AUTHOR(S):

OSAWA, Naoya

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ORIGINAL ARTICLE

Provision of small sterile eggs is a circumstance-dependent maternal investment in sibling cannibalism in the ladybird beetle *Harmonia axyridis* (Coleoptera: Coccinellidae) *

Naoya OSAWA (ID)

Laboratory of Forest Ecology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan;
e-mail: osawa.naoya.3z@kyoto-u.ac.jp

Abstract. Many aphidophagous ladybird beetles lay clusters of eggs and sibling cannibalism occurs at hatching. Larvae that hatch early tend to cannibalize undeveloped eggs and those that hatch late. The cannibalized eggs, especially those that are sterile, represent a maternal investment in the cannibal and are regarded as "trophic" eggs, which increase their chances of surviving. The characteristics of cannibalized eggs, however, are poorly studied. In this study, I determined the sizes of eggs that developing and undeveloped eggs within egg clusters of *Harmonia axyridis* (Coleoptera: Coccinellidae). Developing eggs were significantly larger (on average 3.45% larger) than the undeveloped sterile ones, which were mainly small. Furthermore, the hatchability of eggs was also significantly associated with their size, inclusive of the maternal effects. These results indicate that the production of relatively small sterile eggs functions as circumstance-dependent maternal investment in improving the survival of the first instar larvae to hatch in each egg cluster.

INTRODUCTION

Temporal variation in reproductive investment, i.e., maternal egg provisioning, has a substantial effect on offspring fitness. Therefore, the subject has received much attention from evolutionary biologists. Maternal egg provisioning results in the production of eggs of different sizes, which directly influence the performance of offspring in many taxa (e.g., Ameri et al., 2019).

The egg is the starting material for embryogenesis and the size of a hatchling is directly related to the size of an egg at fertilization (Polilov, 2015). It is suggested that in ladybird beetles egg size is possibly constrained by the minimum size at which the first instar larvae can capture active prey and complete their development (e.g., Stewart et al., 1991). This finding suggests that egg size is determined by the availability of food for offspring. In addition, egg size is likely to be the least variable reproductive trait and cluster size the most variable (Dixon & Guo, 1993). Therefore, variations in egg size have consequences for the fitness of females.

Rather than universal allometric constants, the place where eggs are laid is more important in the evolution of insect egg size and shape (Church et al., 2019). The study by Church et al. (2019) indicates that egg size is not correlated with adult body size, but the evolution of parasitism and aquatic oviposition helps explain the diversity in the size and shape of insect eggs among species. Thus, the biotic and abiotic environmental factors at the oviposition site may affect the size and shape of insect eggs.

In general, the adaptive function of trophic egg-laying is regarded as an extended parental investment in offspring (e.g., Alexander, 1974; Polis, 1981). The maternal investment per offspring should be ideally equal between small offspring with trophic eggs and large offspring without any additional parental investment within species (e.g., Baur, 1990; Dixon, 2000). However, the evolutionary factors promoting trophic egg-laying, instead of larger eggs, are still not fully understood.

Noriyuki et al. (2012) theoretical study of maternal fitness identified two alternative strategies: (1) production of small offspring with trophic eggs, and (2) production of large offspring without any additional investment. These authors conclude that small egg size with trophic egg-laying is favoured in heterogeneous environments where mothers cannot adjust egg size. Moreover, Noriyuki et al. (2012) assume that both trophic and viable eggs are the

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same size because no general trend in size is reported for ladybird beetles.

All females provision their eggs with nutrient-rich yolk, but the degree of egg provisioning varies dramatically within and across species (Balshine, 2012). Young born in challenging environments that hatch from large eggs have a higher probability of survival than those from small eggs (Nager & van Noordwijk, 1992). However, not all females of a species produce eggs of the same size and egg size may be influenced by many factors, including clutch size, female's phenotypic quality, environmental conditions such as food availability and density, and the predictability of the environmental conditions (Smith & Fretwell, 1974; Christians, 2002; Kindsvater et al., 2011).

