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Effects of zinc acquired through the plant-aphid-ladybug food chain on the growth, development and fertility of *Harmonia axyridis*

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HIGHLIGHTS

- Zinc accumulation was found at all three trophic levels in a broad bean–aphid–ladybug system.
- Accumulated zinc negatively affected growth and survival of *H. axyridis*.
- It also reduced fertility indicators, including relevant gene expression.

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ABSTRACT

Heavy metal pollution is an increasingly serious problem in agricultural ecosystems. Zinc accumulation in the food chain may harm the physiological functions of organisms, including herbivorous and predatory insects. Its effects on development and reproduction in *Harmonia axyridis* are largely unknown. In this study, five Zn solutions (25, 50, 100, and 150 mg/kg) plus control (0 mg/kg) were used to treat broad beans and to water the resulting seedlings. Aphids fed on these seedlings were eaten by *H. axyridis* ladybugs. Zn accumulation was found at all three trophic levels. Compared with the control group, ladybugs in the 25, 50, and 100 mg/kg groups had significantly reduced weight gain from the 4th instar to adulthood. Pupae and larvae (instars 1–4) in the 150 mg/kg group had the lowest survival of any group; pupal mortality in the 100 mg/kg group was significantly higher than that in the control group. Under Zn stress, female adults had inhibited expression of *Vg1*, *Vg2* and *VgR*, reducing egg production and hatchability. Zn thus negatively affected their fertility. These results provide a theoretical basis for future exploration of soil heavy metal pollution impacts in ecosystems.

1. Introduction

Zinc is a relatively common metal with an average concentration of 50 mg/kg soil and a range of 10–300 mg/kg soil (Barceloux, 1999). According to the National Standard of the People's Republic of China (GB15618-2018), when pH ≦ 6.5, the limit value of Zn pollution in agricultural land is 200 mg/kg. Soil acts as an important environmental medium and can mediate human exposure to pollutants (Davis et al., 2009; Liu et al., 2018). In China, sewage irrigation, sludge application, and mining and smelting operations for metallic ores are the dominant sources of soil metal pollution (Chen et al., 1999; Zhu et al., 2018). Statistics show that cadmium is the most critical toxic metal accumulated in Chinese agriculture soils (Wang et al., 2015; Zhao et al., 2015; Yang et al., 2019). Interestingly, Zn could be used to reduce the toxic effects of Cd in plants (Rizwan et al., 2019). In recent years, human input of heavy metals into agricultural soils has increased (Huang et al., 2015; Guan et al., 2018). Globally, the potential adverse effects of heavy metal pollution in agricultural soils has drawn increasing public attention (Lu et al., 2015; Hu et al., 2018; Wang et al., 2019). The mobility of Zn in anaerobic environments is poor, and severe Zn contamination therefore occurs primarily near point sources of Zn release

Abbreviations: qRT-PCR, quantitative real-time PCR; Vg, vitellogenin; VgR, vitellogenin receptor.

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(Barceloux, 1999). Whether natural or anthropogenic, heavy metals released into agricultural soil can adversely affect human health through the food chain (Cloquet et al., 2006; Tóth et al., 2016; He et al., 2019). For example, Zn deficiency as well as Zn excess result in severe immune system disturbances that can cause significant health problems (Maywald et al., 2017).

Zn is an essential component of thousands of plant proteins, although it is toxic in excess (Broadley et al., 2007; Noulas et al., 2018). When soil levels exceed the processing capacity of the environment, metals affect plants directly and often accumulate within them (Mo et al., 2002; Hu and Fu, 2007). Plants are thus the means of exposure for herbivores. Metals can also accumulate in higher trophic-level organisms via the food chain (Chen et al., 2010). Aphids are harmful pests and can severely damage crop plants (Rehman et al., 2014). *Harmonia axyridis* functions naturally as a biological control agent, preying on aphids and minimizing crop losses (Koch, 2003; Vandereycken et al., 2013). Such natural biocontrol of pests by insect predators is of great economic importance, yet is at risk if these predators are adversely affected by toxic metals.

Metal pollution causes genetic changes in insects, induces apoptosis, affects cell viability and proliferation, and affects their growth and reproduction (Amdam et al., 2007). Zn is one of the most important trace elements, because it participates in a variety of cellular processes including reproduction, DNA synthesis, behavioral responses, bone formation, growth, and wound healing (Barceloux, 1999). Zn concentrations above organisms' physiological limits are potentially toxic and can affect insect reproduction (Nursita et al., 2005; Augustyniak et al., 2008; Shu et al., 2009b). In oviparous animals, including *H. axyridis*, vitellogenin (Vg) is the main egg storage protein precursor, and is involved in the maturation and development of oocytes (Zhang et al., 2017). It is therefore a critical factor for insect reproduction. *Vg* genes and cDNAs have been extensively reported in many insects, including Lepidoptera, Diptera, Hymenoptera, and Hemiptera (Hatakeyama et al., 1990; Raikhel et al., 2005; Swevers et al., 2005; Raviv et al., 2006). Vg is one of the main nutrient sources incorporated into developing eggs. Vg content in the ovary depends not only on its synthesis, but also on uptake by the vitellogenin receptor (VgR) into oocytes during vitellogenesis (Zhao et al., 2018). In *H. axyridis,* Du and Zeng (2016) reported two vitellogenin genes with a similarity of only 38.73%, indicating that they are different genes.

In this study, we aimed to explore how Zn accumulates in the food chain and its toxic effects on the useful predatory insect *H. axyridis*. To achieve this goal, we simulated a natural plant–aphid–predatory insect food chain using the broad bean *Vicia faba* L., the aphid *Aphis medicaginis*, and the ladybug *H. axyridis*. Zn pollution was simulated using different concentrations of Zn solution, applied to the beans. The Zn^{2+} content of broad beans, aphids and ladybugs, and the effects of observed Zn levels on ladybug survival and reproductive parameters, were measured.

