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Effects of long-term cold storage on maternal and progeny fitness of laboratory-reared *Harmonia axyridis* adults

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Abstract Long-term cold storage is a commonly used technique in mass rearing of natural enemies, but it brings a diversity of negative effects, especially for the laboratory-reared biocontrol agents. *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is an important natural enemy of aphids. In this study, the laboratory-reared *H. axyridis* adults were stored at 6 °C for up to 120 days, and the accompanying effects on maternal and progeny fitness were evaluated and compared with the non-stored beetles (control). Before storage, the newly emerged adults were fed with β-carotene-amended artificial diet for ten days and then acclimated at 15 °C for two days. The mass and trehalose content of the beetle were inversely proportional to the storage duration. The survival rates of the cold-stored beetles decreased as the storage duration increased. However, the survival rates were still above 50% after 120 days in storage (DIS-120). The DIS-120 beetles had a similar fecundity but a significantly longer pre-oviposition period than the control beetles. The egg hatch rates of the DIS-120 beetles were above 60% during the first six days and then dramatically decreased to near zero after day 13. However, the egg viability of the fertility-reduced beetles could be partially restored to approximately 40% by re-mating with non-stored partners. The F1 offspring of the DIS-120 beetles also had a sustained high fecundity but significantly lower egg hatch rate than the F1 offspring of the control beetles. This study demonstrated that the laboratory-reared *H. axyridis* could be cold stored for approximately 120 days, and their reproductive capability was still acceptable.

Keywords Cold storage · *Harmonia axyridis* · Fertility · Laboratory-reared population · Biological control

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Introduction

Low temperature storage is a commonly used technique in mass rearing of natural enemies to prolong the period over which biocontrol agents can be held and remain viable (Etzel and Legner 1999). However, somatic damages caused by direct and/or indirect chilling can accumulate with the prolonged cold exposure (Rojas and Leopold 1996; Lalouette et al. 2011). Significantly lower survival rates following a long-term cold storage have been reported (Rathee and Ram 2018). However, sublethal effects of

long-term storages have not been studied adequately (Sakaki et al. 2019).

Sublethal effects of cold storage generally include delayed oviposition, decreased fecundity and fertility, and reduced parasitism or voracity (Rathee and Ram 2014; Sakaki et al. 2019). For example, the parasitism and fecundity of *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae) were dramatically reduced by 70% and 73%, respectively, and 44% of the females became sterilized if immature wasps were stored at 4.5 °C and 7.5 °C for 40 days (Chen et al. 2008a). For tachinid parasitoids, the longevity and fecundity of *Exorista larvarum* (L.) (Diptera: Tachinidae) were greatly reduced when puparia were stored at 15 °C for four weeks (Benelli et al. 2018). Moreover, some specific sublethal effects caused by cold storage could be passed to the next generation, so called "transgenerational effects" (Hackermann et al. 2008). For example, the delayed development, decreased fecundity, and reduced longevity were observed in the F1 generation of *G. ashmeadi* if their parents were cold stored at 10 °C for more than 20 days (Chen et al. 2008b). However, the influence of cold storage on the development of predators has not been reported as much as those reported on parasitoids (Sakaki et al. 2019; Zhang et al. 2019).

The multicolored Asian ladybird beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), native to Asia, is an important predator of many pests, especially of various species of aphids (Koch 2003). This beetle is also reported as a notorious invasive species due to its excellent adaptability to key environmental factors, such as temperatures (Brown et al. 2011; Camacho-Cervantes et al. 2017). Several studies have evaluated the storage efficiency of field-collected pre-overwintering *H. axyridis* adults and showed that their survival rates generally decreased with increasing storage duration at various temperatures, e.g. − 3 °C and 0 °C (Ruan et al. 2012), 5 °C (Takahashi et al. 2019), and 6 °C (Awad et al. 2013). However, the individuals that survived generally had a high post-storage fecundity, e.g. 21 eggs per day after eight months of storage at 6 °C (Awad et al. 2013), around 25 eggs per day after 90 days of storage at 0 °C (Ruan et al. 2012) and at 3 °C (Du et al. 2016). In addition, the eggs produced by cold-stored adults also had a high hatchability (70–80%) (Awad et al. 2013).

Studies have shown that laboratory-reared *H. axyridis* individuals were more sensitive to cold stress as compared to field-collected individuals (Berkvens et al. 2010; Wu et al. 2016). Although the field-collected overwintering *H. axyridis* adults could be stored at − 5 °C for up to 200 days (Watanabe 2002), the logistics of low-temperature storage has been shown to be a critical limitation for large-scale application of this beetle in pest management (Tang et al. 2017; Wu et al. 2016). We recently found that storing laboratory-reared unmated *H. axyridis* adults at 6 °C for 60 days after allowing them to feed on the pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphidoidea) for four days prior to storage resulted in a survival rate of approximately 50% and a very low egg hatch rate (19.4%). However, the artificial diet was shown to be a more fruitful pre-storage nutrition and gave a relatively higher survival rate (around 70%) and a superior egg viability (28.1%). Even though, no living adult were noted after 90 days of the storage (Sun et al. 2019).

