

**Mechanism and function of synchronized hatching
in Pentatomidae (Insecta: Heteroptera)**

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March 2018

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General introduction

Synchronized hatching is a widely observed phenomenon in animals laying egg clutches. Embryos in a clutch can hatch synchronously due to their responses to environmental cues, such as water floods (Anderson & Williamson 1976) and predatory attack (Warkentin 1995). Mothers of egg clutches induce synchronized hatching by providing hatching cues, for example, in a crab *Sesarma haematocheir* (Decapoda: Sesarmidae) (Saigusa 1992, 1993). In some species, however, hatching seems to be synchronized without such external cues: Embryos are caused to hatch synchronously by their clutch mates. For example, newly hatched larvae of the large white butterfly, *Pieris brassicae* (Lepidoptera: Pieridae), assist hatching of the adjacent eggs by biting and tearing the egg chorion, and thus hatching of the entire clutch is synchronized (Long 1955).

Embryos of other animals synchronize hatching with clutch mates without such hatching assistance. In these species, an important issue has been the mechanism by which embryos inside the eggs achieve synchronization. Researchers have revealed that embryos respond to various cues from their clutch mates by altering the hatching time in various manners. In precocial birds like the bobwhite quail, *Colinus virginianus* (Galliformes: Odontophoridae), embryos respond to acoustic cues from clutch mates by either accelerating or retarding the development for synchronization of hatching (Vince 1964, 1966, 1968). In the Murray River turtle, *Emydura macquarii* (Pleurodira: Chelidae), hatching is synchronized within a clutch because less developed embryos accelerate the development against advanced embryos (Spencer et al. 2001; McGlashan et al. 2012). It is presumed that the turtle embryos respond to changes in heart rates of the clutch mates that are conveyed as vibrations or sounds (Loudon et al. 2013).

It is well recognized that synchronization of hatching has evolved when they must migrate as a sibling group. After synchronized hatching, larvae of *P. brassicae* migrate to a

feeding site together (Long 1955). Chicks of precocial birds leave the ground nest simultaneously with their parents and, if hatching is not synchronized, the young before and after hatching receive inefficient parental care because the parents cannot guard both the eggs and highly mobile chicks at the same time (Woolf et al. 1976). Hatchlings of *E. macquarii* emerge from an underground nest simultaneously, and this seems to save energy at the emergence and reduce predation risks (Spencer et al. 2001; McGlashan et al. 2012).

However, recent studies indicate that synchronization of hatching also have functions other than facilitating group migration. In the pig-nosed turtle, *Carettochelys insculpta* (Cryptodira: Carettochelyidae), when the nest begins to be flooded in the wet season, some submerged embryos are firstly induced to hatch by hypoxia. Then, the others soon follow them by responding to vibration from the earlier-hatching siblings to avoid drowning (Webb et al. 1986; Doody et al. 2012). In this case, we recognize that the earlier-hatching siblings play a social role to warn about a risk, and remaining embryos respond to the signal by the earlier-hatching siblings to synchronize hatching, thereby avoiding the risk. In the painted turtle, *Chrysemys picta* (Cryptodira: Emydidae), less developed embryos synchronize hatching with advanced embryos by hatching prematurely, although hatchlings stay and overwinter inside the nest (Colbert et al. 2010). Colbert et al. (2010) presumed that the premature hatching allows the hatchlings to acquire better positions within the nest. Thus, synchronized hatching has been reported where there is competition among siblings for resources. As found in these examples, young animals adapt to various social contexts among siblings through synchronization of hatching, and thereby cope with some constraints. It is important to study functions of synchronized hatching for understanding constraints and adaptive strategies in early stages of animals that start life with clutch mates.

In the present study, I address the mechanism and function of synchronized hatching in Pentatomidae (Insecta: Heteroptera), which includes some stink bugs and shield bugs. Females of this family lay their eggs densely on a plant surface in a clutch size of several to one

hundred eggs, the number varying with species (Kiritani & Hokyo 1965). In general, embryos develop with no parental care, and therefore do not receive behavioral control over timing of the hatching from parents in contrast to sub-social relatives in Cydnidae and Parastrachiidae (Mukai et al. 2012, 2014). Previous studies by Morimoto (1965) and Lockwood and Story (1985a) showed, in a few species of Pentatomidae, that embryos hatch by responding to cues from clutch mates. Because of these hatching responses, hatching of the entire clutch seems to be synchronized. However, the precise mechanism of the synchronized hatching is not understood. After hatching, first-instar nymphs of this family do not move away from the natal egg clutch but form a characteristic, dense aggregation that persists for a few days close to the egg clutch (Kiritani 1964; Saito et al. 1964). The function of the synchronized hatching is not to facilitate mass migration and, therefore, is unclear. Furthermore, Morimoto (1965) and Lockwood and Story (1985a) did not focus on the commonality of hatching synchronization in Pentatomidae, although such a viewpoint may help understand diversity and evolution of this behavioral trait.

In Chapter 1, I worked on the commonality of hatching synchronization in Pentatomidae using eight species of this family and showed substantial differences in hatching synchronization among these species. In Chapter 2, I investigated the function of the hatching synchronization. I hypothesized a factor responsible for evolution of the hatching synchronization, and examined the factor in the eight species with varying synchronization. In Chapter 3, I looked into the mechanism of the hatching synchronization. I clarified a hatching cue used by an embryo to synchronize hatching in *Halyomorpha halys* that shows the highest synchronized hatching pattern.

Chapter 1

Commonality and interspecific variation of hatching synchronization

Introduction

In Pentatomidae, some species are known to show an egg hatching response to clutch mates. In one species of this family, *Eurydema rugosum*, when hatching patterns were compared between intact egg clutches and eggs individually detached from egg clutches, hatching proceeded more synchronously when eggs remained attached to each other (Morimoto 1965). From these results, Morimoto (1965) suggested that an intact egg clutch shows a synchronized hatching pattern because some kind of cue(s) associated with earlier hatching stimulate successive hatchings. Similarly, Lockwood and Story (1985a) showed in *Nezara viridula* that two single detached eggs required more time for hatching than two attached eggs, and suggested the presence of a hatching response to clutch mates. In another species, *Halyomorpha halys*, a synchronized hatching pattern has been noted only by observation of intact egg clutches (Taylor 2016). In the latter two species, however, the extent to which hatching of an egg clutch is accelerated through the hatching response has not been elucidated, and thus it is unclear whether the effect is similar or different among species within Pentatomidae.

To clarify the commonality and interspecific variation of such a response among pentatomids, I observed hatching of intact egg clutches and eggs individually detached from egg clutches in eight species among different genera, including the three species mentioned above: *Aelia fieberi*, *Dolycoris baccarum*, *E. rugosum*, *H. halys*, *N. viridula*, *Palomena angulosa*, *Piezodorus hybneri*, and *Plautia stali*. I expect that, in Pentatomidae, the synchronized hatching mediated by a hatching response to clutch mates is an adaptation of embryos to some social contexts among siblings in their early developmental stages. Experiments in this chapter may

potentially unveil complexity underlying these social contexts.

Materials and methods

Insect rearing and preparation of egg clutches

Adults of *A. fieberi*, *D. baccarum*, *E. rugosum*, *H. halys*, *N. viridula*, *Pa. angulosa*, *Pi. hybneri*, and *Pl. stali* were collected from the field in Kyoto, Osaka, and Hyogo Prefectures, Japan, in 2014-2016 to obtain egg clutches. These adults were provided with water and oviposition sites made of drawing paper in plastic cases under LD 16 : 8 h at 25°C. Raw seeds of peanut, *Arachis hypogaea*, and dried seeds of soybean, *Glycine max*, were fed to *D. baccarum*, *H. halys*, *N. viridula*, *Pa. angulosa*, *Pi. hybneri*, and *Pl. stali*; dried seeds of barley, *Hordeum vulgare*, and supplementary dried seeds of a wild rye, *Elymus tsukushiensis*, to *A. fieberi*; and dried seeds of a mustard, *Brassica juncea*, to *E. rugosum*. Egg clutches laid within 24 h were cut out with their ovipositional substrates, and then kept in a plastic container at 25°C and 80% or higher relative humidity until they were used for experiments described below. Although Kiritani and Hokyō (1965) stated that, in Pentatomidae, the clutch size (i.e., the number of eggs in a single egg clutch) is largely fixed according to the species, in my laboratory-reared insects, egg clutches with a wide range of sizes were observed for each species. To minimize possible variation in results originating from this size variation, egg clutches used for this study were limited to a particular range of clutch size that included frequently observed sizes: 12 ± 2 for *A. fieberi* and *E. rugosum*; 14 ± 2 for *Pa. angulosa* and *Pl. stali*; 28 ± 4 for *D. baccarum*, *H. halys*, and *Pi. hybneri*; and 84 ± 13 for *N. viridula*.

Recording of hatching patterns

The following preparations for observation were conducted within 2 days before estimated hatching of egg clutches. Intact egg clutches were fixed with a piece of double-stick tape on the bottom of a clear plastic case (36×36 mm, 14 mm height), together with wet cotton to prevent desiccation. Detachment of individual eggs from egg clutches was carried out with a razor blade and forceps under a binocular stereomicroscope (S8 APO, Leica Microsystems, Wetzlar, Germany). However, this method alone was not successful for egg clutches of *D. baccarum*, *N. viridula*, or *Pi. hybneri* due to the combinations of their fragile chorions and tight attachment between eggs. For these three species, the attachment between eggs was loosened by moistening beforehand on wet cotton for 10-30 minutes. Each of the detached eggs was then separately attached to a piece of drawing paper (5×8 mm) using starch glue, and individually fixed using a piece of double-stick tape in a well (9.85 mm diameter, 17.50 mm depth) of a flat-bottom, 48-well culture plate (AGC Techno Glass, Shizuoka, Japan), at the end of a plastic column (5 mm diameter, 10 mm height) that had been attached at the center of the bottom of the well. To prevent desiccation of the egg, 200 μ l water was poured into the well. The above detaching procedures seemed to have little or no effect on the hatching of embryos in all the species examined, because hatching rates were not significantly different between intact egg clutches and eggs individually detached from egg clutches (Mann-Whitney *U* test, $P > 0.1$).

Subsequently, temporal patterns of hatching were recorded for intact egg clutches and eggs individually detached from egg clutches using time-lapse photography with a digital camera (CX6, Ricoh, Tokyo, Japan and P310, Nikon, Tokyo, Japan) from the top view at 25°C under constant light (photon flux density of approximately $3.6 \mu\text{mol m}^{-2} \text{s}^{-1}$, FL15D, Toshiba, Tokyo, Japan). In intact egg clutches, hatching of an egg was sometimes concealed by nymphs that had previously hatched and were covering the top surface of the egg. Thus, the hatching time of each egg was defined as the time when its hatchling was first identified in photographs. The number of hatchings was determined every 5 min for 6 h after the first hatching by analyzing the photographs taken at intervals of 1 min for intact egg clutches and 5 min for eggs individually

detached from egg clutches. Both in intact egg clutches and eggs individually detached from egg clutches, hatching was usually completed within 6 h in all the species examined. Egg clutches in which 10% or more eggs did not hatch within this period were excluded from analyses. To determine temporal patterns of hatching, cumulative hatching rates were calculated as proportions relative to the total number of hatchings at 6 h.

Statistical analyses

To examine differences in temporal hatching patterns between intact egg clutches and eggs individually detached from egg clutches, time-treatment interaction effects were analyzed by two-way repeated measures ANOVA. Eight time points of the hatching patterns were used for the analyses. The initial time point was determined as 5 min and the final point was selected for each species so that all the interval between the points become equal and mean cumulative hatching rates of both intact clutches and eggs individually detached from clutches exceeded 90% between the seventh and eighth points (145 min for *Pa. angulosa*, *Pi. hybneri*, and *Pl. stali*, 180 min for *A. fieberi* and *E. rugosum*, and 215 min for *D. baccarum*, *H. halys*, and *N. viridula*. The hatching rates were arcsine transformed for variance stabilization. Greenhouse-Geisser corrections were used to calculate *P*-values because sphericity assumptions for the ANOVA tests were violated (Greenhouse & Geisser 1959).

To examine whether the second hatching occurs predominantly in an egg adjacent to the first hatching egg, the observed frequency of the second hatching occurring next to the first hatching egg was compared with the expected frequency when the second hatching was assumed to occur randomly within an egg clutch, using a one-tailed binomial test. Because hatching was recorded at 1-min intervals, sometimes both an egg next to the first hatching egg and another egg hatched within the same interval. In these cases, all possibilities were taken into account to calculate *P*-values.

