Egg Cracking Vibration as a Cue for Stink Bug Siblings to Synchronize Hatching

Jun Endo[1], Takuma Takanashi[2], Hiromi Mukai[2], Hideharu Numata[1,3,*]

[1]Graduate School of Science, Kyoto University, Kyoto, 606-8502, Japan

[2]Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, 305-8687, Japan

[3]Lead Contact

[*]Correspondence: numata@ethol.zool.kyoto-u.ac.jp

SUMMARY

Egg clutches of many animals hatch synchronously due to parental control [1, 2] or environmental stimulation [3, 4]. In contrast, in some animals, embryos actively synchronize their hatching timing with their siblings for facilitating adaptive behavior in sibling groups, such as mass migration [5, 6]. These embryos require synchronization cues that are detectable from eggs and indicative of when the siblings hatch, for example, pre-hatching vocalizations in birds and crocodiles [7, 8]. Previous studies, using methods including artificial presentation of non-specific mechanical stimuli, demonstrated that vibrations or other mechanical forces caused by sibling movements are cues used by some turtles and insects [9-13]. However, there is no evidence about which movements of tiny embryos or hatchlings, among multiple possibilities, can generate mechanical cues actually detectable through eggs. Here we show that embryos of the brown marmorated stink bug, *Halyomorpha halys*, synchronize hatching by responding to single pulsed vibrations generated when siblings crack open their egg shells. An egg-cracking vibration seems to be transmitted to distant eggs within a clutch and still maintain its function as a cue, thus leading to the highly synchronized hatching pattern previously reported [14]. In this species, it is possible that embryos attempt to hatch with short lags after earlier-hatched siblings to avoid egg cannibalism by them [14]. The present study illustrates the diversity of social information use by animal embryos for success in the sibling group.

Key words: synchronization cue, social information use, playback experiment, sibling group, egg cannibalism, brown marmorated stink bug, *Halyomorpha halys*

RESULTS AND DISCUSSION

The Type of Hatching Cue

We first examined whether embryos of *Halyomorpha halys* synchronize hatching by responding to mechanical cues that are transmitted from the siblings. In several stink bugs of Pentatomidae (Heteroptera) including *H. halys*, previous studies have proposed that the use of this type of cue facilitates synchronized hatching of an egg clutch, which is laid on a plant surface, typically on a leaf [14-16]. In Pentatomidae, eggs in a clutch are attached to each other with hardened maternal secretions [17], and therefore vibrations or other mechanical forces exerted by siblings can probably spread to nearby eggs.

To determine the type of synchronization cue(s), we paired *H. halys* eggs (almost spherical shaped, approximately 1.4 mm diameter when viewed from the top) originating from the same clutch (typically containing 28 eggs) and fixed them on a piece of drawing paper in various arrangements (Figure 1A), and then examined whether one egg could synchronize hatching with the other egg. As a baseline for comparison, we used pairs of two adjacent eggs that remained naturally attached (group I), i.e., miniature egg clutches. In these pairs, at 15 min after the hatching of the first egg, the hatching rate of the second egg exceeded 90%. From this result, synchronized hatching in a pair of eggs was defined using the criterion that the second egg hatched within 15 min after the first egg hatched. Synchronized hatching was also observed in more than 90% of pairs of separate eggs artificially attached to each other (group II, Figure 1A). Because the proportion of synchronized hatching was not significantly different between these two groups, it seems that the cue is conveyed between two eggs

that were attached artificially at least as well as between those attached naturally. In pairs of separate eggs that were placed at a distance of 1 mm (group III) and in pairs of separate eggs that were individually fixed on two pieces of paper and isolated from each other to interrupt the cue transmission (group IV), the proportion of synchronized hatching was significantly and markedly lower than the proportions of groups I and II (Figure 1A). If the cue were either an air-borne sound or volatile chemical(s), the proportion of group III would have not been much different from the proportions of groups I and II. Therefore, the cue seems not to be transmitted through the air but rather by the attachment between eggs, implicating vibrations or other mechanical forces. Because the proportion of synchronized hatching was slightly but significantly higher in group III than in group IV, even if the eggs are not attached to each other, the cue is probably conveyed through the egg substrate (drawing paper in the present experiments), although only to a small extent. Thus, it is likely that the synchronization cue is vibrations produced by an embryo or a hatched nymph, but not other mechanical forces.

However, it remained possible that air-borne stimuli were more attenuated in group III than in groups I and II due to the longer distance between the two eggs in the former. If vibrations transmitted from one egg stimulate hatching of the other, the synchronization will be facilitated when the transmission is enhanced. To examine this possibility, we observed hatching in pairs of separate eggs that originated from the same clutch and were fixed on a piece of drawing paper at a distance of 1 mm without or with a bridge (a small piece of mechanical pencil lead) between the eggs (group V and VI, respectively) (Figure 1B). The proportion of synchronized hatching was significantly higher in group VI. This indicates that, in clutches of *H. halys*, vibrations are transmitted from embryos or hatched nymphs to unhatched embryos and used as cues to

synchronize hatching. Based on the above results, transmission of the vibrational cues scarcely depends on the egg substrate, but highly depends on the attachment between eggs, at least when eggs are laid on drawing paper.

