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Obstructs-equipped apparatus reduces cannibalism and improves larval survival of the Coccinellid, *Harmonia axyridis* (Coleoptera: Coccinellidae)

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Abstract

Background: *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is an important biocontrol agent in native region of Asia, while its high propensity for cannibalism causes great obstacles in mass production. Provisioning obstructs in rearing containers could decrease the cannibalism of ladybird beetles. In this study, three different patterns of obstructs (Con-A, Con-B, and Con-C) were designed and equipped in plastic Petri dishes (95.38 cm³) as rearing units, and their efficiency for *H. axyridis* larval survival was tested. The potential of the high-density rearing was then evaluated using the optimal units with 16, 24, or 32 larvae per unit (named 16-L, 24-L, or 32-L, respectively).

Results: Larval survivals in obstructs-equipped units were generally higher than those in control, and significantly increased with the Con-C units (39.8% versus 74.2% at adult stage). With the Con-C units, the survivals were significantly higher at 16-L density (82.5%) than those at 24-L density (62.5%), but both were non-significantly different from those at 32-L density (70.0%). The weights of newly emerged adults (fit with the expected sex ratio of 1:1) at the higher densities were lower than those at 16-L density.

Conclusions: The results demonstrate that *H. axyridis* can be reared at a higher density (≈ 0.336 larvae/cm³) in a constrained unit and highlight the effects of obstructs in reducing cannibalism and improving insect survivals.

Keywords: *Harmonia axyridis*, Cannibalism, Mass rearing, Larval survival, Obstructs

Background

Biological control approaches are well-established methodologies for the control of pest population in some agricultural systems (Mills and Heimpel 2018). Among them, the augmentative type is shown to play a great role in reducing pesticide use (van Lenteren and Bueno 2003), and its typical feature is the mass releasing of natural enemies into infested field to obtain an immediate control of pests (van Lenteren 2012). In this type of pest management system, space-saving in rearing natural

enemies and maximizing of rearing density are vital to meet the quantity demand (Riddick and Wu 2015). More importantly, optimal rearing density in suitable units can decrease the maintenance cost (Mamay and Mutlu 2019). This is more crucial for the natural enemies that have cannibalism behaviors. Cannibalism, a common behavior in Coccinellidae (Osawa 2002), has been identified as a key factor for the population dynamics, and a major problem in the mass-rearing of coccinellid species (Obrycki and Kring 1998).

The multicolored Asian ladybird beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), a notorious aggressive invasive species (Roy et al. 2016), is an important augmentation biological control agent in the native distribution region of Asia (Brown et al. 2008).

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Earlier studies demonstrated that *H. axyridis* larvae had a stronger cannibalism behavior than many other aphidophagous ladybirds and was independent from prey abundance (Reznik et al. 2017) but increased with larval density (Michaud 2003). In field, the mortality of 4th instar larvae (93.30%) was higher than those of the other stages (less than 50.51%) due to the food storage and cannibalism (Osawa 1992a). Taken together, increasing rearing density without decreasing pre-imaginal development appeared to be very difficult for the mass rearing of coccinellids larvae, like *H. axyridis* (Omkar and Pathak 2009). In addition, *H. axyridis* larvae, especially the 4th instar, are highly voracious (Paul et al. 2015) and take a long period to complete their development. For example, fed on the pea aphid, *Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae), the mean duration of each stage of *H. axyridis* at 26 °C was 2.5 days for the 1st instar, 1.5 days for the 2nd instar, 1.8 days for the 3rd instar, and 4.4 days for the 4th instar (LaMana and Miller 1998).

It has been shown that rearing density of several coccinellid species could be increased with the units that provide refuges (i.e., hiding places) (Riddick and Wu 2015). Thus, mass rearing of *H. axyridis* larvae, with a high density, would be achieved by designed obstructs in rearing units that can decrease larval-larval or larval-pupal encounter frequency.

In this study, three types of obstructs were first designed and used in plastic Petri dishes rearing units. The rearing efficiencies were evaluated by mix populations of the cowpea aphid, *Aphis craccivora* Koch and the pea aphid, *A. pisum* (Hemiptera: Aphididae). After that, the optimal type was selected out and used for determining the potential for a high-density rearing of *H. axyridis*.

