Eur. J. Entomol. **106**: 607–610, 2009
http://www.eje.cz/scripts/viewabstract.php?abstract=1494
ISSN 1210-5759 (print), 1802-8829 (online)

The presence of micropyles in the shells of developing and undeveloped eggs of the ladybird beetle *Harmonia axyridis* (Coleoptera: Coccinellidae)

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Key words. Coccinellidae, *Harmonia axyridis*, hatchability, infertile egg, morphology, scanning electron microscopy, sibling cannibalism, trophic egg

Abstract. In Hymenoptera and Heteroptera, the absence of micropyles is one criterion for categorizing an egg as trophic. Undeveloped eggs are observed in more than 90% of the egg clusters of the ladybird beetle *Harmonia axyridis* Pallas. Traditionally, these undeveloped eggs are regarded as "trophic eggs." The surfaces of the eggs of *H. axyridis* were examined using scanning electron microscopy and the presence of micropyles in the shells of developing and undeveloped eggs determined. Micropyles are circularly distributed around the top of eggs and present in both developing and undeveloped eggs. The number of micropyles in the shells of developing and undeveloped eggs did not differ significantly. Our results indicate that the undeveloped eggs of *H. axyridis* have micropyles, suggesting that the mechanisms regulating the production of undeveloped eggs in *H. axyridis* differ from those resulting in the production of trophic eggs by Hymenoptera and Heteroptera.

INTRODUCTION

Many female insects store the sperm received from one or more males in a sperm storage organ called a spermatheca (Gullan & Cranston, 2000). In insects, eggs are fertilized as they pass down the median oviduct and vagina. Specifically, sperm enter the egg via one or more micropyles, which are narrow canals that pass through the eggshell (Gullan & Cranston, 2000). Therefore, the structure and number of micropyles play an important role in the fertilization of eggs in insects. The number of micropyles varies in insects; most dipteran eggs have only a single terminal micropyle (e.g., Chapman, 1998), whereas the ladybird beetle *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae) has 25 to 45 micropyles arranged in a ring at the posterior end of the egg (Hinton, 1981). Interestingly, the number of micropyles can also vary within a species (e.g., Chapman, 1998).

In Hymenoptera, females can reproduce regardless of whether they are mated, and the unfertilized haplodiploid eggs develop into males (Hölldobler & Wilson, 1990). In ants, breeders and workers are morphologically specialized; workers have lost the ability to mate and produce diploid offspring in most species, whereas in many other species, workers have retained ovaries and can produce male eggs or trophic eggs, which are non-viable and morphologically distinct from reproductive haploid eggs (e.g., Camargo-Mathias & Caetano, 1995). In fact, in the ant *Gnamptogenys menadensis* (Mayr) (Hymenoptera: Formicidae) mated workers produce reproductive eggs, whereas virgin workers in colonies without a queen only lay small trophic eggs that lack micropyles (Gobin et al., 1998). Thus, morphological specialization in the form of the presence/absence of micropyles occurs throughout Hymenoptera. Kudo et al. (2006) report similar differences in the eggs of the subsocial burrower bug *Adomerus triguttulus* (Motschulsky) (Heteroptera: Cydnidae).

In the ladybird beetle *Harmonia axyridis* Pallas, females lay clusters of eggs and sibling cannibalism of eggs occurs in more than 90% of the egg clusters (Osawa, 1989). This type of sibling cannibalism may result from two proximate factors: the eggs hatch asynchronously (Kawai, 1978; Osawa, 1992) and/or there are undeveloped eggs in the egg cluster (Kawai, 1978). Because they have no reproductive value, undeveloped eggs may function as nurse or trophic eggs for the larvae that hatch from the other eggs (e.g., Polis, 1981). The undeveloped eggs have been traditionally regarded as "infertile eggs" (e.g., Kawai, 1978; Osawa, 1992; Perry & Roitberg, 2005). This type of sibling cannibalism occurs widely among insect species (e.g., Stevens, 1992; Hodek & Honěk, 1996; Kudo et al., 2006).