In this study, an optimized pre-storage nutritional supplementation procedure was adopted to test the possibility of achieving a more than 90 day cold storage for laboratory-reared *H. axyridis*. Firstly, a β-carotene-amended artificial diet was supplied as pre-storage nutrition since it has been shown that carotenoids confer a variety of benefits to insects and are essential components in their diet (Heath et al. 2013). In addition, a prolonged pre-storage rearing period was adopted since longer periods of nutrient supplementation might be conducive for *H. axyridis* to obtain more energy reserves (Deng 1982; Sun et al. 2019). Then, the adults were stored at 6 °C for up to 120 days. The post-storage survivals as well as the reproductive performances in F0 and F1 generation were measured to evaluate the effects of long-term cold storage on maternal and progeny fitness. Trehalose may play an important role in cold hardiness of insects (Shi et al. 2016). Thus, the dynamic changes of the trehalose content in the surviving individuals after different periods of storage were measured.

Material and methods

Insects

Three pairs of *H. axyridis* f. *succinea* were collected at late spring of 2019 from the broad bean *Vicia faba* L. field (co-infested with the aphids *Aphis craccivora*

Koch and *A. pisum* (Hemiptera: Aphididae)) in Gansu Agricultural University, Lanzhou, Gansu Province, China. The aphids were reared on the broad bean seedlings in nylon cages (50 × 50 × 50 cm). The ladybird beetles were maintained with both the species of aphids for more than eight generations in the laboratory. The larvae of *H. axyridis* were reared in specially designed containers (95.38 cm^3) with the density of 32 larvae per container (Sun et al. 2021). The newly emerged adults were used for cold storage treatments. The colony of another aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), was established on pepper *Capsicum frutescens* L. seedlings and used as the food for cold-stored *H. axyridis* adults to evaluate their reproductive performances. This aphid causes significant damages to agricultural crops (Umina et al. 2014), and the experiments described in this study could be used to determine the reproductive performance of cold-stored *H. axyridis* adults when they are used to control *M. persicae*. All insects were kept in an insectary (25 ± 1 °C with 60% RH, and a L:D 16:8 photoperiod).

Preparation of artificial diet and pre-storage feeding

Before the cold storage treatments at 6 °C, the laboratory-reared *H. axyridis* adults were fed with a β-carotene-amended artificial diet. The diet was prepared according to the methods described by Sun et al. (2019) but with an additive β-carotene. Briefly, β-carotene (> 98.0%) (Yuanye Biotechnology Company, Shanghai, China) was fully dissolved in the solvent of lin-seed oil and olive oil (1:1.4 ratio by volume) to get a concentration of 474 μg ml^{-1}. All ingredients were weighed and thoroughly mixed in a beaker (100 ml) and stored at − 20 °C until use.

Newly emerged adults were reared in plastic Petri dishes (9 cm in diameter) with the density of 6–8 adults per dish (females and males were separately kept in different dishes to prevent mating). Three diet patches (each was approximately 0.2 ml in volume) were provided in each dish and refreshed every other day. The adults were continuously reared for ten days in an incubator (RDN-300D-5, Ningbo Southeast Instrument Co., LTD, Zhejiang, China) at 25 ± 1 °C with 60% RH and a L:D 16:8 photoperiod. Before cold storage, the ten-day old adults were transferred to another incubator and kept at 15 ± 1 °C for two days of cold acclimation (Sun et al. 2019). Then the 13-day old adults were stored for up to 120 days.

Cold storage treatments

The adults (181 females and 182 males) were individually weighed using an AE224C electronic balance (SDPTOP, China) and kept in a small plastic Petri dish (3 cm in diameter). Five dishes were fastened together with a parafilm and placed in a carton box (30 × 10 × 10 cm) and then transferred to a 6 °C refrigerator (HYCD-205, Qingdao Haier Special Electric Appliance Co., LTD, Shandong, China) under full darkness for different days in storage (DIS), i.e., 30, 60, 90, and 120 days, namely DIS-30 (47 females and 38 males), DIS-60 (42 females and 35 males), DIS-90 (41 females and 51 males) and DIS-120 (51 females and 58 males). During storage, the beetles were not fed but transferred back to 15 ± 1 °C for 1 h intermittent recovery every 30 days (Sun et al. 2019). After storage for specific days, the survival rates were determined at 25 ± 1 °C. Live individuals were weighed again to investigate the weight loss during the cold storage. Sixteen live individuals of either sex were collected, frozen and stored at − 80 °C for biochemical analysis as described below. The 13-day old adults that were subjected to the same feeding and cold acclimation procedures as the cold-stored beetles were used as non-stored control in following measurements.