Vibration generated by egg shell cracking

All eggs in a clutch of *H. halys* complete embryonic development in 5-6 d and, even without the synchronization cues, hatch within 4 h at 25°C [14]. In this species, it is probable that hatching is synchronized as follows: Earlier-hatching eggs among a clutch generate a specific vibrational cue during the hatching behavior, and this cue immediately induces hatching of later-hatching eggs at a stage shortly before spontaneous hatching. We hypothesize that the vibrational cue is generated by cracking open the egg shell. Egg shell-cracking by siblings has been regarded as one of the possible movements that generates vibrational or acoustic cues for synchronizing hatching in some turtles and insects [10, 12, 14, 18]. In the present study, we used pairs of two adjacent eggs that remained attached and were fixed on a piece of drawing paper, and recorded the vibration transmitted from the first-hatched egg to the adjacent egg when the shell of the former cracked, using a laser Doppler vibrometer (Figure 2A).

In our observation on hatching in pairs of adjacent eggs that remained attached, the eggs always opened along a circular hatching line where an operculum joins the body of the egg shell. To open the egg, the embryo repeatedly pushed a T-shaped structure (egg burster) located on the middle of the forehead against the operculum, a process which could be observed through the operculum as tiny movements from the dorsal to ventral side. Eventually, the egg cracked 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 (Video S1). We analyzed displacement of embryos inside the eggs using video tracking of the compound eyes and detected a sharp movement in the first-hatched egg at the moment of cracking and also in the adjacent egg in a very synchronous manner (n = 3) (Figures 2B, S1A, and S1B; Video S1). This implies that a certain vibration was generated by the egg cracking and conveyed to the adjacent egg. After the egg cracking, the nymph wriggled out of the egg while extending the slit toward both sides. Cracking of the first-hatched egg was immediately followed by cracking of the adjacent one, with an interval of 110 ± 48 s (mean ± SD, n = 18). Because the first-hatched nymph always remained inside the egg during this interval, it is not possible that movements of fully emerged nymphs generate the vibrational cues.

We recorded a single pulsed vibration that was transmitted to the adjacent unhatched egg at the moment when the first-hatched egg cracked (Figures 2C and S1C). The vibration generated by egg cracking had a short duration of 3.0 ± 0.4 ms (mean ± SD, n = 8) with a peak-to-peak amplitude of 60.0 ± 21.3 mm/s (mean ± SD). Spectral analyses revealed that the vibration typically had, compared to the background noise, a broad frequency range from approximately 100 Hz to more than 10 kHz (Figures 2D-2F and S1D). A dominant frequency peak mostly lay in high frequencies of more than 5 kHz (Figures 2D and 2F). Some peaks also existed at lower frequencies between 100-400 Hz (Figures 2D and 2E); such peaks were usually not distinctive and were sometimes absent (Figure 2F). In one recording, there were two dominant peaks at 112 Hz and 18.7 kHz (Figure 2E). Within the frequency range of more than 5 kHz, the mean peak frequency (± SD) was 15.4 ± 7.8 kHz.

Embryonic Responses to Vibration Playback

Next we conducted a playback experiment to examine whether embryos at a stage slightly before spontaneous hatching respond to an egg-cracking vibration. The experiment was performed as follows: 26-28 separate eggs originating from the same clutch were individually fixed on cover glasses, which enable efficient vibration transmission from a vibration exciter (Figure S2). Hatching of the eggs was checked every 10 min. When two or more eggs had started hatching, we presumed that the other unhatched eggs were slightly before spontaneous hatching. Within the next 10 min, the following procedures were conducted: Four unhatched eggs individually fixed on the cover glasses were placed on a vibration exciter and exposed to playback of one of four vibration recordings, i.e., two recordings of egg-cracking vibrations and two corresponding recordings of background noise (Figure 3A). Then, in the same way, each of the other three recordings was individually played back to four eggs. Four egg clutches were used for this experiment, and therefore each of four recordings was individually played back to 16 eggs in total.

The two recordings of an egg-cracking vibration induced hatching within 15 min significantly more frequently than did the corresponding background noise recording (Figure 3B). Within 15 min, more than 60% of eggs hatched in the groups exposed to an egg-cracking vibration, whereas only a small portion of eggs (6.3% and 18.8%) hatched in the groups with background noise. Accordingly, we conclude that, in clutches of *H. halys*, a single vibration caused by cracking in the earlier-hatching egg induces other eggs to hatch immediately; hatching of these eggs generates additional cues. Because of this successive hatching and cue generation, hatching of the entire clutch is synchronized.

Transmission Efficiency of the Cue

In *H. halys*, a whole clutch shows a highly synchronized, explosive hatching pattern (Video S2), and this may be because a hatching cue is conveyed from one egg to adjacent (directly attached) eggs and also distant (indirectly attached) eggs via some intervening eggs [14]. Regarding this possibility, we examined how far a hatching cue can be conveyed between two eggs, via intervening eggs, and still maintain its function as a cue. We grouped two living eggs and zero, one, two or three freeze-killed eggs originating from the same clutch and fixed them on a piece of drawing paper in a line so that the latter were sandwiched between the former (Figure 4). Then, in each egg set, we examined whether hatching of the pair of living eggs was synchronized, i.e., occurred within 15 min. Besides the above four types of egg sets, we prepared pairs of eggs that were separately fixed on two pieces of paper and isolated from each other as a control.