Methods

Insects

The ladybird beetle, *H. axyridis* was obtained from continuous rearing colony in the laboratory and reared on the mix population of *A. craccivora* and *A. pisum*. The two co-infested aphids were collected from field and maintained on the broad bean seedlings. All insects were maintained in an insectary (25 ± 1°C, 60% RH, and 16:8 L:D). Three pairs of newly emerged adults were reared in a plastic Petri dish unit (9 cm in diameter, 1.5 cm high; BKMAM, China) and supplied with sufficient aphids as food and 2 broad bean leaves for oviposition. The daily eggs produced were incubated in a new Petri dish for 3 days with an immersed cotton ball for keeping moisture. Egg hatching was monitored with an interval of 12 h, and the neonate larvae were collected for subsequent rearing.

Design of obstructs

It was assumed that the larval movement and the larval-larva encounter of *H. axyridis* could be restricted at

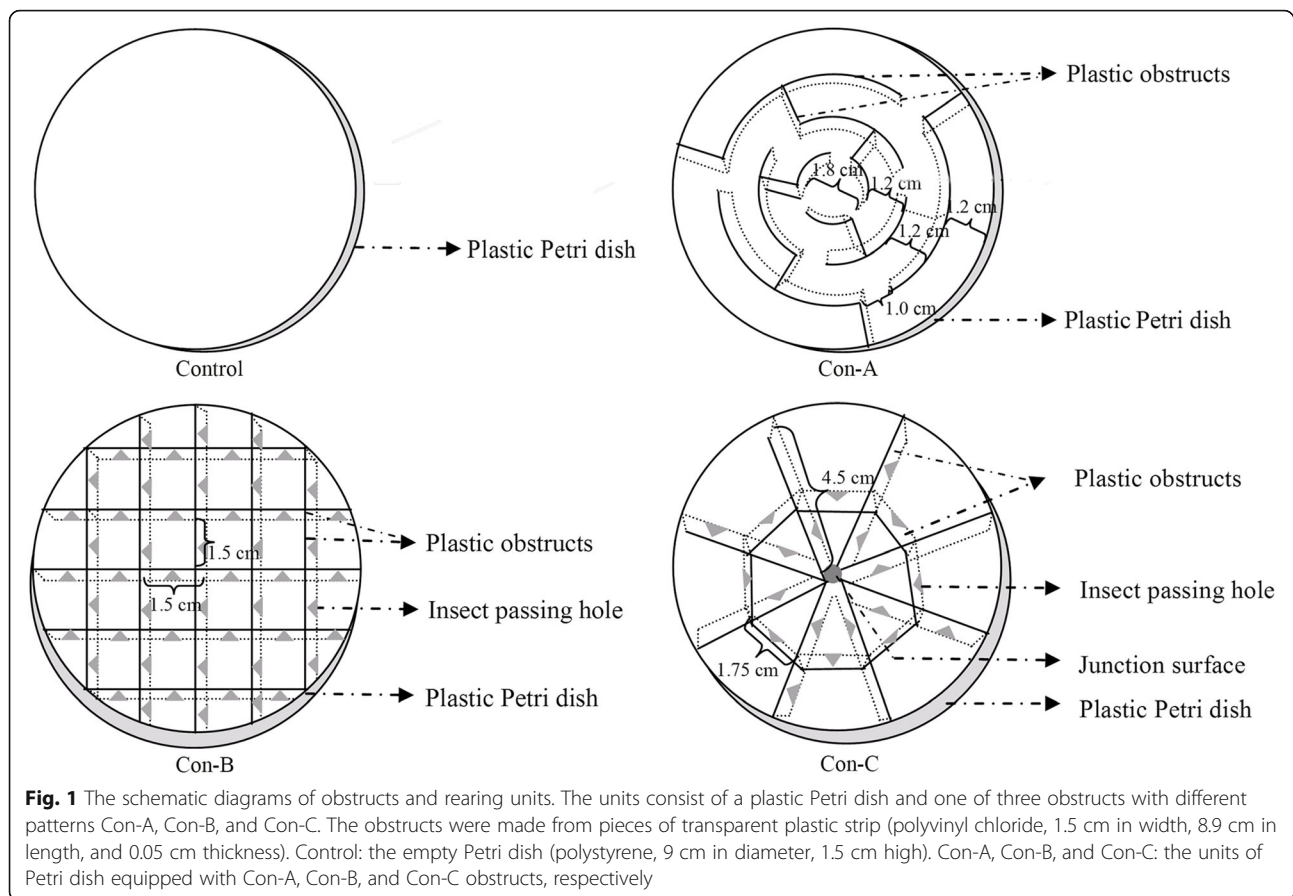
different degrees with obstructs so that the cannibalism could be reduced. In order to evaluate their efficiency in reducing cannibalism, in this study, three different patterns of obstructs (polyvinyl chloride) were designed (Con-A, Con-B, and Con-C, Fig. 1) and equipped in transparent plastic Petri dishes (polystyrene, 9 cm in diameter and 1.5 cm high with the total volume of 95.38 cm³) as rearing units (Fig. 1). The obstructs were made from pieces of transparent plastic strips (1.5 cm in width, 8.9 cm in length, and 0.05 cm in thickness). The design of the Con-A obstruct was from the inspiration of maze which provides many refuge spaces. The Con-B obstruct provided many quadrate divisions (1.5 × 1.5 cm). The Con-C obstruct had 16 divisions with radial strips connected with another strip on the central section, and it had less restriction for larval movement than the Con-B obstruct. The Con-B and Con-C obstructs had insect passing holes (equilateral triangle in shape with 0.5 cm in length of side) on each side. The obstructs were glued together by a hot melt adhesive and each placed into a plastic Petri dish as a complete rearing unit. The surface area of Con-A, Con-B, and Con-C units was approximately 291 cm², 495 cm², 312 cm², respectively, and the surface area of the empty plastic Petri dish (control) was approximate 106 cm².

