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ORIGINAL ARTICLE

Identification of vibrational signals emitted by embryos of the migratory locust *Locusta migratoria* (Orthoptera: Acrididae) that induce synchronous hatching

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Abstract. Eggs of the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae), hatch synchronously when in a pod, but only sporadically when kept separately. Here, we aimed to detect the vibrational stimuli emitted by eggs that initiate synchronous hatching. First, we recorded the vibrations emitted by an egg pod and single eggs. One bout of vibrations consisted of 2 to 46 vibrations. The total number and amplitude of vibrations in single eggs increased as the time to hatch decreased. Eggs kept separately were continuously subjected during the last 2 days before hatching to recordings of vibrations from a single egg. Recordings made during the last 2.5 h before hatching caused these eggs to hatch significantly earlier than those not subjected to this treatment, the control. In contrast, eggs subjected to recordings made 8 to 10 h before hatching significantly delayed their hatching relative to the controls, which indicates that synchronous hatching of eggs is induced by age-dependent changes in vibrations from neighbouring eggs. Exposure to one large bout of vibrations (consisting of 40 vibrations in 101 s) was sufficient to induce synchronous hatching in eggs kept separately when applied 5 h before hatching, but not 36 or 11.5 h before hatching. Visual inspection of the spectra indicated that the vibrations had two peaks at about 100 Hz and 1.5 kHz. Only exposure to the latter altered the hatching time of eggs. The embryo moved the posterior part of its abdomen when emitting the vibrations. These results indicate that the synchronous hatching of locust eggs is induced when the embryos emit particular vibrations.

INTRODUCTION

Synchronous hatching has various advantages for animals laying batches of eggs, including the shortening of the time interval in which hatchlings as a group can be attacked by predators, swamping predators on emergence, reduction of egg cannibalism among siblings, helping hatchlings to escape from the ootheca or in forming an aggregation and enabling hatchlings to start feeding as a group (Morimoto & Sato, 1962; Provine, 1976, 1977, 1981; Arnold & Wassersug, 1978; Dehn, 1990; Warkentin et al., 2006; Nishide & Tanaka, 2016; Endo & Numata, 2017; Endo et al., 2019). Synchronous hatching is well researched and is reported in both vertebrates and invertebrates (Spencer et al., 2001; Saigusa, 2002; Ikeda et al., 2006; Vergne & Mathevon, 2008; Webster et al., 2015; Aubret et al., 2016). However, the mechanisms that underlie synchronization are not well understood.

In insects, eggs are often laid in masses, which hatch synchronously. However, there is little information on the mechanisms that underlie synchronous hatching in insects, with the exception of a few sub social species in which hatching synchrony is induced by specific types of parental behaviour, such as spraying water or vibrating eggs (Ohba, 2002; Mukai et al., 2012, 2014). However, most insect species do not attend their eggs, and there are only a few studies on the mechanism that underlie synchronous hatching in such species with the exception of the cockroach (Provine, 1976) and several species of bugs (Kiritani, 1964; Endo & Numata; 2017).

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In the desert locust, *Schistocerca gregaria*, eggs in pods hatch more synchronously than those kept individually (Nishide et al., 2017a). In this locust, some of the eggs start hatching when they are mechanically stimulated by shaking (Bernays 1971; Nishide & Tanaka, 2016). However, such treatment does not necessarily promote synchronous hatching in test egg populations.

In the migratory locust, *Locusta migratoria* L. (Orthoptera: Acrididae), eggs separated by a few millimetres do not hatch synchronously whereas those kept in contact do (Tanaka, 2017). Eggs kept in contact with other eggs killed by freezing shortly before hatching, hatch asynchronously, which provides no evidence for the involvement of chemical stimuli in controlling synchronous hatching (Tanaka et al., 2018). In contrast, separated eggs can hatch as synchronously as those kept in contact with one another when they are connected by a piece of metal wire, which indicates that synchronous hatching may be controlled by a vibrational stimulus produced by the embryos (Tanaka et al., 2018). Interestingly, separated eggs vibrationally stimulated by Mozart piano music hatch more rapidly than those that are not (Tanaka et al., 2018). In this case, however, synchronous hatching is observed only when the music started shortly before hatching, indicating that the embryo becomes ready to respond to such stimuli but hatch only after reaching a certain stage, which is called the standby stage (Tanaka et al., 2018).

To explain the mechanism that controls synchronous hatching in the migratory locust, we previously proposed a two-step hypothesis (Tanaka et al., 2018), in which an embryo shortly before hatching is assumed to enter a quiescent period at the end of embryogenesis and monitor neighbouring eggs for synchronous hatching. This hypothesis was based on the results of previous studies (Padgham, 1981; Tanaka, 2017). In the first step, the embryos receive a vibrational signal from neighbouring eggs and enter the standby stage simultaneously. In the second step, the embryos at this stage hatch promptly when they receive another signal from neighbouring eggs. Thus, there are two signals, one controlling the time of entry into the standby stage and one for synchronous hatching, although neither signal is required for hatching, because single eggs can hatch successfully. An embryo before the standby stage cannot hatch in response to the second signal but can hatch later spontaneously. However, these signals have not been identified. Our current study was designed to resolve this problem.