Results

Effect of hatching response

Temporal patterns of hatching were compared between intact egg clutches and eggs individually detached from egg clutches for eight species in Pentatomidae to test the effect of hatching response to clutch mates on the hatching patterns (Fig. 1). Although the duration of the egg period and the total number of hatchings may also influence the hatching pattern, these parameters were not significantly different between intact egg clutches and eggs individually detached from egg clutches (Table 1). The hatching patterns significantly differed depending on the presence or absence of the contact between eggs for *A. fieberi*, *H. halys*, *N. viridula*, *Pi. hybneri*, and *Pl. stali*, but not for *D. baccarum*, *E. rugosum*, or *Pa. angulosa* (Fig. 1). Intact egg clutches of *A. fieberi* hatched less synchronously than eggs individually detached from egg clutches during the latter half of the hatching progress (Fig. 1A), although the reason for this is unclear. In *H. halys*, *N. viridula*, *Pi. hybneri*, and *Pl. stali*, hatching proceeded more synchronously throughout the hatching progress when eggs were attached to each other (Fig. 1D, E, G, H). Accordingly, I concluded that a hatching response to clutch mates existed in these four species.

In particular, the temporal patterns of hatching showed a sharp contrast between intact egg clutches and eggs individually detached from egg clutches in *H. halys*. Hatching proceeded gradually when eggs were not attached to each other: Within the first 15 min, only a small proportion of eggs in an egg clutches (median, 3.8%, interquartile range [IQR], 3.7-7.4%) started hatching, and the hatching rate exceeded 95% at 200 min (median, 96.3%, IQR, 86.8-100.0%; Fig. 1D). However, hatching spread rapidly when eggs were attached to each other (Fig. 2): 15 min after the first hatching, the hatching rate reached more than 95% (median, 96.4%,

IQR, 96.3-100%; Fig. 1D). Compared with *H. halys*, hatching was less accelerated in *N. viridula*, and far less accelerated in *Pi. hybneri* and *Pl. stali* via the attachment between eggs (Fig. 1D, E, G, H).

Spatial patterns of hatching

In intact egg clutches of some species, hatching seemed to spread from hatched eggs toward adjacent ones successively. To assess this spatial pattern of hatching, I examined whether a second hatching was more likely to occur at an egg adjacent to the first hatching egg than would be predicted by random hatching (Table 2). The results showed that such a tendency was evident in *N. viridula*, *Pi. hybneri*, and *Pl. stali*, uncertain in *H. halys*, and improbable in *A. fieberi*, *D. baccarum*, *E. rugosum*, and *Pa. angulosa*. Hatching of *H. halys* spread as follows: After the first hatching (Fig. 2A), hatching of not only the adjacent eggs but many peripheral eggs followed immediately and almost simultaneously (Fig. 2B). Then, eggs that were positioned far from the earliest-hatching one in the egg clutch followed with a slight delay (Fig. 2C).

Discussion

Interspecific variations in temporal hatching patterns

In a few species of Pentatomidae, a hatching response to clutch mates is known to be an important factor that affects the temporal pattern of hatching within an egg clutch (Morimoto 1965; Lockwood & Story 1985a). In this chapter, I found that the hatching response varies depending on the species in Pentatomidae, with a wide spectrum of effects on the hatching patterns. Among the eight species examined, the hatching response clearly occurred in *H. halys*,

N. viridula, *Pi. hybneri*, and *Pl. stali*, resulting in more synchronized egg hatching. In *H. halys*, remarkably, the hatching response greatly accelerated the hatching and consequently led to highly synchronized patterns. However, no evidence of such a response was found in *A. fieberi*, *D. baccarum*, *E. rugosum*, or *Pa. angulosa*. My findings in *E. rugosum* contradict those of Morimoto (1965) that hatching of intact egg clutches proceeds faster than hatching of eggs individually detached from egg clutches, although the observation temperature that Morimoto used (27°C) was different from that used here (25°C). I cannot exclude the possibility that a hatching response exists in *E. rugosum* also, but its effect may be observable only in particular conditions or differ substantially among individuals in the species.

Through the more synchronized hatching resulting from the hatching response to clutch mates, embryos or nymphs of Pentatomidae presumably adapt to some social context among siblings. The interspecifically different hatching patterns indicate that the social context varies greatly among the species in Pentatomidae. Especially, the social context of *H. halys* seems to be considerably different from that of the other species, and forces the embryos to hatch within a very short period. To suggest directions for further studies, I discuss the possible mechanisms producing the interspecifically different hatching patterns and the adaptive functions of synchronized hatching in Pentatomidae.

Mechanisms of hatching stimulation

In *N. viridula*, *Pi. hybneri*, and *Pl. stali*, hatching spread from the first egg toward the adjacent ones. Although such a tendency was not as clear in *H. halys*, eggs near the first hatching were likely to hatch earlier than distant ones. Thus, these four species that possess the hatching response showed some kind of spreading hatching patterns. On the other hand, I could not observe such hatching patterns for the other four species in which the hatching response was not detected (i.e., *A. fieberi*, *D. baccarum*, *E. rugosum*, and *Pa. angulosa*). Accordingly, I consider

that the spreading hatching patterns are associated with a hatching response. To produce such hatching patterns, the most simply understood mechanism would be a hatching response to a cue associated with the preceding hatching, supporting the speculation by Morimoto (1965) and Lockwood and Story (1985a). In egg clutches of Heteroptera, there are some examples of embryonic responses to physical cues or signals at hatching. In some sub-social bugs of Cydnidae and Parastrachiidae, synchronized hatching is due to a rapid response of embryos to a maternally provided vibration signal (Mukai *et al.* 2012, 2014). In the giant water bug, *Kirkaldyia* (= *Lethocerus*) *deyrolli* (Belostomatidae), the mechanical shock of embryonic moulting is presumed to stimulate moulting of adjacent embryos (Ohba 2002). During the hatching process of individual eggs in Pentatomidae, an embryo ruptures the operculum, and wriggles out of the egg chorion. After hatching, the first-instar nymph steps on its clutch mates by walking over the egg clutch. These physical movements probably produce some distinctive vibrations or pressures that are transmitted to other embryos, and thus potentially stimulate their hatching, as previously speculated (Morimoto 1965; Lockwood & Story 1985a).

We can explain the proximate causes of the interspecific variation in the effectiveness of the hatching responses by differences in embryos' responsiveness to a cue or differences in the intensity and timing of generation of the cue. Alternatively, differences in the egg clutch structure may partially explain the variation as follows: Hatching was more accelerated in *N. viridula* than in *Pi. hybneri* despite the fact that the processes of hatching spread were similar between them. In both species, eggs are arranged in such a manner that every three adjacent eggs form an equilateral triangle (i.e., hexagonal or triangular lattice). However, eggs of *N. viridula* have contact with a larger number of sibling eggs than those of *Pi. hybneri* on average, because the eggs are lined in more rows in the former species (Fig. 2E, G). Thus in *N. viridula*, hatching cues may be transmitted to more eggs simultaneously. Compared with the hatching of *N. viridula*, *Pi. hybneri*, and *Pl. stali*, hatching of *H. halys* showed the unique features that the first hatching was soon followed by hatching of many nearby eggs, and the nearest eggs were

often not the fastest followers. These observations indicate that a cue reaches distant eggs in *H. halys* but only adjacent eggs in the other species.

Adaptive functions of synchronized hatching

It is likely that pentatomids adapt to different social contexts among siblings through species-specific synchronized hatching patterns during the early developmental stages. Based on several types of early sibling associations known in this family, several hypotheses to explain the interspecifically different adaptations are conceivable as follows:

In this family, newly hatched nymphs form a tight nymphal aggregation persisting for a few days on or around a natal egg clutch (Kiritani 1964). This aggregation is important for the nymphs' growth and survival (Kiritani 1964; Kiritani & Kimura 1966; Nielsen *et al.* 2008). Moreover, this aggregation also helps them to cope with low temperature, desiccation, predators, and drop out from plants (Lockwood & Story 1986). Synchronized hatching can profit hatchlings through accelerating formation of the aggregation, as Morimoto (1965) predicted. Thus, if hatchlings need to aggregate more rapidly, more synchronized hatching is favoured.

Just after hatching, nymphs of Pentatomidae roam on the egg clutch for a certain period, and this behavior is known to be associated with acquisition of symbiont resources (Abe *et al.* 1995), which are contained in maternal secretions smeared over the egg surface (e.g., *H. halys*, Taylor *et al.* 2014; *N. viridula*, Prado *et al.* 2006). A necessity for external digestion has been proposed for the acquisition process, and cooperative behavior seems to help it (Taylor 2016). Conversely, competition can occur between hatchlings if the symbiont resource is limited. In both circumstances, it is advantageous to hatch without delay after hatching of other clutch mates.

In insects whose hatchlings cannibalize intra-clutch eggs, when hatching occurs more synchronously, fewer eggs are eaten by the previously hatched nymphs (Fox 1975; Crespi 1992).

Synchronized hatching might function to avoid cannibalism if the hatchlings are egg-cannibalistic. Such cannibalism is known in multiple Pentatomomorpha families, such as Lygaeidae (Root & Chaplin 1976) and Cydnidae (Nakahira, 1994; Kudo & Nakahira, 2004). In Pentatomidae, egg cannibalism by older nymphs and adults has been reported (Kiritani *et al* 1966; Stam, 1987; Iverson *et al.* 2016). However, it had long been believed that first-instar nymphs of this family do not feed at all (e.g., *H. halys*, Lee *et al.* 2013; *N. viridula*, Kiritani 1964; Lockwood & Story 1986). Although a recent study provided evidence of plant feeding by *N. viridula* as determined by detection of a marker bacteria that had infected to a food source (Esquivel & Medrano 2014), there are no reports of egg cannibalism by hatchlings in this family. However, I observed preliminarily in some pentatomid species, including *H. halys*, that hatchlings suck unfertilized eggs or unhatched fertilized eggs, if any, in the natal egg clutch. It will be important to examine the variation of this cannibalistic tendency along with its relation to species-specific hatching patterns in Pentatomidae.

In conclusion, my findings imply that there are interspecifically different physiological, environmental, or social constraints in the early lives of Pentatomidae. Functional studies of the different synchronized hatching patterns will unveil these constraints.

Chapter 2

Relationship between synchronized hatching and the onset time of egg feeding

Introduction

In some species of Pentatomidae, when an egg clutch begins hatching, cues from earlier-hatched nymphs spread to the surrounding embryos. Then, the cues directly trigger their hatching response, and consequently lead to synchronized hatching (Morimoto 1965; Lockwood & Story 1985a). In Chapter 1, I confirmed this phenomenon in four species of different genera, *Halyomorpha halys*, *Nezara viridula*, *Piezodorus hybneri*, and *Plautia stali*: Compared to the case when eggs within their egg clutch are individually detached and isolated to interrupt transmission of hatching cues, hatching proceeds significantly faster in intact clutches. However, such hatching synchronization was not found in other four species examined, *Aelia fieberi*, *Dolycoris baccarum*, *Eurydema rugosum*, and *Palomena angulosa*, indicating lack of a hatching response. Therefore, not all the species in Pentatomidae show the synchronized hatching patterns. Even among the former four species, the intensity of synchronizing effects on hatching is different.

It is unknown why the hatching synchronization varies from non-synchronized to highly synchronized species in Pentatomidae. Presumably, only the synchronized species have a factor promoting the evolution of the synchronized hatching. The factor can be different among the synchronized species. However, previous studies have not revealed the factor responsible for the evolution of the hatching synchronization in the early life stage of Pentatomidae (Morimoto 1965; Lockwood & Story 1985a). After hatching, the first-instar nymphs of this family do not move away from the natal egg clutch but form a characteristic, dense aggregation that persists for a few days closed to the egg clutch (Kiritani 1964; Saito et al. 1964).

Accordingly, it is unlikely that hatching synchronization differs depending on whether hatchlings must migrate in a group or not.

In Chapter 1, I assumed that, in Pentatomidae, hatching synchronization would be associated with sibling egg cannibalism, i.e. cannibalism of later-hatching eggs by earlier-hatching siblings. In this cannibalism, the benefits and costs vary with standpoints as follows: (1) The cannibals obtain nutrition and increase survival at the expense of its kin. (2) The victims of the cannibalism sacrifice themselves to increase survival of their kin. (3) Their mother profits from increased survival of its offspring but lose other offspring (O'Connor 1978). According to the kin selection theory, the above three beneficiaries have different thresholds of the increase in survival for the sibling cannibalism to be favorable (Hamilton 1964a, b), and, importantly, the victims or both the victims and the mother exclusively incur negative fitness consequences due to the cannibalism in some cases (Osawa 1992). These cases would favor evolution of mechanisms to inhibit the cannibalism. It is possible to assume that later-hatching embryos inhibit cannibalism by the earlier-hatching clutch mates via hatching in response to their cues before the onset of the cannibalistic attack.