In ladybird beetles, the morphology of the micropyles has only been examined in a few species: *A. bipunctata* (Mazzini, 1975; Hinton, 1981), *Epilachna vigintioctomaculata* Motschulsky (Katakura, 1988), *Illeis indica* Timberlake (Kumar et al., 2000) and *Scymnus sinuanodulus* Yu et Yao (Lu et al., 2002). However, detailed information on the formation of micropyles during the development an egg and whether undeveloped eggs are analogous to Hymenopteran trophic eggs does not exist for any taxon other than Hymenoptera and Heteroptera. Therefore, in this study, the development of micropyles in the shell of the eggs of the ladybird beetle *H. axyridis* was examined.

MATERIAL AND METHODS

Laboratory observations

Three female adults of *Harmonia axyridis* were collected on 10 June 2007 from a peach tree, *Prunus persica* Betsch (Rosales: Rosaceae), infested with *Myzus varians* Davidson and *Hyalopterus pruni* (Geoffroy) (Hemiptera: Aphididae), in the Botanical Gardens of Kyoto University (35°02´N, 135°47´E). These *H. axyridis* females were provided with a surplus of aphids (*H. pruni*) in plastic Petri dishes (7 cm diameter, 2 cm deep) and kept under natural conditions. The Petri dishes were checked daily, all eggs laid counted and the number of eggs per cluster recorded for each female (total no. of clusters = 5, total no. of eggs = 108). After removing the beetles, each egg batch was kept individually in a Petri dish labelled with the female's code and date of oviposition. Eggs were maintained under natural conditions and checked more than twice daily. A total of five egg clusters were collected from the three females. In cases of sibling cannibalism in aphidophagous ladybird beetles, the cannibals eat two types of sibling eggs within a cluster: undeveloped eggs and developing eggs whose development lags behind that of the other eggs (Kawai, 1978; Osawa, 1992). Any cannibalized egg that contained an embryo, which is indicated by its dark coloration, was classified as a developing egg. Eggs that had a light yellow colour when the other eggs in the cluster hatched were classified as undeveloped eggs (Brown, 1972; Osawa, 1992). Thus, we used colour as the criterion for determining the developmental status of the eggs.

Under natural conditions, all undeveloped and late developing eggs in a cluster are usually eaten by the first larvae to hatch within approximately 8 h after their hatching (Kawai, 1978), which is defined as the moment when the head of a larva emerges from an eggshell (Osawa, 1992). Larvae typically start to cannibalize eggs soon after hatching (ca. 1 h). In order to prevent sibling cannibalism and focus on the developing and undeveloped eggs, 5 clusters of eggs were examined under a stereomicroscope (Carl Zeiss® SV 11 Apo) and larvae removed as soon as they hatched. However, because the larvae began cannibalizing eggs shortly after hatching, we may have missed a few. To reduce the disruptive effect of this cannibalistic behaviour clusters of eggs were kept at approximately 5°C for 1 h. After refrigeration, the clusters were observed under a stereomicroscope in the laboratory for a total of 8 h. This procedure did not kill any of the larvae or developing eggs but temporarily reduced both the rate of cannibalism and egg development, which both increased when the clusters were returned to laboratory conditions. The observations of a total of 104 eggs in five clusters identified 84 developing and 20 undeveloped eggs, from which 18 eggs from three females (four eggs from each of two different females and 10 eggs from a third) were randomly chosen (developing eggs: $n = 10$, undeveloped eggs: $n = 8$). Biological material is usually desiccated prior to sputter-coating and scanning under an electron microscope. However, the *H. axyridis* eggs are too soft for this procedure. Therefore, the 18 eggs were air-dried and then sputter-coated with gold and observed under a scanning electron microscope (JEOL® JSM6600) at 5 kV.

