axyridis} showed to be applicable for releasing to control filed pests due to the fact that the egg hatchability can be recovered when their parents were re-mated with a wild partner.

\textbf{Material And Methods}

\textbf{Insects}

The ladybird beetles \textit{H. axyridis} were from continuous laboratory rearing colony and they were reared with the mixture of \textit{Aphis craccivora} Koch (Hemiptera: Aphididae) and \textit{Acyrthosiphon pisum} Harris (Hemiptera: Aphididae) that co-infesting on broad bean seedlings. Large number of \textit{H. axyridis} larvae were reared with these two aphids and used for producing adults for cold storage. In addition, the colony of \textit{Myzus persicae} (Sulzer) (Hemiptera: Aphididae), a serious pest of many crops\textsuperscript{46}, was established on pepper seedlings to evaluate the post-storage fitness of \textit{H. axyridis}. All these insects as well as their host plants were maintained in an insectary (24 ± 1°C, 60% RH, and 16: 8 L: D).

\textbf{Preparation of pre-storage diets}

The fresh pork liver-based artificial diet was prepared following the methods described in Sun \textit{et al}.\textsuperscript{1}, but with the additional additive of carotenoids. Briefly, the standard (> 98.00%) (Yuanye Biotechnology Company, Shanghai, China) was weighted and fully dissolved in the lin-seed oil + olive oil (1 + 1.4 by volume) using an ultrasonic cleaner to get a solution of 475 µg/ml. After that, 1.9 ml β-carotene oily solution was added into the basic components. All ingredients were fully stirred in a beaker (100 ml), and the prepared diet was divided into groups (kept in 20 ml plastic vials) and stored in a −20°C refrigerator until use. The diet without any carotenoid additives was used as control.

\textbf{Cold storage treatments}

The cold storage procedures were also similar to those reported by Sun \textit{et al}\textsuperscript{1}. Step 1, newly emerged \textit{H. axyridis} adults were reared in plastic Petri dishes (9 cm in diameter) with the density of 6–8 in each. These adults were respectively fed with sufficient β-carotene-amended artificial diet or control diet for 10 days (respectively named Ha-Car and Ha-CK). During this period, the diet was refreshed every other day. Step 2, the adults were transferred to a climate chamber (15 ± 1°C) for cold acclimation for 2 days (continue feeding with diet). Step 3, at day 13, adults were individually weighed with an AE224C electronic balance (SDPTOP, China) and then transferred to a small plastic Petri dish (3 cm in diameter).
Five or six plastic Petri dishes were placed one on top of another and fastened together with a parafilm. Step 4, the Petri dishes were packed in a carton box (30×10×10 cm) and stored at 6°C under full darkness in a refrigerator (6°C). The storage durations were set as 30, 60, 90, or 120 days, and the storage treatments as well as number of individuals used in each treatment were shown in Table 1. Step 5, following different days in storage (DIS), the adults were transferred back to 25 ± 1°C, and their survivals were calculated following the equation:

\[
\text{survival rate} = \frac{\text{(survival number after storage)}}{\text{(total number of adults stored)}} \times 100\%
\]

The alive individuals were weighed again to calculate the weight loss during storage (pre-storage weight – post-storage weight), and 10–13 individuals of both sexes were stored in a − 80°C refrigerator for measuring nutrition. Specially, during storage, the ladybird beetles were transferred back to 15 ± 1°C for one-hour intermittent recovery every 30 days.

**Determination of glycogen and trehalose content**

The glycogen and trehalose content in survived adults were determined using a glycogen and trehalose assay kit (Comin Biotechnology Co., Ltd. Suzhou, China). To obtain enough samples, the Ha-Car females and males at same DIS were incorporated and 3–4 individuals (elytra discarded, almost 0.05 g) were used as one replicate. The extractions were conducted following the instructions, and the absorption at 620 nm was measured four times for each replicate. In total, 6 and 8 replicates were respectively conducted for measuring glycogen and trehalose content.