Hatching was synchronized in pairs of eggs significantly more frequently when transmission of the hatching cue passed through one to three killed eggs than when the eggs were isolated (Figure 4). Furthermore, the frequency of synchronized hatching did not differ significantly among pairs of eggs in which the cue transmission was direct or passed through one to three killed eggs. Together with the above findings about the vibrational cue for hatching, these results indicate that the vibration can be transmitted through at least three eggs in contact in a line between adjacent ones and retain its function as a cue. Although eggs are not always arranged in such a manner in clutches of *H. halys*, we consider that an egg-cracking vibration is conveyed from one egg to distant ones and used to synchronize hatching.

Mechanism and Significance of Responses to Vibrations

Some animals have evolved so that embryos achieve hatching synchronization by responding to sibling cues for reasons such as facilitating ensuing mass migration [5, 6] or taking advantage of sibling competition for resources [19]. The synchronization is facilitated through developmental adjustment [7, 18, 19-21], immediate hatching [9-11], or both [13, 22]. Potentially, the sibling-cued immediate hatching produces more synchronized hatching patterns. The present study revealed that the highly synchronized hatching pattern in *H. halys* is promoted by immediate hatching responses to vibrational cues generated by cracking of the earlier-hatched eggs. The egg cracking is a vigorous process, and the generated vibration is transmitted to and detected by many other embryos within a clutch, also causing the highly synchronized pattern. Transmission efficiency of vibrational cues or signals deeply depends on substrate material [23-25]. Hard egg shells of *H. halys*, which are tightly attached to each other, may not attenuate the egg-cracking vibration much.

Although how insect embryos detect vibrations is unknown, it is possible that embryos of *H. halys* respond to egg-cracking vibrations through the same vibration-detection pathway as used in adults. Adults of Pentatomidae have various vibration receptors; physiological properties have been suggested for some of them, including receptor organs located on the legs and antennae, and hair sensilla on the body [26, 27]. These receptors seem to have sensitivities to vibrations with low frequencies, no more than 1 kHz, and to be required for sexual communication using plant- or air-borne vibrational signals with frequencies in this range [26-29]. Also in *H. halys*, the same low-frequency vibrations constitute some known communication signals [30], and therefore can probably be detected at least in the adult stage. Thus, it is probable that embryos of *H. halys* respond to low-frequency components of the egg-cracking

vibrations, although we cannot exclude the possibility of their responses to the high-frequency components. In subsocial bugs of related families, the embryos evidently respond to low-frequency vibrations: They hatch immediately in response to a maternal vibration, and this is reproduced by presenting artificial vibrations of several tens of Hz [31, 32]. In *H. halys*, low-frequency components of the egg-cracking vibrations from 100-400 Hz may facilitate the embryonic responses.

Vibration-cued hatching has been much studied in the case of cues generated by egg predators. In some frog species, such as the red-eyed treefrog, *Agalychnis callidryas*, premature embryos hatch soon after receiving substrate-borne vibrations generated by snake predators, thereby escaping from them [4, 33]. It is known that, in *A. callidryas*, embryos avoid hatching prematurely when they receive vibrations from harmless sources like wind and rain, by responding only to specific frequency properties and temporal patterns involved in snake vibrations [34-36]. Embryos of *H. halys* should also discriminate among vibrations to successfully synchronize hatching. In general, plant-dwelling animals detect vibrations generated by wind or rain, which predominantly contain rather low-frequency components below 100 Hz [36-38]. Compared to such vibrations, the egg-cracking vibrations are likely to have different frequency properties, and this, as well as their extremely short durations, may contribute to the discrimination.

Hatching synchronization in *H. halys* seems not to have evolved to promote migration together with siblings, because nymphs of Pentatomidae do not move away from the natal egg clutch for several days after hatching [39]. In some insects, siblings in the same clutch have a special form of a predator-prey relationship: Earlier-hatched individuals cannibalize sibling eggs, which are immobile and easy prey that provide

valuable nutrient supplements [40]. Nymphs of *H. halys* also cannibalize unhatched eggs, if any remain in their clutch [14]. Moreover, the cannibalism starts at a very early stage after hatching. Although all viable eggs have usually hatched by then because of the highly synchronized hatching, it is presumed that, without hatching synchronization, embryos might fail to hatch before cannibalism starts, and therefore be killed (Endo, J. & Numata, H. in prep).Therefore, in this species, hatching synchronization allows embryos to avoid egg cannibalism by siblings. The embryos use vibrational information from siblings just as frogs like *A. callidryas* use that from heterospecific egg predators [4, 33]. Thus, in *H. halys*, embryonic responses to social information are essential for early success. The egg-cracking vibration may be the only or first available information, or it may be more detectable and/or discriminable than other information.

ACKNOWLEDGEMENTS

We thank Yoshito Suzuki and Akira Mori for helpful advice on this study, Elizabeth Nakajima for linguistic corrections, and Karen Warkentin and three anonymous reviewers for valuable comments that improved the manuscript.