Larval rearing with different units

Sixteen newly hatched *H. axyridis* first instar larvae were gently transferred into one rearing unit and supplied with sufficient *A. craccivora* and *A. pisum*. The aphids were daily refreshed with an approximate weight of 180, 240, 400, and 1000 mg for 1st, 2nd, 3rd and 4th instar larvae, respectively. Number of survived individuals as well as their development stages were checked and recorded daily until all adults emerged. Percentage of survived individuals in each unit was calculated against the total number of beetles in the unit. After feeding and recording, the unit was sealed by a parafilm (1.2 cm in width) to avoid insect escaping. After pupation (all pupation finished on day 13 after rearing), the distribution of pupae on each surface of the unit (bottom, lid, and wall of the Petri dish and on obstructs) was recorded. Eight replicates were conducted for each type of the rearing unit.

Evaluation of the potential for high-density rearing

In this experiment, 16, 24, or 32 neonate larvae were respectively transferred to one Con-C equipped unit with the density of 0.168, 0.252, and 0.336 larvae/cm³, respectively (abbreviated as 16-L, 24-L, and 32-L density, respectively). At 16-L density, similar number of aphids was provided as described above, while approximate 1.5 and 2 times more aphids were respectively provided to 24-L and 32-L density. The above number of aphids would be sufficient for high-density rearing of *H.*



axyridis as that the predation rates of aphids showed to be decreased with rearing density (Gao et al. 2020). Once adult emergence started, the number of adults emerged in each day was recorded and calculated as percentage relative to the total number larvae tested with the following equation: $(\text{number of adults emerged}) / (\text{total number of adults obtained}) \times 100$. The weight of newly emerged adults (24–36 h old) was measured using an AE224C electronic balance (SDPTOP, China) with an accuracy of 0.1 mg, and the female and male ratio was recorded. Ten replicates were conducted for each rearing density.

Data analysis

All data analysis was performed using SPSS 19.0 statistical software. The differences among different treatments were analyzed with one-way analysis of variance (ANOVA) for the data that met the assumptions of normality (Levene's test). For the data that failed to meet the assumptions of normality, the non-parametric Kruskal-Wallis analysis of variance (K-W ANOVA) was used. Means were separated with Tukey HSD test ($P < 0.05$). The comparison between the observed sex ratio and the expected one (1:1) was conducted by the chi-square test. For 24-L and 32-L densities, the difference