In Pentatomidae, hatchlings of some of the above-mentioned species are egg-cannibalistic and feed on unhatched eggs, if any, in their natal clutches (Chapter 1). Therefore, in this chapter, I hypothesize that the hatching responses to clutch mates have evolved, in some species of Pentatomidae, to escape from sibling egg cannibalism. In a species in which the hatchlings start egg feeding before all the eggs within the clutch complete hatching, synchronized hatching may have evolved so that later-hatching embryos could avoid being eaten. From these viewpoints, I first examine the onset time of egg feeding by using eight species mentioned above with different hatching synchronization. Then, I evaluate whether embryos of the synchronized species (*H. halys*, *N. viridula*, *Pi. hybneri*, and *Pl. stali*) incur risk of the cannibalism unless they show hatching responses and whether embryos of the non-synchronized species (*A. fieberi*, *D. baccarum*, *E. rugosum*, and *Pa. angulosa*) have no risk of the cannibalism.

Materials and methods

Insects and egg clutches

Adults of *A. fieberi*, *D. baccarum*, *E. rugosum*, *H. halys*, *N. viridula*, *Pa. angulosa*, *Pi. hybneri*, and *Pl. stali* were collected from the field in Kyoto, Osaka and Hyogo Prefectures, Japan, in 2014-2016. To obtain egg clutches, these adults were maintained under a long day photoperiod of LD 16:8 at $25 \pm 1^\circ\text{C}$ in plastic cups, provided with water and oviposition sites made of drawing paper. Foods were supplied as in Chapter 1. Egg clutches were collected with their oviposition substrates within 24 h of oviposition. Eggs were kept at 25°C and 80% or higher relative humidity unless otherwise mentioned. Clutches of particular size ranges were used as in Chapter 1: 12 ± 2 for *A. fieberi* and *E. rugosum*; 14 ± 2 for *Pa. angulosa* and *Pl. stali*; 28 ± 4 for *D. baccarum*, *H. halys*, and *Pi. hybneri*; and 84 ± 13 for *N. viridula*. These ranges nearly correspond to frequently observed clutch sizes in these species.

Onset time of egg feeding

To observe egg feeding behavior, embryonic development was delayed in a small portion of eggs within a clutch so that the first-instar nymphs could feed on after hatching. Within 54 h of collection of an egg clutch, the eggs were detached from its periphery by approximately 15% of the clutch size using a razor blade and forceps under a stereomicroscope (S8 APO; Leica Microsystems, Wetzlar, Germany). These eggs were transferred to $10 \pm 1^\circ\text{C}$ to retard their embryonic development, and then returned to the original clutch kept at 25°C within 2 days before estimated hatching onset. For convenience of observation, the eggs were arranged at nearly equal distances at the periphery of the egg clutch using starch glue. This treated egg

clutch was fixed on the bottom of a clear plastic case (36 × 36 mm, height 14 mm) using a piece of double-stick tape, together with wet cotton to prevent desiccation. Hatching and subsequent egg feeding behavior were recorded by taking a photograph every 5 min from the top view with a digital camera (CX6; Ricoh, Japan or P310; Nikon, Tokyo, Japan). In egg feeding, a first-instar nymph first held a victim egg and, as the nymph sucks the pigmented embryo in the egg shell, the egg dents or changes its color, or both. The first egg holding behavior that resulted in these egg changes within 24 h after the first hatching was recorded on photographs, and the time was regarded as the onset of egg feeding.

Frequency of sibling egg cannibalism in H. halys

A female/male pair was kept in a plastic cup, and egg clutches were collected for 20 days after the first oviposition of fertilized eggs. One or 2 days before estimated hatching of the egg clutch, each constituting egg was checked: If the compound eyes and the egg buster were observed through the operculum of the egg shell, the embryo was judged as normally developing. Nymphs aggregating on their natal egg clutch were eliminated 24-48 h after hatching to inspect the internal unhatched eggs for traces of egg feeding.

Nutritional effects of egg feeding in H. halys

Growth and survival of nymphs were compared between the treatment group in which a nymph ate a single sibling egg soon after hatching and the control group with no egg feeding. Eggs of seven clutches were individually detached on the collection day, and then approximately one-third eggs of each clutch were transferred to 10 ± 1 °C to retard their development for use as victim eggs of the treatment group. The remaining eggs were separately adhered to a piece of drawing paper (15 × 15 mm) using starch glue and allowed to develop normally to obtain

hatchlings for the test. A single egg with delayed development was provided in close to some of these eggs within 24 h before hatching, so that the nymphs could consume it immediately after hatching. The other normally developing nymphs were provided with no eggs. From the former group (the total number of 61), 39 hatchlings that actually consumed the provided egg by the next day of hatching were selected for the treatment group. From the latter group (the total number of 52), 36 hatchlings were selected for the control group, so that nearly the same number of hatchlings from each clutch were included in the both groups.

In both groups, each nymph was kept at 25 °C in a small plastic case (86 mm diameter, 40 mm depth). Wet cotton was provided as a water source since 1 day before hatching, and replenished every day. A half-cut raw peanut was provisioned at 2-4 days intervals since the next day of hatching. Developmental stages of the nymphs were examined daily until the day of adult eclosion or the 72nd day of hatching. All individuals that survived more than 72 days without eclosion died in the final (5th) instar. The sex and body weight were examined on the day of adult emergence.

Results

Egg feeding by newly hatched nymphs

When I provided some sibling eggs with artificially delayed development to an egg clutch for feeding the newly hatched nymphs, nymphs of most species examined actually ate the provided eggs. However, the onset time of egg feeding was remarkably different among these species (Fig. 3). In highly synchronized species, *H. halys* and *N. viridula*, the median onset time was earlier than in the other species, although the difference was not statistically significant between *N. viridula* and *Pa. angulosa* or between *N. viridula* and *Pi. hybneri*. No egg feeding was observed in any egg clutch in *E. rugosum* and approximately a half of egg clutches in *A.*

fieberi for 24 h after the hatching onset.

Behavioral sequences of newly hatched nymphs of *H. halys* and *Pl. stali* until they start egg feeding are shown in Fig. 4. In general, first-instar nymphs of Pentatomidae acquire symbiont bacteria on the egg surfaces during their stay on the natal egg clutches (Prado & Almeida 2009; Hirose et al. 2012). In some species including *H. halys*, nymphs hatching from the egg shells immediately start roaming on the natal egg clutch, and this behavior is associated with the symbiont acquisition (Calizotti & Panizzi 2014; Taylor 2016). Also in my observation, the emerging nymphs of all species examined roamed on the natal egg clutch (*H. halys*, Fig. 4B; *Pl. stali*, Fig. 4G). Then, typically after cuticle of all the nymphs was pigmented, the nymphs settled in close to the egg clutch and became motionless, namely they formed a dense aggregation (*H. halys*, Fig. 4E; *Pl. stali*, Fig. 4H). While the nymphs aggregated, they were usually motionless but sometimes resumed roaming on the egg clutch simultaneously. In some or all clutches of *Pi. hybneri*, *Pl. stali*, *A. fieberi*, *D. baccarum*, and *Pa. angulosa*, egg feeding started when the nymphs resumed roaming after all the nymphs within the clutch became motionless at least once for 5 min (*Pl. stali*, Fig. 4H, I). In *H. halys* and *N. viridula*, however, egg feeding always started during the initial roaming process before the nymphs first became motionless (*H. halys*, Fig. 4C-E).

Risk of sibling egg cannibalism

I conducted the following estimation, based on the relationship between the onset time of egg feeding, and temporal hatching patterns of the intact or detached egg clutches in the eight species examined (Fig. 3), which described in Chapter 1. In the four synchronized species, I evaluated whether an embryo incurs a risk of being eaten by the earlier-hatched siblings if the embryo does not hatch by responding to the siblings. For this purpose, I estimated, for each species, a probability that an embryo without a hatching response remains unhatched at the onset

of egg feeding (Fig. 3, the probability is expected to coincide with a rate of unhatched eggs at the same time in detached egg clutches). In highly synchronized species, *H. halys* and *N. viridula*, it was expected that such an embryo incurs a high risk of the cannibalism due to their early onset of egg feeding. At the median value of the onset time of egg feeding, the probability of being unhatched was 57.1% and 51.9% (median) in *H. halys* and *N. viridula*, respectively. However, both in mildly synchronized species, *Pi. hybneri* and *Pl. stali*, this probability was 0% and, therefore, the risk of the cannibalism was expected to be very low even if an embryo does not show a hatching response. In *H. halys* and *N. viridula*, almost all the embryos in intact egg clutches were estimated to hatch before the onset of egg feeding (Fig. 3). Accordingly, I concluded that embryos of *H. halys* and *N. viridula* can mostly prevent the risk of the cannibalism as far as they hatch in response to the earlier-hatched siblings. Also in egg clutches of non-synchronized species, *A. fieberi*, *D. baccarum*, *E. rugosum*, and *Pa. angulosa*, the embryos seem to be imposed a little or no risk of the cannibalism because almost all the embryos were estimated to hatch before the onset of egg feeding (Fig. 3). I observed no egg feeding in *E. rugosum* in this experiment.

Cannibalism frequency in H. halys

To verify that embryos of *H. halys* avoid sibling egg cannibalism in intact egg clutches, an actual frequency of the cannibalism was observed. Nine females laid 24 clutches (1-5 clutches per female, 660 eggs in total) with clutch sizes of 28 ± 4 eggs for the first 20 days, which accounted for 36.4% of the total number of the egg clutches (62.5% of the total number of the eggs). In 5 of 24 egg clutches, 1-5 eggs (12 eggs in total, 1.8%) were cannibalized by adults before collection. When these eggs were excluded, the development rate per egg clutch was 96.4% (median, interquartile range [IQR], 94.8-100.0%). In 13 egg clutches, there were 1-10 eggs (34 eggs in total, 5.2%) that did not develop normally and thus did not hatch or

synchronize hatching with normally developed clutch mates. Among these 34 eggs, 33 eggs (97.1%) were eaten by hatched siblings. In contrast, normally developed eggs were seldom eaten by the siblings: The number of victims of sibling egg cannibalism was only 9 of 614 eggs (1.5%, 1-3 eggs in 5 egg clutches). The hatching success of normally developed eggs per clutch was 100.0% (median, IQR, 100.0-100.0%, $n = 24$).

*Nutritional effects of egg feeding in *H. halys**

The above results indicate the possibility that highly synchronized hatching of *H. halys* is an embryonic strategy not to be a victim of the egg feeding. To discuss the evolution of the highly synchronized hatching and the egg feeding in this species, I examined whether and how the egg feeding benefits the nymphs nutritionally. I compared the growth and survival between the individuals that fed on a single egg soon after hatching (treatment group) and those that fed on no eggs (control group). Figure 5 shows the duration of nymphal instars between the two groups for individuals that emerged as adults. In the first instar, the duration was significantly shorter in the treatment group than in the control group (Mann-Whitney U test, $U = 496$, $P < 0.001$). The durations of the second to fifth instar did not differ significantly between the groups (Mann-Whitney U test, $P > 0.05$). The mortality also did not differ significantly between the groups for each instar (Fisher's exact test, $P > 0.05$). Median body weight of adult females and males were 0.177 g (IQR, 0.156-0.223 g, $n = 12$) and 0.143 g (IQR, 0.119-0.153 g, $n = 13$) in the control group, and 0.207 g (IQR, 0.186-0.214 g, $n = 11$) and 0.152 g (IQR, 0.142-0.158 g, $n = 17$) in the treatment group, respectively. For the body weight of both sexes, significant differences were not detected between the groups (Mann-Whitney U test, females: $U = 51$, $P = 0.37$; males: $U = 69$, $P = 0.08$). Collectively, by consuming a single egg soon after hatching, nymphs seem to profit only from shortening of the duration in the first instar.