Determination of trehalose content

The trehalose contents were measured using an assay kit (Comin Biotechnology Co., Ltd. Suzhou, China) (Wu et al. 2020). Briefly, four elytra discarded individuals of almost 0.05 g (two females and two males together) were used as one replicate. In total, eight replicates were used for each treatment. The samples were triturated by an OSE-Y30 electric tissue grinder (TIANGEN Biotech Co., Ltd., Beijing, China) and extracted for the trehalose following instructions. The content was determined by measuring the absorption at 620 nm and calculated following the equation provided in instructions.

Evaluation of the post-storage reproductive capabilities

Six pairs of live adults following 120 days in storage (DIS-120) and the non-stored control beetles were separately reared with daily refreshed *M. persicae* in plastic Petri dishes (9 cm in diameter) at 25 ± 1 °C. The egg production of paired individuals was monitored once a day for 15 days. The eggs were collected and incubated in new plastic Petri dishes, and their hatch rates were then determined (the number of hatched eggs per total number of laid eggs).

Re-mating cold-stored beetles

On the 16th day after oviposition, either females or males of the DIS-120 beetles were paired and allowed to re-mate with 15–20 days old unmated non-stored partners from laboratory colony and respectively named as ReM-♀ (females re-mating, six pairs) and ReM-♂ (males re-mating, five pairs). Once oviposition initiated, their egg production as well as the egg hatch rates were recorded as described above for six days. The pairs of the control beetles were continuously recorded with the same procedure and used as control group.

Reproductive capabilities of F1 offspring

The neonate larvae from eggs produced by the DIS-120 pairs at day 8–9 were reared with *M. persicae* at 25 ± 1 °C (named F1-DIS-120). The larvae from the control beetles were reared with the same procedure and used as control (named F1-Control). The newly emerged adults were paired and reared with *M. persicae*. The pre-oviposition periods, the egg production as well as the egg hatch rates were recorded for ten days. In total, ten pairs were used in F1-DIS-120 group as well as in F1-Control group.

Data analysis

All data analyses were conducted with R software (R Core Team 2020). Percentages of survival after different durations of storage were compared with a test of proportions (prop.test). Pairwise comparisons were carried out with the function pairwiseOrdinalIndependence from the rcompanion package ($p < 0.005$). One-way analysis of variance (ANOVA) was used to test the difference of weight losses of the cold stored beetles, and the pre-oviposition period, egg number and egg hatch rate of the re-mating and control beetles. The means were separated using Tukey's HSD test ($p < 0.05$). The non-parametric Kruskal–Wallis test was used for the whole given measurement of trehalose contents ($p < 0.05$). When a significant difference was detected, the Mann–Whitney U-test with Bonferroni correction was used for multiple comparisons (Ghazy et al. 2014). To compare the reproductive performance of beetles that experienced 120-day storage at 6 °C (DIS-120) and those non-stored control, a paired *t*-test was used to compare the pre-oviposition period, egg number and egg hatch rate in F0 as well as in F1 generation ($p < 0.05$) (Ruan et al. 2012). Before analyses, data on the percentage of egg hatch rates were arcsine square-root transformed, but untransformed data are presented.

Results

Post-storage survivals

The survival rates greatly decreased from above 90% to around 50% along with an increase of cold storage duration from 30 to 120 days (female: $\chi^2 = 33.650$, $df = 3$, $p < 0.001$; male: $\chi^2 = 19.448$, $df = 3$, $p < 0.001$). Specifically, the survival rates of DIS-90 and DIS-120 beetles were significantly lower than those of DIS-30 and DIS-60 beetles. However, no significant difference was detected between DIS-90 and DIS-120 beetles (Fig. 1).

Weight loss during cold storage

The weight loss of DIS-90 and DIS-120 female beetles were significantly higher than those of DIS-60 and DIS-30 female beetles ($F_{3,\ 119} = 59.010$, $p < 0.001$). With respect to the male beetles, the weight losses of DIS-60, DIS-90 and DIS-120 beetles were all significantly higher than that of DIS-30 beetles. There was also a significant difference in the weight loss between DIS-120 and DIS-60 or DIS-90 beetles ($F_{3,\ 129} = 23.590$, $p < 0.001$) (Fig. 2).