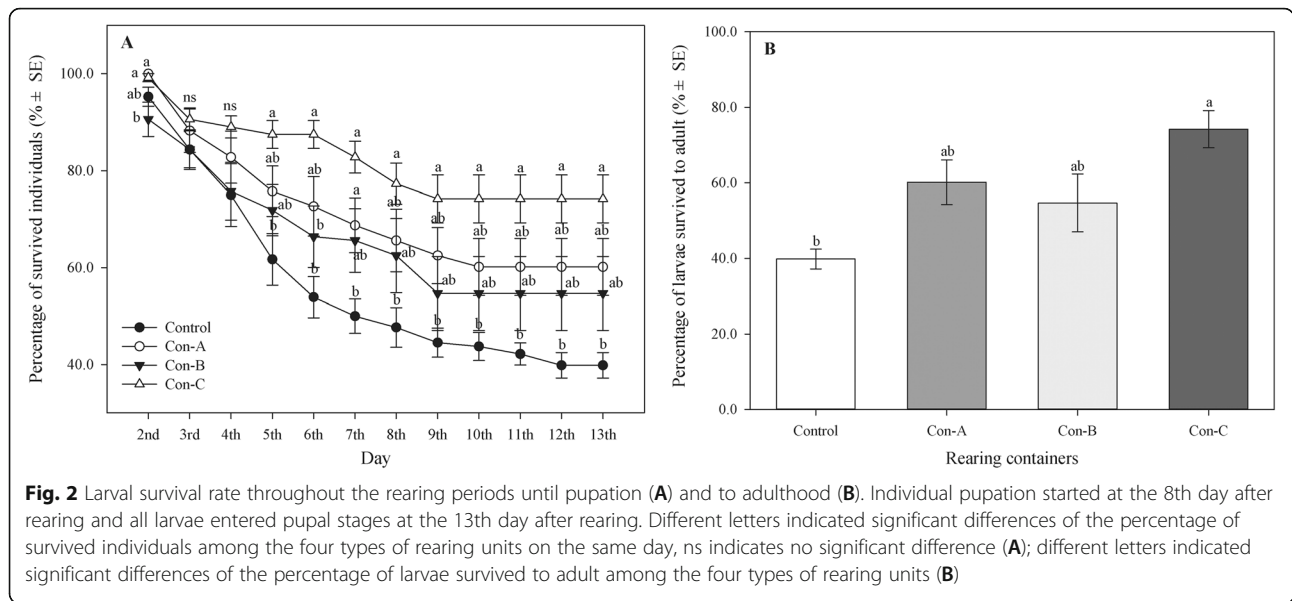
of the percentage of adults emerged at day 5 were compared with independent sample t test ($P < 0.05$).

Results

Screening of obstructions

Under the density of 16 larvae per unit, the survival of the larvae that reared in the units with either the Con-A or the Con-B obstruct was non-significantly different from that reared in the control unit (Petri dish without any obstructions), while it was significantly higher with the Con-C unit from day 5 (3rd and 4th day: $\chi^2 = 1.8$ and 3.4 , $P = 0.332$ and 0.621 ; the remaining days: $\chi^2 = 10.2$ – 13.9 , $P = 0.003$ – 0.017) (Fig. 2A). The larval survivals tended to be stable from day 8 in the units with obstructions. However, the larval survivals in the control unit decreased sharply throughout the whole rearing periods and also decreased faster than those in the units with obstructions (Fig. 2A). Consequently, the percentage of larvae that survived to adult in the Con-C units (74.2% of 16 neonate larvae) was significantly higher than the control (39.8% of 16 neonate larvae) ($\chi^2 = 13.2$, $P = 0.004$) and also relatively higher than those in the Con-A and Con-B units (Fig. 2B).

In the control units, more than 80% pupae were distributed on the bottom of the Petri dish. However, in