Trophic eggs are mainly reported in eusocial insects (e.g., Sakagami, 1982; Hölldobler & Wilson, 1990; Crespi, 1992), but also in some non-social insects, Coleoptera (e.g., Crespi, 1992; Perry & Roitgerg, 2006), Neuroptera (Henry, 1972), Orthoptera (West & Alexander, 1963) and Heteroptera (Hironaka et al., 2005; Kudo et al. 2006). These trophic eggs usually lack micropyles (e.g., Gobin et al., 1998; Kudo et al., 2006). However, in the ladybird beetle *Harmonia axyridis* (Coleoptera: Coccinellidae), both developing and undeveloped eggs have micropyles (Osawa & Yoshinaga, 2009), which indicates that mechanisms involved in the production of "trophic" eggs in *H. axyridis* are different from those in other taxa. Many authors have reviewed this type of sibling cannibalism (e.g., Stevens, 1992; Hodek & Honěk, 1996; Dixon, 2000; Perry & Roitberg, 2006). For the latter developing eggs the altruistic behaviour of being a victim is seen to be beneficial when the victim is cannibalized by a full sibling when aphids are scarce (Osawa, 1992a). Furthermore, there are theoretical studies on the role of sterile eggs, particularly in terms of female resource provisioning (Noriyuki et al., 2012) and that related to the existence of male-killers (Noriyuki et al., 2016). However, characteristics of sterile eggs are less documented.

In this study, I recorded differences in developing and undeveloped eggs in terms of egg size.

MATERIALS AND METHODS

Ladybird beetles and eggs

Eggs of *H. axyridis* were obtained from a total of 10 females collected in the Botanical Garden of Kyoto University, Kyoto (135°47′E, 35°02′N) in May 2008. Larvae were reared to the adult stage in plastic cups (13 cm wide, 10 cm high) at 25°C, 16L : 8D and ca. 70% relative humidity. To eliminate the possible effects of food on egg and cluster size, the larvae were fed with a surplus of frozen *Ephestia kuehniella* Zeller eggs (Beneficial Insectary® Inc., Redding, CA). Newly emerged and unmated *H. axyridis* females (n = 27) and males (n = 27) were randomly chosen, and each pair was individually reared in a plastic Petri dish with a surplus of frozen eggs at 25°C, 16L : 8D, and ca. 70% relative humidity. The body length (to the nearest 0.01 mm) of all the females and males was measured using digital callipers (Mitutoyo® CD67-S15PS). All the egg batches were coded for each female and 10 to 20 eggs from each of five different clutches laid by each female were detached from an egg cluster and put on a filter paper with ca. 1 cm of tape glue using a tiny brush, indi-

vidually marked and kept at 25°C, 16L : 8D and ca. 70% relative humidity. When the number of eggs in a clutch was fewer than 10, then all eggs in that clutch were measured. Both egg height (*h*) and width (*r*) were measured using a micrometre attached to a stereo microscope (Zeiss® SV-11 Apo) to the nearest 0.025 mm. Eggs were checked daily for five days and developmental conditions were noted as either: (1) developing with an embryo with blackish colour inside and those with the head of a larva emerging from an egg shell (i.e., hatched egg); (2) undeveloped without an embryo and of a pale-yellow colour (Osawa & Yoshinaga, 2009). Egg size was estimated using the equation $hr^2\pi/6$ (μl) (Takakura, 2004). For all the eggs, the egg hatchability was checked daily and recorded. After checking egg size, all the female adults were dissected under a stereo microscope and their number of ovarioles recorded.

Elimination of the effect of male-killer on hatchability

Male eggs infected by male-killing bacteria are killed and the male embryos are present in the undeveloped eggs of *H. axyridis* (e.g., Majerus et al., 1998; Noriyuki et al., 2014). Thus, the hatchability of eggs could be strongly influenced by a male-killer infection. In fact, the theoretical and empirical studies indicate that cluster size is larger, egg size smaller and proportion of "trophic" egg is lower in male-killer infected females of *H. yedoensis*, i.e., a sibling species of *H. axyridis*, than in uninfected females (Noriyuki et al., 2016). Therefore, in order to clarify the role of female maternal investment via undeveloped eggs for offspring in *H. axyridis* the male-killer effect should be eliminated.