2. Materials and methods

2.1. Test insects

H. axyridis and *A. medicaginis* populations already maintained in our laboratory were used, bred at 25 ± 1 °C with a photoperiod of L14: D10, and a relative humidity of 50–75%.

2.2. Zn treatment and application through three trophic levels

$ZnCl_2$ (CAS: 7646-85-7) was diluted with water to a Zn mass ratio of 0 (deionized water only; control), 25, 50, 100, and 150 mg/kg. Broad bean seeds were soaked each of these solutions for 12 h then planted in the soil, watered using the corresponding solutions every four days. When the broad bean seedlings grew to about 4–5 cm, aphid adults were transferred onto the seedlings as the F0 generation; their offspring nymphs being considered the F1 generation. F1 aphid adults were then transferred to new broad bean seedlings (treated with the same Zn concentration), where they produced F2 nymphs; these were treated in the same way as F1 on reaching adulthood. Hatching ladybug larvae were fed with F0, F1 and F2 generation aphids and their development was observed. To ensure accurate and straightforward recording, each ladybug was placed in a 25 × 95 mm *Drosophila* vial sealed with gauze, and fed daily with more than 100 aphids.

2.3. Determination of Zn^{2+} content

Samples were collected for determination of Zn^{2+} content as follows: stems and leaves from broad bean seedlings on reaching approximately 10 cm in height; aphid adults in generations F1 to F3; and 1-day-old 4^{th} instar larvae, 1-day-old pupae, and 7-day-old adult ladybugs. Each experiment was repeated three times.

The samples were vacuum dried at 60 °C for 24 h in Pyrex test tubes. Each 500 mg dry sample was digested in 10 ml boiling 65% nitric acid (CAS: 7697-37-2) and 1 ml concentrated perchloric acid (CAS: 7601-90-3) (Baker Analyzed reagent; Baker, Deventer, Holland) (Xia et al., 2006; Shu et al., 2009a). When the fume was white and the solution was completely clear, the samples were cooled to room temperature. After filtering through filter paper, the clear solution was transferred to a volumetric flask and made up to 50 ml with deionized water. Its Zn^{2+} content was estimated using an inductively coupled plasma–atomic emission spectrometer (ICP–AES, Thermo Jarrell Ash, USA). Concentrated nitric acid and perchloric acid were used as the blank control. Zn^{2+} content was calculated as (C × 50)/500 mg, where C is the Zn^{2+} content detected by ICP-AES.

2.4. Ladybug developmental duration, weight and survival rate

Developmental duration was recorded for all larvae surviving at each growth stage. An electronic balance AL204 (Mettler Toledo, Shanghai, China) was used to measure the weight of all surviving individuals on their first day as 4^{th} instar larvae, the first day of pupation, and the first day of adulthood. Weight gain was measured as adult weight minus 4^{th} instar larval weight. For the control and the groups under 25, 50, 100, and 150 mg/kg treatments, the initial hatchling numbers were 35, 32, 35, 38, and 37, respectively, and the number of 1^{st} instar larvae used to record developmental duration and measure body weight were 34, 30, 33, 36, and 31, respectively.

2.5. Ladybug reproduction

Following completion of development into adults, the preoviposition, total eggs produced, and egg hatchability was determined for ladybug females. Males and females emerging on the same day were caged together. Each mating pair was extracted, placed in an individual plastic box, and fed aphids of the appropriate treatment type ad lib. There were 13 pairs of mating adults in the 150 mg/kg Zn treatment group, and 14 pairs in each of the other four groups. Preoviposition, being the time from emergence to oviposition, was recorded for each female adult. The resulting eggs were counted and removed into plastic boxes daily. The number of successfully hatching eggs was recorded daily, to obtain the hatching rate. We counted the number of eggs laid by a single female from day 1 to day 7.

2.6. RNA extraction and first-stand cDNA synthesis

Three fresh *H. axyridis* adults on day 4, day 7 and day 10 were respectively collected in an Eppendorf tube which was repeated 3 times Then total RNA was extracted using Trizol reagent (Thermo Scientific, Shanghai, China). Subsequently, the quality of the extracted RNA was detected by 1% agarose gel electrophoresis and RNA concentration was measured using NanoDrop™ 2000 (Thermo Scientific, Shanghai, China). First-strand cDNA was synthesized using a PrimeScript RT® with gDNA Eraser kit (TaKaRa, Dalian, China).

2.7. Quantitative real-time PCR (qRT-PCR)

The *Vg1* (Accession ID: KU761584), *Vg2* (Accession ID: KY032002) and *VgR* (Accession ID: KY032000) gene sequences were downloaded from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), and the primers for qRT-PCR were designed using Primer Premier 5 and DNA Star software (Table 1). *Ha-rp49* (*ribosomal protein 49* gene, AB552923) was used as an internal reference gene. The *Ha-rp49* forward primer was GCGATCGCTATGGAAAACTC (5′−3′) and the reverse primer was TACGATTTTGCATCAACAGT (5′−3′) (Kuwayama et al., 2014). The 20 μl qRT-PCR system consisted of 10 μl SYBR® Premix Ex Taq (TaKaRa, Dalian, China), 7 μl DEPC-treated water (Thermo Scientific, Shanghai, China), 1 μl each of forward and reverse primer, and 1 μl template cDNA. A C1000™ Thermal Cycler (BioRad, California, USA) was used to perform qRT-PCR under the following conditions: pre-denaturation at 95 °C for 3 min, 39 cycles of 10 s at 95 °C, 30 s at 57 °C, 30 s at 65 °C. The fluorescence signal was collected at 65 °C. The data were analyzed using a relative quantitative method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Results were expressed as the mean ± standard deviation (SD) or the mean ± standard error (SE) of independent replicates ($n \geq 3$). The data were analyzed using IBM SPSS statistics v20 software. Statistical significance was defined as $P < 0.05$. Tukey's test of one-way ANOVA were performed to test the significance of differences among treatments. All figures and tables were produced using Microsoft Office 2013 and SigmaPlot 10.0 software.