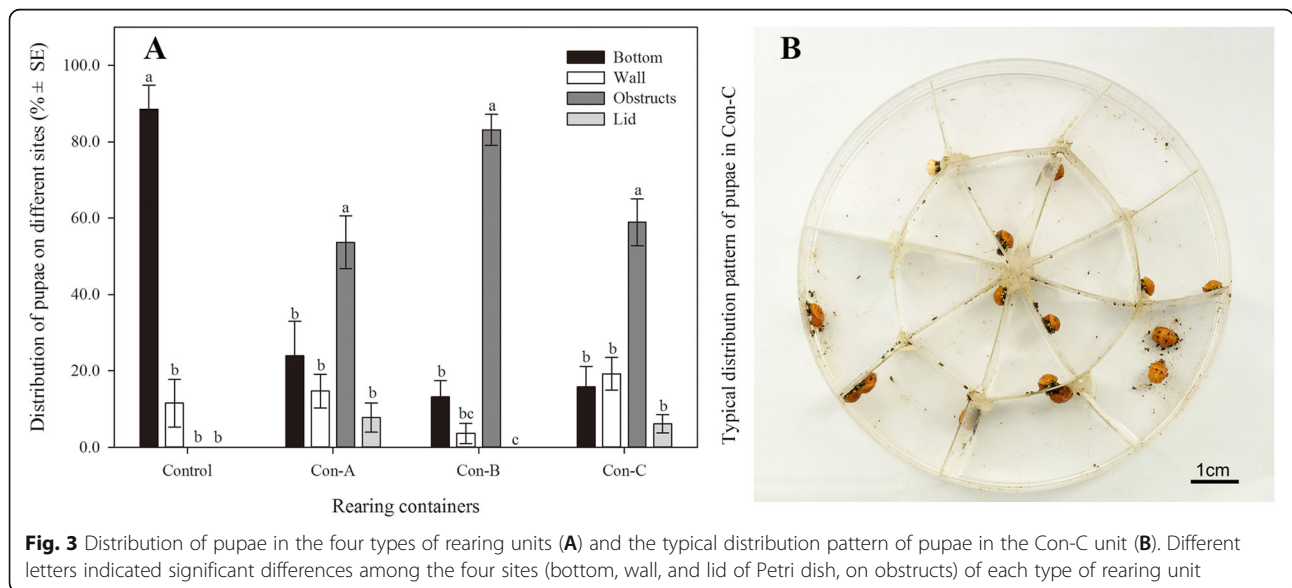


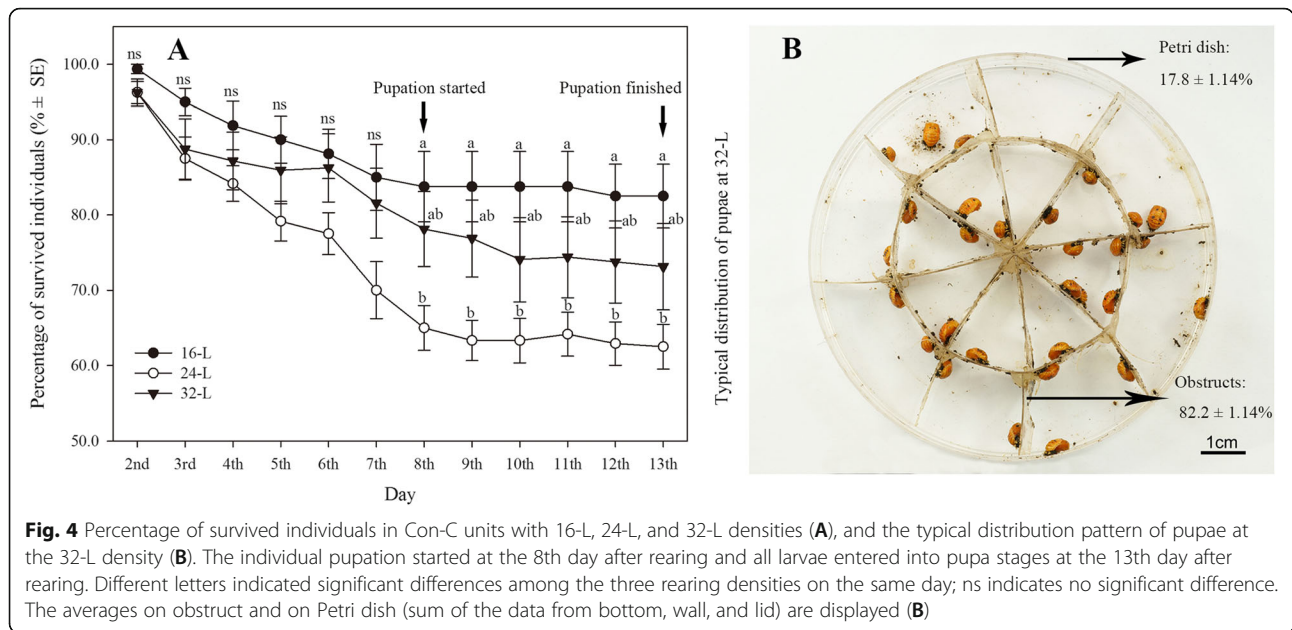
the Con-A, Con-B, and Con-C units, significantly more larvae selected obstructs as pupation sites than those selected the bottom, lid, and wall of the Petri dish ($\chi^2 = 13.6\text{--}26.2$, $P < 0.001$) (Fig. 3A). The typical distribution of pupae in the Con-C unit was shown in Fig. 3B, with an average of 58.92% on obstructs and 41.08% (sum of the data from bottom, wall, and lid) on the Petri dish.