We first searched for specific vibrational signals emitted by masses and single eggs of the migratory locust. After detection, recording and analysis of the signals, we found temporal variations in the size and frequency of vibrations that were potentially involved in the control of synchronous hatching. To determine the roles of these stage-specific signals, we played back recordings of some of the vibrations to stimulate eggs in isolation. We also determined the frequency range of the vibrational signal that was involved in the control of hatching time. Finally, we established how the embryos emitted the vibrational signals. Here, we describe the results of these observations and discuss the mechanism controlling synchronous hatching in this locust.

MATERIALS AND METHODS

Insects

The strain of migratory locust and rearing method used in this study are the same as used previously (Tanaka, 2017). The locusts originated from Okinawa, Japan and were fed leaves of *Bromus catharticus*, *Dactylis glomerata* and *Sorghum vulgare* depending on the season and reared at 30°C under a 12 h photoperiod in the Tsukuba laboratory of the National Institute of Agro-biological Sciences at Ohwashi (NIASO). Adults were reared in groups of 100–200 individuals in wooden-framed cages (42 × 24 × 42 cm) covered with nylon screens. Egg pods laid in moist river sand (about 15% moisture content) in plastic containers (diameter 13 cm; depth 7.5 cm) were washed with tap water within 24 h of deposition and kept in clean moist sand in Petri dishes (9 × 2 cm) at 30°C until required. Eggs from this strain do not enter embryonic diapause at 30°C and hatch in 14 or 15 days (Tanaka, 2017). All experiments were carried out at 30°C under continuous illumination at NIASO.

Methods of recording the vibrations emitted by eggs

To record the vibrations that might be emitted by locust eggs, we pierced an egg pod containing about 50 eggs with a steel insect pin (No. 000, Ento S.R.O., Pardubice, Czech Republic) connected to a stethoscope (CHO-4, Nol Co., Tokyo, Japan) and microphone (MHM-M18; Nakabayashi Co., Tokyo, Japan) connected to an amplifier (AT-PMXP, Audio-Technica Co., Tokyo, Japan) and a PC (Fig. 1). It is possible that the pin pierced some of the eggs in the egg pod. The vibrations were recorded continuously for 2 or 3 days before hatching and processed using music software (Choroku ver. 1.28A; ©2005 PINO). The egg pod was also videotaped continuously under a digital microscope (Dino-Lite. AM4113TS, AnMo Electronics Co., Hsinchu, Taiwan) in order to record the time of hatching (Fig. 1A). For recording, the root of

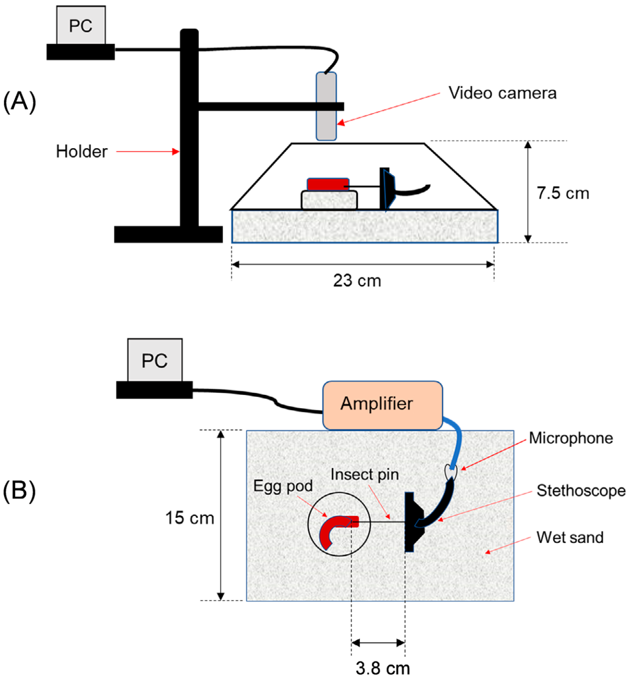

Fig. 1. Device for recording the vibrations emitted by the eggs of the migratory locust. A – side view; B – view from above.