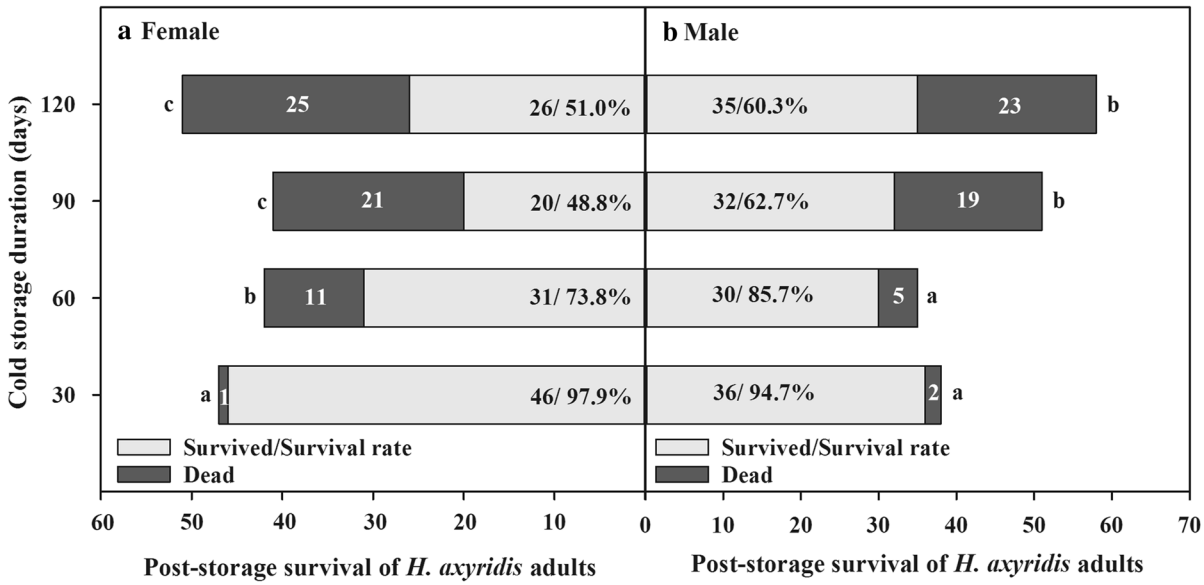

Fig. 1 Survival of *H. axyridis* female (**a**) and male (**b**) adults following different durations of cold storage at 6 °C. The values labelled in dark column are the number of dead adults, while the values/percentages in grey column represent the number of surviving adults and corresponding survival rates. Different lowercase letters indicate significant differences among different storage durations of either female or male survivals ($p < 0.05$)

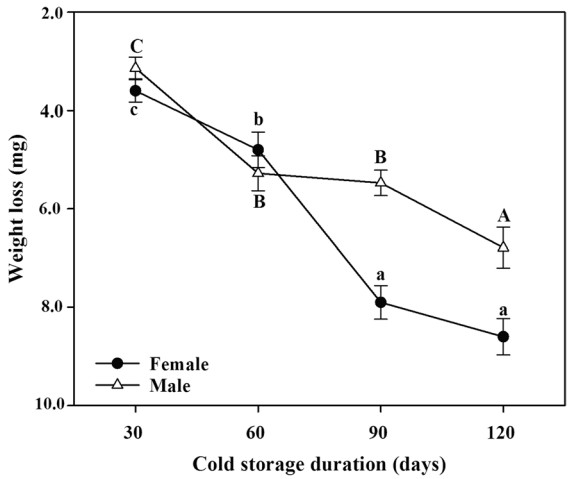

Fig. 2 Weight losses (means ± SE) of *H. axyridis* adults following different durations of cold storage at 6 °C. Different lower-case and upper-case letters indicate the significant differences in the weight losses of females and males, respectively, at different cold storage durations ($p < 0.05$)

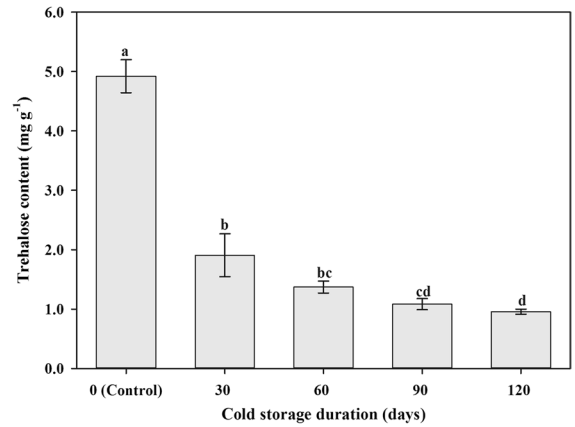

Fig. 3 The trehalose content (means ± SE) in *H. axyridis* adults following different durations of cold storage at 6 °C. Different lower-case letters represent significant differences among different storage durations ($p < 0.05$)

Dynamic changes of trehalose content

The trehalose contents of the individuals that survived from cold storage were below 2.00 mg g^{-1}, which were significantly lower than that of the control beetles (4.92 mg g^{-1}). DIS-120 beetles also had a significantly lower trehalose content than DIS-30 and DIS-60 beetles ($\chi^2 = 28.358$, $df = 4$, $p < 0.001$) (Fig. 3).