**Evaluation of post-storage reproductive capabilities**

F0 generation. In order to determine the effects of long-term cold storage on reproductive capabilities of *H. axyridis*, the alive-adults of Ha-Car and Ha-CK following 90 and 120 days of storage were paired and separately reared in plastic Petri dishes (9 cm in diameter, 1.5 cm high). Sufficient *M. persicae* infesting on pepper leaves was supplied as food and refreshed every day. Their oviposition was monitored, and, once egg production initiated, the eggs in each Petri dish was carefully counted and incubated in another plastic Petri dish with an immersed cotton ball for keeping moisture. Specially, at day 5, 8, 11, and 14, 6–8 eggs were randomly selected from the egg cluster of each pair (respectively named Ovi-5, Ovi-8, Ovi-11, and Ovi-14) and used for determination of the number of micropyles. The hatching of eggs was monitored once a day, and newly hatched larvae were immediately picked out to avoid cannibalizing on unhatched eggs. Egg hatch rates were calculated following the equation:

\[
\text{egg hatch rate} = \frac{\text{(number of hatched larvae)}}{\text{(Total number of eggs)}} \times 100\%
\]

In total, 6–7 pairs were used for each treatment and their egg production within 15 days was recorded.
In this study, we found that egg hatch rates gradually decreased with prolonged oviposition period and finally decreased to zero at day 14 and 15. Here, we specially designed a re-mating experiment to test whether the egg viability could be recovered. That was, at day 16, six females or five males (one male dead) each was re-mated with a normal unmated male or female (15–20 days old) (these two treatments were respectively named ReM-[♀] and ReM-[♂]), and their egg production as well as hatch rates were recorded for another 7 days. The average egg production and hatch rates were calculated as described above. At day 8, 10 eggs from each pair were also randomly selected for determination of micropyles (named ReM-8).

**F1 generation.**

In order to test whether long-term cold storage had transgenerational effects on reproductive capabilities of F1 offsprings, the neonate larvae of Wa-Car-120 (from eggs produced at day 8–9) were reared with *M. persicae* until pupation. The newly emerged adults were paired and reared with *M. persicae* in a plastic Petri dish. The pre-oviposition period, egg production and hatch rates within 10 days were recorded, and those larvae from pairs that had not been cold stored were used as control. In total, 10 pairs were used in each treatment. Specially, after recording of 10 days, at day 11, the eggs from F1 offsprings were collected for determining the number of micropyles (named F1-11).

**Determination of the number of micropyles**

Number of micropyles located on the egg shell was examined with Hitachi S-3400N scanning electron microscope (SEM) (Hitachi Science Systems, Ltd., Japan). For SEM photographing, the sample preparations were following the methods described by Sun et al. Briefly, the collected egg samples were immediately transferred to a 1.5 ml centrifuge tube containing 10% ethanol. Then, they were dehydrated in a graded series of 30%, 50%, 70%, 80%, 90% ethanol for 20 min each and in 100% ethanol, twice for 30 min. Specimens were transferred to a mixed solution of ethanol and tert-butanol (3:1, 1:1, and 1:3, by volume) for 15 min each, and finally to 100% tert-butanol for 30 min. After that, the specimens were dried with a freeze-drier (VFD-21S, SHINKKU VD, Japan) for 2 hr. The dried specimens were mounted on aluminum stubs and coated with gold/palladium (40/60) in a high-resolution sputter coater (MSP-1S, SHINKKU VD, Japan). In total, 80 available images were selected for each treatment to determine the number of micropyle located on the top area of each egg.