To eliminate the effect of male-killer bacteria in the present study, hatching percentages of eggs laid by individual females reared in 2008 were compared with that of other field-collected females of *H. axyridis*. These females either produced only female progeny (i.e., were infected), or both male and female progeny (i.e., were uninfected). Females (n = 9) of *H. axyridis* were collected form the Botanical Garden of Kyoto University, Kyoto (135°47′E, 35°02′N) from April to June, 2010 and 2012, and were individually reared to check the hatchability of their eggs. Six females produced male and female progeny and three only female progeny. In total, 94 egg batches (male-killer uninfected, n = 76; male-killer infected, n = 18) were examined and the hatchability of the eggs of male-killer infected *H. axyridis* females was recorded (mean ± S.E., 27.2094 ± 7.0261, 95% CL Upper 41.5%, n = 18). Therefore, females with a hatchability less than 41.5% were regarded as infected with male-killer and not included in the analysis of egg size.
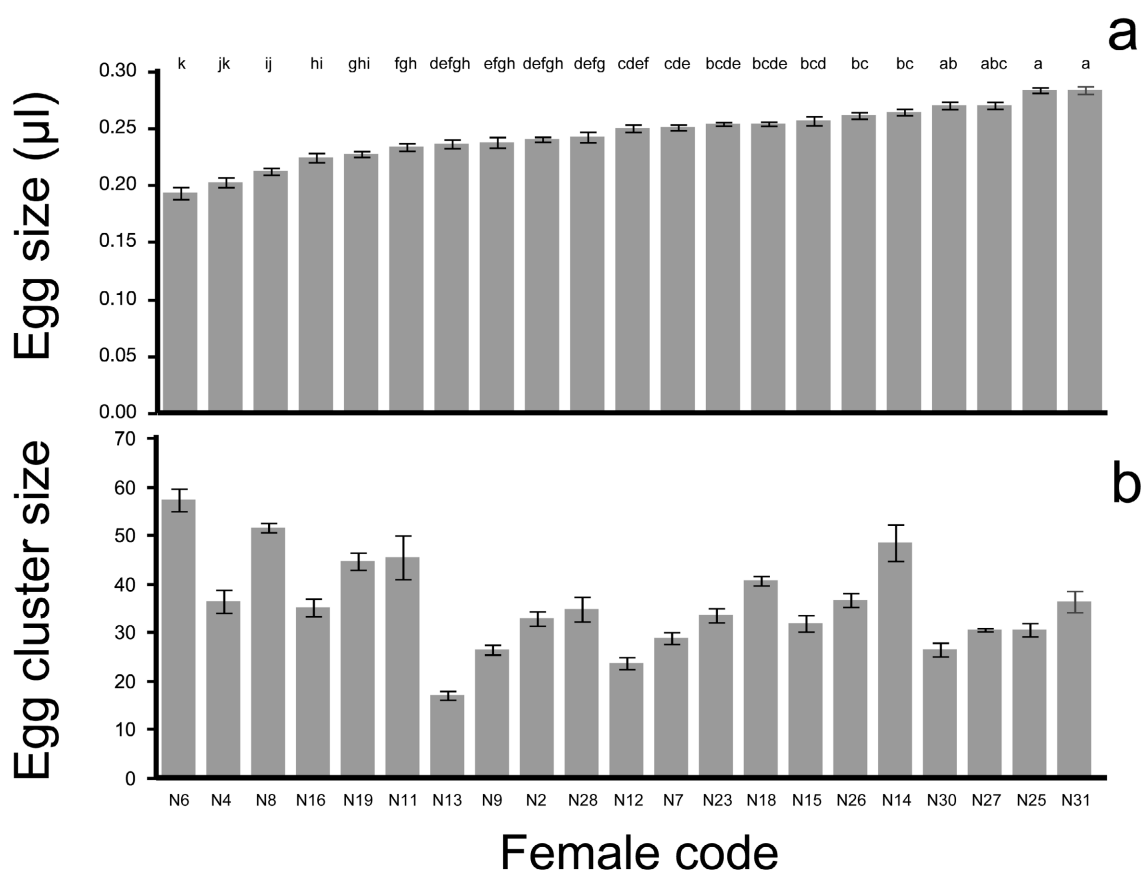
In total, 933 *H. axyridis* eggs (802 developing eggs, among which 689 hatched, and 131 undeveloped eggs) derived from 21 of the 27 females collected in 2008 were used in the following analysis, four females were excluded because they were infected with male-killer and two because they laid fewer than five egg clusters.