AUTHOR CONTRIBUTIONS

Conceptualization, J.E. and H.N.; Methodology, J.E., T.T., and H.M.; Formal Analysis, J.E., T.T., and H.M.; Investigation, J.E. and T.T.; Resources, J.E. and H.N.; Writing – Original Draft, J.E.; Writing – Review & Editing, J.E., T.T., H.M., and H.N.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Christy, J. H. (2011). Timing of hatching and release of larvae by brachyuran crabs: patterns, adaptive significance and control. Integr. Comp. Biol. *51*, 62–72.
2. He, J., Chen, Q., Wei, Y., Jiang, F., Yang, M., Hao, S., Guo, X., Chen, D., and Kang, L. (2016). MicroRNA-276 promotes egg-hatching synchrony by up-regulating brm in locusts. Proc. Natl. Acad. Sci. USA *113*, 584–589.
3. Webb, G. J., Choquenot, D., and Whitehead, P. J. (1986). Nests, eggs, and embryonic development of *Carettochelys insculpta* (Chelonia: Carettochelidae) from Northern Australia. J. Zool. *1*, 521–550.
4. Warkentin, K. M. (1995). Adaptive plasticity in hatching age: a response to predation risk trade-offs. Proc. Natl. Acad. Sci. USA *92*, 3507–3510.
5. Warkentin, KM, and Caldwell, M. S. (2009). Assessing risk: embryos, information, and escape hatching. In Cognitive Ecology II: The Evolutionary Ecology of Information Processing and Decision-Making, R. Dukas and J. M. Ratcliffe, ed. (Illinois: University of Chicago Press), pp 177–200.
6. Warkentin, K. M. (2011). Environmentally cued hatching across taxa: embryos respond to risk and opportunity. Integr. Comp. Biol. *51*, 14–25.
7. Vince, M. A. (1966). Artificial acceleration of hatching in quail embryos. Anim. Behav. *14*, 389–394.
8. Vergne, A. L. and Mathevon, N. (2008). Crocodile egg sounds signal hatching time. Curr. Biol. *18*, 513–514.
9. Provine, R. R. (1976). Eclosion and hatching in cockroach first instar larvae: a stereotyped pattern of behaviour. J. Insect Physiol. *22*, 127–131.

10. Ohba, S. (2002). Synchronized mechanism and its meaning in the egg hatching of the giant water bug, *Lethocerus deyrolli* (Heteroptera: Belostomatidae). Jpn. J. Entomol. (New Ser.) *5*, 157–164 (in Japanese with English abstract).
11. Doody, J. S., Stewart, B., Camacho, C., and Christian, K. (2012). Good vibrations? Sibling embryos expedite hatching in a turtle. Anim. Behav. *83*, 645–651.
12. Nishide, Y. and Tanaka, S. (2016). Desert locust, *Schistocerca gregaria*, eggs hatch in synchrony in a mass but not when separated. Behav. Ecol. Sociobiol. *70*, 1507–1515.
13. Tanaka, S., Sakamoto, H., Hata, T., and Sugahara, R. (2018). Hatching synchrony is controlled by a two-step mechanism in the migratory locust *Locusta migratoria* (Acrididae: Orthoptera): Roles of vibrational stimuli. J. Insect Physiol. *107*, 125–135.
14. Endo, J. and Numata, H. (2017). Effects of embryonic responses to clutch mates on egg hatching patterns of Pentatomidae (Heteroptera). Physiol. Entomol. *42*, 412–417.
15. Morimoto, N. (1965). Synchronization of hatching in an egg mass of the cabbage stink bug, *Eurydema rugosa*. Jpn. J. Appl. Entomol. Zool. *9*, 125–126 (in Japanese).
16. Lockwood, J. A. and Story, R. N. (1985). Photic, thermic, and sibling influences on the hatching rhythm of the southern green stink bug, *Nezara viridula* (L.). Environ. Entomol. *14*, 562–567.
17. Southwood, T. R. E. (1956). The structure of the eggs of the terrestrial Heteroptera and its relationship to the classification of the group. Trans. Royal Entomol. Soc. London *108*, 163–221.

18. Spencer, R. J., Thompson, M. B., and Banks, P. B. (2001). Hatch or wait? A dilemma in reptilian incubation. Oikos *93*, 401–406.

19. Colbert, P. L., Spencer, R.J., and Janzen, F. J. (2010). Mechanism and cost of synchronous hatching. Funct. Ecol. *24*, 112–121.

20. McGlashan, J. K., Spencer, R. J., and Old, J. M. (2012). Embryonic communication in the nest: metabolic responses of reptilian embryos to developmental rates of siblings. Proc. Biol. Sci. *279*, 1709–1715.

21. Aubret, F., Blanvillain, G., Bignon, F., and Kok, P. J. (2016). Heartbeat, embryo communication and hatching synchrony in snake eggs. Sci. Rep. *6*, 23519.

22. Tanaka, S. (2017). *Locusta migratoria* (Orthoptera: Acrididae) embryos monitor neighboring eggs for hatching synchrony. J. Orthopt. Res. *26*, 103–115.