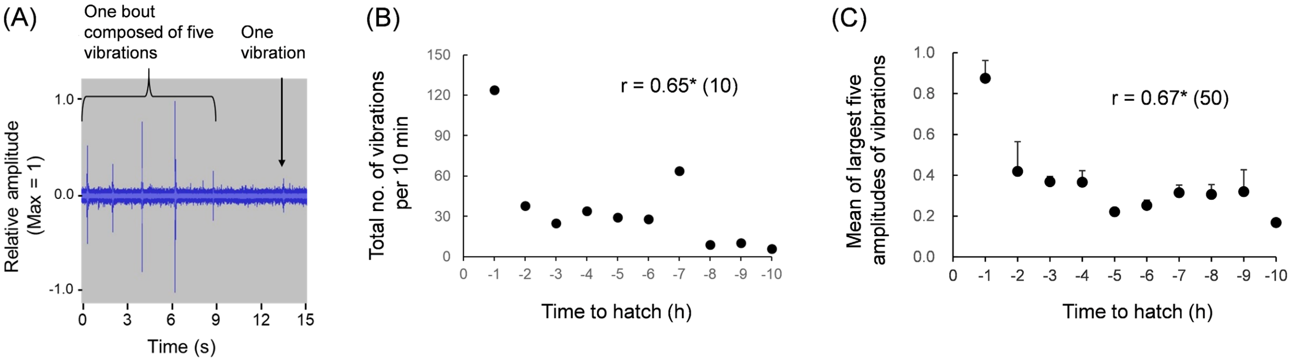

Fig. 2. Vibrations recorded from an egg pod of the migratory locust. A bout of five vibrations with different amplitudes and a single vibration are shown (A). Temporal changes in the total number of vibrations (B) and mean (+ SD) of the largest five amplitudes of the vibrations (C). The data in (B) and (C) are based on the measurements made during a 10 min period in each of the 10 h before hatching. Numbers in parentheses indicate *n*. Asterisks indicate that the correlation was significant at the 5% level.

the pin was kept in the air. The egg pod was placed horizontally on a wet sand mount in a Petri dish (5 cm diameter, 1.2 cm deep), which was placed on the wet sandy floor of a rectangular plastic container (15 cm × 23 cm × 7.5 cm deep) with a glass cover (Fig. 1B). The recordings were made at 30°C under continuous illumination in an air-conditioned room. Vibrations from single eggs were recorded in the same way, except that the recording pin was placed horizontally across an egg lying on a wet sand mount in a small glass Petri dish (1 cm diameter, 1 cm deep). In some cases, eggs were pre-treated with a chlorine bleaching agent (Kitchen Haiter, Kao Co., Tokyo, Japan) for 15 to 20 s and rinsed with tap water so that the movements of the embryos could be easily seen through the chorion. Our preliminary observations indicated that treatment with chlorine had no marked effect on hatching time (data not shown). Using the same device, we also recorded background noise by placing the tip of the pin on the wet sand mount without eggs. The background noise was subtracted from the recordings made in the presence of eggs by using the noise reduction function of audio editing software (Audacity ver. 2.1.1.; ©Team Audacity). In this system, relative amplitudes were determined by designating the maximum amplitude of vibration for each recording as 1.0.

Playback experiments

To determine the specific role of the vibrations recorded at different times before hatching they were played back using music software (Windows Media Player, ver. 10, Microsoft Co.) and transmitted via a stainless steel wire to each of 12 eggs on a moist sand mount held in the playback apparatus previously described (Tanaka et al., 2018), except for the use of a vibrational speaker (DN-B004WH, DeviceNet Co., Tokyo, Japan; Fig. S1A) instead of a sound speaker. We used the recordings from one egg that was arbitrarily chosen from 15 eggs that produced vibrations during the last 10 h before hatching. Background noise was removed from the recordings before playback experiments using the noise reduction function in Audacity. The volume was precisely controlled using software, Volume^2, produced by Irza Alexandr (Ver. 1.1.4.347). We recorded the vibrational signals transmitted along the stainless steel wire from the speaker and confirmed by visual inspection that they were similar to those recorded from the eggs (Fig. S2). Unless otherwise stated, 12 eggs, aged 12 days post-laying, were removed from an egg pod, placed separately on the sand mount and subjected continuously to the vibrations until they hatched. Another 12 eggs were also individually placed on another sand mount and not subjected a recording of the vibrations, the control (Fig. S1B). A third group of 12 eggs was placed as a group in a pit (7 mm in diameter; 1 cm in height) in moist sand in a Petri dish (6 cm in diameter; 1.2 cm in height). All eggs came from the same pod and were housed in the same rectangular container (30 × 24 × 21 cm deep) with a transparent lid. They were photographed from above using a digital camera every 30 min until no more hatching was observed. The photographs were analysed later on a monitor to determine when the eggs hatched.

Analysis of vibrations

The characteristics of the vibrations recorded from the eggs were analysed using Audacity. We confirmed that vibrations were not detected when no eggs were monitored, that is, we could distinguish vibrations from the background noise. By listening to the sounds transformed from the recorded vibrations, vibrations that produced characteristic sounds were easily separated from the background noise. Fig. 2A illustrates an example of a bout of vibrations recorded from an egg pod. We arbitrarily regarded two vibrations occurring within 3 s as a bout or part of a bout. One bout consisted of 2 to 40 vibrations with varying amplitudes. The numbers of bouts consisting of three or more vibrations and the largest five amplitudes were determined during a 10 min period in each of the 10 h before the hatching of an egg pod. For single eggs, the number of vibrations per bout, the total number of vibrations per hour, the largest amplitude of the vibrations in each bout and the lengths of the intervals between vibrations in each bout (length of bout / no. of vibrations – 1) were determined. The amplitude of the vibrations recorded was influenced by the pressure exerted by the recording pin on an egg, which was difficult to control. Therefore, the largest amplitude of vibration in each recording (i.e. per egg pod or egg) was designated as 1.0, and the relative amplitudes of the other vibrations recorded from egg pods and eggs were then calculated. To compare the amplitudes for different eggs, the largest five amplitudes in bouts from each egg were used.