3. Results

3.1. Zn accumulation among the three trophic levels

As the treatment concentration increased, it was found that the Zn^{2+} content in broad bean stems and leaves also increased (Fig. 1A). According to Fig. 2B, it can be obtained that at the treatment concentration of 150 mg/kg, the Zn^{2+} content in the F1, F2, and F3 generations of aphids was higher than that at other treatment concentrations. And in the control group 0 mg/kg, the F1, F2, and F3 generations of aphids had the lowest Zn^{2+} content.

Ladybug 4th instar larvae did not differ in Zn content under different treatment concentrations. Among the pupae, the Zn^{2+} content was the highest in the 100 mg/kg group and lowest in the control group; there was no difference in Zn^{2+} content between the remaining three treatment groups. Compared with the control group, the Zn-treated adult ladybugs contained significantly more Zn, the difference being largest for the 100 and 150 mg/kg groups. Within each treatment group, the adult Zn^{2+} content of adult was the highest, followed by that of the pupae (Fig. 1C).

3.2. Effects of Zn treatment on ladybug survival

At each developmental stage, some ladybugs died in all groups, the 150 mg/kg group having the fastest reduction rate. The 100 mg/kg group had fewer deaths in the early stages, but high mortality during pupation resulted in the lowest overall survival. The 25 and 50 mg/kg groups had higher survival rates, similar to that in the control group (Fig. 2).

3.3. Effects of Zn treatment on ladybug weight and developmental duration

It can be seen from Table 2 that pupal weight exceeded adult weight, which in turn exceeded the 4th instar larval weight. Insect weight was only affected by the treatment, compared with the control group, in the 100 mg/kg group. The weights of 4th instar larvae and adults in the 100 mg/kg group were significantly lower than those in the control group (Table 2). Ladybug weight gains between day 1 of the 4th instar and day 1 of adulthood in the 25, 50, and 100 mg/kg groups were significantly lower than that in the control group (Fig. 3).

There was no significant difference in the development stage lengths between treatment and control for the 25 and 150 mg/kg treatments. In the 50 mg/kg group, the periods spent as 1st instar larvae and pupae, and between 1st instar day 1 and adult day 1, were significantly shorter than those in the control group. In the 100 mg/kg group, only the pupal stage was significantly shorter than in the control group (Table 3).

3.4. Effects of Zn treatment on ladybug fertility

The preoviposition time in the 150 mg/kg group was significantly longer than that in the control group, and that in the 50 mg/kg group was the shortest (Fig. 4A). All Zn-treated females except for the 150 mg/kg group laid significantly fewer eggs than the control group (Fig. 4B). The egg hatching rates in the 25 and 50 mg/kg groups did not differ from that in the control group; those in the 100 and 150 mg/kg groups were significantly lower than that in the control group (Fig. 4C).

3.5. Effects of Zn treatment on the expression levels of genes

The internal control *Ha-rp49* was clearly expressed in female ladybugs on days 4, 7 and 10 following adult emergence (Fig. 5D). Other than for the vitellogenin receptor gene *VgR* in the 150 mg/kg Zn-treated group on day 7, the relative expression levels of *Vg1*, *Vg2*, and *VgR* on days 4 and 7 were significantly lower than those in the control group (Fig. 5). On day 10, the relative expression levels of

Table 1
Primers for *Vg* and *VgR* genes of *Harmonia axyridis* used in qRT-PCR.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
HaVg1	GCAACAGAGTCCGTGGTCTTT	GCTGCTTTCACCGTTCTTCAA
HaVg2	CAATCAAAACTCAAGCAAGGAGA	GTCAAAAACTGGATGGACAACAA
HaVgR	TGTAGGAGGCGAAGCAATGAT	TGGGATGTGACAGGGAAATAA