Statistical analysis

Because the variances did not differ significantly among treatments (O'Brien's unequal variance test: $p = 0.5548$), one-way analyses of variance (ANOVA) were performed to test for differences in the number of micropyles in the shells of developing and undeveloped eggs. The effect of mother on the number of micropyles was analyzed using Wilcoxon/Kruskal-Wallis nonparametric tests because the sample sizes were small ($n < 5$). All statistical analyses were conducted using JMP® Discovery Software (SAS Institute, 2000).

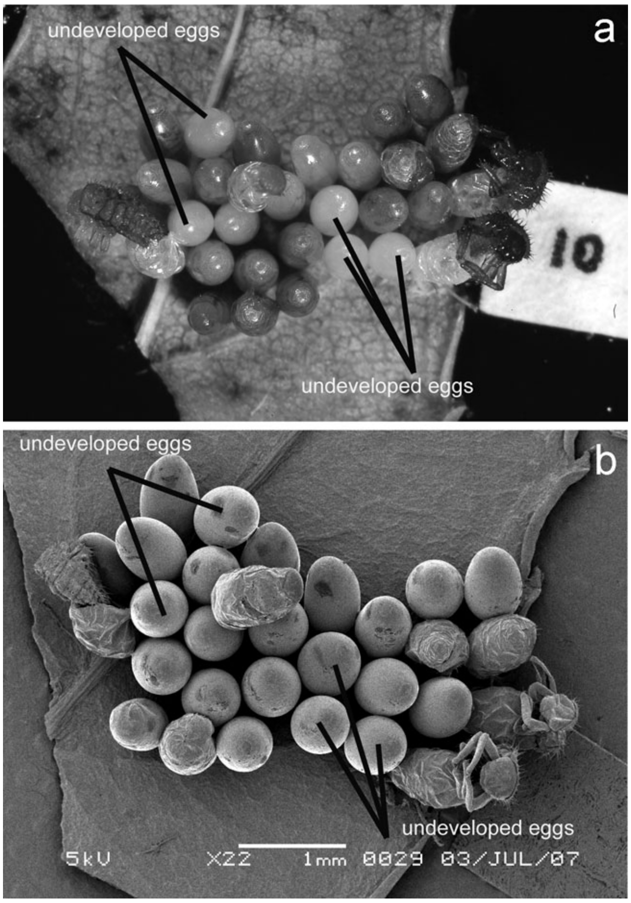

Fig. 1. Whole view of *H. axyridis* eggs before (a) and after (b) sputter-coating. Five hatching, twenty developing and five undeveloped eggs in an egg cluster. Lines indicate the five undeveloped eggs.

RESULTS

Micropyles (ca. 2 µm in diameter; no. of micropyles: 19.83 ± 2.90, mean ± S.E., $n = 18$) were distributed in a circle at the top of each *H. axyridis* egg (Figs 1a, b and 2a, b). There was no significant difference in the number of micropyles (one-way ANOVA: $F_{1,16} = 0.2857$, $p = 0.6003$) in the shells of developing (19.50 ± 0.94, $n = 10$) and undeveloped (20.25 ± 1.05, $n = 8$) eggs. The identity of the mother had no significant effect on the number of micropyles (Wilcoxon/Kruskal-Wallis nonparametric test: $df = 2$, $\chi^2 = 2.0946$, $p = 0.3509$).

DISCUSSION

Trophic eggs are non-developing eggs or egg-like structures produced for offspring consumption (e.g., Hölldobler & Wilson, 1990). In the ladybird beetle, *Harmonia axyridis*, which lays clusters of eggs, the first larvae to hatch eat any eggs in the cluster that have not hatched (e.g., Kawai, 1978; Osawa, 1992). The eggs that fail to develop are often regarded as "infertile eggs" (e.g., Kawai, 1978; Osawa, 1992; Perry & Roitberg, 2006) although their infertility is rarely confirmed; the eggs are