Spectral analysis of specific vibrations in three typical bouts of vibration obtained from different eggs was done using Audacity. As described below, the spectra of specific vibrations ranged widely. In the present study, spectra were divided into low-frequency (< 270 Hz) and high-frequency (≥ 270 Hz) ranges that were then separately played back to determine which range was important in controlling the hatching time of eggs using the low-pass and high-pass filter in Audacity.

Statistical analysis

The relationships between the various parameters and the time before hatching were analysed using Pearson's correlation analysis. Hatching times were compared using Tukey's multiple comparison analysis after ANOVA. Variances were compared by

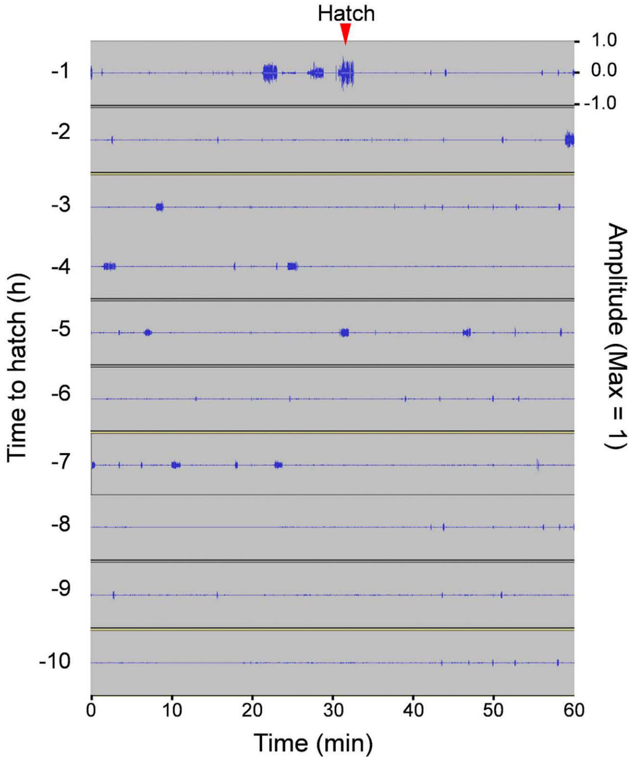

Fig. 3. Patterns in vibrations recorded from a single egg of the migratory locust during the last 10 h before hatching. Background noise was removed using the noise reduction function in Audacity. Arrow indicates the beginning of hatching.

using an *F*-test. A *P*-value of < 0.05 (two-sided) was considered statistically significant for all tests except for variances (one-sided).

RESULTS

Vibrations recorded from an egg pod

Fig. 2B shows the vibrations recorded from an egg pod during the last 10 h before hatching. The total number of vibrations including those in bouts and single vibrations, during a 10 min period in every hour from 1 to 10 h before hatching, varied between 6 and 64 up until 2 h before hatching and then increased to 124 1 h before hatching (Fig. 2B). A significant correlation was also recorded between the two variables from 10 to 2 h before hatching ($R = 0.48$; $n = 9$; $P = 0.015$). The relative amplitudes of the vibrations increased greatly shortly before hatching (Fig. 2C). The recording of the egg pod started 46 h before hatching and the first bout of vibrations was detected at 22 h before hatching (data not shown).

Vibrations recorded from single eggs

Possible overlapping of the vibrations emitted by two or more eggs in an egg pod (Fig. 2) made the analysis difficult. Therefore, we recorded the vibrations from single eggs. Fig. 3 shows the vibration patterns recorded from an egg during the last 10 h before hatching. The frequency and relative amplitude of the vibrations appeared to increase as the egg approached hatching time. To confirm this, we ana-