23. Elias, D. O., Mason, A. C., and Hoy, R. R. (2004). The effect of substrate on the efficacy of seismic courtship signal transmission in the jumping spider *Habronattus dossenus* (Araneae: Salticidae). J. Exp. Biol. *207*, 4105-4110.

24. McNett, G. D., and Cocroft, R. B. (2008). Host shifts favor vibrational signal divergence in *Enchenopa binotata* treehoppers. Behav. Ecol. *19*, 650-656.

25. Hill, P. S. (2009). How do animals use substrate-borne vibrations as an information source? Naturwissenschaften *96*, 1355-1371.

26. Čokl, A. (2008). Stink bug interaction with host plants during communication. J. Insect Physiol. *54*, 1113–1124.

27. Čokl, A., Laumann, R. A., and Stritih, N. (2017). Substrate-borne vibratory communication. In Stink bugs: Biorational control based on communication processes, A. Čokl and M. Borges, ed. (Florida: CRC Press), pp 145–184.

28. Kavčič, A., Čokl, A., Laumann, R. A., Blassioli-Moraes, M. C., and Borges, M.

(2013). Tremulatory and abdomen vibration signals enable communication through air in the stink bug *Euschistus heros*. PLoS ONE *8*, e56503.

29. Nishino, H., Mukai, H., and Takanashi, T. (2016). Chordotonal organs in hemipteran insects: unique peripheral structures but conserved central organization revealed by comparative neuroanatomy. Cell Tissue Res. *366*, 549–572.
30. Polajnar, J., Maistrello, L., Bertarella, A., and Mazzoni, V. (2016). Vibrational communication of the brown marmorated stink bug (*Halyomorpha halys*). Physiol. Entomol. *41*, 249–259.
31. Mukai, H., Hironaka, M., Tojo, S., and Nomakuchi, S. (2012). Maternal vibration induces synchronous hatching in a subsocial burrower bug. Anim. Behav. *84*, 1443–1448.
32. Mukai, H., Hironaka, M., Tojo, S., and Nomakuchi, S. (2014). Maternal vibration: an important cue for embryo hatching in a subsocial shield bug. PLoS ONE *9*, e87932.
33. Warkentin, K. M. (2011). Plasticity of hatching in amphibians: evolution, trade-offs, cues and mechanisms. Integr. Comp. Biol. *51*, 111–127.
34. Warkentin, K. M. (2005). How do embryos assess risk? Vibrational cues in predator-induced hatching of red-eyed treefrogs. Anim. Behav. *70*, 59–71.
35. Warkentin, K. M., Caldwell, M. S., and McDaniel, J. G. (2006). Temporal pattern cues in vibrational risk assessment by embryos of the red-eyed treefrog, *Agalychnis callidryas*. J. Exp. Biol. *209*, 1376–1384.
36. Caldwell, M. S., McDaniel, J. G., and Warkentin, K. M. (2009). Frequency information in the vibration-cued escape hatching of red-eyed treefrogs. J. Exp. Biol. *212*, 566–575.

37. Guedes, R. N. C., Matheson, S. M., Frei, B., Smith, M. L., and Yack, J. E. (2012). Vibration detection and discrimination in the masked birch caterpillar (*Drepana arcuata*). J. Comp. Physiol. A *198*, 325-335.

38. Tsubaki, R., Hosoda, N., Kitajima, H., and Takanashi, T. (2014). Substrate-borne vibrations induce behavioral responses in the leaf-dwelling cerambycid, *Paraglenea fortunei*. Zool. Sci. *31*, 789-794.

39. Kiritani, K. (1964). The effect of colony size upon the survival of larvae of the southern green stink bug, *Nezara viridula*. Jpn. J. Appl. Entomol. Zool. *8*, 45–53.

40. Stevens, L. (1992). Cannibalism in beetles. In Cannibalism: ecology and evolution among diverse taxa, eds M. A. Elgar and B. J. Crespi, ed. (Oxford: Oxford University Press), pp 256–276.

41. Boersma, P. and Weenink, D. (2016). Praat: doing phonetics by computer [Computer program]. Version 6.0.22, http://www.praat.org/

42. R Core Team (2016). R: A language and environment for statistical computing. Version 3.3.2 (R Foundation for Statistical Computing), http:// www.r-project.org/

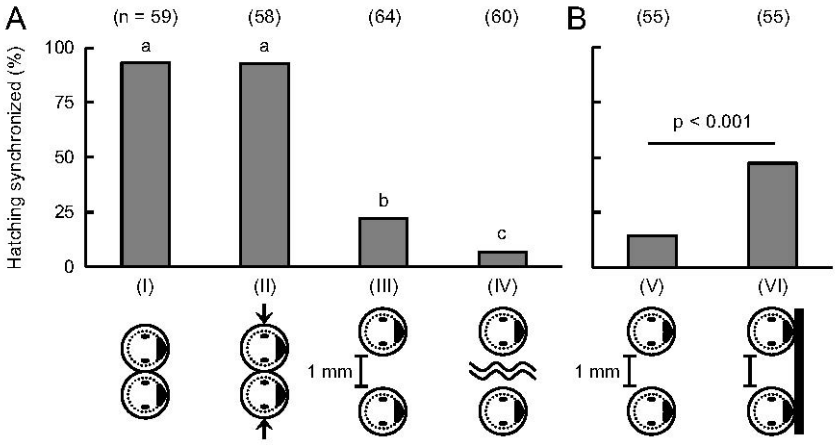

Figure 1. Proportion of synchronized hatching in pairs of *Halyomorpha halys* eggs attached, separated, or bridged

A pair of eggs that originated from the same clutch was fixed on a piece of drawing paper (15 × 15 mm). Synchronized hatching was defined as both eggs hatching within 15 min. *n* = numbers of pairs tested.