Discussion

Function of highly synchronized hatching

Egg clutches of *H. halys* and *N. viridula* show highly synchronized hatching via embryonic responses to earlier-hatched siblings (Chapter 1). In the present chapter, I expected that without a hatching responses embryos cannot hatch by the time when egg feeding starts in the egg clutch, and thus they have a risk of cannibalism by the earlier-hatched siblings. The obtained results indicate a possible conflicting relationship among siblings in a same egg clutch. Without a hatching response, each embryo in an egg clutch should hatch at spontaneous timing determined by the development rate and endogenous clock. Probably because of fluctuation in the development rate and the period of the endogenous clock, hatching of eggs constituting a clutch is distributed over a certain period, which coincides with hatching duration of detached eggs. Whereas some embryos with earlier spontaneous hatching time have a chance of egg cannibalism, others with later hatching time have a risk of being eaten by earlier siblings. Kutcherov (2015) presumed that, in the case that hatchlings prefer to cannibalize the unhatched sibling eggs and embryos prefer to avoid being cannibalized by the earlier-hatching siblings, it becomes advantageous for both of them to hatch earlier. This advantage would select a relatively fast, uniform embryonic development rate that is close to the species-specific physiological limit. I consider that such a selective advantage possibly compacts the hatching duration. The later-hatching embryos with a risk of cannibalism can reduce the risk if they hatch by responding to earlier hatching of siblings. In *H. halys* and *N. viridula*, I expect also that almost all the eggs evade the cannibalism in intact egg clutches, which have the hatching response. Accordingly, we can consider the hatching response of both species as an avoidance strategy of embryos against sibling egg cannibalism. In intact egg clutches of *H. halys*, normally developed embryos mostly succeeded in hatching without being eaten.

In some animals, embryos hatch by responding to attacks by predators to escape from them (Warkentin & Caldwell 2009; Warkentin 2011), as known in the red-eyed tree frog *Agalychnis callidryas* (Anura: Hylidae) against its snake predator (Warkentin 1995). Here I show that also a cannibalistic clutch mate can be a predator which evolves such a so-called escape hatching. However, in contrast to the cases of the hatching responses to external predators, it is also possible that avoidance of predation is not the driving force of the evolution, as discussed below.

Costs and benefits of sibling egg cannibalism

The above scenario supposes that egg feeding of *H. halys* and *N. viridula* has evolved to prey on later-hatching siblings. For the evolution of cannibalism on viable sibling eggs, the benefits must exceed the costs (Elgar & Crespi 1992). In general, when killing viable siblings, the cannibals will lose not only their inclusive fitness but also group function, which would have received when not killing (Stevens 1992).

Insects in Pentatomidae have gregarious nymphs. In the first instar, nymphs maintain aggregation in close to their natal clutches for a certain period (Kiritani 1964; Saito et al. 1964). In and after the second instar also, they live more or less in groups on food plants (Hokyo & Kiritani 1962). Such group formation promotes survival and growth (Kiritani 1964; Kiritani & Kimura 1966; Kiritani et al. 1966; Nielsen et al. 2008), although instar-specific dependence to the group seems to be different among the species (Kiritani & Kimura 1966). Particularly, the aggregation during the first instar has several specific adaptive functions (Lockwood & Story 1986).

In this chapter, I examined, in *H. halys*, how egg feeding (a single egg) in the early first instar benefits the nymphs nutritionally, and showed significant reduction in nymphal duration of the first instar. This result indicates that the cannibals acquire potential but limited

nutritional advantage against non-cannibals. However, Eickwort (1973) suggests that seemingly marginal nutritional advantage is enough for the evolution of sibling egg cannibalism by newly hatched individuals: Because of high mortality during the immature period, even if benefit of the cannibalism is only a small reduction of nymphal duration in this period, it greatly decreases exposure to mortality factors and thus significantly increases a chance to adult emergence. Furthermore, in many insects, such cannibalism is considered to be especially advantageous for individuals in the first instar because they often suffer specifically high mortality and low food availability (Stevens 1992). In *H. halys*, because the first instar is a period with relatively high mortality both in outdoor and constant temperature conditions (Haye et al. 2014; see Nielsen et al. 2008 also), the observed reduction of nymphal duration in this period can bring adaptively significant consequences to the cannibals. However, there is no evidence that the first-instar nymphs of Pentatomidae suffer starvation risk, because it is widely accepted that these nymphs do not feed on plants and, to become the second instar, do not require uptake of nutrition but only water (e.g. *N. viridula*: Kiritani 1964; Lockwood & Story 1986). Esquivel and Medrano (2014) recently demonstrated plant feeding in *N. viridula*; nevertheless, I consider that the plant feeding is improbable before dispersing from the natal clutches. In this context, it is interesting that many species of this family show egg feeding in the early first instar. Then, an important question is why the newly hatched nymphs actively eat eggs even though they seem not to eat plants. I expect that the nymphs would have some costs in an attempt to feed on plants. For the newly hatched nymphs, the natal egg clutch provides an important tactile cue to form an aggregation (Lockwood & Story 1985b). Therefore, the nymphs would be unable to maintain the aggregation when leaving egg clutch for search of plant foods. Eggs may serve as valuable food sources all the more because the nymphs can eat them without leaving egg clutch.

Interspecific variation and evolution of hatching synchronization

Egg feeding starts earlier in highly synchronized species than in non-synchronized species, *A. fieberi*, *D. baccarum*, and *Pa. angulosa* (no egg feeding in *E. rugosum*). More importantly, in all highly synchronized species, egg feeding starts early enough to impose a risk of being eaten on the embryos without a hatching response. Thus, I consider that only highly synchronized species show an early egg feeding onset with biological significance, and therefore synchronized hatching must have evolved in association with the early onset to some degree. Currently I can discuss the evolution of both traits based on phylogenetic relationship among Pentatomidae only partially. Yuan et al. (2015) conducted phylogenetic analyses among species of the infraorder Pentatomomorpha, including some from Pentatomidae, by using several mitogenomic datasets, and proposed the phylogenetic affinity of (*Dolycoris* + (*Halyomorpha* + (*Eurydema* + *Nezara*))). According to this phylogenetic relationship, my results indicate at least two evolutionary origins of either highly synchronization with early egg feeding onset or non-synchronization with non-early onset. However, I should point out that, in mildly synchronized species (*Pi. hybneri* and *Pl. stali*), egg feeding does not start early enough to impose risk of being eaten even on the embryos without a hatching response. The synchronized hatching of these species may have functions other than avoiding sibling egg cannibalism. My results partially support the hypothesis that the evolution of synchronized hatching has been driven by risk of the cannibalism in Pentatomidae, although some other driving forces can also exist.

I show the following three possible hypotheses for explaining why the early egg feeding evolved in some of the examined species, but not in the others. However, I exclude *E. rugosum* from the discussion because this species does not show egg feeding at all.

The first hypothesis is that these species have different cost-benefit balances of killing and consuming viable sibling eggs. Namely, sibling egg cannibalism is adaptive in *H. halys* and *N. viridula*, but maladaptive in *A. fieberi*, *D. baccarum*, *Pa. angulosa*, *Pi. hybneri*, and *Pl. stali*. In this hypothesis, hatchlings of the latter species do not start egg feeding until viable siblings finished hatching so that they will not kill the siblings. They do egg feeding because, in the natal

clutch, there can be some available inviable eggs, such as unfertilized, abnormally developed, or parasitized eggs. Consumption of parasitized eggs is reported in *Oncopeltus* (Pentatomomorpha: Lygaeidae) (Root & Chaplin 1976). I observed, in all of the latter five species, that hatchlings started egg feeding after spending a motionless period in close to the natal egg clutch, but did not in the former two species. The motionless period of the hatchlings may possibly represent that they avoid consuming viable eggs.

In the second hypothesis, cannibalism of viable eggs is also adaptive in the five species as well as *H. halys* and *N. viridula*. In this hypothesis, these five species could not evolve early egg feeding because, for example, they require a relatively long time for developing some organs essential for egg feeding. It is likely that hatchlings need some time for cuticle sclerotization to become competent to pierce egg shells using the stylet. Even in *H. halys* and *N. viridula*, egg feeding starts at least approximately 1 h after the first hatching and, by that time, the cuticle has been considerably pigmented (*H. halys*, Fig. 4C). It is conceivable that, in the other five species, the hatchlings require more time for their stylets to be available, although cuticle pigmentation have been almost completed before the onset of egg feeding in these species, typically (*Pl. stali*, Fig. 4I).

In the third hypothesis, cannibalism of viable eggs is maladaptive in all the species including *H. halys* and *N. viridula* and egg feeding has evolved to utilize inviable eggs within the natal clutch. In this hypothesis, the highly synchronized hatching of *H. halys* and *N. viridula* was originally selected for some functions other than avoiding egg cannibalism. Then, the early egg feeding onset was selected because it was advantageous in competition among siblings for inviable eggs. However, if this hypothesis is valid, alternative hypotheses is needed to explain the substantial interspecific variation in hatching synchronization. It remains unclear why egg feeding onset greatly differs even among the species except highly synchronized ones and why *E. rugosum* shows no egg feeding.

Detailed examination of the cost-benefit balance of cannibalism will provide a hint

for further discussion on the association between the hatching synchronization and the onset time of egg feeding in Pentatomidae. It is worthwhile, in this family, to explore traits associated with the early onset of egg feeding and examine whether these traits favor cannibalism of viable eggs. A larger clutch size is one of the possible traits favorable for cannibalism because, in larger clutches, cannibalism may less negatively affect group function of nymphs than in smaller clutches. Kiritani and Kimura (1966) showed in *N. viridula* with a relatively large clutch size of 80-100 eggs, that, when the number of nymphs in a group rises to more than 10 individuals, it does not improve survival and development in the early nymphal stages (Kiritani & Kimura 1966).

Chapter 3

Mechanism of highly synchronized hatching in *Halyomorpha halys*

Introduction

In insects that produce egg clutches, embryos are often induced to hatch synchronously by the clutch mates. In the large white butterfly, *Pieris brassicae*, Long (1955) reported that newly-hatched larvae eat egg shells of the clutch mates and thereby facilitate and possibly induce their hatching. In other insects, embryos do not receive such hatching assistance from clutch mates. In a cockroach, *Periplaneta americana*, Provine (1976) speculates that, when some embryos start hatching, they cue other embryos to hatch. In the desert locust, *Schistocerca gregaria*, Nishide and Tanaka (2016) recently showed that embryos obtain hatching cues from the clutch mates. In the giant water bug, *Kirkaldyia* (= *Lethocerus*) *deyrolli*, Ohba (2002) pointed out that similar cue transmission underlies synchronized embryonic molting. These authors demonstrated that the cues are mechanical stimuli probably derived from movements of the embryos or newly-hatched insects. Accordingly, I presume that embryos of many insects respond to mechanical cues from the clutch mates to synchronize hatching or hatching-related behavior. However, it is unclear which movements can actually generate such cues.

In the brown marmorated stink bug, *Halyomorpha halys*, hatching is strikingly synchronized due to embryonic responses to some cues from the clutch mates: Approximately 28 eggs constituting a clutch typically complete hatching within 20 min at 25°C (Chapter 1). In the embryos of Pentatomidae, responses to mechanical cues has not been examined but are likely because embryonic responses to this type of cues have been shown in some species of Heteroptera. Besides the above example in *K. deyrolli* (Ohba 2002), in some sub-social species of Cydnidae and Parastrachiidae, embryos respond to a maternal vibration by immediate hatching (Mukai et al. 2012, 2014).

Before and after the moment of hatching, insects in Pentatomidae can convey various mechanical stimuli to the unhatched siblings. Generally, insect hatching occurs when an embryo repeatedly exerts pressure against the inside of the egg to open the way to the outside (Chapman 1998). Fabre (1903) described that, in Pentatomidae, an embryo inside the egg presses a T-shaped structure (egg buster) against the edge of the egg cap (operculum) and effectively breaks it open. The newly-hatched nymph wriggles out of the egg and finally starts leg movement. Because eggs in a clutch are attached to each other via hardened maternal secretions (Southwood 1956), during this series of events some vibrations or pressures probably spread to nearby eggs and can potentially stimulate their hatching. This chapter aims at better understanding the mechanism of the synchronized hatching in *H. halys*. I examined whether the embryos respond to mechanical cues from the clutch mates and, if this is the case, which movements of the embryos or nymphs generate the cues. Furthermore, I investigated the cue transmission to ask why the entire clutch hatches so synchronously.

Materials and methods

Insect rearing and general handling of eggs

Adults of *H. halys* were collected from the field in Kyoto and Hyogo Prefectures, Japan, in 2014, 2016, and 2017. These adults and their offspring were used to obtain egg clutches. Adults and nymphs were fed on raw seeds of peanut and dried seeds of soybean at $25 \pm 2^\circ\text{C}$ under a LD 16:8 photocycle. Pieces of drawing paper were provided as oviposition substrates.