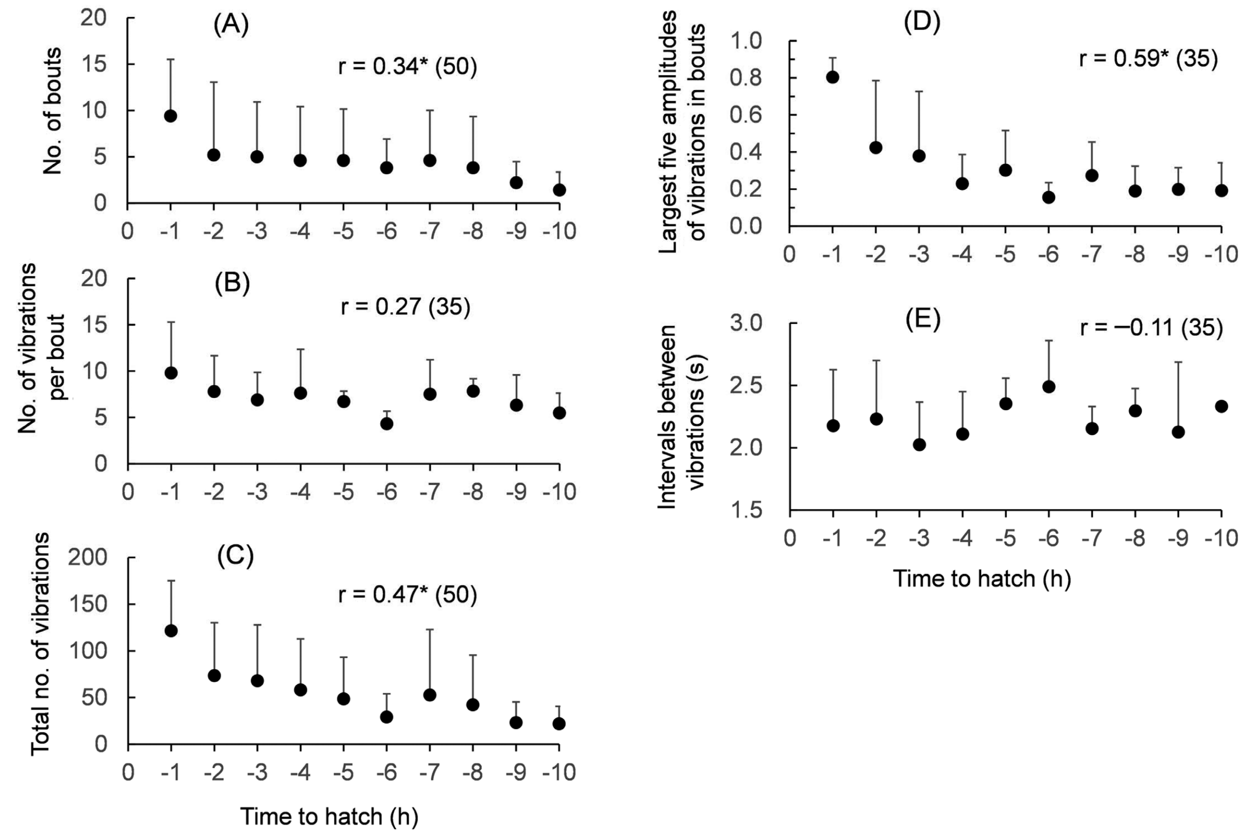

Fig. 4. Characteristics of the vibrations recorded from five eggs of the migratory locust obtained from different pods and kept singly. Temporal changes in the mean number of bouts (A), the mean numbers of vibrations per bout (B), the total number of vibrations per hour (C), the mean of the largest five amplitudes of the vibrations observed in bouts (D) and the mean intervals between vibrations in each bout (E). Bars indicate one SD. Numbers in parentheses indicate number of points. Asterisks indicate that the correlation is significant at the 5% level.