(A) Comparison among pairs of adjacent eggs that remained attached (I), separate eggs that were attached together (II), separate eggs that were placed at a distance of 1 mm (III), and separate eggs that were isolated from each other on different pieces of paper (IV). Different letters above the columns indicate significant differences ($P < 0.05$, Fisher's exact test with Holm's correction for multiple tests).

(B) Comparison between pairs of separate eggs placed at a distance of 1 mm in the absence (V) or in the presence (VI) of a bridge made by a small piece of mechanical pencil lead attached between the eggs. *P* value: Fisher's exact test.

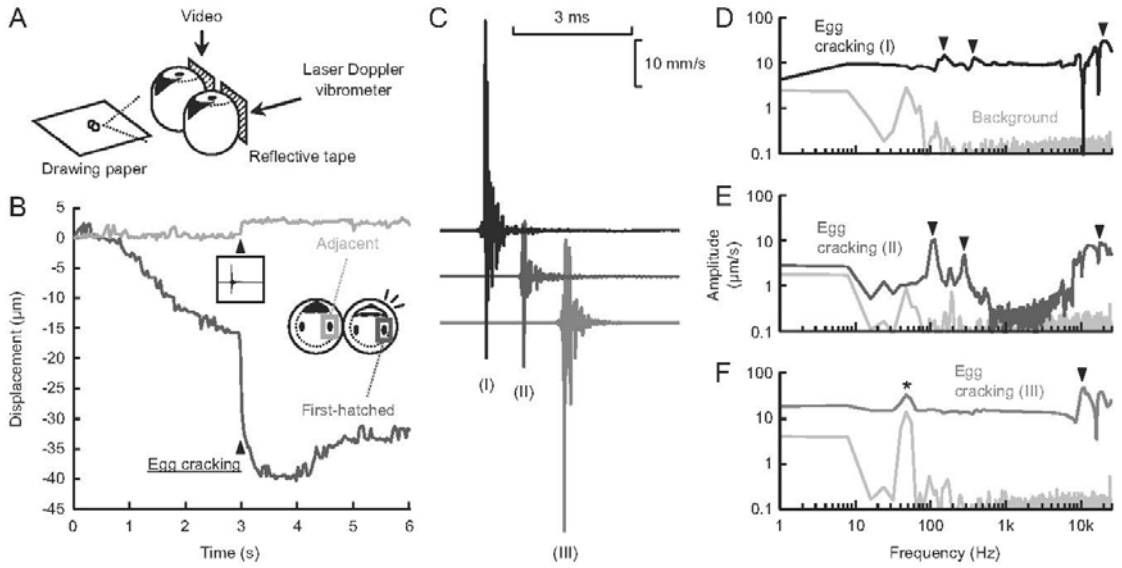

Figure 2. Vibration transmitted to the adjacent egg at the moment of egg shell cracking in *Halyomorpha halys*

(A) Diagram of the experimental setup for vibration recording by a laser vibrometer and simultaneous video recording of a pair of adjacent eggs.

(B) Displacement of embryos inside the eggs revealed by video tracking of the compound eyes. A sharp movement was detected in the first-hatched egg at the moment of cracking and also in the adjacent egg in a highly synchronous manner, implying vibration transmission to the adjacent egg.

(C) Typical oscillograms of single pulse vibrations that were transmitted to the adjacent egg at the moment of egg cracking.

(D-F) Power spectra that correspond to oscillograms I, II, and III in (C), respectively. Power spectra of background noise are also shown in light color. Frequency peaks characteristic of the egg-cracking vibrations are shown by arrowheads. Note that the amplitude differences between the largest peaks and other peaks with arrowheads in (D) and (E) are less than 4 dB. A frequency peak indicated by an asterisk in (F) represents

background noise.

See also Video S1 and Figure S1.

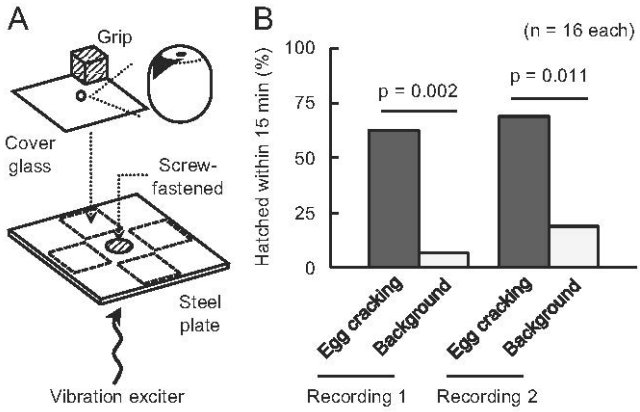

Figure 3. Embryonic responses to playback of vibrations caused by egg shell cracking in *Halyomorpha halys*

(A) Diagram of the experimental setup for vibration playback using 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). The distance between an egg and the center of the plate was approximately 16 mm.