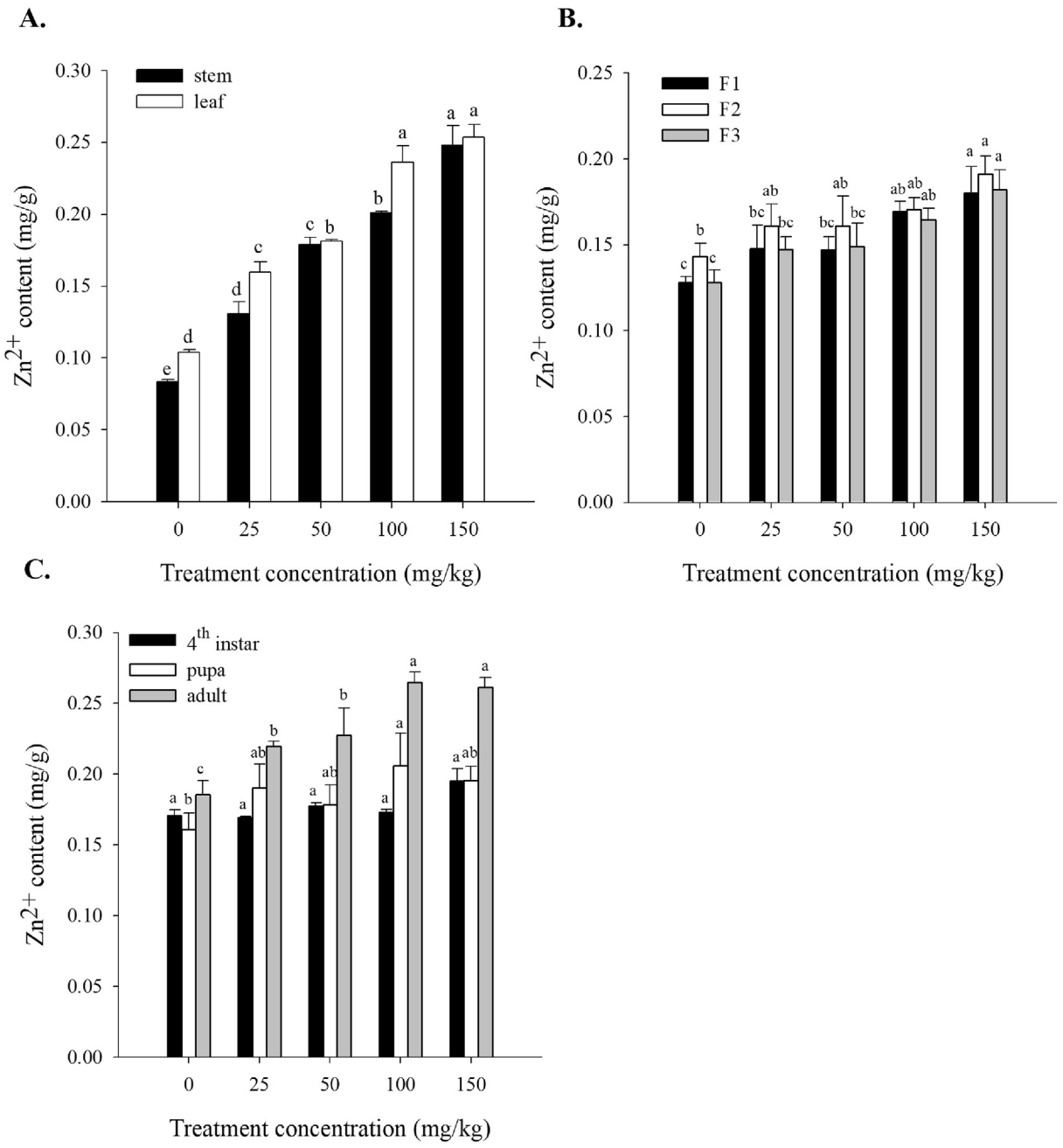

Fig. 1. Zn accumulation in *Vicia faba* stem and leaf, the F1–F3 generations of *Aphis medicaginis*, and the 4th instar larvae, pupae, and adults of *Harmonia axyridis*. (A) *V. faba* (broad bean) seeds were soaked for 12 h in aqueous Zn solutions at concentrations of 0 (deionized water only; control), 25, 50, 100, and 150 mg/kg, planted in soil and then watered with the corresponding solutions. The Zn^{2+} concentrations in the stem and leaf at 10 cm plant height were determined. (B) *A. medicaginis* adults were transferred onto 4–5 cm *Vicia faba* seedlings as the F0 generation. Subsequent F1 to F3 generation adults were collected for Zn^{2+} content determination. (C) F1 to F3 generation aphid adults were fed to hatching larva of *H. axyridis*. We collected 1-day-old 4th instar larvae, 1-day-old pupae, and 7-day-old adults for determination of Zn^{2+} content. Results shown are means (±SD) of three replicates. Different lowercase letters indicate significant differences at the $P < 0.05$ level from Tukey's test, with a > b > c > d.

Vg1 and *VgR* in the 100 mg/kg group were significantly lower than those in the control group, whereas those in the other three treatment groups were significantly higher. In addition, the expression levels of all three genes were higher on the 10th day than on the 4th day.

4. Discussion

4.1. Bioaccumulation of Zn in plants-aphids-ladybugs

Xie et al. (2014) found that broad bean seeds and seedlings could accumulate Zn following treatment. Similar results were obtained in this study (Fig. 1A), indicating that the treatment of heavy metal stress in this study was successful, and the subsequent experimental results were reliable.

In the published literature, there are several studies involving the transfer of heavy metals through soil–plant–insect food chains. The content of Zn and Cu increases slightly with the rising trophic level (Zhuang et al., 2009). Blasco et al. (2019) found that 100 μm $ZnSO_4$ treatment caused significant accumulation of Zn in plant leaves and roots, compared to controls. Thus, the consensus is that plants exposed to high Zn levels will accumulate it. Root levels are

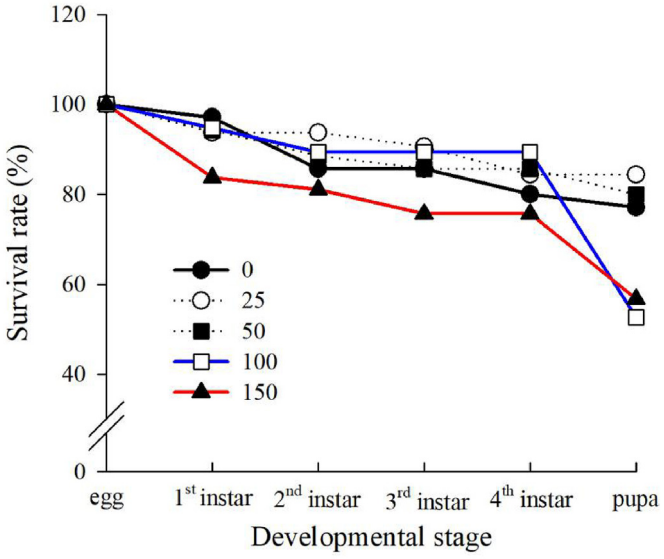

Fig. 2. Percentage survival of *Harmonia axyridis* under different Zn treatments.

Table 2
Weight of *Harmonia axyridis* under different Zn^{2+} concentrations.

Zn^{2+} concentration (mg/kg)	Weight(mg)		
	4th instar	Pupa	Adult
0	9.7 ± 0.32 ab	21.8 ± 0.66 a	19.1 ± 0.60 a
25	10.1 ± 0.34 a	21.2 ± 0.68 a	18.0 ± 0.56 ab
50	9.9 ± 0.39 a	20.8 ± 0.61 a	17.7 ± 0.55 ab
100	8.5 ± 0.28 b	19.5 ± 0.61 a	16.6 ± 0.45 b
150	9.3 ± 0.37 ab	21.5 ± 0.64 a	18.6 ± 0.52 ab
F	3.97	1.75	2.80
df	151	128	123
P	0.004	0.14	0.029

Results are expressed as the mean ± SE. Different lowercase letters indicate significant differences at the $P < 0.05$ level from Tukey's test, with a > b > c.