Egg clutches laid within 24 h were collected and kept in the same temperature and light conditions. Within 2 days before hatching, eggs were detached from the clutches with a razor blade and forceps. This procedure produced separate eggs or pairs of two adjacent eggs that remained attached. In the following experiments, these detached eggs were fixed on

substrates with their anterior surfaces upside using starch glue. The eggs were kept at $25 \pm 2^{\circ}\text{C}$ under constant light throughout the experiments. Eggs of this species are almost spherical shape with a diameter in the top view of approximately 1.4 mm (Kobayashi 1956).

Tests for the type of the hatching cue

Two experiments were designed to determine the type of the cues that are emitted from clutch mates and are used to synchronize hatching. In both experiments, eggs of the same clutches were paired and variously treated. In each treatment, it was examined whether one egg can synchronize hatching with the other egg.

The first experiment included the following four treatments: (1) Two adjacent eggs that remained attached were fixed on a piece of drawing paper (15×15 mm); (2) Two separate eggs were fixed together on a piece of paper with their side faces attached to each other using starch glue; (3) Two separate eggs were fixed together on a piece of paper at a distance of 1 mm; (4) Two eggs were separately fixed on two pieces of paper and isolated from each other. Eighteen clutches were used and three or four pairs were produced from each clutch for each of the four treatments.

The second experiment included the following two treatments: (1) Two separate eggs were fixed together on a piece of paper at a distance of 1 mm; (2) Two separate eggs were fixed on a piece of paper in the same way and bridged by a lead for mechanical pencils (0.3 mm diameter, 4 mm length, HRF3G-20-B; Pilot, Tokyo, Japan) that was attached to the side faces of the two eggs in parallel with them using starch glue. Eight clutches were used and six or seven pairs were produced from each clutch for each of the two treatments.

Pieces of paper carrying egg(s) were individually placed in a clear plastic case ($36 \times 36 \times 14$ mm). Then, hatching was recorded every 5 min from the top view by a digital camera (P310; Nikon, Tokyo, Japan). Synchronized hatching in a pair of eggs was defined that the

second egg hatched within 15 min after the first egg hatched, based on the fact that, in pairs of two adjacent eggs that remained attached, hatching rate of the second egg exceeded 90% at 15 min after the hatching of the first egg.

Vibration recording and analysis

I hypothesized that embryos of *H. halys* hatch in response to a vibration generated when a nearby clutch mate breaks the egg open. In *S. gregaria*, such a substrate-borne vibration is regarded as one of the possible mechanical cues that can be used to synchronize hatching (Nishide & Tanaka 2016). In a heteropteran *K. deyrolli*, an air-borne vibration arises when an embryo opens the egg (Ohba 2002), and my preliminary observation showed that this is also the case in *H. halys*. I assume, therefore, an embryo of this species can transmit vibrations to unhatched siblings mainly through contact between eggs.

I focused on a vibration that is transmitted from the adjacent clutch mate when it ruptures the egg. To record such a vibration, a pair of two adjacent eggs that remained attached were fixed on a piece of drawing paper (15 × 15 mm), and one was targeted for vibration recording, referred to as “target egg”, and the other was used to generate a vibration by egg rupture, “adjacent egg”. Egg vibrations were detected using a laser Doppler vibrometer (LV-1720A, Ono Sokki, Yokohama, Japan) with a 20 kHz low-pass filter, by directing its laser beam at the target egg (Fig. 6A). Laser reflection was enhanced by a small piece of reflective tape (1 × 1 mm). Output signals from the vibrometer were sent to a computer using a data acquisition hardware (LAN-XI type 3160-A-042; Brüel & Kjær, Nærum, Denmark) and monitored in real time using the PULSE Labshop software (Brüel & Kjær) for vibration recording. At the same time, the hatching behavior was observed and recorded using a digital video camera (HDR-CX630V; Sony, Tokyo, Japan) attached to a binocular stereomicroscope (M651 Leica Microsystems, Wetzlar, Germany) (Fig. 6A). Then, vibration recording was attempted at the

moment of the egg rupture if hatching started earlier in the adjacent egg than in the target egg. Twenty pairs from five egg clutches were used for the recording trials, and 18 of them were observed until hatching.

The recordings were converted to WAV file format and used to analyze the duration of the vibrations with the PRAAT software (www.praat.org). To analyze spectral characteristics of the recorded vibrations, power spectra were computed by PULSE Labshop using fast Fourier transform (Hanning window, 12.8kHz bandwidth, 1600 lines). Recordings used here were 0.1 s segments that included each vibration by egg rupture. Power spectra were also obtained for background noise that was recorded in target eggs just before hatching of the adjacent eggs (1-75 min before hatching) and used as controls for playback experiment. Recordings of dead eggs ($n = 2$) showed that the power spectra of dead eggs were very similar to those of background noise.

Playback experiment

Vibration recordings were played back to eggs slightly before hatching to examine whether embryos respond to a vibration produced by egg rupture. For this purpose, two recordings were converted to uncompressed WAV file format and trimmed to 1 s segments containing each of the vibrations using the SASLab Pro software (Avisoft, Glienicke, Germany). In addition, two corresponding recordings of background noise were edited in the same way for control files. Signals of the edited files were sent from a computer to a vibration exciter (type 4809; Brüel & Kjær) using the LAN-XI with its signal generating function. The exciter was connected to a power amplifier (type 2718, Brüel & Kjær) and equipped with a flat steel plate (5×5 cm, 2.3 mm thick) by a screw (5 mm diameter, 8 mm length) for a position to place eggs. Outputs from the exciter were adjusted as follows: For the vibration by egg rupture, the amplitude range (minimum to maximum) was approximated to the average value of the recorded

vibrations. For the background noise, the peak amplitude was approximated to the background level of the vibration by egg rupture that was played.

The playback experiment was performed as follows: 26-28 eggs originating from the same clutch were individually fixed on cover glasses (15×15 mm, 0.13-0.16 mm thick). A piece of polychloroprene tape (5×5 mm, 5 mm thickness) had been attached on the cover glass as a grip for convenience of holding. The eggs were separately placed on pieces of polyurethane tape, which had been attached on a plastic tray, so that vibrations would not spread from some eggs to others. Hatching of the eggs was checked every 10 min until two or more eggs started hatching. Within the next 10 min, the four recordings (two vibrations by egg rupture, two background noise) were played back once to four eggs before hatching, respectively: Four eggs fixed on the cover glasses were placed on the exciter, exposed to playback of one of the recordings, and placed back on the tray (Fig. 6B). These procedures were repeated for the other three recordings. Then, hatching was recorded every 5 min from the top view by a digital camera (P310). Four egg clutches were used for this experiment. Therefore, each recording was played back to 16 eggs in total.

Tests for transmission efficiency of the hatching cue

If the vibration by egg rupture serves as a hatching cue, the following experiment was conducted to examine how far the vibration can reach from one egg to the other within an egg clutch maintaining the function as the cue. Two living eggs and one, two or three separate dead eggs were fixed on a piece of paper (drawing paper, 15×15 mm) in a line so that the latter was sandwiched between the former. Each egg was attached to the next egg(s) at the side faces using starch glue. Then, in each egg set, it was examined whether the egg at one end can synchronize hatching with the egg at the other end. Besides above three types of egg sets, two types of controls were used: two separate eggs that were fixed together on a piece of paper with their

side faces attached to each other using starch glue and two eggs that were separately fixed on two pieces of paper and isolated from each other. Ten clutches were used and one or two egg sets were produced from each clutch for each of the five types of egg sets. The pieces of paper carrying the egg(s) were individually placed in a clear plastic case ($36 \times 36 \times 14$ mm), and then hatching was recorded every 5 min from the top view by a digital camera (P310).

RESULTS

Type of the hatching cue

Synchronized hatching in a pair of eggs was defined that one egg hatched within 15 min after the other egg hatched. Synchronized hatching was observed in more than 90% of pairs of adjacent eggs that remain attached (group A) and pairs of separate eggs that were attached together (group B) (Fig. 7). Because the proportion of synchronized hatching was not different significantly and almost the same between the two groups, it seems that the cue can be conveyed between two eggs that were attached artificially at least as much as those attached naturally. In pairs of eggs that were placed at a distance of 1 mm (group C) and in pairs of eggs that were isolated to interrupt the cue transmission (group D), the proportion of synchronized hatching was significantly lower than the groups A and B (Fig. 7). If the cue is either air-borne sound or volatile chemicals, hatching would be mostly synchronized in the group C. Therefore, the cue seems not to be transmitted through the air but the attachment between eggs, and thus vibrations or pressures. The proportion of synchronized hatching was significantly higher in the group C than in the group D. This possibly means that the cue is vibrations that are produced by an embryo or a hatched nymph and sometimes conveyed through the egg substrates (drawing paper) and stimulate hatching.

However, it remains possible that air-borne stimuli were more attenuated in the group

C than in the groups A and B due to the longer distance between the two eggs in the formers. If vibrations transmitted from one egg stimulate hatching of the other, the synchronization will be facilitated when the transmission is enhanced. In this context, I observed hatching in pairs of separate eggs that were placed at a distance of 1 mm with or without a bridge between the eggs. The proportion of synchronized hatching was significantly higher when two eggs were bridged (Fig. 8). This means that, in clutches of *H. halys*, some kind of vibration is transmitted from embryos or hatched nymphs to unhatched embryos and used as a cue to synchronize hatching.

Vibration by egg rupture

In my observation on hatching in pairs of adjacent eggs that remained attached, the eggs always opened along a circular hatching line where the operculum joins the body of the chorion. To open the egg, the embryo repeatedly pushed the egg-buster against the operculum, which could be seen through the operculum as tiny movements from the dorsal to ventral side. Eventually, the egg ruptured vigorously at a small part of the hatching line with which the egg-buster had had contact, thus resulting in a slit on the egg surface. Thereafter the nymph wriggled out of the egg extending the slit to the both sides. Egg rupture of the first-hatched egg was immediately followed by the second one with the interval of 110 ± 48 s (mean $\pm$ SD, $n = 18$). Because the first-hatched nymph always remained inside the egg during this interval, I deny the possibility that movements of fully emerged nymphs generate the vibrational cues.

I recorded a single pulsed vibration that was transmitted from the adjacent egg at the moment of the egg rupture (Fig. 9A-C). The vibration by egg rupture had a short duration of 3.0 ± 0.4 ms (mean $\pm$ SD, $n = 8$) and an amplitude range (minimum to maximum) of 60.0 ± 21.3 mm/s (mean $\pm$ SD). Spectral analyses revealed that the vibration typically had a broad frequency range from low to high frequencies, when compared to background noise (Fig. 9D-F). A dominant frequency peak was mostly more than 5 kHz (e.g. 20.5 kHz in Fig. 9D; 10.7 kHz in

Fig. 9F), except the one where a peak at 112 Hz dominated a peak at 18.7 kHz (Fig. 9E). When limited to frequency ranges more than 5 kHz, the mean peak frequency ($\pm$ SD) was 15.4 ± 7.8 kHz. Some peaks existed also at lower frequencies between 100-400 Hz (Fig. 9D, E), although such peaks were mostly not distinctive and sometimes absent (Fig. 9F).

Embryonic responses to vibration playbacks

Two recordings of a vibration by egg rupture induced hatching significantly more frequently within a short period of 15 min than the corresponding background noise recording (Fig. 10). Within 15 min, more than 60% eggs hatched in the groups with a vibration by egg rupture, whereas only a small portion of eggs (6.3% and 18.8%) hatched in the groups with background noise. Accordingly, I conclude that, in clutches of *H. halys*, an embryo hatches in response to a single egg vibration caused by rupture of the earlier-hatching egg.

Transmission efficiency of the hatching cue

Hatching was synchronized, i.e. occurred within 15 min, in pairs of separate eggs significantly more frequently when transmission of the hatching cue was interrupted by one to three dead eggs than when the eggs were isolated (Fig. 11). Furthermore, the frequency of synchronized hatching did not differ significantly among the pairs of eggs in which the cue transmission was interrupted by no or one to three dead eggs. Together with the above findings on a vibrational cue for hatching, these results indicate that the vibration can be transmitted over at least three eggs in a line in contact between adjacent ones and function as a cue.