**Data analysis**

All data analyses were conducted with R software (version 4.0.2). Post-storage survivals were analyzed using a generalized log-linear model (function glm. model) with pre-storage diet treatments (Ha-Car and Ha-CK) and cold storage durations (DIS) as independent fixed factors. Weight loss during cold storage was analyzed using two-way analysis of variance (ANOVA). If the interactions of pre-storage diets and storage durations were significant, one-way ANOVA were then used to examine the effects of one factor within each level of the other factor. For the parameters number of micropyles (after log10 transformation), daily average fecundity and fertility in F0 and F1 generation, and pre-oviposition period
in F1 generation, one-way ANOVA was used to analyze the differences among different treatments; while the non-parametric Kruskal-Wallis test was used to analyze the differences of glycogen and trehalose content and the pre-oviposition period in F0 generation.

**Declarations**

**Data Availability**

The datasets generated and analyzed during the current study are available in the figshare repository, https://doi.org/10.6084/m9.figshare.13663613.

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**Author Contributions**

Y.X.S. and C.Z.L. designed the research; Y.X.S., Y.N.H., and M.L.L. performed the research; and Y.X.S., Y.N.H., and S.S.W wrote the paper. All authors reviewed the manuscript.

**Competing Interests**

The authors declare no competing interests.

**References**

1. Sun, Y. X., Hao, Y. N., Liu, C. Z. & Wang, S. S. Artificial diet is fruitful pre-storage nutrition for long-term cold storage of laboratory-reared *Harmonia axyridis* (Pallas) adults. *Biol. Control* **139**, 104075, https://doi.org/10.1016/j.biocontrol.2019.104075 (2019).
2. Rathee, M. & Ram, P. Impact of cold storage on the performance of entomophagous insects: an overview. *Phytoparasitica* **46**, 421-449 (2018).
3. Storey, K. B. & Storey, J. M. Freeze tolerance in animals. *Physiol. Rev.* **68**(1), 27-84 (1988).
4. Sakaki, S., Jalali, M. A., Kamali, H. & Nedved, O. Effect of low-temperature storage on the life history parameters and voracity of *Hippodamia variegata* (Coleoptera: Coccinellidae). *Eur. J. Entomol.* **116**,
5. Rathee, M. & Ram P. Effect of cold storage of *Aenasius bambawalei* Hayat (Hymenoptera: Encyrtidae) during pupal stage on its key biological characteristics. *J. Bio. Control* **28**(2), 11-17 (2014).

6. Seyahooei, M. A., Mohammadi-Rad, A., Hesami, S. & Bagheri, A. Temperature and exposure time in cold storage reshape parasitic performance of *Habrobracon hebetor* (Hymenoptera: Braconidae). *J. Econ. Entomol.* **111**(2), 564-569 (2018).

7. Lessard, E. & Boivin, G. Effect of low temperature on emergence, fecundity, longevity and host-feeding by *Trichogramma brassicae*. *BioControl* **58**, 319-329 (2013).

8. Chen, W. L., Leopold, R. A. & Harris, M. O. Cold storage effects on maternal and progeny quality of *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae). *Biol. Control* **46**(2), 122-132 (2008).

9. Häckermann, J., Rott, A. S., Tschudi-Rein, K. & Dorn, S. Cold stored ectoparasitoid of *Cydia* fruit moths released under different temperature regimes. *BioControl* **53**, 857-867 (2008).

10. Chen, W. L., Leopold, R. A. & Boetel, M. A. Cold storage of adult *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae) and effects on maternal and progeny fitness. *J. Econ. Entomol.* **101**(6), 1760-1770 (2008).

11. Ö zgökçe, M. S., Atlıhan, R. & Karaca, I. The life table of *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) after different storage periods. *J. Food Agric. Environ.* **4**(1), 282-287 (2006).

12. Senal, D., Demirozer, O. & Karaca, I. Investigation on the storage possibilities of *Rhyzobius lophantae* Blaisdell (Coleoptera: Coccinellidae) at different temperatures and periods. *Phytoparasitica* **45**, 175-182 (2017).

13. Brown, P. M. J. *et al*. The global spread of *Harmonia axyridis* (Coleoptera: Coccinellidae): distribution, dispersal and routes of invasion. *BioControl* **56**, 623-641 (2011).