typically higher than that of aboveground parts, as shown by many authors (e.g. Green et al., 2010; Gupta et al., 2016; Dar et al., 2017; Blasco et al., 2019; Song et al., 2019). Although root Zn accumulation was not measured in this study, stem Zn levels were found to be higher than leaf levels (Fig. 1A). This is consistent with the results of Malecka et al. (2019), who found that mustard stems accumulated more Zn than leaves. Differential Zn accumulation in different plant organs has been explored in the published literature. Firstly, it is related to plant defense. Stolpe et al. (2017) has reported that heavy metal super-enrichment in phloem may be effective against aphids, and excessively metal-enriched plants can specifically increase heavy metal concentration in response to the attack of herbivores that suck on the phloem, as evidenced, for example, by the higher concentration of heavy metals in young leaves compared to old leaves. Secondly, it is related to the transport of Zn in plants. Gupta et al. (2016) describes the relevant processes, in which Zn is taken up mainly as the divalent cation (Zn^{2+}) by plant roots, driven by hyperpolarization of RCPM, which is mediated by the activity of the RCPM H^+-ATPase system. Zn then passes through the epidermis, cortex, endodermis and pericycle before reaching the xylem. Translocation continues via the symplastic and apoplastic routes; once Zn enters the phloem, further translocation to various plant organs and developing sinks is mediated. Moreover, mobility of Zn is higher in phloem than xylem (Gupta et al., 2016).

Aphid *Sitobion avenae* can absorb metals from wheat plants,

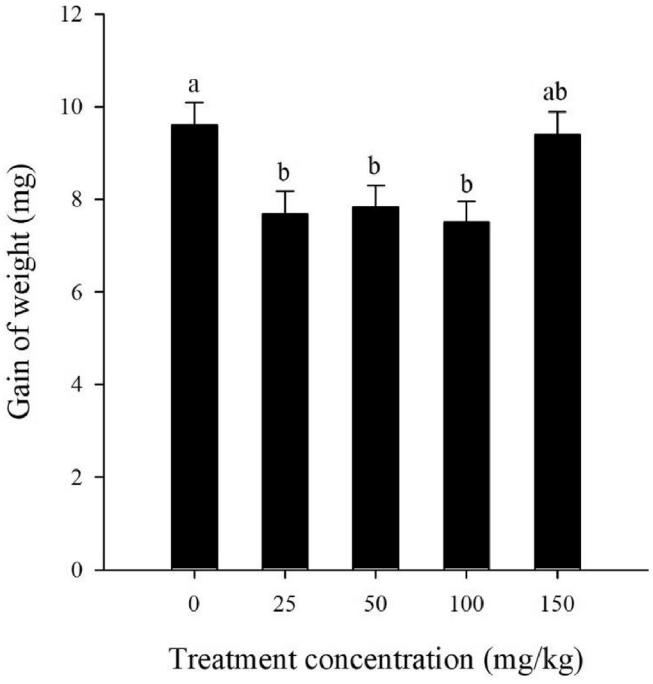

Fig. 3. Weight gain of *Harmonia axyridis* under different Zn treatments. Weight gain was measured as the adult weight (day 1) minus the 4th instar larval weight (day 1). Results shown are means (±SD) of three replicates. Different lowercase letters indicate significant differences at the $P < 0.05$ level from Tukey's test, with a > b.

including Zn and Cd (Green et al., 2003). Our results showed that Zn in broad beans can be transferred to and accumulated in aphids (Fig. 1B). Dar et al. (2017) conducted experiments on the transfer of Zn and Cd in the food chain and found that when mustard is treated with Zn^{2+} solution, aphids and ladybugs also accumulate heavy metals in their bodies. A similar study by Green et al. (2010) found that Zn content in aphids fed on wheat exposed to Zn was higher than in the control group. In addition, the present study found that the accumulation of Zn^{2+} in aphids was related to the treatment concentration, and that there was little difference across generations (Fig. 1B). Indeed, Zn was freely transferred from the shoots to aphids and there was a consistent level of biomagnification in all the aphid populations (Green et al., 2003, 2005, 2006, 2010; Green and Tibbett, 2008). We speculate that this is related to the rapid reproduction and overlapping generations of aphids.

Feeding on contaminated host plants can cause harmful metals to accumulate in aphid body tissues and affect absorption through the food chain (Zhuang et al., 2009). The results obtained by Naikoo et al. (2019) showed that the lead accumulated in newly emerged adult ladybugs increased with rising treatment concentration. This is very similar to our results (Fig. 1C). Varying Zn^{2+} content in ladybugs at different developmental stages is related to the stage-related discharge of toxic heavy metals from insects (Dar et al., 2015; Zhou et al., 2015; Sang et al., 2018). However, their experimental results differ from those obtained in this study. For example, Dar et al. (2015) found that the Zn^{2+} level in adults is lower than that in the pupal stage because accumulated heavy metals are eliminated when the pupae are discarded during emergence (Dar et al., 2015); Luo et al. (2019) found that, of its growth stages, *Ostrinia furnacalis* larvae had the highest accumulated Cd from their food, followed by pupae and adults. In our study, the accumulation of Zn^{2+} in 4th instar larvae and pupae was less obvious than that in adults (Fig. 1C). This difference may be caused by differences in the accumulation of heavy metals between recently emerged and

Table 3
Developmental period of *Harmonia axyridis* under different Zn^{2+} concentrations.