Egg size and hatchability

To evaluate the relationship between egg size and hatchability, egg size was divided into three categories: (1) large, ≥ 0.2677 μl, 25% of all the eggs; (2) medium, ≥ 0.2266 μl, < 0.2677 μl, 50%; (3) small, < 0.2266 μl, 25%.

Statistical analysis

One-way analysis of variance (ANOVA) and the Tukey-Kramer HSD test were used to analyse egg size and egg cluster size per female. *T*-tests were used to analyse egg size of developing and undeveloped eggs. Nominal logistic regression analysis was used to analyse the effect of a particular mother (code) and egg size on hatchability of eggs because egg size differed for each female (see Fig. 1). The likelihood ratio chi-square test was used

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Fig. 1. The average egg sizes (a) and egg cluster sizes (b) of each the females of *H. axyridis* studied. Vertical lines indicate S.E. Different letters indicate statistical difference at *P* = 0.05 level of significance based on Tukey-Kramer HDS.

to analyse the number of developing and undeveloped eggs per egg size class. Linear regression analysis was used to reveal the relationships between (1) female body size and total number of ovarioles per female, (2) female body size and egg size and, (3) number of ovarioles per female and minimum size of eggs that hatched per female. All statistical analyses were conducted using JMP® Discovery Software (SAS, 2018).

RESULTS

Mean egg size differed significantly among females (one-way ANOVA: $F_{20,932} = 48.3697$, $R^2 = 0.5147$, $P < 0.0001$) (Fig. 1a). The largest mean egg size for an individual female (mean ± S.E. = 0.2837 ± 0.0004 µl in female code N31) is 46.77% larger than the smallest mean egg size (0.1933 ± 0.0003 in N6) (Fig. 1). However, mean cluster size was only marginally significantly different among females (one-way ANOVA: $F_{20, 95} = 1.5383$, $R^2 = 0.2909$, $P = 0.0935$) (Fig. 1b).

Size of developing (mean ± S.E. = 0.2456 ± 0.0012 µl, $n = 802$) and undeveloped eggs (0.2374 ± 0.0029, $n = 131$) (*t*-test; $t = 2.6580$, $df = 931$, $P < 0.0080$) differed significantly with the developing eggs 3.45% larger than undeveloped eggs (Fig. 2). Including the effect of females (i.e., the female code), the hatchability of eggs was also significantly associated with egg size (nominal logistic regression: $\chi^2 = 123.3775$, $df = 41$, $R^2 = 0.1630$, $P < 0.0001$ for the whole model; mother code: $df = 20$, likelihood ratio $\chi^2 = 68.1058$, $P < 0.0001$; egg size [mother code]: $df = 21$, likelihood ratio $\chi^2 = 39.4994$, $P = 0.0086$).