14. Roy, H. E. & Brown P. M. J. Ten years of invasion: *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) in Britain. *Ecol. Entomol.* **40**, 336-348 (2015).

15. Camacho-Cervantes, M., Ortega-Iturriaga, A. & Del-Val, E. From effective biocontrol agent to successful invader: the harlequin ladybird (*Harmonia axyridis*) as an example of good ideas that could go wrong. *PeerJ* **5**, e3296, 10.7717/peerj.3296 (2017).

16. Gao, Q. *et al*. Differences in the development of internal reproductive organs, feeding amount and nutrient storage between pre-diapause and pre-reproductive *Harmonia axyridis* adults. *Insects* **10**(8), 243, 10.3390/insects10080243 (2019).

17. Zeng, B. *et al*. Effect of long-term cold storage on trehalose metabolism of pre-wintering *Harmonia axyridis* adults and changes in morphological diversity before and after wintering. *PLoS One* **15**(3), e0230435, 10.1371/journal.pone.0230435 (2020).

18. Brown, P. M. J. *et al*. *Harmonia axyridis* in Europe: spread and distribution of a non-native coccinellid. *BioControl* **53**, 5-21 (2008).
19. Koch, R. L. The multicolored Asian lady beetle, *Harmonia axyridis*: a review of its biology, uses in biological control, and non-target impacts. *J. Insect Sci.* **3**(32), 1-16 (2003).

20. Perve, A. & Omkar. Ecology and biological control application of multicoloured Asian ladybird, *Harmonia axyridis*: a review. *Biocontrol Sci. Tech.* **16**(2), 111-128 (2006).

21. Watanabe, M. Cold tolerance and myo-inositol accumulation in overwintering adults of a lady beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae). *Eur. J. Entomol.* **99**, 5-9 (2002).

22. Shi, Z. *et al.* Two novel soluble trehalase genes cloned from *Harmonia axyridis* and regulation of the enzyme in a rapid changing temperature. *Comp. Biochem. Phys. B* **198**, 10-18 (2016).

23. Galvan, T. L., Koch, R. L. & Hutchison, W. D. Impact of fruit feeding on overwintering survival of the multicolored Asian lady beetle, and the ability of this insect and paper wasps to injure wine grape berries. *Entomol. Exp. Appl.* **128**(3), 429-436 (2008).

24. Berkvens, N., Bale, J. S., Berkvens, D., Tirry, L. & De Clercq, P. Cold tolerance of the harlequin ladybird *Harmonia axyridis* in Europe. *J. Insect Physiol.* **56**(4), 438-444 (2010).

25. Wu, M. J. *et al.*, The super cooling point change of *Harmonia axyridis* under low temperature stress and its cold-resistance genes' expression analysis. *Sci. Agric. Sin.* **49**(4), 677-685 (2016).

26. Britton, G., Armitt, G. M., Lau, S. Y. M., Patel, A. K. & Shone, C. C. Carotenoid Chemistry and Biochemistry. (Oxford: Pergamon Press, 1982).

27. Heath, J. J., Cipollini, D. F. & Stireman, J. O. III. The role of carotenoids and their derivatives in mediating interactions between insects and their environment. *Arthropod-Plant Inte.* **7**, 1-20 (2013).

28. Sun, Y. X., Hao, Y. N. & Liu, T. X. A beta-carotene-amended artificial diet increases larval survival and be applicable in mass rearing of *Harmonia axyridis*. *Biol. Control* **123**, 105-110 (2018).

29. Ruan, C. C., Du, W. M., Wang, X. M., Zhang, J. J. & Zang, L. S. Effect of long-term cold storage on the fitness of pre-wintering *Harmonia axyridis* (Pallas). *BioControl* **57**, 95-102 (2012).