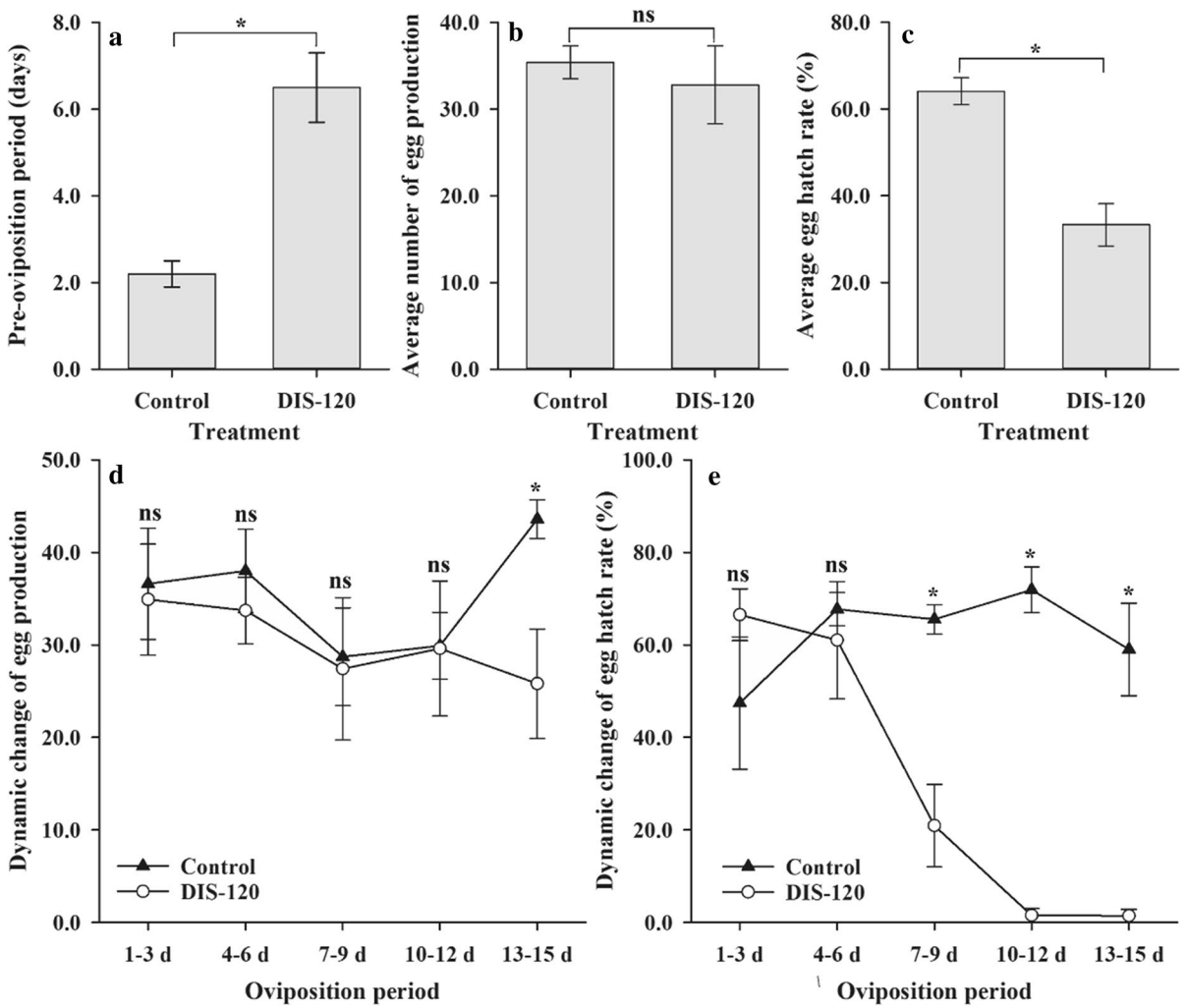

Fig. 4 Reproductive capabilities (means ± SE) of *H. axyridis* adults after the cold storage at 6 °C for 120 days (DIS-120). Pre-oviposition period (**a**), daily average egg number (**b**) and egg hatch rate (**c**) within 15 days of oviposition period, and dynamic changes of three-day average egg production (**d**) and hatch rate (**e**) over 15 days. In **a**–**c**, the asterisk represents a significant difference in daily averages between DIS-120 and control beetles ($p < 0.05$). In **d** and **e**, the asterisk represents a significant difference in the three-day average data between DIS-120 and control beetles ($p < 0.05$). ns means no significant difference ($p > 0.05$)