Zn^{2+} concentration (mg/kg)	Developmental stage (day)						
	1st instar	2nd instar	3rd instar	4th instar	Pupa	1st-adult	Egg (F_2)
0	3.1 ± 0.6 a	1.9 ± 0.3 a	2.1 ± 0.4 ab	4.6 ± 0.7 ab	3.8 ± 0.2 a	15.3 ± 1.0 ab	2.3 ± 0.3 a
25	2.9 ± 0.6 a	1.8 ± 0.4 a	2.0 ± 0.4 b	4.6 ± 0.5 ab	3.7 ± 0.2 ab	15.0 ± 1.1 bc	2.4 ± 0.2 a
50	2.6 ± 0.6 b	1.9 ± 0.3 a	2.0 ± 0.3 b	4.3 ± 0.5 b	3.6 ± 0.2 b	14.3 ± 0.9 c	2.4 ± 0.3 a
100	3.1 ± 0.6 a	2.0 ± 0.4 a	2.3 ± 0.4 a	4.8 ± 0.7 a	3.6 ± 0.2 b	15.6 ± 1.2 ab	2.5 ± 0.3 a
150	3.2 ± 0.7 a	2.0 ± 0.3 a	2.3 ± 0.4 a	4.9 ± 0.5 a	3.7 ± 0.2 ab	15.9 ± 0.9 a	2.6 ± 0.2 a

Results are expressed as the mean ± SD. Different lowercase letters indicate significant differences at the $P < 0.05$ level from Tukey's test, with a > b > c.

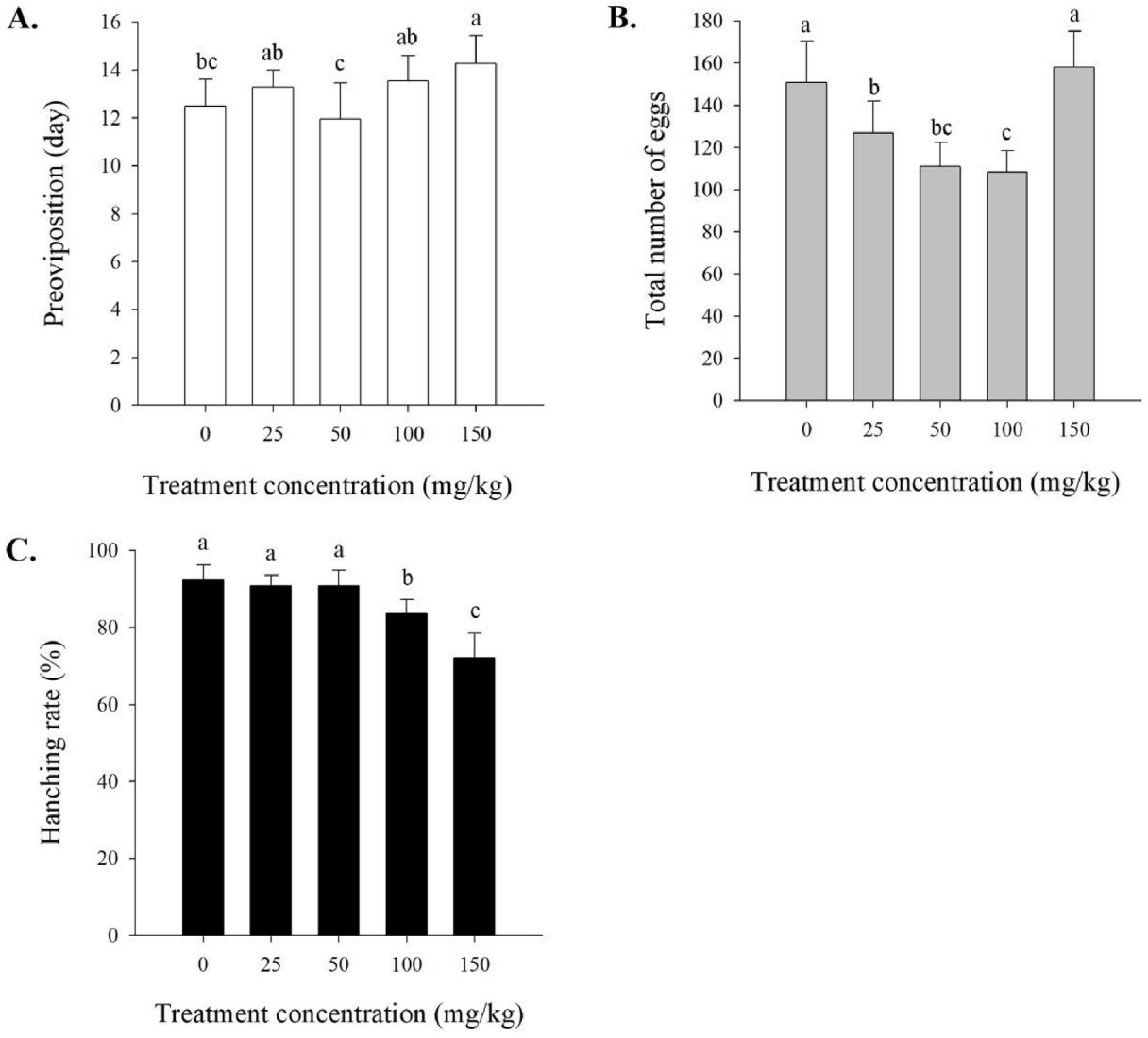

Fig. 4. Effects of Zn treatment on the fertility of *Harmonia axyridis*. (A) The duration of preoviposition in female adults; (B) the total number of eggs per female; (C) the hatching rate (%) of eggs. Results shown are means (±SD) of three replicates. Different lowercase letters indicate significant differences at the $P < 0.05$ level from Tukey's test, with a > b > c.

mature adults. Zhou et al. (2015) explained the mechanism of Pb removal by the silkworm (*Bombyx mori*) as loss of Pb absorbed into the gut epithelium following cellular renewal in the alimentary tract and loss during metamorphosis, noting that Pb which has penetrated into other tissues through the gut epithelium is retained. This mechanism could result in varying bioaccumulation rates for different metals, depending on their toxicity and retention capacity, and on the method by which the insects acquire the heavy metals (Butt et al., 2018). Further experiments are needed to investigate this possibility.