The sizes of developing and undeveloped eggs differed significantly, with a higher percentage of large eggs developing than small eggs (The likelihood ratio chi-square test for number of eggs: $\chi^2 = 7.3910$, $P = 0.0248$) (Fig. 3).
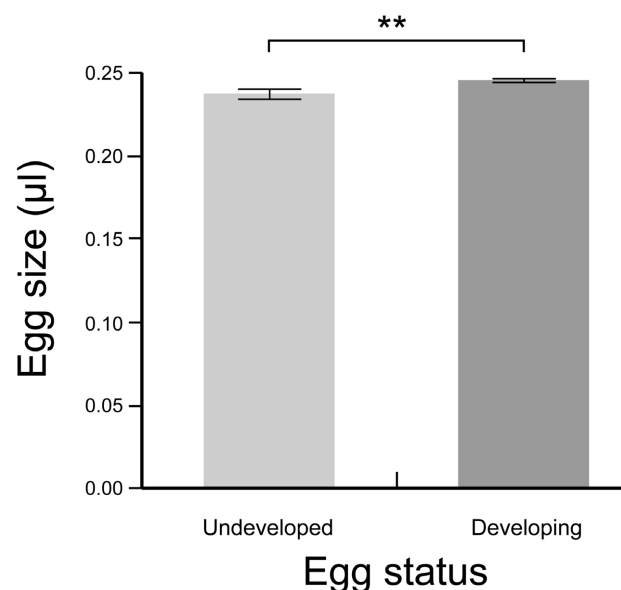
Fig. 2. The difference in the size of eggs of *H. axyridis* that were undeveloped (bright grey) and developing (darker grey). Vertical lines indicate S.E. Asterisks (**) indicate statistical significance at *P* = 0.01 level of significance based on a *t*-test.
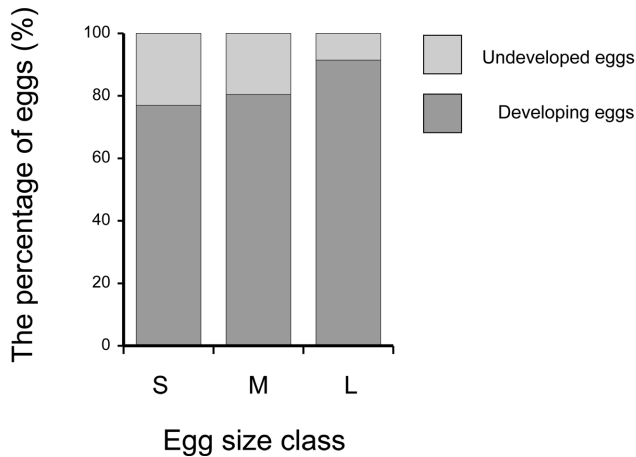
Fig. 3. The percentage of eggs of *H. axyridis* that were undeveloped (bright grey) or developing (dark grey) in three egg size classes.

There was a significant relationship between female body size and the number of ovarioles per female (one-way ANOVA: $F_{1, 20} = 5.8669$, $R^2 = 0.2359$, $P = 0.0256$) (Fig. 4a). A significant but weak relationship was recorded between female body size and egg size (one-way ANOVA: $F_{1, 932} = 21.3011$, $R^2 = 0.0224$, $P < 0.001$) (Fig. 4b). Furthermore, there was a significant relationship between the total number of ovarioles per female and the minimum size of the eggs that hatched (one-way ANOVA: $F_{1, 20} = 4.5111$, $R^2 = 0.1919$, $P = 0.0470$) (Fig. 4c).

DISCUSSION

This study shows that egg size differed significantly among females (Fig. 1a), while egg cluster size was only marginally significant among females (Fig. 1b). Interestingly, this finding is not consistent with the expectation that egg size is likely to be the least variable reproductive trait and cluster size the most variable (Dixon & Guo, 1993), which indicates that egg size as well as egg cluster size vary greatly within species. Variations in progeny size among species and throughout populations within species are attributed to variations in natural selection (e.g., Fox & Czesak, 2000). Furthermore, the variations in egg size within and among clutches may be a bet-hedging strategy to minimize variation in fitness (e.g., Philippi & Seger, 1989). The above indicate that the large variation of egg size recorded in this study (maximum egg size is 46.77% larger than the minimum size) may be an evolutionary consequence of individual-based natural selection in their habitats. Changes in cluster size observed in this study may be due to variations in the ovariole numbers per female and the oviposition timing caused by differences in the developmental speed of female oocytes even when provided with a surplus and constant food supply.