The reproduction of cold-stored beetles

The DIS-120 beetles had a significantly longer pre-oviposition period than the control beetles ($t = -4.716$, $df = 5$, $p = 0.005$) (Fig. 4a). During the 15 day oviposition period, the DIS-120 beetles had an average fecundity of almost 30 eggs per day, similar to that of the control beetles ($t = 0.465$, $df = 5$, $p = 0.662$) (Fig. 4b). However, the average egg hatch rate of DIS-120 beetles was significantly lower than that of the control beetles ($t = 3.354$, $df = 5$, $p = 0.020$) (Fig. 4c). The three-day average egg number of DIS-120 beetles was similar to that of the control beetles from day 1 to day 12 ($t = 0.028$–0.742, $df = 5$, $p = 0.492$–0.979), and became significantly lower than that of the control beetles from day 13 to day 15 ($t = 2.999$, $df = 5$, $p = 0.030$) (Fig. 4d). In addition, the three-day average egg hatch rate of DIS-120 beetles was above 60.00% from day 1 to day 6, similar to those of the control beetles (day 1–3: $t = -1.186$, $df = 5$, $p = 0.289$; day 4–6: $t = 0.272$, $df = 5$, $p = 0.797$), but

it sharply decreased in the following period from day 7 to day 9, and finally reached to near zero from day 10 to day 15, which were significantly lower than those of the control beetles ($t = 4.246–9.580$, $df = 5$, $p = 0.0002–0.008$) (Fig. 4e).

Effect of re-mating on the reproduction of cold-stored beetles

About two days after the pairing and re-mating, the females began to lay eggs. During the six day oviposition period, the number of eggs produced by females of the re-mating ReM-♂ and ReM-♀ were not significantly different from that of the control beetles ($F_{2, 14} = 0.642$, $p = 0.541$) (Fig. 5a). The eggs from re-mated beetles re-gained their viability with the average egg hatch rates similar to that of the control beetles ($F_{2, 14} = 0.222$, $p = 0.803$) (Fig. 5 b). The three-day average egg production at day 1–3 and day 4–6 was not significantly different between ReM-♂ and ReM-♀ beetles, which was also similar to that of the control beetles (day 1–3: $F_{2, 14} = 2.867$, $p = 0.251$; day 4–6: $F_{2, 14} = 0.171$, $p = 0.845$) (Fig. 5c). In addition, no significant difference in the three-day average egg hatch rate was detected among ReM-♂, ReM-♀ and the control beetles (day 1–3: $F_{2, 14} = 0.543$, $p = 0.593$; day 4–6: $F_{2, 14} = 0.609$, $p = 0.558$) (Fig. 5d).

Reproductive capabilities of F1 offspring

The F1-DIS-120 females took almost 6.9 days of pre-oviposition development and thereafter produced an average of 34.5 eggs per day within ten days of oviposition period, which were similar to those of the F1-Control beetles (pre-oviposition period: $t = 0.605$, $df = 9$, $p = 0.560$; egg number: $t = 0.530$, $df = 9$, $p = 0.609$). However, the average egg hatch rate of F1-DIS-120 beetles was significantly lower than that of the F1-Control beetles ($t = 4.590$, $df = 9$, $p < 0.001$) (Table 1).

Discussion

This study helps to clarify the effects of long-term cold storage on laboratory reared biocontrol agent, *H. axiridis*, the Asian ladybird beetle. We found that a relatively high proportion of adults was alive (above 50%) after 120-day cold storage (DIS-120), and the beetles that survived also had unaffected fecundity compared to the non-stored control beetles. However, these beetles had a significantly reduced fertility. In addition, an obvious transgenerational effect was detected in the fertility of their F1 offspring. Interestingly, the egg viability of the DIS-120 beetles could be restored by re-mating with non-stored partners.

After 30 days of storage at 6 °C, up to 90% adults were alive, but the survival rates decreased to about 50% after 120 days of storage. Past studies have proven that energy consumption, water loss and chilling injuries were the main reasons for death during cold storage (Hance et al. 2007; Rathee and Ram; 2018; Sun et al. 2019). Trehalose is the main blood sugar in most insects (van Handel 1969) and provides important protection during low temperature exposure (Zeng et al. 2020). In this study, the body weights as well as the trehalose contents in live individuals greatly decreased with the increasing of storage durations, indicating a rapid consumption of body energy reserves. It is worth noting that in our previous study, no *H. axyridis* adult survived after 90 days of storage if they were fed with artificial diet for only four days before the cold storage (Sun et al. 2019). The difference in the survival rates could be caused by the duration of pre-storage feeding, and the results from this study confirmed that prolonging pre-storage feeding duration can greatly increase the post-storage survival of *H. axyridis*. Besides, there was a difference in the diets (with or without the supplementation of β-carotene), suggesting that an appropriate pre-storage food can improve the cold resistance of *H. axyridis* as proposed in our previous study (Sun et al. 2019).