4.2. Effects of heavy metals on insect growth and development

Toxic heavy metals can affect insect survival. Kafel et al. (2014) have shown that the survival rate of *Spodoptera exigua* exposed to Cd and Zn was significantly reduced. The results of our study are

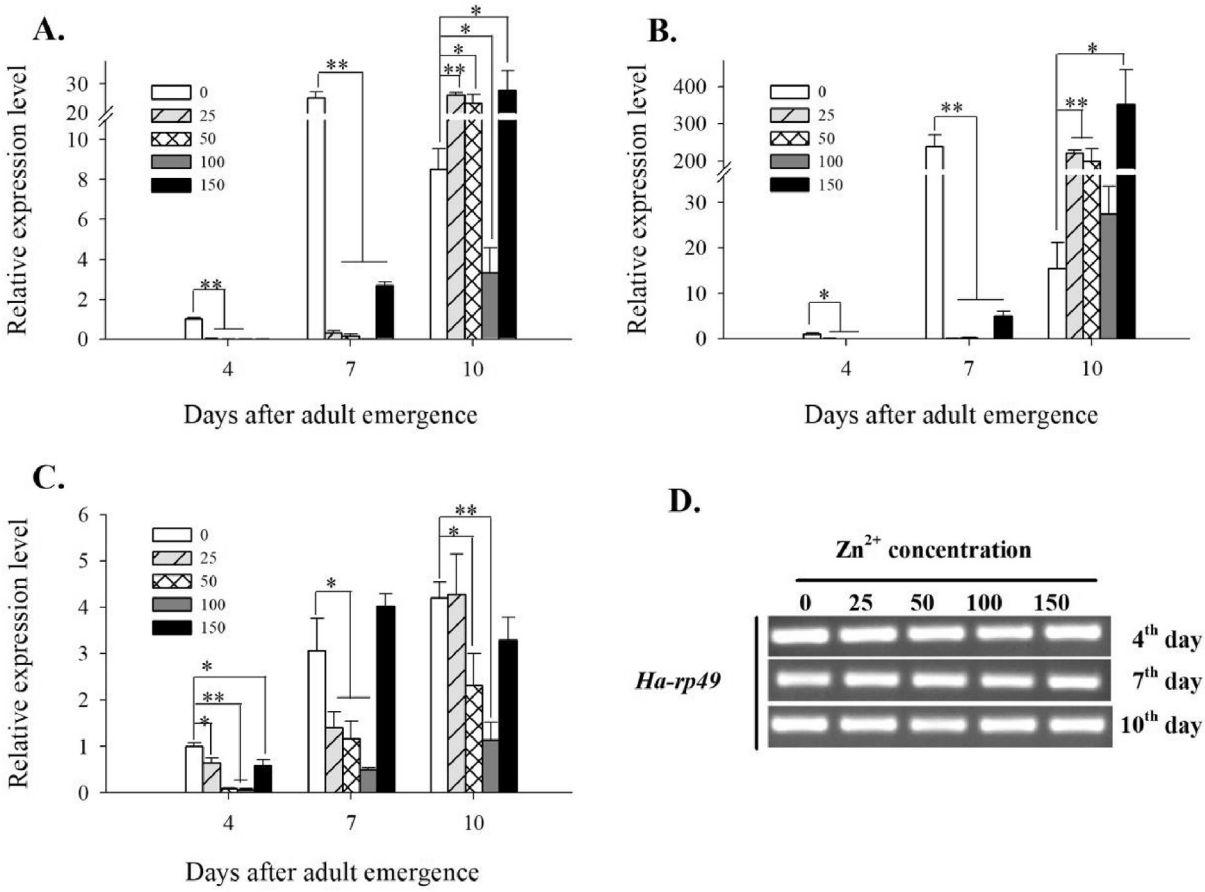

Fig. 5. Effects of Zn treatment on vitellogenin-related gene expression in *Harmonia axyridis*. Relative expression levels of *Vg1* (A), *Vg2* (B), and *VgR* (C) genes at days 4, 7, and 10 after emergence of female adults; and (D) agarose gel electrophoresis image of the reference gene *Ha-rq49*. Results shown are means (±SD) of three replicates. $^{**}P < 0.01$,$^{*}P < 0.05$ (Tukey's test).

surprising, in that larval survival was higher for the 25 mg/kg group and the 100 mg/kg group than in the control group. During pupation, significantly more ladybug deaths occurred in the two high-concentration (100 and 150 mg/kg) groups (Fig. 2). Multiple experiments have found that *Cryptolaemus montrouzieri* has a higher mortality rate at the beginning and later stages of the experiment due to heavy metal stress and the end of the individual's own life (Xia et al., 1999; Sana-Ullah et al., 2011; Green and Walmsley, 2013; Sang et al., 2018). All insects have a detoxification system, but this system cannot detoxify heavy metals at higher concentrations (Safaee et al., 2014). Al-Momani and Massadeh (2005) speculated that higher concentrations of heavy metals can cause the expression of metallothionein to be impaired, resulting in the accumulation of a large number of metal in the body, and ultimately adversely affecting insect health. In summary, different concentrations of the heavy metal Zn have different degrees of influence on the survival potential of *H. axyridis*, and appropriate concentrations are beneficial to their survival.

Heavy metals absorbed by insects have a significant impact on the insects' growth rate, survival and physiology (van Ooik et al., 2007; Baghban et al., 2014). In this study, we found that Zn did not significantly change the developmental duration of *H. axyridis*, but, importantly, did slightly reduce the periods spent as first-instar larvae and pupae, and between 1st instar day 1 and adult day 1, compared to the control group (Table 3). According to Mirčić et al. (2013) the effect of heavy metals on the developmental period of insects is affected by age, For example, when *Lymantria dispar* L. larvae were given feed containing Cd, 4th, 5th, and 6th instar larvae showed delayed development and 3rd and 4th instar larval weights were reduced. Prolongation of insect larval and pupal development times may be explained as follows. Firstly, heavy metals are directly toxic to larvae (Ding and Wang, 2006). Secondly, heavy metals affect the fetus inside the egg (Safaee et al., 2014). Thirdly, heavy metals can reduce the space between mitochondrial cristae, which can cause reduced oxidative phosphorylation and ATP synthesis (El-Sheikh et al., 2010), which in turn affects pupal morphogenesis and organogenesis. It is likely that almost no effect on the developmental period of *H. axyridis* was seen in the present study because the heavy metal concentration was too low, or Zn specifically is not very toxic to this species.