DISCUSSION

Mechanism of synchronized hatching

Results in this chapter show that, in *H. halys*, egg hatching is cued by a short vibration generated when the clutch mate ruptures the egg during hatching. When the first egg ruptures in a clutch, the emitted vibrational cue causes hatching of nearby eggs, which generates additional cues. Because of this successive hatching and cue generation, the entire clutch hatches synchronously. In this species, when eggs in a clutch are individually detached and isolated so that the embryos do not receive hatching cues, the last hatching occurs approximately 4 h after the first one at 25°C (Chapter 1). Taken together with the embryonic development time of 5-6 days at this temperature, embryos slightly before beginning spontaneous hatching seem to respond to the cues to hatch in intact clutches.

According to results in Chapter 1, hatching of another stink bug, *Nezara viridula*, is also synchronized within a clutch probably due to cue transmission but less synchronized than in *H. halys*. In *N. viridula*, after the first hatching, the second one predominantly occurs at the adjacent of the first egg. On the other hand, this tendency is not observed in *H. halys*. The results in Chapter 1 suggested that this is probably because a hatching cue can pass from one egg to distant ones as well as the adjacent ones within a clutch, and the results in this chapter confirmed this idea. I speculate that, in a clutch of *N. viridula*, a hatching cue produced by one egg stimulate only the adjacent ones. Difference in reaches of the hatching cues may be one reason why the hatching is more synchronized in *H. halys* than in *N. viridula*.

Lockwood and Story (1985a) used pairs of adjacent eggs in *N. viridula* and found that two eggs hatch with a longer interval when the first-hatched nymph is removed from the egg after its operculum lifted. Accordingly, hatching cues of *N. viridula* seem to arise after egg rupture, and thus differ from those of *H. halys* which are generated at the moment of egg rupture. Earlier generation of the cues in *H. halys* may be another reason of the more synchronized hatching. In conclusion, embryos in different species possibly use cues generated by different

movements of embryos or nymphs to synchronize hatching.

Escape from sibling cannibalism

Nymphs of *H. halys* begin to prey on unhatched eggs with a short delay after hatching. In Chapter 2, I considered that later-hatching embryos advance the timing of hatching to escape from the sibling cannibalism and thus hatching of an entire clutch is synchronized. Potentially, synchronized hatching is caused when earlier-hatching nymphs facilitate hatching of the siblings for their own benefits, as shown in a lepidopteran species (e.g. Long 1955). However, results in this chapter revealed that hatching of *H. halys* is synchronized because later-hatching embryos hatch by responding to vibrations accompanied by hatching of the earlier siblings. This mechanism coincides with the above evolutionary story of synchronized hatching.

In some animals, embryos assess predation risk by receiving cues generated by nearby predators and thereby alter the timing of hatching to reduce the risk (Warkentin & Caldwell, 2009; Warkentin 2011a). The hatching response of *H. halys* seems to be the same anti-predator defense. Vibrational cues provide information on the risk by conspecifics in *H. halys*, and the risk by heterospecifics in other animals. In several frog species, such as the red-eyed tree frog, *Agalychnis callidryas*, embryos are often attacked by predators like snakes and wasps. When the embryos receive vibrations from the predators, they immediately hatch to avoid being eaten, even if they are not fully developed (Warkentin 2011b). In insects after hatching, it has been shown that air-borne or substrate-borne vibrations are used to assess risk by heterospecific predators and parasitoids (e.g. Tautz & Markl 1978; Gnatzy & Kämper 1990; Bacher et al. 1997). My findings provide further evidence that insect embryos respond to vibrations, thus emphasizing the importance to study such use of vibrations in a stage before hatching.

Detection of the vibrational cue

Vibrations by egg rupture have a very short duration and a broad frequency range. Although it is still unclear how embryos of *H. halys* detect such vibrations, we can explain that the embryos use vibrational sensory organs same as used during communication between adult males and females. In many species of Pentatomidae, adults before copulation emit vibrational song signals with frequency ranges below 1 kHz and dominant frequencies around 100 Hz (e.g. Kon et al. 1988; Čokl et al. 2001). These signals are conveyed via plants and received by leg vibrational sensory organs (Čokl 2008; Nishino et al. 2016). Song signals of *H. halys* have peak frequencies which mostly lie between 50-80 Hz and do not exceed 400 Hz (Polajnar et al. 2016). Therefore, at least in the adult stage, this species can detect vibrations of low frequency around this range. In sub-social relatives of the family Cydnidae and Parastrachiidae, it is evident that embryos detect low-frequency vibrations. Embryos hatch by responding to maternal vibrations, and these responses are also induced by artificial vibrations of several tens of Hz (Mukai et al. 2012, 2014). Collectively, it is probable that embryos of *H. halys* respond to low-frequency components of the vibrations by egg rupture. Although not evident in results in this chapter, the vibrations seem to contain relatively high energy at lower frequencies between 100-400 Hz, and this may facilitate the embryonic responses. I cannot exclude the possibility that the embryos respond to high frequencies above 10 kHz, where the vibrations by egg rupture have main energy. However, in *N. viridula*, the leg vibratory receptor neurons hardly respond to frequencies above 5 kHz (Čokl 1983).

As mentioned above, immature embryos of the frog *A. callidryas* hatch when they receive vibrations by predators like snakes. Because immature hatching incurs some costs, the embryos usually do not respond to vibrations from harmless sources (Warkentin 2005). It is interesting to ask, in *H. halys*, whether embryos show such discrimination of vibrations. *A. callidryas* achieves this by responding only to vibrations with specific frequency properties and temporal patterns (Warkentin et al. 2006; Caldwell et al. 2008). I speculate that embryos of *H.*

halys discriminate vibrations, if they do, in a very different way from *A. callidryas* because the vibrations by egg rupture do not have temporal patterns that can appear in vibrations that last for such a long time as several seconds (Warkentin et al. 2006). In this context, it is important to examine vibrational properties that *H. halys* embryos use to recognize the sibling cue.

General discussion

In the present study, I revealed new aspects on the mechanism and function of synchronized hatching in Pentatomidae. Hatching is highly synchronized in *H. halys* and *N. viridula*. This seems an embryonic strategy to escape from cannibalism by earlier-hatching siblings because, in these species, newly hatched nymphs start egg feeding early. It is unclear why the onset time of egg feeding varies with species, which may provide important issues in behavioral ecology, as discussed in Chapter 2. In Chapter 2, I demonstrated that, in *Pi. hybneri* and *Pl. stali*, the mildly synchronized hatching has not evolved due to the same factor as in *H. halys* and *N. viridula*. For fully understanding the interspecific variation in the hatching synchronization in Pentatomidae, it is also important to study the function of the mildly synchronized hatching. In Chapter 1, I proposed two possible adaptive functions of synchronized hatching besides avoiding sibling egg cannibalism. These are worth testing in *Pi. hybneri* and *Pl. stali*.

As an embryonic strategy to avoid cannibalistic attacks from siblings, accumulation of a defensive chemical is known in insects (Kirk 1988). A hatching response as the similar strategy is the recently proposed idea as a possible adaptive function of synchronized hatching in the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae) (Nishide & Tanaka 2016). In a conventional view, mothers of eggs are predominantly control the hatching synchronization so that they can obtain the best fitness consequences from the subsequent sibling cannibalism as shown (in birds, Stoleson & Beissinger 1995; Mock & Parker 1997, in insects, Crespi 1992; Dickinson 1992; Perry & Roitberg 2005a, b). Mothers of some birds induce asynchronous hatching within a clutch by dispersing timing of oviposition, and this causes the younger, smaller hatchlings to be cannibalized by the older, larger ones (Ingram 1959). In insects, mothers of the green lacewing *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) somehow control the hatching synchronization of the eggs that are placed close to each other, to adjust frequencies of

egg cannibalism according to availability of aphid prey (Fréchette & Coderre 2000).

The present study shows possible participation of embryos (potential victims) in the control of hatching synchronization and sibling cannibalism (Chapter 2). However, it is doubtless that mothers of eggs still take the initiative in controlling synchronization via hatching responses, because transmission of cues for the responses should deeply depend on how the mothers arrange their eggs within a clutch. A hatching response reducing sibling egg cannibalism has two aspects, i.e. an embryonic escape from the cannibalism and a component of maternal control of the cannibalism.

It is worth testing, in other insects, whether embryos hatch in response to clutch mates to avoid sibling cannibalism. In some species of Coccinellidae (Coleoptera), many eggs in a clutch hatch synchronously, i.e. within 10 min after the first hatching, and the rest of the eggs are often cannibalized (Michaud & Grant 2004). Although such a synchronized hatching implies hatching responses to earlier-hatching clutch mates and avoidance of sibling cannibalism, Michaud and Grant (2004) did not mention these possibilities.

I clarified, for the first time in insects, a mechanical cue from clutch mates used for synchronized hatching (Chapter 3). My finding provides a base for future studies on how embryos achieve synchronization of hatching in noisy environment, as discussed in Chapter 3. In Chapter 3, I pointed out a possibility that cues used in embryos for synchronized hatching are different between *H. halys* and *N. viridula*. According to the results in Chapter 2, the embryos synchronize hatching probably to avoid sibling cannibalism. These two species may possibly have evolved from an ancestral species that have no synchronized hatching and have independently acquired different cues for avoidance of cannibalism. Other evolutionary scenarios are also possible, but it is nevertheless interesting to ask why the two species in the same family would respond to different cues. I consider that available cues may differ depending on egg structure or egg arrangement manner in a clutch, both of which are highly variable among Pentatomidae (Southwood 1956).

In conclusion, the present study revealed complex relationships among siblings of early developmental stages in Pentatomidae and, more generally, gave new directions in research on the mechanism and function of synchronized hatching mediated by embryonic responses to clutch mates. Such responses can function as an adaptive strategy against cannibalistic siblings. This finding emphasizes diverse social contexts underlying evolution of hatching synchronization.

Acknowledgements

I am grateful to Dr. Hideharu Numata and Dr. Akira Mori, Kyoto University, for their guidance throughout this study, and Dr. Takuma Takanashi and Dr. Hiromi Mukai, Forestry and Forest Products Research Institute, for their suggestions and assistance in vibration recording and playback. I appreciate Dr. Minoru Ishii, Osaka Prefecture University, and Dr. Yoshito Suzuki for their critical comments during the preparation of this thesis. I am also thankful to Kazuhiko Kuge and Masaho Haikata for their assistance in collecting insects at the experimental farm of Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center, and Dr. Elizabeth Nakajima for linguistic corrections in Chapter 1. I would like thank Dr. Hiroko Udaka, Kyoto University, and colleagues in Laboratory of Ethology, Kyoto University, for their helpful advice and encouragement.

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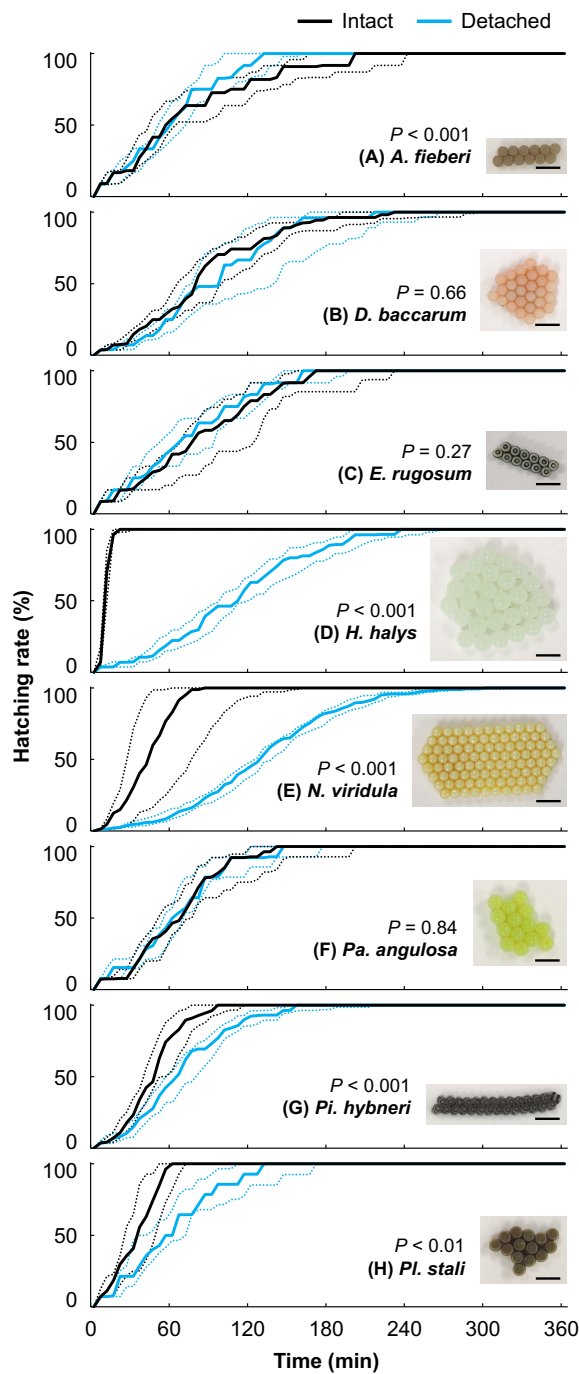


Fig. 1. Comparison of hatching patterns between intact egg clutches and eggs individually detached from egg clutches of eight species in Pentatomidae at 25°C. (A) *Aelia fieberi*, (B) *Dolycoris baccarum*, (C) *Eurydema rugosum* (D) *Halyomorpha halys*, (E) *Nezara viridula*, (F) *Palomena angulosa*, (G) *Piezodorus hybneri*, and (H) *Plautia stali*. The median (solid line), the first quartile (lower dotted line), and the third quartile (upper dotted line) are shown for the hatching rate at 5 min intervals. *P*-values indicate statistical differences in hatching patterns between intact egg clutches and eggs individually detached from egg clutches (two-way repeated measures ANOVA with the Greenhouse-Geisser correction). Inset photographs show egg clutches of these species. Scale bar, 2 mm. See Table 1 for further information on egg clutches used.