This study showed that developing eggs were significantly larger than undeveloped eggs, and the percentage of eggs that did not develop was greater for small than large eggs (Figs 2 and 3). In addition, because all undeveloped eggs in a cluster were eaten by siblings, small eggs (many of which did not develop) may have been produced by fe-
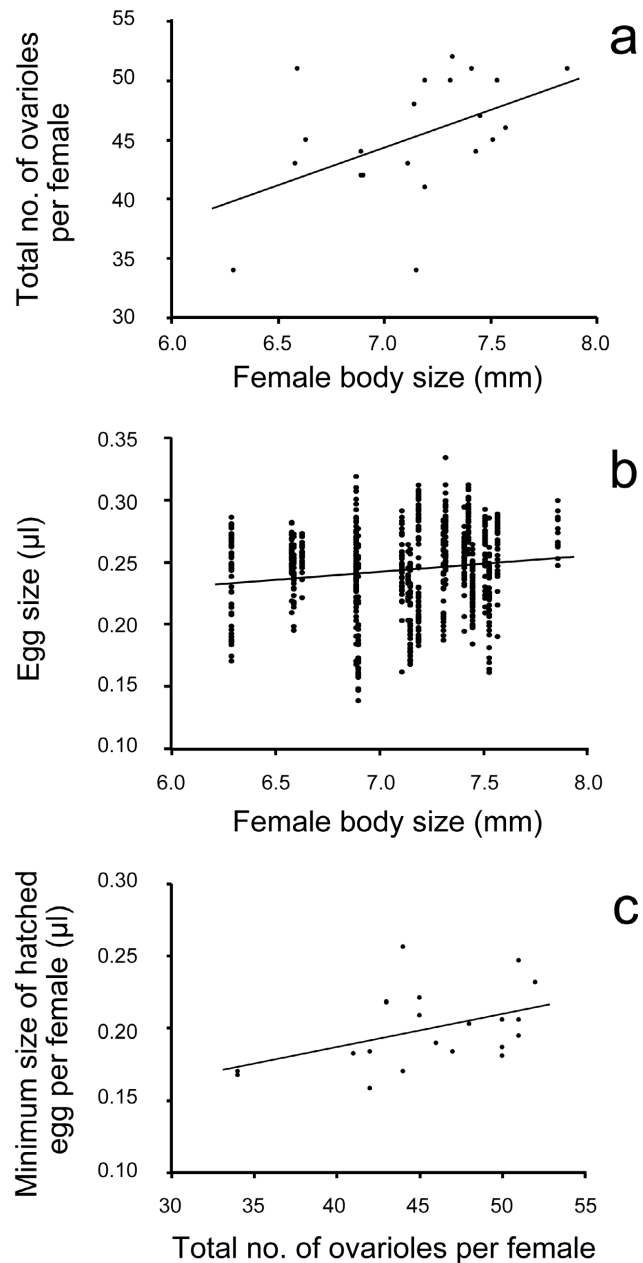
Fig. 4. The relationships for *H. axyridis* between female body size and total number of ovarioles per female (a), female body size and egg size (b), and total number of ovarioles per female and minimum size of the eggs that hatched per female (c).

males to be cannibalized as "trophic" eggs. In a detailed laboratory experiment, Perry & Roitberg (2005) found that the ladybird beetle *H. axyridis* uses information from prey encounters to manipulate the proportion of "trophic" eggs in a cluster. In a field study on *H. axyridis*, the relationship between cluster size and the percentage of sibling cannibalism per cluster (i.e., an indicator of "trophic" egg production per cluster) is positive and that between the distance from a cluster to the nearest aphid colony (i.e., an indicator of food availability for offspring) and cluster size is also positive, whereas the percentage of sibling cannibalism per cluster did not change in relation to the distance from a cluster to the nearest aphid colony (Osawa, 2003). This circumstantial evidence indicates that *H. axyridis* females

in the field may have a weak ability to manipulate the proportion of "trophic" eggs in a cluster and thereby provide future food for their offspring. However, the reduction in this ability in the field may due to the presence of male-killer affecting the behaviour of females.

The number of ovarioles is generally an indicator of potential reproductive output (e.g., Stewart et al., 1991; Roff, 1993). Furthermore, egg size influences the survival of progeny and has a large effect on fitness (e.g., Fox, 1994; Fox & Czesak, 2000). In this study there was a significant positive relationship between female body size and the number of ovarioles (Fig. 4a). The same results are reported within (e.g., Dixon & Guo, 1993; Togashi et al., 2020) and between (e.g., Stewart et al., 1991; Honěk, 1993; Hodek & Honěk, 1996; Dixon & Hemptinne, 2001) species. This study also indicated that there is a positive relationship between female body size and egg size (Fig. 4b). These results suggest that large females have the potential for laying larger and more eggs, implying that large females may have higher fitness than small ones. These advantages result in a selection for large female size. A comparison of two ladybird beetles, *Anisolemnia dilatata* and *Coccinella septempunctata* (Agarwala & Dixon, 2017), indicates a trade-off between egg hatch time and egg size, which indicates that large eggs might have longer hatching times than smaller ones even in the same species.