(B) The percentage of eggs that hatched within 15 min after exposure to playback of an egg-cracking vibration or background noise. Two recordings of an egg-cracking vibration and their corresponding recordings of background noise were used; Recordings 1 and 2 correspond to oscillograms I and II of Figure 2C, respectively. *P* values: Fisher's exact test. *n* = numbers of eggs tested.

See also Figure S2.

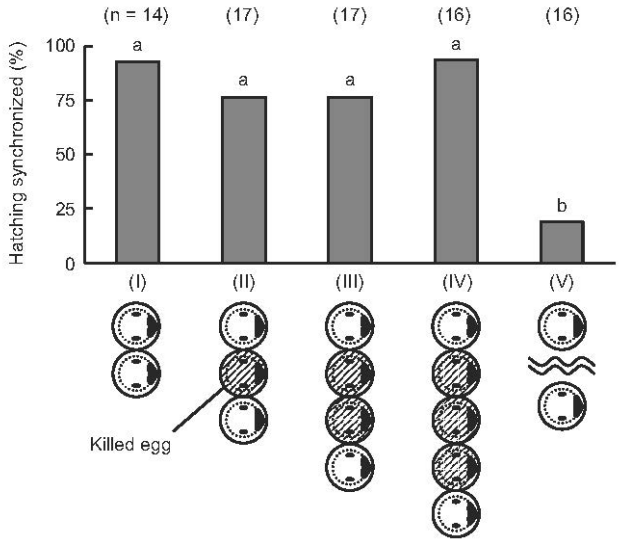

Figure 4. Proportion of synchronized hatching in two living *Halyomorpha halys* eggs separated by freeze-killed egg(s)

Living and killed eggs originated from the same clutch and were fixed on a piece of drawing paper (15 × 15 mm). Synchronized hatching was defined as both living eggs hatching within 15 min. Two living eggs that sandwiched zero (I), one (II), two (III), or three (IV) killed eggs, and two living eggs that were isolated from each other on different pieces of paper (V) were used. Different letters above the bars indicate significant differences ($P < 0.05$, Fisher's exact test with Holm's correction for multiple tests). n = numbers of pairs tested. See also Video S2.

STAR★METHODS

KEY RRESOURCES TABLE

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hideharu Numata (numata@ethol.zool.kyoto-u.ac.jp)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Adult males and females 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 peanuts and soybeans at 25 ± 2°C under 16-h light and 8-h darkness. 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 to produce separate eggs or pairs of adjacent eggs that remained attached. Then, these detached eggs were fixed on pieces of drawing paper (15 × 15 mm) or cover glasses (15 × 15 mm, 0.13-0.16 mm thickness). Starch glue was used to attach eggs to these substrates or to other eggs. Eggs were kept at 25 ± 2°C under constant light throughout the experiments. The sex of embryos inside the

eggs was not determined by keeping them until adult eclosion, because the highly synchronized hatching is the phenomenon observed in a whole clutch including both male and female embryos [14], and therefore embryonic responses to hatching cues should not be affected by the sex.

METHOD DETAILS

Tests for the Type of Hatching Cue

Eggs originating from the same clutch were paired and variously treated in two experiments (see *The Type of Hatching Cue*). The first experiment included four treatments, as shown in Figure 1A. Eighteen clutches were used, and three or four pairs were produced from each clutch for each of the four treatments. The second experiment included two treatments, as shown in Figure 1B. In one of the treatments, a bridge was constructed between a pair of separate eggs. A piece of mechanical pencil lead (0.3 mm diameter, HRF3G-20-B; Pilot) cut into 4 mm length pieces was used for the bridge and attached to the sides of the two eggs 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 drawing paper carrying egg(s) were individually placed in a clear plastic case (36 × 36 × 14 mm), and hatching was recorded every 5 min from the top view by a digital camera (P310; Nikon).

Vibration Recording and Analysis

A pair of two adjacent eggs that remained attached were fixed on a piece of drawing paper; one was targeted for vibration recording and the recording was attempted if the

non-target egg hatched first, transmitting a vibration caused by cracking of its egg shell. Egg vibrations were detected using a laser Doppler vibrometer (LV-1720A; Ono Sokki) with its laser beam directed at the target egg (Figure 1A). Vibrations with frequencies above 20 kHz were filtered using a low-pass filter because preliminary recording indicated no conspicuous peaks in this frequency range. Laser reflection was enhanced by a small piece of reflective tape (1 × 1 mm). The distance between the reflective tape and sensor head of the vibrometer was approximately 200 mm. Output signals from the vibrometer were sent to a computer using data acquisition hardware (LAN-XI type 3160-A-042; Brüel & Kjær) and monitored in real time using PULSE Labshop (ver. 18.1.1.9; Brüel & Kjær) for vibration recording. Vibration velocities were obtained by converting voltages on the data acquisition hardware. At the same time, the hatching behavior was observed and recorded from the top view using a digital video camera (HDR-CX630V; Sony) attached to a binocular stereomicroscope (M651; Leica Microsystems) (Figure 1A). Eighteen pairs from five egg clutches were observed until hatching for the recording trials, and recordings were successfully obtained in eight of them.