In fact, in response to a polluted environment, larval weight is more important to survival than the growth rate (van Ooik et al., 2007). In this study, in order to eliminate the differences between individual ladybugs, the weight gain from the 4th instar to the adult stage was calculated. On this basis, the treated insects gained less weight than the control insects (Fig. 3). This is consistent with other similar studies, such as that of Płachetka-Bożek et al. (2018b), in which *Spodoptera litura* exposed to high concentrations of Cd showed a significant reduction in larval weight. In *Coccinella transversalis*, adult body weight decreased after Pb treatment (Naikoo et al., 2019). The study of van Ooik et al. (2007) found that the larval relative growth rate and pupal weight of *Epirrita autumnata* on birch trees contaminated with heavy metals was significantly lower than those on uncontaminated birch trees.

These results may be related to the additional energy demands of metal detoxification, which is reflected in certain trait changes, such as weight loss (Morkunas et al., 2018). In addition to weight, the size of other parts of the insect's body, such as the elytra, the tibia and the back of the femur, are affected (Maryański et al., 2002).

4.3. Effects of heavy metals on insect fertility

Published papers show that heavy metal pollution can reduce the fertility of insects (Culliney and Pimentel, 1986; Sang et al., 2018; Peterson et al., 2019), which is manifested in prolonged preoviposition, shortened egg-laying periods and reduced egg numbers (Xie et al., 2014; Bixler and Schnee, 2018; Płachetka-Bożek et al., 2018a; Sang et al., 2018; Hu et al., 2019). Almost identical results were obtained in our study in the 25 and 100 mg/kg concentration groups (Fig. 4A and B). Moreover, Shu et al. (2009b) found that the fecundity and hatchability of female adults of *Spodoptera litura* decreased at high Zn concentrations. It can be concluded that heavy metals can delay the synthesis of vitellogenin peptides, thus delaying ovarian maturation and inhibiting vitellogenesis (Cervera et al., 2005, 2006). The observed expression levels of *Vg1*, *Vg2*, and *VgR* in this study corroborate this at the molecular level (Fig. 5). Many studies report that the fertility of *Spodoptera litura* exposed to Zn, Pb or Cd causes significant inhibition of *Vg* gene expression in females (Shu et al., 2009a, 2009b; Zhao et al., 2016; Płachetka-Bożek et al., 2018a). A study also found that *Vg* gene expression levels in both the Cd-treated and control groups were time-dependent (Płachetka-Bożek et al., 2018b), which is consistent with the results of our study (Fig. 5). It is worth mentioning that the gene expression level on the 10^{th} day was found to be higher than that on the 4^{th} day (Fig. 5). This should be a normal phenomenon, because with the extension of development time, females show specificity for gene expression, which increases first and then decreases (Zhao et al., 2018). Previous experiments also explored the reduction of *Vg* expression due to Zn in the second trophic level species *A. medicaginis* (Xie et al., 2014). In fact, in addition to its effects on *Vg*, Cd exposure may negatively affect the expression of genes related to reproduction in *Drosophila melanogaster* (*enok, dally* and *dpp*) and trigger the transcription of defense-related genes (*hsp70*, *gstd2*, and *gstd6*) (Hu et al., 2019). Clarification of the molecular mechanisms operating in insects relating to heavy metals requires further investigation.

Surprisingly, the females in the 50 mg/kg group laid eggs before the other four groups (Fig. 4A). Given the developmental calendar discussed earlier, we speculate that 50 mg/kg of Zn^{2+} will shorten the developmental period at some stages, including the preoviposition period. *Ostrinia furnacalis* given food containing Cd was shown to lay eggs at a similar rate to that of the control group, but the hatching rate was significantly reduced (Luo et al., 2019). This result is in agreement with the results for the 150 mg/kg group in this study (Fig. 4B and C). We speculate that in order to cope with the emergency situation of substantial heavy metal stress, females increase the probability of survival of offspring by laying a large number of eggs, but the quality of these eggs is poor. Luo et al. (2019) found that after one or both parents were treated with Cd, the hatching rate was significantly lower than that in the control group. This shows that both the maternal and paternal gametes may be damaged by heavy metals. Sun et al. (2016) reported that high concentrations of nickel (Ni) can damage the quality of *Spodoptera litura* sperm bundles. In addition, Osman et al. (2015) found that exposure to Cd can cause deformation of chromatin in oocytes, leading to necrosis and shape changes, which can damage the *Blaps polycresta* female gonad. It has also been suggested that Cd can affect vitellogenin formation, resulting in reduced vitellogenin synthesis and egg production (Płachetka-Bożek et al., 2018a). Overall, the heavy metal Zn has a negative effect on the fertility of *H. axyridis*.

5. Conclusions

In summary, when exposed to Zn, broad beans, aphids, and ladybugs in the food chain accumulated Zn. The accumulated Zn has a lesser impact on the developmental period of *H. axyridis* than on its weight and survival. Finally, Zn negatively affects fertility by reducing egg production and hatching rate and the expression level of the *Vg1* and *Vg2* vitellogenin genes and the vitellogenin receptor gene *VgR*. Our results provide a theoretical basis for future exploration of soil heavy metal pollution on ecosystems. Further experiments are needed to explore the excretion system of aphids and ladybugs on Zn, so as to reveal a clearer response mechanism of heavy metals.

Authorship contribution statement

Zuokun Shi: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Investigation. Shasha Wang: Methodology, Formal analysis, Writing - original draft, Writing - review & editing. Biying Pan: Methodology, Software, Data curation, Writing - original draft, Writing - review & editing. Yongkang Liu: Methodology, Investigation. Yan Li: Methodology, Investigation, Validation. Shigui Wang: Resources, Writing - review & editing, Supervision. Su Wang: Conceptualization, Visualization, Supervision. Bin Tang: Validation, Visualization, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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