Sibling cannibalism often occurs in *H. axyridis* with early hatching larvae eating undeveloped sterile and late developing eggs (Kawai, 1978; Osawa, 1992a). Furthermore, non-sibling cannibalism also occurs in *H. axyridis* in the field in which larvae from a different egg cluster cannibalize eggs mainly in the middle and late period of oviposition and when egg clusters are close to an aphid colony and are large (Osawa, 1989, 2003). Thus, large eggs with a long developmental period in large clusters are at a greater risk of non-sibling cannibalism, especially late in the period of oviposition and near aphid colonies. Therefore, large females are not always the most fit in nature and the reverse is true for small females, which may be important when there are large variations in female body size in the field (e.g., Osawa et al., 2015).

This study revealed a significant relationship between the total number of ovarioles and the minimum size of eggs that hatch egg per female (Fig. 4c). Combining the positive correlation between female body size and total number of ovarioles (Fig. 4a) and that between female body size and egg size (Fig 4b), indicates that large females provided more direct (i.e., egg size) and indirect (i.e., sibling cannibalism large sterile eggs) investment in offspring than small females. In ladybird beetles, egg size is probably constrained by the minimum size at which the first instar larvae can capture active prey and complete their development before prey becomes scarce (Stewart et al., 1991). The results of this study revealed the minimum size of eggs that hatched varied greatly in size among females (the largest egg is 61.85% larger than the smallest) (Fig. 4c). Generally, the production of eggs that do not develop has a cost, although the cost of producing small eggs that

do not develop is lower than that of large developing egg. Moreover, these eggs are a target for a natural selection because sterile eggs have no reproductive value and their production directly decreases female fitness. However, all the sterile eggs were always cannibalized by the early hatching siblings in an egg cluster and played an essential role in increasing their survival (Osawa, 1992a) and fitness (Osawa, 2002). Therefore, the production of these sterile eggs is adaptive. A small undeveloped egg may not develop due to internal physiological defects, meaning these eggs function as "trophic" eggs.

It is often observed that upon darkening and hardening of the cuticle and legs, first instar larvae of *H. axyridis* that emerge early in an egg cluster eat the undeveloped and more slowly developing eggs (e.g., Osawa, 1992a). The order in which eggs are laid, the shape of a cluster (a linear vs. a cluster) and the position in a cluster (inside vs. edge) had no effect on the likelihood of being cannibalized (Roy et al., 2007). In addition, 24.33% of the eggs subject to sibling cannibalism were undeveloped (Osawa, 1992a) and sibling cannibalism occurred in more than 90% of clusters, but not 100% (Osawa, 1989). Therefore, females do not always invest in sterile eggs and cannot provide such eggs for specific larvae. The distribution of sterile eggs in a cluster, i.e., the probability of these eggs being near to the early hatching larvae, may determine the consequence of this maternal investment. In the field, *H. axyridis* females oviposit before aphid densities peak, however, severe food shortages often occur during larval development (e.g., Osawa, 1992b, 1993, 2000). Furthermore, sibling cannibalism promotes more rapid development and faster development may be adaptive for resource tracking as the cannibals are more likely to survive and pupate before aphids become scarce (Osawa, 2002). Thus, small sterile eggs were mostly only available for early hatching larvae, i.e., the larvae with highest probability of survival in an egg cluster. This maternal investment is highly circumstance-dependent, but more certain and effective than a large egg with long developmental time and higher risk of predation, as it results in the faster development of the cannibals. The circumstance-dependent maternal investment by aphidophagous ladybirds in eggs that do not develop is an important bet-hedging strategies in oviposition for tracking fluctuations in resources.

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