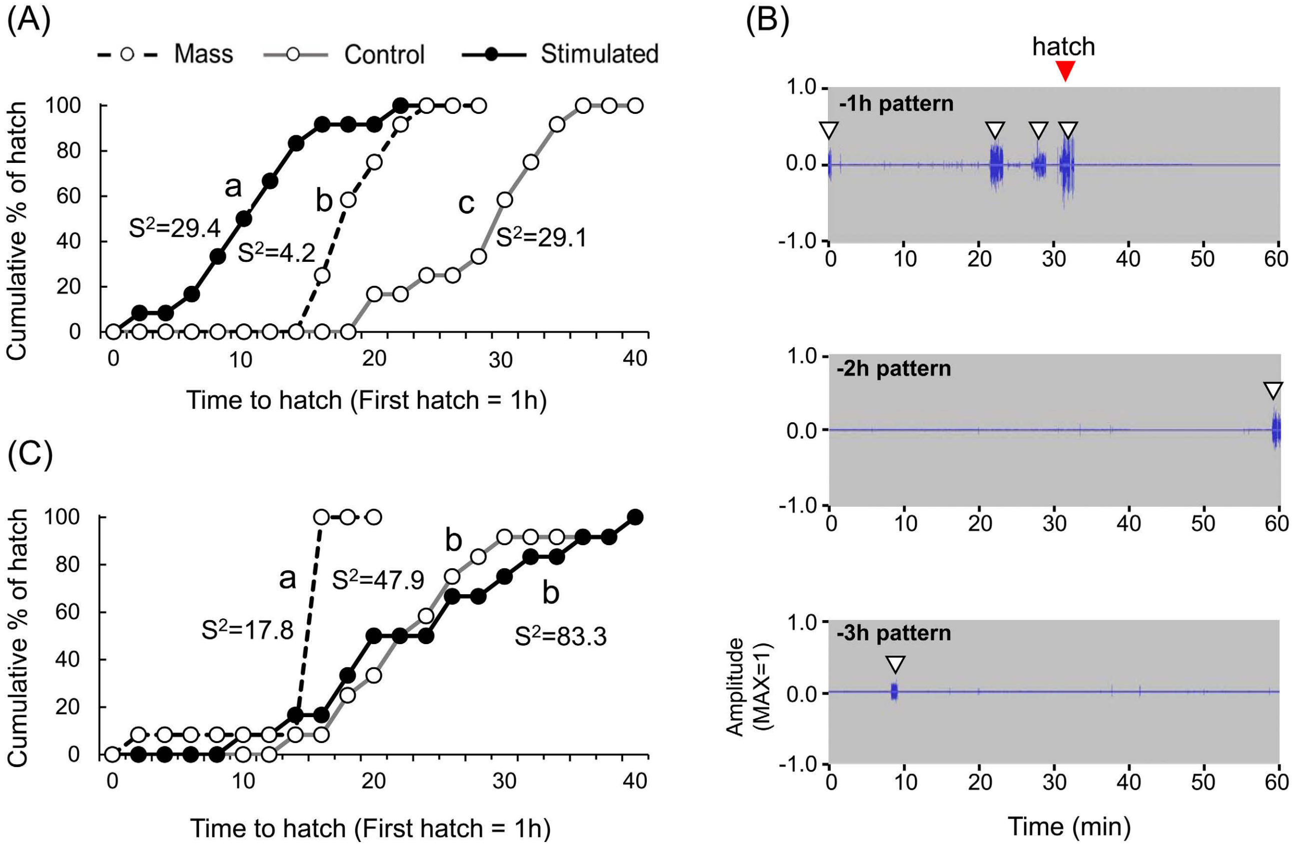

Fig. 5. Effects of recordings of the vibrations emitted by an egg of the migratory locust during the last 2.5 h before hatching on the hatching times of eggs kept separately (A). Twelve-day-old eggs were separated and subjected to recordings of the vibrations emitted by a single egg (B) until hatching. Some eggs were also kept separately or in a mass and not subjected to recordings of the vibrations, the controls. In another experiment (C), eggs were similarly treated but the recordings were played back after the major bouts of vibrations (containing more than four vibrations or vibrations with a maximum amplitude of > 0.15) were deleted using Audacity. In (B), white arrowheads indicate the major bouts. In (A) and (C), $n = 12$. Different letters in each panel indicate a significant difference at the 5 % level using Tukey's multiple-comparison test. Variance (S^2) is shown for each treatment. In (B), the red arrowhead indicates the beginning of hatching.

lysed the temporal changes in the characteristics of the vibrations from five single eggs derived from different pods. The numbers of bouts containing three or more vibrations increased as the time of hatching approached, and a significant correlation was recorded between the two variables (Fig. 4A). The number of vibrations per bout also showed a similar pattern but the correlation was not significant ($P = 0.12$; Fig. 4B); however, the largest number of vibrations per bout per hour increases as the time to hatching decreased ($R = 0.71$; $n = 10$; $P < 0.05$; data not shown). The longest bout recorded consisted of 46 vibrations and occurred during the last hour before hatching. The total numbers of vibrations per hour increased significantly as the time to hatching decreased (Fig. 4C). The amplitudes of the vibrations recorded in each bout, and the largest five values recorded for each egg during each hour were analysed in relation to the time before hatching. The mean value was small at 10 h before hatching but increased close to the time of hatching; a significant positive correlation was recorded between amplitude and time before hatching (Fig. 4D). The intervals between vibrations within a bout were not significantly correlated with time (Fig. 4E). The first bout for each of the five eggs tested was recorded at 25, 21, 18, 16 and 13 h, respectively, before hatching.

Role of the vibrations

To determine the role of the vibrations in the synchronous hatching of eggs, 12-day-old eggs kept separately were continuously subjected to vibrations recorded from an egg during the last 2.5 h before it hatched (Fig. 5A, B). The control group consisted of similarly aged eggs kept separately but not subjected to a recording of the vibrations. The time to hatching of control eggs was more variable and significantly later than recorded for the mass of eggs (Tukey's multiple comparison test; $P < 0.05$; Fig. 5A); this is consistent with our previous results (Tanaka, 2017). In contrast, the eggs subjected to a recording of the vibrations hatched significantly earlier than those that were not (Tukey's multiple comparison test; $P < 0.05$) or those kept in a mass (Tukey's multiple-comparison test; $P < 0.05$). However, the variance in hatching times of the two groups of eggs kept separately, regardless of whether they were subjected to a recording of the vibrations or not, was similar (F-test; df = 11, 11; $P = 0.49$). The results indicate that recordings of the vibrations produced by an egg during the last 2.5 h before hatching advanced hatching but did not induce synchronous hatching. Similar results were obtained using another set of eggs (data not shown).