The pre-oviposition period of the DIS-120 beetles was significantly longer than that of the control beetles. This may be because the cold-stored *H. axyridis* adults need a period of time to restore the ovarian development as reported in *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) (Davis and Kirkland 1982). However, once egg production initiated, the DIS-120 beetles had a relatively stable and high daily fecundity (around 30 eggs per day) that was similar to beetles after 60-days storage in our previous study (Sun et al. 2019). Moreover, a sustainable and high fecundity was reported in the field collected pre-overwintering adults, e.g. six months at 6 °C (Awad et al. 2013) and 120–150 days at 3 °C (Ruan et al. 2012; Du et al. 2016). It is possible that

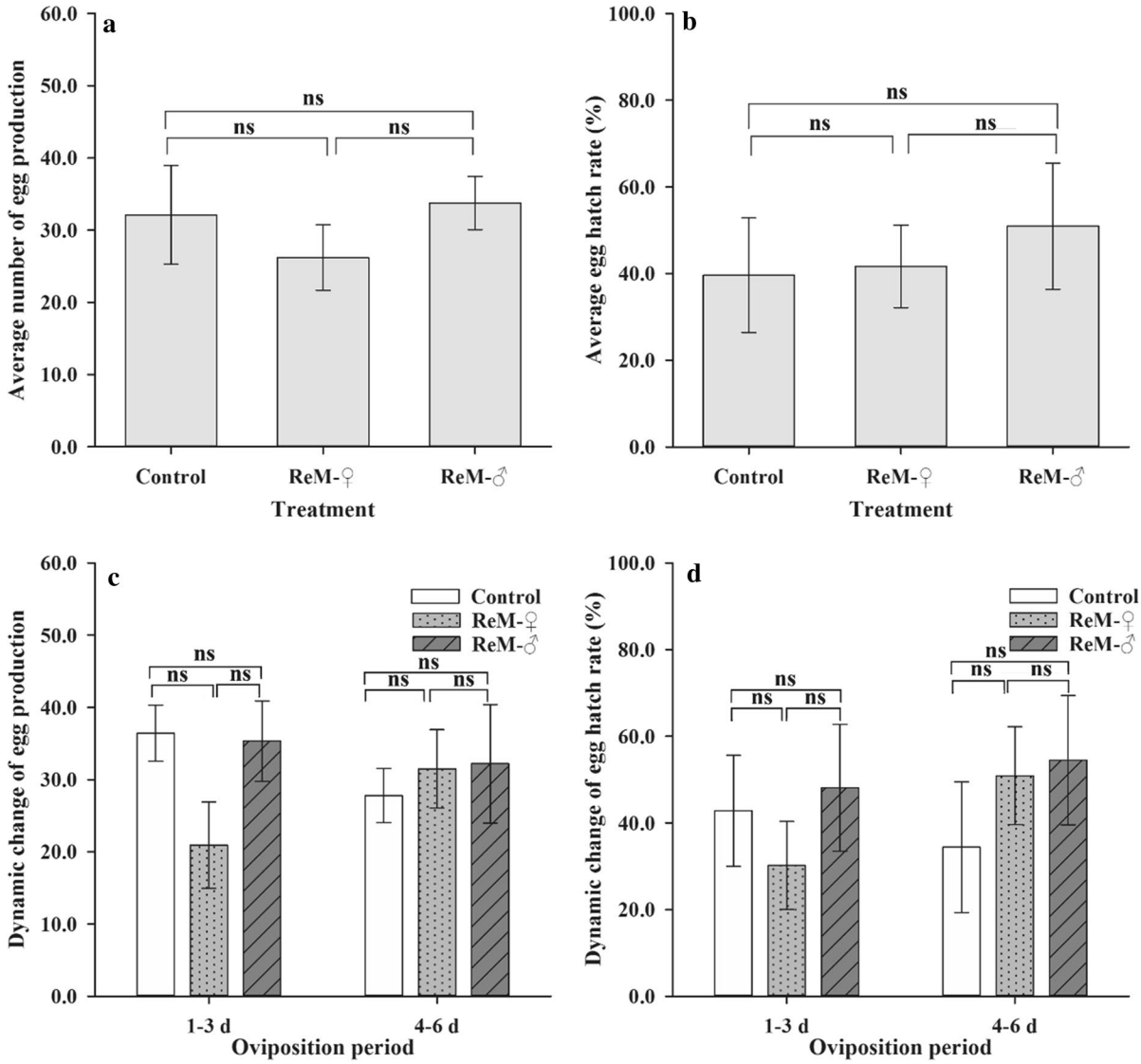

Fig. 5 Reproductive capabilities (means ± SE) of cold-stored individuals following re-mating with a non-stored unmated partner. The average egg production (**a**) and hatch rate (**b**) within six days of oviposition period, and the dynamic changes of three-day average egg number (**c**) and hatch rate (**d**) over 6 days. ReM-♀ and ReM-♂ respectively represents the re-mating pairs of cold-stored female × non-stored partner and cold-stored male × non-stored partner. ns indicates no significant difference between either ReM-♀ or ReM-♂ and control beetles, and also between ReM-♀ and ReM-♂ ($p > 0.05$)

low temperature experiences could not effectively influence the fecundity of *H. axyridis*. However, the fertility of DIS-120 beetles was above 60% during the first six days and then dramatically decreased to near zero at day 10–15. Consequently, the average egg hatch rate within 15 days was only 33.30%, which was greatly lower than that of the field collected pre-overwintering adults (70–80% lasting for more than three months) (Awad et al. 2013). These results confirmed that the laboratory-reared *H. axyridis* are more sensitive to low temperature exposure. Past studies have shown that short day and fluctuating low temperature conditions during the pre-overwintering periods could delay the oviposition of *H. axyridis* females and speed up the development of pre-diapausing stages (Reznik and Vaghina 2013; Reznik et al. 2015). Thus,