Evaluation of density-dependent rearing efficiency

When 16, 24, and 32 neonate larvae per unit were reared in the Con-C units, which produced the best larval survival (Fig. 2), non-significant difference in the survival percentage was detected during the first 7 days ($F_{2, 27} = 1.5\text{--}3.4$, $P = 0.050\text{--}0.250$). Until pupation is finished,

the larval survivals at the 16-L density were significantly higher than those at the 24-L density ($\chi^2 = 6.7\text{--}8.7$, $P = 0.013\text{--}0.035$), and the larval survivals at the 32-L density were non-significantly different from either. Finally, average number of pupae were observed as 13.2 (82.5% of 16 neonate larvae), 15.0 (62.5% of 24 neonate larvae), and 22.4 (70.0% of 32 neonate larvae) at the 16-L, 24-L, and 32-L density, respectively, on the 13th day. In addition, the survival percentages decreased sharply during the first 8 days, and stabilized afterward at all densities (Fig. 4A). At the rearing density of 32-L, the typical distribution of pupae was shown in Fig. 4B with an average of 82.2% on obstructs and 17.8% on Petri dish (sum of the data from bottom, wall, and lid). Almost all pupae





successfully developed to adult, so adult emergence rate was not separately recorded again and assumed that all pupae developed to adults.

At the 16-L density, the adult emergence lasted for 4 days and nearly equally distributed in each day (relatively lower at day 4) ($F_{3, 36} = 1.7, P = 0.178$). However, at either the 24-L or the 32-L density, the adult emergences lasted for 5 days. In addition, at the 2 higher densities, relatively higher proportions of adults emerged during the first 3 days, which were significantly higher than those on the 5th day (24-L: $F_{4, 45} = 6.5, P < 0.001$; 32-L: $\chi^2 = 23.4, P < 0.001$) (Table 1). On day 1–4, the adult

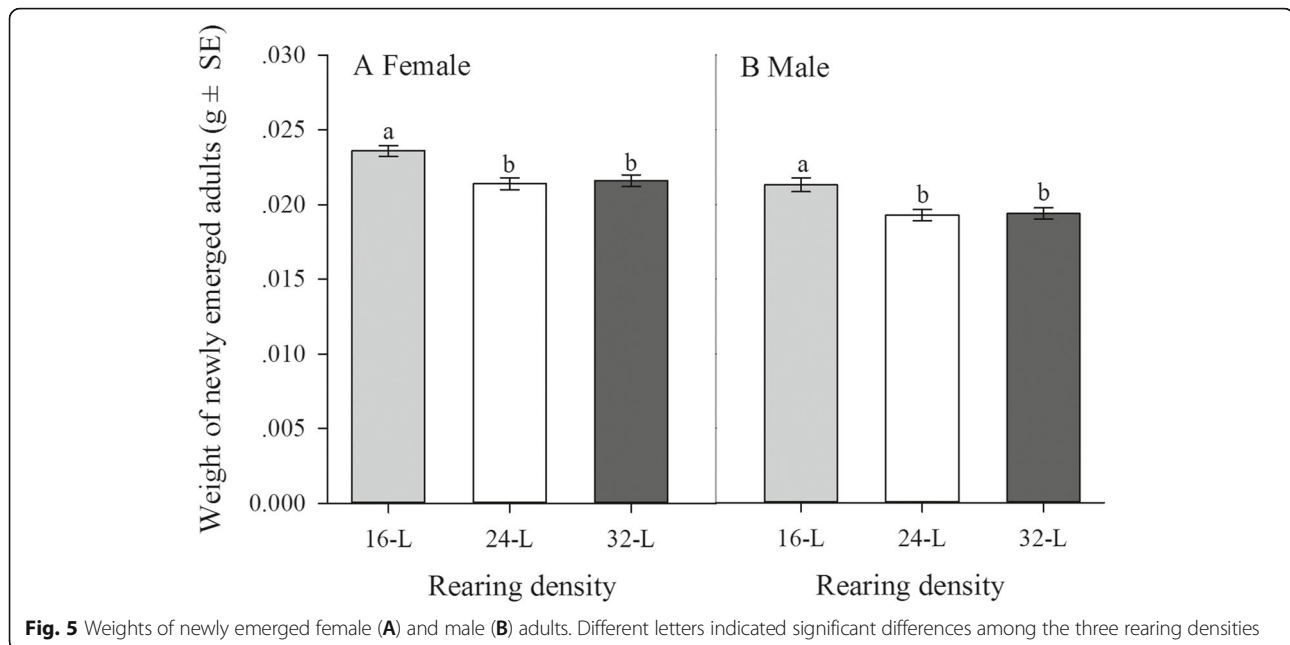
emergence percentages at the 3 rearing densities were similar ($F_{2, 27} = 0.026–0.571, P = 0.571–0.974$). On day 5, non-significant difference in adults' emergence was detected between the rearing densities of 24-L and 32-L ($t = -0.077, df = 18, P = 0.939$) (Table 1).