Table 1. Comparison of various parameters between intact egg clutches and eggs individually detached from egg clutches of eight species in Pentatomidae at 25°C.

Species	<i>n</i>		Clutch size ^a		Egg period (day) ^a		No. of hatching eggs in 6 h ^a	
	Intact	Detached	Intact	Detached	Intact	Detached	Intact	Detached
<i>Aelia fieberi</i>	15	20	12 [11-12]	12 [11-13]	5 [5-6]	5 [4-6]	12 [11-12]	12 [11-13]
<i>Dolycoris baccarum</i>	16	13	28 [26-31]	28 [25-29]	5 [4-5]	5 [4-6]	27.5 [24-29]	27 [25-29]
<i>Eurydema rugosum</i>	18	19	12 [11-12]	12 [11-12]	6 [5-6]	6 [5-6]	12 [11-12]	12 [11-12]
<i>Halyomorpha halys</i>	15	15	28 [27-28]	28 [26-28]	5 [5-5]	5 [5-6]	28 [27-28]	27 [25-28]
<i>Nezara viridula</i>	13	10	83 [72-96]	84.5 [75-96]	6 [5-7]	6 [5-6]	81 [69-94]	80.5 [71-93]
<i>Palomena angulosa</i>	12	9	14 [12-14]	14 [14-14]	6 [5-7]	6 [5-6]	14 [12-14]	14 [13-14]
<i>Piezodorus hybneri</i>	20	15	27.5 [24-32]	28 [25-32]	5 [4-6]	5 [4-5]	27 [23-32]	28 [24-32]
<i>Plautia stali</i>	22	11	14 [14-14]	14 [14-15]	4 [4-5]	4 [4-5]	14 [13-14]	14 [13-15]

^aThe values are not significantly different between intact egg clutches and eggs individually detached from egg clutches (Mann-Whitney *U* test, *P* > 0.05). Data are shown as median and minimum/maximum value.

Table 2. Comparison between observed frequencies of the second hatching occurring in an egg adjacent to the first hatching egg of an intact egg clutches and those expected by random hatching in eight species in Pentatomidae.

Species	<i>n</i>	Observed frequency	Expected frequency	<i>P</i> ^a
<i>Aelia fieberi</i>	15	0.27	0.32	0.76
<i>Dolycoris baccarum</i>	16	0.06-0.13 ^b	0.16	0.74-0.94 ^b
<i>Eurydema rugosum</i>	18	0.39	0.32	0.33
<i>Halyomorpha halys</i>	15	0.13-0.27 ^b	0.12	0.096-0.55 ^b
<i>Nezara viridula</i>	13	0.69-0.77 ^b	0.07	< 0.001
<i>Palomena angulosa</i>	12	0.42	0.29	0.26
<i>Piezodorus hybneri</i>	20	0.35	0.14	0.02
<i>Plautia stali</i>	22	0.45	0.22	0.01

^a *P*-values were calculated using one-tailed binomial tests.

^b The values are shown as possible ranges because the second hatching could not be exactly determined in some of the egg clutches.

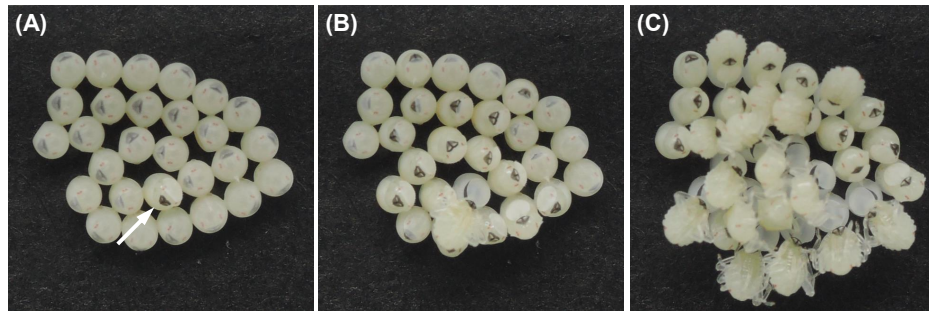


Fig. 2. Highly synchronized hatching in an intact egg clutch of *Halyomorpha halys*. (A) The first egg has just started to hatch (arrow). The egg buster (black triangle) and the compound eyes (red spots) can be seen in unhatched eggs. (B) After 5 min, the whole body of the first nymph has emerged from the egg chorion and several other eggs have started to hatch. (C) After 10 min, more than half of the nymphs have emerged from the egg chorions and the remaining eggs have also started to hatch.

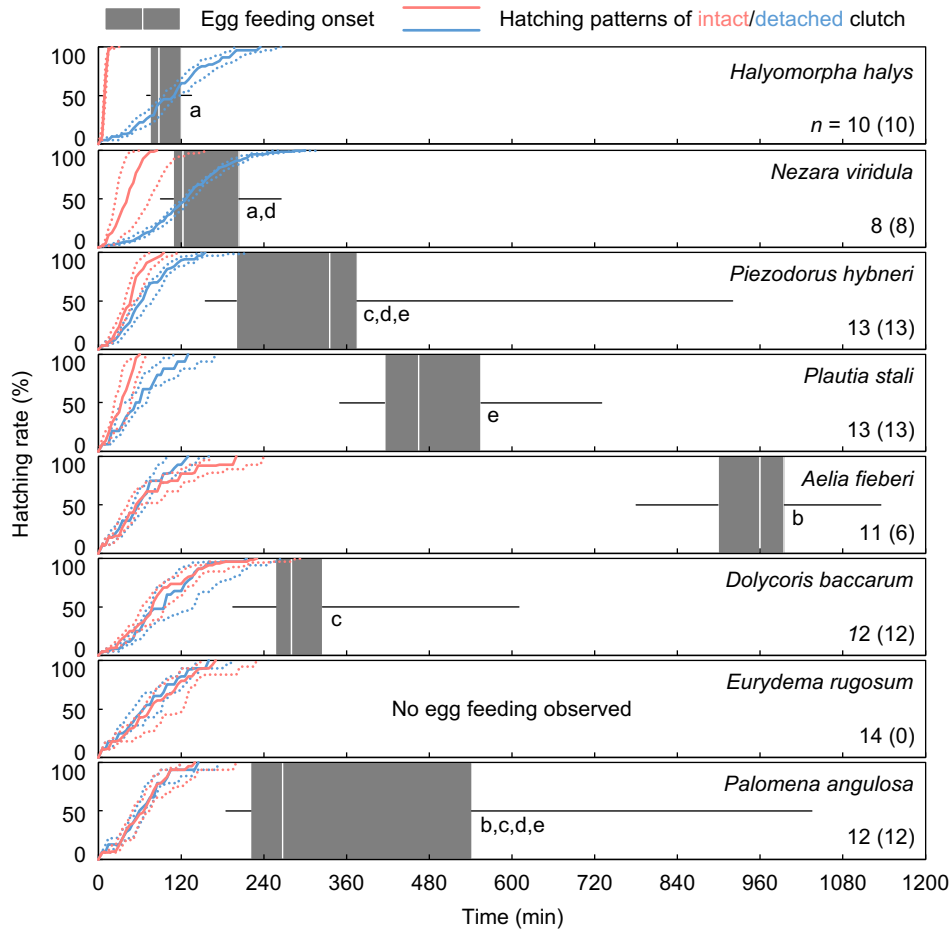


Fig. 3. Relationship between the onset of egg feeding and temporal hatching patterns of intact or detached egg clutches (Fig. 1) in eight species of Pentatomidae. Highly synchronized species, *Halyomorpha halys* and *Nezara viridula*; mildly synchronized species, *Piezodorus hybneri* and *Plautia stali*; and non-synchronized species, *Aelia fieberi*, *Dolycoris baccarum*, *Eurydema rugosum*, and *Palomena angulosa*. Box-and-whisker plots with different letters indicate statistical differences in the onset of egg feeding (Steel-Dwass test, $P < 0.05$). The numbers of observed egg clutches and those in which egg feeding was actually observed are shown under the species name (the latter are in parentheses). For the temporal hatching patterns, the median (solid line), the first quartile (lower dotted line), and the third quartile (upper dotted line) are shown for the rates of unhatched eggs within a clutch at 5 min intervals. In *H. halys* and *N. viridula*, it was expected that an embryo can frequently be unhatched at the onset time of egg feeding if not hatching by responding to clutch mates, based on the rate of unhatched eggs in the detached clutches at the same time. However, this was not the case in *Pi. hybneri* and *Pl. stali*. In intact clutches of all the species examined, almost all the eggs seem to complete hatching at the onset time of egg feeding.

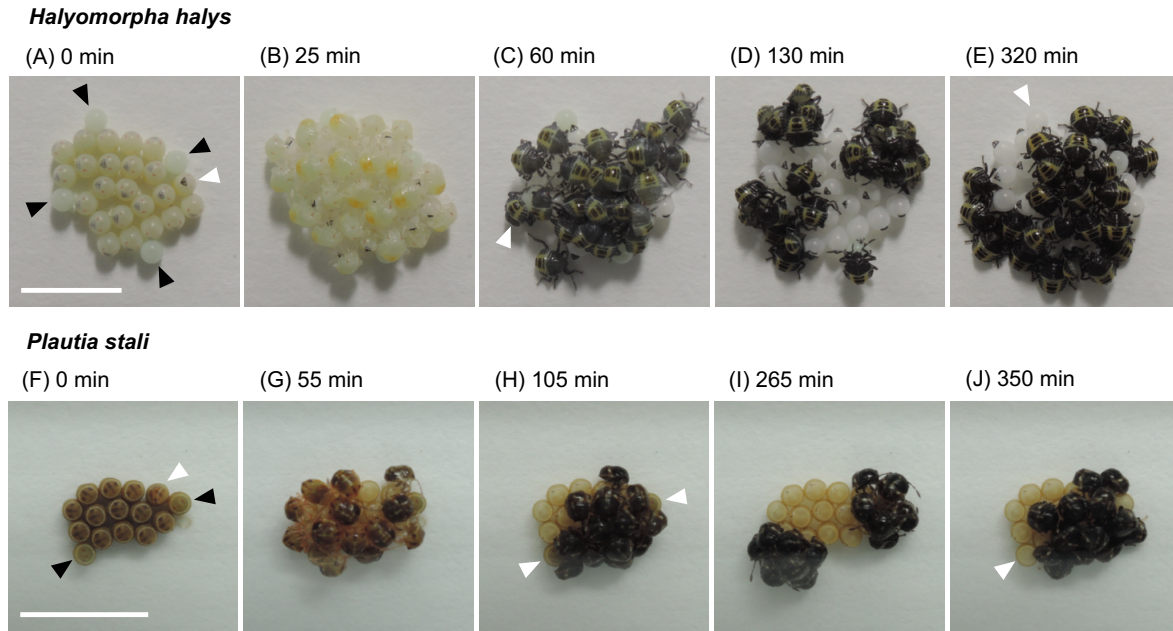


Fig. 4. Behavioral sequences after hatching in relation to egg feeding behavior. (A-E) *Halyomorpha halys* and (F-J) *Plautia stali*. (A, F) The first egg has just started to hatch (white triangle). Some eggs with experimentally delayed development had been provided beforehand at the periphery of the egg clutches (black triangle) so that the newly hatched nymphs could feed on. (B, G) After 25 and 55 min, all the eggs of *H. halys* and *Pl. stali* have hatched except the provided eggs, respectively, and most of the nymphs have already egressed from the egg shells and started roaming on the natal clutches. (C) In the egg clutch of *H. halys*, after 60 min, one nymph has started holding one of the provided eggs (white triangle) to eat it. The cuticle of the nymphs has been considerably pigmented. (D) After 130 min, all the provided eggs have been subjected to egg feeding. All the nymphs are observed aggregating around the provided eggs. (E) After 320 min, egg feeding has finished and the nymphs have settled in close to the egg clutch for the first time and become motionless. (H) In the egg clutch of *Pl. stali*, after 105 min, the provided eggs have not been eaten yet (white triangle) but the nymphs have become motionless. The cuticle pigmentation of the nymphs has almost completed. (I) After 265 min, the nymphs have become active and aggregated around the provided eggs to eat them. (J) After 350 min, egg feeding has finished and the nymphs have become motionless again. Scale bar = 5 mm.