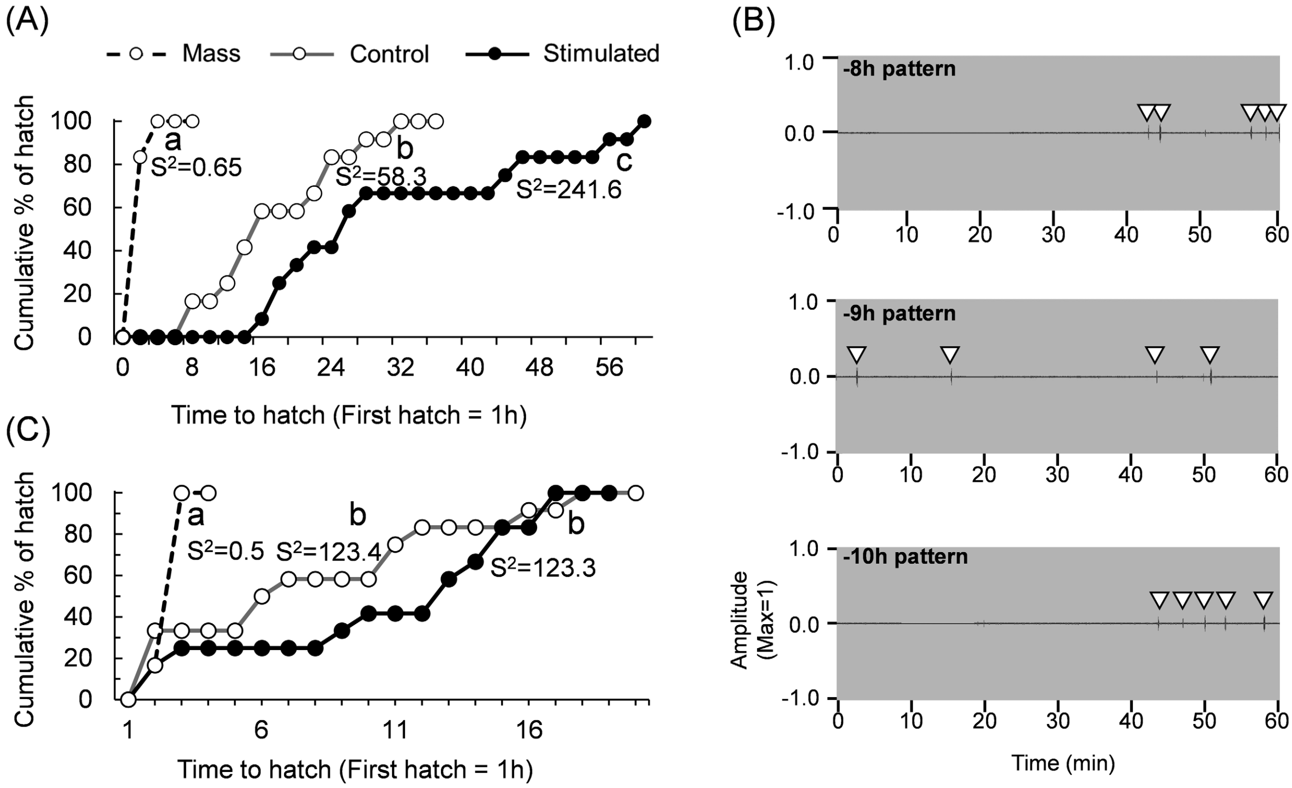

Fig. 6. Effects of recordings of vibrations emitted by eggs of migratory locusts 8 to 10 h before hatching on the hatching time of eggs kept separately (A). Twelve-day old eggs were separated and subjected to the recordings shown in (B) until they hatched. Some eggs were kept separately or in a group but not subjected to the recordings, the controls. In another experiment (C), eggs were similarly treated except that the recordings were played back after the major bouts of vibrations (containing more than four vibrations or vibrations with a maximum amplitude of > 0.08) were deleted using Audacity. In (B), white arrowheads indicate the major bouts. In (A) and (C), *n* = 12. Different letters in each panel indicate a significant difference at the 5 % level using Tukey's multiple-comparison test. Variance (S^2) is shown for each treatment.

In another experiment, the same recordings were used after the bouts containing more than four vibrations were replaced by no sound using Audacity in order to determine the role of the bouts on the hatching time of eggs (Fig. 5B). As shown in Fig. 5C, the eggs subjected to the modified recording showed a similar response to those not subjected a recording of the vibrations in terms of both the mean hatching time (Tukey's multiple-comparison test; $P > 0.05$) and variance (*F*-test; df = 11, 11; $P = 0.19$), indicating the importance of the bouts of vibrations in advancing the hatching time.