For the newly emerged adults, the weights of both females and males were significantly higher at the 16-L density than those at the 24-L and the 32-L density (female $F_{2, 192} = 8.5, P < 0.001$; male $F_{2, 180} = 6.1, P = 0.003$), but there was non-significant difference in the adults' weights between the latter 2 densities (Fig. 5). In addition, at the densities of 16-L and 24-L, relatively

Table 1 Percentage of adults emerged at different periods and the ratio of females and males, with an expected ratio of 1:1

Adult emergence period	Rearing densities		
	16-L	24-L	32-L
	Percentage of adults emerged (%)		
Day 1	29.84 ± 6.4 Aa	32.31 ± 5.78 Aab	28.39 ± 6.20 Aab
Day 2	31.22 ± 6.9 Aa	33.69 ± 5.45 Aa	29.43 ± 4.95 Aa
Day 3	28.00 ± 6.39 Aa	18.60 ± 5.84 Aabc	25.77 ± 2.98 Aab
Day 4	13.73 ± 4.05 Aa	12.30 ± 5.70 Abc	13.15 ± 3.26 Abc
Day 5		3.11 ± 1.30 Ac	3.26 ± 1.43 Ac
	Female and male ratio		
Number of females	57	63	79
Number of males	43	52	87
χ^2	1.960	1.052	0.386
P	0.162	0.305	0.535

Different lowercase letters indicate significant differences in the percentages of adults emerged at different days; same uppercase letters indicate no significant difference among different rearing densities at each adult emergence period ($P < 0.05$)



more females were harvested than males, but more males were harvested than females at the 32-L density. Even so, at the three rearing densities, the female and male ratio was non-significantly different from the expected ratio of 1:1 (Table 1).

Discussion

As expected, providing obstructs as a part of rearing units can effectively improve the survival of *H. axyridis* larvae, which have genetic inheritance for the propensity of cannibalism (Wagner et al. 1999). Survival rate decreased sharply with time in the rearing unit without any obstruct, but it decreased moderately in the unit equipped with obstructs, indicating a decrease of the cannibalism of *H. axyridis* by using the obstructs. Specially, throughout the whole rearing periods, the larval survivals in the unit equipped with Con-C obstruct (312 cm²) were relatively higher than those in the units with either decreasing the surface area to 291 cm² by the Con-A obstruct or increasing the surface area to 495 cm² by the Con-B, demonstrating the efficiency of obstructs was a pattern-dependent rather than surface area (Mamay and Mutlu 2019). Such results might be caused by some unrevealed behaviors of *H. axyridis* larvae, and more studies, e.g., video monitoring, need to be conducted to fully reveal the mechanism. Finally, the rate of individuals reaching to adult increased from 39.8% in empty Petri dish (control unit without any obstructs) to 74.0% in the unit equipped with the Con-C obstruct, when the initial larval density was 16. In this study, the adult survival rate without any obstructs (39.8% at 0.168 larvae/cm³) was lower than 46.7–53.8% that reported by Sun et al. (2019) under similar densities (0.15

larvae/cm³), where *H. axyridis* larvae were fed on the eggs of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). For *H. axyridis*, Reznik et al. (2017) reported that the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) was more easily to cause density-dependent effects than the eggs of the grain moth, *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae). Thus, prey type might be also an important factor affecting rearing efficiency, and in the future, optimal prey should be considered to further improve the efficiency of high-density rearing with the Con-C unit.