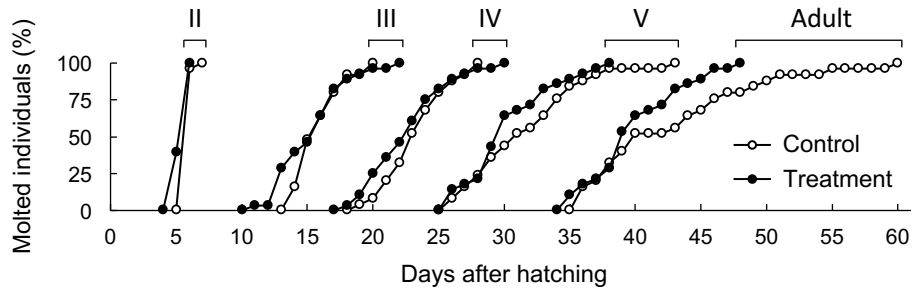


Fig. 5. Duration of *H. halys* nymphs that fed on a single egg soon after hatching (treatment group: $n = 25$) and no eggs (control group: $n = 28$) shown for each instar as cumulative percentages of molted individuals. Within each experimental group, because no significant sexual differences were detected in the duration of each instar (Mann-Whitney U test, $P > 0.1$), the data of females and males were combined.

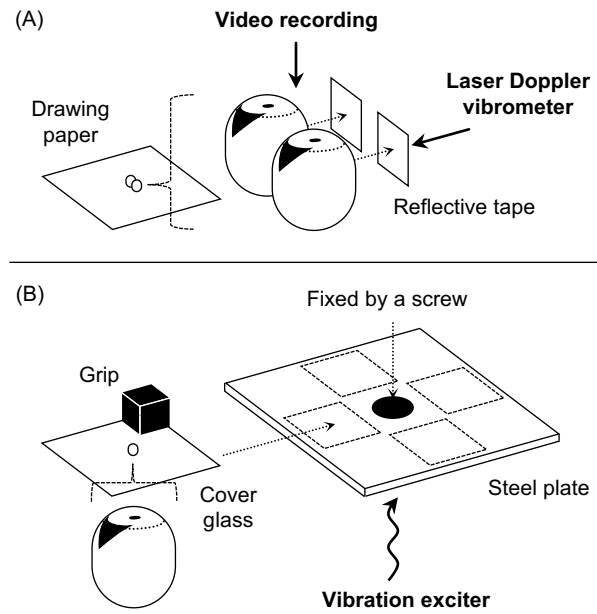


Fig. 6. Diagrams of the experimental setup. (A) Vibration recording by a laser vibrometer and simultaneous video recording. (B) Vibration playback by an exciter. Before playing back one vibration recording, four cover glasses each carrying a single egg were placed simultaneously on a steel plate (dashed squares).

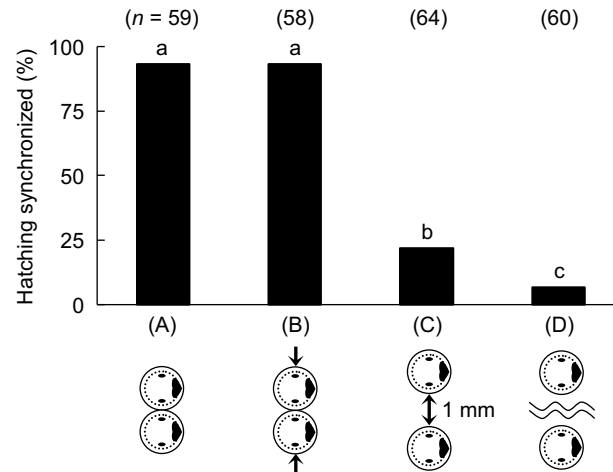


Fig. 7. Proportion of synchronized hatching among pairs of eggs originating from a same clutch and variously treated. Synchronized hatching was defined that both eggs hatched within 15 min. (A) Pairs of adjacent eggs that remained attached. (B) Pairs of separate eggs that were attached together. (C) Pairs of eggs that were placed at a distance of 1 mm. (D) Pairs of eggs that were isolated from each other. Pieces of drawing paper (15 × 15 mm) were used for egg substrates. Different letters above the columns show significant differences ($P < 0.05$, Fisher's exact test with Holm correction for multiple tests).

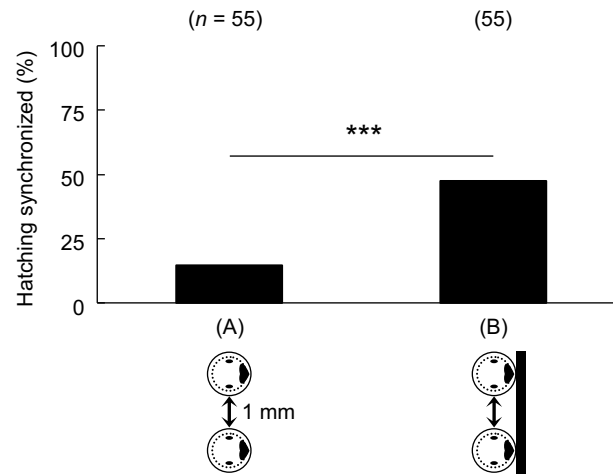


Fig. 8. Proportion of synchronized hatching among pairs of eggs originating from a same clutch and placed at a distance of 1 mm with or without a bridge between the eggs. Synchronized hatching was defined that both eggs hatched within 15 min. (A) Without a bridge. (B) With a bridge by a lead for mechanical pencils. Pieces of drawing paper (15 × 15 mm) were used for egg substrates. ***, a significant difference ($P < 0.001$, Fisher's exact test).

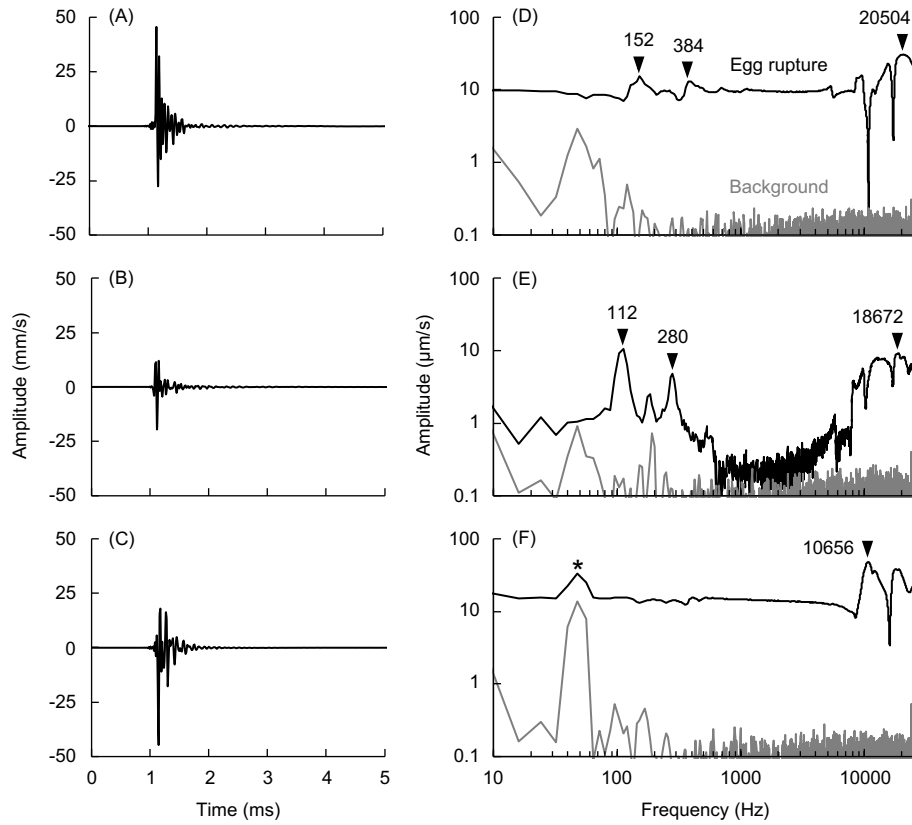


Fig. 9. Typical examples of single pulse vibrations that were transmitted from the adjacent egg at egg rupture. (A-C) Oscillograms. (D-F) Power spectra that correspond to the oscillograms shown at left. Power spectra of background noise are also shown in gray. Frequency peaks characteristic of the vibrations by egg rupture are shown by arrowheads with the frequency values. A frequency peak indicated by an asterisk in (F) represents background noise.

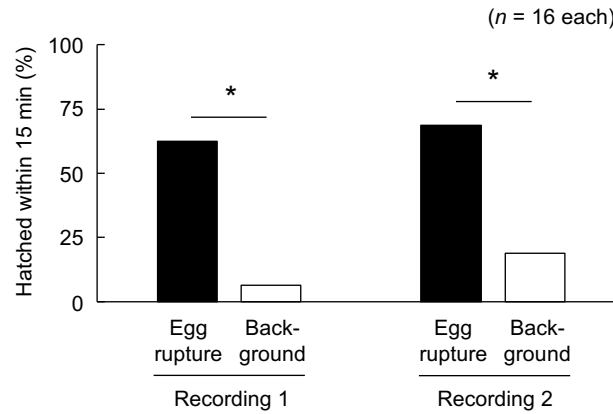


Fig. 10. Percentage of eggs that hatched within 15 min after exposed to playback of a vibration by egg rupture (black column) or background noise (white column). Two recordings of a vibration by egg rupture and their corresponding recordings of background noise were used; Recordings 1 and 2 correspond to Fig. 9A and Fig. 9B, respectively. *, a significant difference ($P < 0.05$, Fisher's exact test with Holm correction for multiple tests).

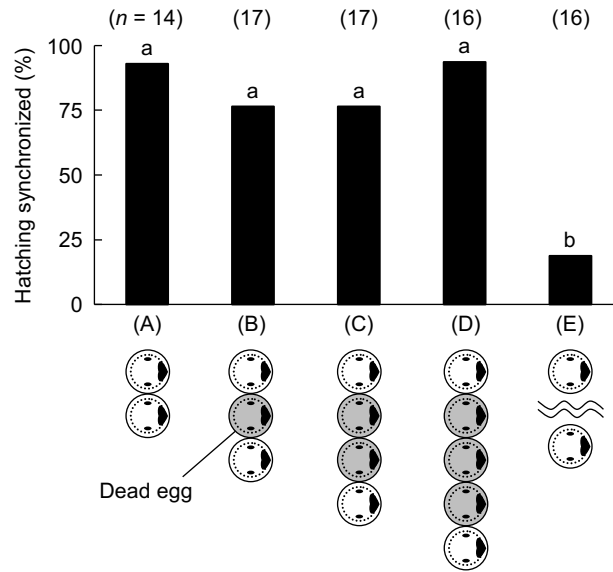


Fig. 11. Frequency of synchronized hatching among pairs of eggs that originated from the same clutch and were treated variously. Synchronized hatching was defined that both eggs hatched within 15 min. (A) Pairs of separate eggs that were attached together. (B-D) Pairs of eggs that were mediated by one dead egg (B), two dead eggs (C), or (D) three dead eggs. (F) Pairs of eggs that were isolated from each other. Pieces of drawing paper (15 × 15 mm) were used for egg substrates. Different letters above the bars show significant differences ($P < 0.05$, Fisher's exact test with Holm correction for multiple tests).