30. Awad, M., Kalushkov, P, Nedvedova, T. & Nedved, O. Fecundity and fertility of ladybird beetle *Harmonia axyridis* after prolonged cold storage. *BioControl* **58**, 657-666 (2013).

31. Takahashi, S. *et al.* Overwintering ability of a flightless strain of the ladybird beetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). *BioControl* **64**, 391-399 (2019).

32. Du, W. M. *et al.* Effect of long-term cold storage on fecundity of pre-wintering *Harmonia axyridis* (Pallas) and the fitness of its progeny. *J. Environ. Entomol.* **38**(2), 286-292 (2016).

33. Deng, D. A. Experiments on feeding with artificial diets and cold storage of *Harmonia axyridis* Pallas. *Insect Knowl.* **19**, 11-12 (1982).

34. Osawa, N. & Yoshinaga, A. The presence of micropyles in the shells of developing and undeveloped eggs of the ladybird beetle *Harmonia axyridis* (Coleoptera: Coccinellidae). *Eur. J. Entomol.* **106**(4), 607-610 (2009).

35. Tauber, M. J., Tauber, C. A. & Gardescu, S. Prolonged storage of *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environ. Entomol.* **22**(4), 843-848 (1993).
36. Hance, T., Baaren, Jv., Vernon, P. & Boivin, G. Impact of extreme temperatures on parasitoids in a climate change perspective. *Annu. Rev. Entomol.* **52**, 107-126 (2007).
37. Clark, K. A. & Lampert, E. Effects of dietary beta-carotene on the melanization response and growth rate of *Trichoplusia ni* (Lepidoptera: Noctuidae). *Environ. Entomol.* **47**(6), 1618-1622 (2018).
38. Atarashi, M., Manabe, Y., Kishimoto, H., Sugawara, T. & Osakabe, M. Antioxidant protection by astaxanthin in the citrus red mite (Acari: Tetranychidae). *Environ. Entomol.* **46**(5), 1143-1150 (2017).
39. Zanga, D. *et al.* Carotenoids moderate the effectiveness of a Bt gene against the European corn borer, *Ostrinia nubilalis.* *PLoS One* **13**(7), e0199317, 10.1371/journal.pone.0199317 (2018).
40. Davis, J. R. & Kirkland, R. L. Physiological and environmental factors related to the dispersal flight of the convergent lady beetle, *Hippodamia convergens* (Guerin-Meneville). *J. Kansas Entomol. Soc.* **55**(1), 187-196 (1982).
41. Gardner, J., Hoffmann, M. P., Pitcher, S. A. & Nyrop, J. P. Recurrent warming to improve cold storage of Trichogrammatids (Hymenoptera: Trichogrammatidae). *Biocontrol Sci. Techn.* **22**(3), 261-270 (2012).
42. McAfee, A. *et al.* Vulnerability of honey bee queens to heat-induced loss of fertility. *Nat. Sustain.* **3**(5), 367-376 (2020).
43. Iossa, G., Gage, M. J. G. & Eady, P. E. Micropyle number is associated with elevated female promiscuity in Lepidoptera. *Biol. Letters* **12**(12), 20160782, 10.1098/rsbl.2016.0782 (2016).
44. Zhao, J. *et al.* Effects of cold acclimation on developmental characteristics and fitness of *Harmonia axyridis* (Coleoptera: Coccinellidae) offsprings. *Acta Entomol. Sin.* **55**(7), 810-815 (2012).
45. Sano, H. DNA methylation and Lamarckian inheritance. *Proc. Jpn. Acad. Sci.* **78**(10), 293-298 (2002).
46. Umina, P. A., Edwards, O., Carson, P., van Rooyen, A. & Anderson, A. High levels of resistance to carbamate and pyrethroid chemicals widespread in Australian *Myzus persicae* (Hemiptera: Aphididae) populations. *J. Econ. Entomol.* **107**(4), 1626-1638 (2014).
47. Wu, M. Y. *et al.* Effects of diallyl trisulfide, an active substance from garlic essential oil, on energy metabolism in male moth *Sitotroga cerealella* (Olivier). *Insects* **11**(5), 270, 10.3390/insects11050270 (2020).
48. Sun, Y. X., Hao, Y. N., Liu, C. Z. & Wang, S. S. Investigating reproductive success of the ladybird beetle *Harmonia axyridis* from the perspective of micropyle variation. *Sci. Rep.* **9**, 12742 (2019). https://doi.org/10.1038/s41598-019-49249-z.