The recordings converted to WAV and TXT file format were prepared and the former was used to analyze the durations of the vibrations with PRAAT (ver. 6.0.22; [41]) and the latter to obtain the peak amplitudes of velocities (m/s, zero-to-peak). To analyze spectral characteristics of the recorded vibrations, power spectra were computed using PULSE Labshop by applying fast Fourier transform (Hanning window, 12.8 kHz bandwith, 1600 lines). Recordings used here were 0.1 s segments that included each vibration caused by eggcracking. Power spectra were also obtained for background noise that was recorded from target eggs within 75 min before hatching of the

first-hatched eggs and used as controls for the playback experiment. Recordings from eggs containing freshly killed embryos (n = 2) showed that the power spectra of these eggs were very similar to those of background noise.

Video Analysis

The video data obtained simultaneously during the vibration recording were analyzed to measure displacement of both embryos at the moment when the first-hatched egg cracked. Six-s video sequences at 30 frames/s containing the moment were used and, for each embryo, movement of one side of the compound eyes was tracked in the X-axis direction using Photron FASTCAM Analysis (ver.1.2.1.1; Photron). Changes in distances from the start position in the video sequences were determined as the displacement.

Playback Experiment

Two vibration recordings were converted to uncompressed WAV file format and trimmed to 1 s segments containing the egg-cracking vibrations, using SASLab Pro (ver. 5.0.23; Avisoft Bioacoustics). 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, 10Hz-20kHz frequency rage; Brüel & Kjær) using PULSE Labshop and 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 (50 × 50 mm, 2.3 mm thickness) by a screw (5 mm diameter, 8 mm length) as a position to place eggs individually fixed on cover glasses (the distance between an egg and the center of the plate was approximately 16 mm). Vibration

transmission through the cover glasses used for egg substrates was tested by generating white noise with frequencies below 12.8 kHz using PULSE Labshop. The noise vibrations were detected at the steel plate or at a cover glass placed on the steel plate (*n* = 3) using LV-1720A. Power spectra of the recorded noise vibrations were obtained using the 120 ms segments by the same method as described above. Outputs of playback signals from the exciter were adjusted as follows: For the egg-cracking vibration, the peak-to-peak amplitude was matched to the mean peak-to-peak amplitude of the recorded vibrations. For the background noise, the peak amplitude was matched to the background level of the egg-cracking vibration that was played.

The playback experiment was performed as described in *Embryonic Responses to Vibration Playback*. On the cover glasses that were used for egg substrates, a piece of polychloroprene tape (5 × 5 mm, 5 mm thickness) had been attached for convenience of gripping. Before eggs were placed on the exciter for playback, they were separately placed on pieces of polyurethane tape (15 × 15 mm, 10 mm thickness). This procedure ensured that egg-cracking vibrations would not spread from some eggs to others. Soon after playback on the exciter, the eggs were placed back on the pieces of the tape, and then hatching was recorded every 5 min from the top view using a digital camera (P310).

Tests for Transmission Efficiency of the Cue

Eggs originating from the same clutches were grouped and treated in five ways as shown in Figure 4 (see *Transmission Efficiency of the Cue*). Freeze-killing was performed at -15°C for 15-30 min immediately after the egg detachment procedure. Ten clutches were used, and one or two egg groups were produced from each clutch for each

of the five treatments. Pieces of drawing paper carrying egg(s) were individually placed in a clear plastic case (36 × 36 × 14 mm), and hatching was recorded every 5 min from the top view using a digital camera (P310).

QUANTIFICATION AND STATISTICAL ANALYSIS

The proportions of synchronized hatching in two eggs or the percentages of eggs that hatched in response to vibrational playback were compared using Fisher's exact test in R (ver. 3.3.2; [42]) with the *fmsb* package. Where multiple comparison analysis was conducted, Holm's correction was applied to adjust *P*-values, as described in figure legends. A significance threshold was set at $P < 0.05$. See figures and figure legends for the statistical details.

Video S1. Real-time video of the moment of egg shell cracking by one of a pair of adjacent *Halyomorpha halys* eggs, related to Figure 2B

The video is shown in synchrony with displacement of an embryo inside the first-hatched egg and the adjacent egg that was measured by video tracking of the compound eye (Figure 2B). The adjacent egg moved at the same time as cracking of the first-hatched egg and, to show this visually, still images obtained from the video 0.1 s before and after the cracking are repeatedly presented. The synchronous movements of the adjacent eggs are represented in the compound eyes and, more clearly, in the reflection of the laser.

Video S2. Time-lapse video of highly synchronized hatching among 28 eggs constituting a clutch of *Halyomorpha halys*, related to Figure 4

Photographs were taken every 30 s and the video was speeded up 150 times by playing at 5 frames/s. The total length of the video sequence was 30 min in real time.