Table 1 Transgenerational effects of long-term cold storage on reproductive capabilities of F1 offspring

	Reproductive performance (means ± SE)		
	Pre-oviposition period (days)	Egg number	Egg hatch rate (%)
F1-DIS-120	6.9 ± 0.4 a	34.5 ± 3.0 a	19.62 ± 5.18 b
F1-Control	7.2 ± 0.3 a	36.1 ± 3.0 a	57.81 ± 5.20 a

For each parameter, different lower-case letters indicate a significant difference between F1-DIS-120 and F1-Control ($p < 0.05$). F1-DIS-120 and F1-Control respectively represents the F1 offspring from eggs produced by the DIS-120 pairs and the control non-stored pairs at day 8–9

simulating similar pre-overwintering conditions in the pre-storage feeding phase might help to achieve more effective storage for laboratory-reared *H. axyridis*.

The reason for gradually decreased fertility of the DIS-120 beetles could be quite complex. It was shown that the reproductive organs of insects were particularly vulnerable to low temperature (Flanders 1938; Denlinger and Lee 1998), and malformation was observed under extreme conditions, e.g. in *Euchalcidia caryobori* (Hanna) (Hymenoptera: Chalcidinae) (Hanna 1935). For *H. axyridis*, the adults are in quiescence physiological status during cold storage (Lombaert et al. 2008) and pathological changes would occur if this status prolonged (Tauber et al. 1984). Actually, the cold-induced quiescence has been reported to decrease the fertile oviposition in *Chrysoperla carnea* (Steph.) (Neuroptera: Chrysopidae) (Chang et al. 1996). In addition, we should notice that the field-collected *H. axyridis* adults used in Awad et al. (2013) were mated before storage, while those in our study were only mated after the storage. The unmated status during storage might be another factor that resulted in the low fertility of DIS-120 beetles. Bueno et al. (2014) has reported that the virgin female *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) had much lower post-storage fecundity than the mated females. In this study, we found that the egg hatch rates of the control beetles also decreased to below 40% at day 22–24 (i.e., at day 4–6 of control group in the re-mating treatments), which were lower than those (above 60% over six weeks) of a laboratory maintained population reported by Lombaert et al. (2008). Thus, some factors related to this strain might also play important roles in decreasing the fertility of the DIS-120 beetles. In coccinellids, the manipulation of host reproduction by bacteria has been widely reported (Hurst and Jiggins 2000; Elnagdy et al. 2011, 2013). Here, we could also propose that the population of *H. axyridis* used in this study was infected by bacteria that might kill a proportion of host embryos and, more importantly, could be activated by the cold treatment.

Our study further showed that the fertility of DIS-120 beetles could be restored by re-mating with non-stored partners. About two days after pairing, the females of two re-mating groups (ReM-♀ and ReM-♂) began to lay eggs with a relatively high fertility within the following six days. However, the mechanisms to explain how egg viability can be partially restored by the re-mating with short-term oviposition interval are unclear, and a prolonged monitoring should be conducted in the future to reveal whether these effects of re-mating are similar to those of the first mating.

Moreover, the negative effect of long-term cold storage on fertility was also observed in the next generation. The average egg hatch rate of F1-DIS-120 beetles was significantly lower than that of F1-Control beetles. These results suggest that the cold storage of maternal generation could cause a change not only at physiological level, but also at gene level. DNA methylation has been supposed to be an important mechanism to pass chilling damage effects from maternal generation to F1 generation (Sano 2002). To our knowledge, this study may be the first report of such negative effects of long-term cold storage on egg hatchability in coccinellids.

In summary, the laboratory-reared *H. axyridis* adults fed with β-carotene-amended artificial diet for ten days and then acclimated at 15 °C for two days could be stored at 6 °C for up to 120 days. Our results demonstrate the possibility to store laboratory-reared natural enemies for a relatively long period and to restore the fertility reduced by the cold storage, which would be very important for augmentative biological control.

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Author contributions YXS and CZL designed the research; YXS, YNH, and SSW conducted experiments and analyzed data. YXS, JJZ and YNH wrote the paper. JJZ made critical revisions and proofreading. All authors read and approved the manuscript.

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

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The following insects used in this study commonly occurred in the agricultural field, e.g. *Harmonia axyridis*, *Aphis craccivora*, *A. pisum* and *Myzus persicae*. The pork liver component in artificial diet was purchased from a market in China, which is a well-received food for many people.

Consent to participate Not applicable.

Consent for publication Not applicable.

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