The studies on coccinellids showed that motionless larvae when molting or partially immobile late 4th instar larvae were most easily attacked by the siblings (Riddick and Wu 2015). However, *H. axyridis* 4th instar larvae were reported to exhibit an avoidance behavior for cannibalism as they tend to avoid conspecific larvae in the olfactometer choice experiments (Rasekh and Osawa 2020). Here, the larval-larval or larval-pupal encounter frequency would be greatly decreased by provisioning the obstructs (pattern-dependent) that can provide more suitable habitats for both ladybird beetle and aphids crawling and roosting, as well as more pupation sites. Significantly more proportion of pupae distributed on the obstructs than on the Petri dishes partially supports the speculations. Osawa (1992b) reported that 14.4% of newly molted *H. axyridis* pupae would be cannibalized at the pupal stage. The larvae of *H. axyridis*, especially the 4th instar were voracious predators. Under high-density conditions, a high-intraspecific competition would be occurred when the larvae reached to 4th instar

due to their voracious behaviors (Paul et al. 2015). Thus, provisioning of obstructs, i.e., increasing the inner surface area and acting as the refuges for larvae and newly molted pupae, would be particularly important for reducing cannibalism in *H. axyridis*.

In addition, with the Con-C obstruct, significantly higher larval survival rates were detected at the 16-L density (0.168 larvae/cm³) than at the 24-L density (0.252 larvae/cm³), while at the 32-L density (0.336 larvae/cm³), the survival rate was between those two and not significantly different from them. More importantly, under the 2 higher densities, a high proportion of individuals survived to adult stage (62.5 and 70.0% for 24-L and 32-L, respectively). These results showed that, even under extreme high-rearing density, there was no clear density-dependent efficiency for larval survival of *H. axyridis* with the rearing unit equipped with Con-C obstruct. This phenomenon might be caused by the fact that 16-L density was very high for the rearing unit with approximate 95.38 cm³.

Increasing population density in mass production of *H. axyridis* might also cause some non-lethal negative effects as the adults need more days for emergence at the 24-L or 32-L densities than those at the 16-L density, and both females and males were also significantly lighter. It was speculated that these negative effects under higher rearing densities might be caused by the disturbance of normal metabolism. For *Coccinella undecimpunctata* L. and *C. novemnotata* Herbst (Coleoptera: Coccinellidae), their adult weights were also generally decreased by increasing of population density (Turnipseed et al. 2014). In addition, decreased weights under high-rearing density might be caused by the changes in feeding regime (feeding intensity) (Reznik et al. 2017) and by the more frequent mechanical interactions, resulting in the stimulation of defense reactions (hemolymph losses) (Sato et al. 2008) and then the negative effects (Bayoumy et al. 2019). Here, the sex ratios of newly emerged adults under all rearing densities were met with the expected ratio (1:1), which was similar to that reported in the three striped ladybird beetle, *Brumoides suturalis* (Fabricius) (Coleoptera: Coccinellidae) (Bista et al. 2012) and in the pink spotted ladybird beetle, *C. maculata* (Riddick and Wu 2015).

Conclusions

The obtained results demonstrated the principle of using purposely designed obstructs in reducing insect cannibalism and highlighted the effects of such obstructs on the improvement of the larval and pupal survival rates of the ladybird beetle *H. axyridis*. Similar rearing unit equipped with obstructs like the Con-C pattern could have great potential in mass-rearing of other natural enemies.

Abbreviations

Con-A, Con-B and Con-C: the units of Petri dish with Con-A, Con-B and Con-C obstructs built-in, respectively; 16-L, 24-L, and 32-L: The rearing density of 16, 24, and 32 larvae in each Con-C unit, respectively

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Authors' contributions

YXS planned the research project, wrote, revised, and edited the manuscript. YNH helped to conduct the experiments and wrote the manuscript. CZL and SSW supervised the research and helped to analyze the data. JJZ made critical revisions to the manuscript. All authors have read and agreed to the final version of the manuscript.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the fgshare repository, <https://doi.org/10.6084/m9.figshare.12581168.v2>.

Declarations

Ethics approval and consent to participate

The materials used in this study were ethically approved and consent to participate was given.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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