**Tables**
Table 1
Number of individuals used in each storage treatment

| Storage treatments | Ha-Car-30 | Ha-Car-60 | Ha-Car-90 | Ha-Car-120 | Ha-CK-30 | Ha-CK-60 | Ha-CK-90 | Ha-CK-120 |
|--------------------|-----------|-----------|-----------|------------|----------|----------|----------|----------|
| Female             | 47        | 42        | 41        | 51         | 41       | 46       | 42       | 48       |
| Male               | 38        | 35        | 51        | 58         | 42       | 41       | 41       | 51       |

Table 2
Transgenerational effects of cold storage on reproductive capabilities of F1 generation

| Reproductive parameters | Pre-oviposition period | Egg number | Egg hatch rate |
|-------------------------|------------------------|------------|---------------|
| F1 generation           | 6.9 ± 0.3 a            | 34.5 ± 3.0 a | 19.6 ± 5.2 b  |
| Control                 | 7.2 ± 0.3 a            | 36.1 ± 3.0 a | 57.8 ± 5.2 a  |

Note: for each parameter, different lowercase letters indicated a significant difference between F1 generation and control.

Figures
Figure 1

Survival of H. axyridis female (A) and male (B) adults after different periods of cold storage at 6 °C. The values labeled in dark column are the number of dead adults, while the values/percentages in grey column represent number of survival adults/survival rate. Different lowercase letters indicate significant differences among four storage periods of females or males in Ha-CK and Ha-Car; asterisk represents a significant difference between Ha-CK and Ha-Car at certain storage period, and ns means no significant difference.
Figure 2

Weight losses of H. axyridis female (A) and male (B) adults after different periods of cold storage at 6 °C. In each graph, different lowercase and uppercase letters represent significant differences among four storage durations in Ha-Car and Ha-CK, respectively (Tukey HSD test, p < 0.05). Asterisk represents a significant difference between Ha-Car and Ha-CK at each storage period, ns means no significant difference (Tukey HSD test, p < 0.05).
The glycogen and trehalose content in H. axyridis adults following different periods of cold storage at 6 °C. Different lowercase and uppercase letters represent significant differences of the trehalose and glycogen content among the treatments, respectively (Kruskal-Wallis test, p < 0.05).
Figure 4

Reproductive capabilities of *H. axyridis* adults after different periods of cold storage at 6 °C. The pre-oviposition period (A), daily egg number (B) and daily egg hatch rate (C) within 15 days of oviposition period. The average egg production and hatch rate of the 15 days (D).
Figure 5

The following 7 days' reproductive capabilities of Ha-Car-120 after re-mating with a new partner (neither mated nor being exposed to cold storage before). The daily egg production (A), average egg number (B) and corresponding hatch rate (C) within 7 days, and the daily hatch rate (D). ns means no significant difference.
Figure 6

The average number of micropyles located on an egg produced by females from different treatments. In the box plots, horizontal line within the box is the median; box indicates the lower and upper quartiles; capped vertical lines are 95% confidence limits, and white dots are outliers. Different letters indicate significant differences (Tukey HSD test, p < 0.05). Ovi-5, Ovi-8, Ovi-11, and Ovi-14 respectively represented treatment oviposition at day 5, 8, 11, and 14; ReM-8 means oviposition at day 8 in re-mating treatment; F1 means oviposition at day 11 in F1 generation.