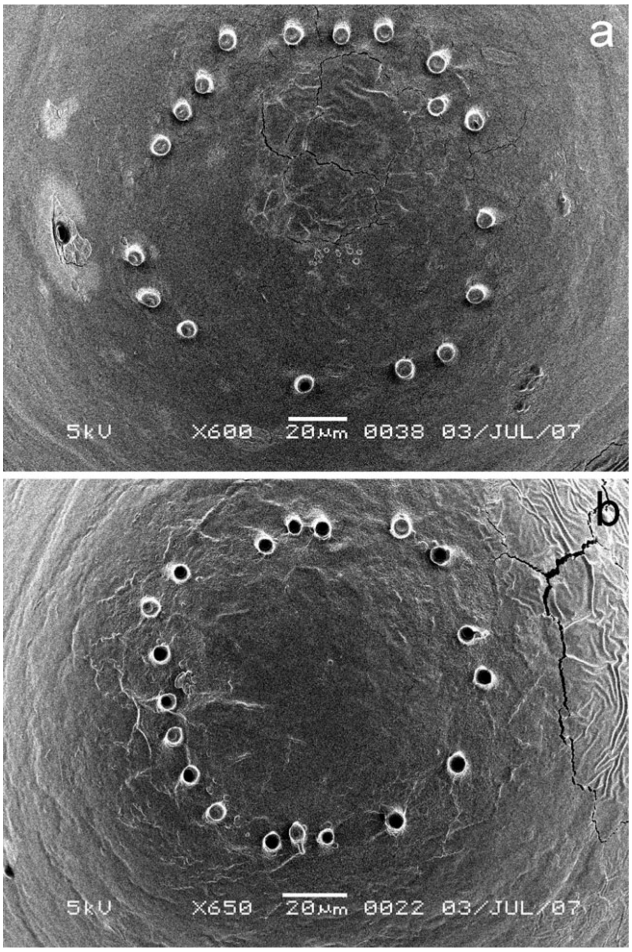

Fig. 2. Scanning electron micrographs of micropyles in the shells of *H. axyridis* eggs; developing egg (a) and undeveloped egg (b).

either infertile or die during early development (Osawa, 2002, 2003). Ecologically, these undeveloped eggs are thought to have evolved and be a form of maternal investment (Osawa, 2003; Perry & Roitberg, 2005).

The morphological differences between developing and undeveloped eggs in *H. axyridis* was examined with particular emphasis on the presence/absence of micropyles in the shells of the eggs. The presence of micropyles is an important criterion, as trophic eggs generally lack micropyles (e.g., in Hymenoptera, Gobin et al., 1998, and Heteroptera, Kudo et al., 2006). In the subsocial burrower bug *Adomerus triguttulus*, viable eggs (i.e., developing eggs) always have approximately five micropyles, whereas trophic eggs have none (Kudo et al., 2006). In contrast, the number of micropyles in the shells of developing and undeveloped eggs of *H. axyridis* did not differ significantly and the two types of eggs types have a similar structure.

Although they did not determine whether the undeveloped eggs were fertile Perry & Roitberg (2005) showed that ladybird beetle mothers (*H. axyridis*) use information from prey encounters to manipulate the proportion of undeveloped eggs in a cluster. Assuming that the undeveloped eggs are unfertilized, our results suggest that *H. axyridis* females may control egg fertility and produce infertile eggs that serve as food for sibling larvae. If this is the case then the fertilization of the eggs must occur at a very late stage in their development, when each egg has a shell complete with micropyles.

ACKNOWLEDGEMENTS. We thank H. Katakura, Hokkaido University, for providing information regarding the scanning electron micrographs, and H. Takeda and M. Fujita, Kyoto University, for their support, suggestions, and encouragement during this research. This study was supported in part by a Grant-in-Aid for Science Research (No. 20405047, to N.O.) from the Japan Society for the Promotion of Science and by the 21st COE Program on "Innovative Food and Environmental Studies Pioneered by Entomomimetic Sciences" from Kyoto University, financed by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Received March 3, 2009; revised and accepted July 31, 2009