Similar experiments were done using recordings of the vibrations from the same egg 8 to 10 h before hatching (Fig. 6). In this case, the eggs subjected to this treatment hatched significantly later than those that were not and those that were kept either in isolation or in a mass (Tukey's-multiple comparison test; $P < 0.05$; Fig. 6A). The variance in the time to hatching of the treated eggs was significantly greater than that of those not treated (controls) and kept separately (*F*-test; df = 11, 11; $P < 0.05$). Replacement of the bouts containing more than four vibrations (Fig. 6B) with no sound caused the eggs to hatch as late as the control eggs that were kept separately (Fig. 6C; Tukey's multiple-comparison test; $P > 0.05$) and with similar variances (*F*-test; df = 11, 11; $P = 0.50$), which indicates the importance of the bouts of vibrations in delaying the hatching time. Similar results were obtained when another set of eggs was used (data not shown).

Factors inducing synchronous hatching

We searched for a specific signal inducing synchronous hatching in this locust. However, no such signal was detected (see Fig. 3). Therefore, we tested the hypothesis proposed in a previous study that a relatively large bout of vibrations emitted by an egg shortly before hatching induces the hatching of other eggs that are ready to hatch in the same mass (Tanaka et al., 2018). We compared the hatching times of eggs kept separately that were subjected to a single large bout of vibrations (recorded during the last hour before hatching; Fig. 7A) at 36, 11.5 and 5 h before the mean hatching time of separately kept control eggs. Exposure to the recording at 36 h and 11.5 h appeared to induce the early hatching of some eggs but not of others, resulting in significantly greater variance in hatching time than recorded for those eggs kept in a mass (Fig. 7B and C; *F*-test; df = 11, 11; $P < 0.05$ each). The mean hatching time of the treated eggs was not significantly different from that of the control eggs kept separately in either case (Tukey's multiple test, $P > 0.05$). In contrast, the eggs kept separately hatched as synchronously as those kept in a mass when they were treated 5 h before the mean hatching time of the controls kept separately, and there was no significant difference in variance recorded in the two treat-

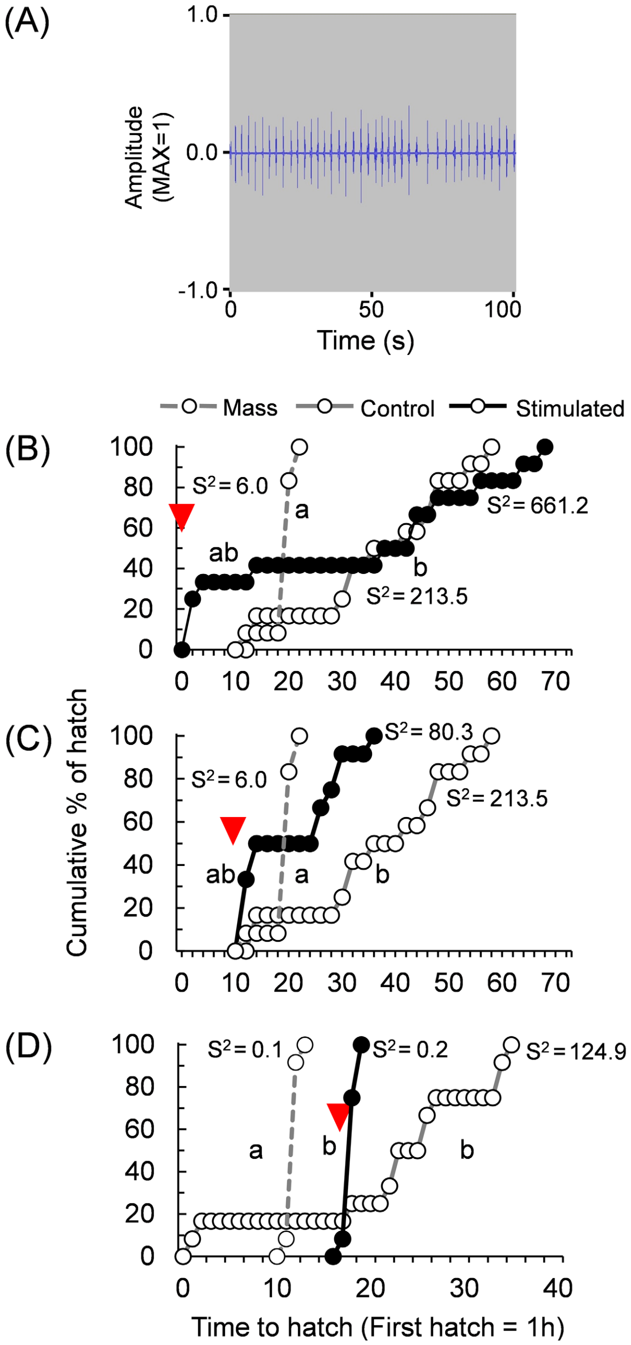

Fig. 7. Effects of the timing of the treatment with a recording of one bout of vibrations on the hatching times of eggs of the migratory locust kept separately. Eggs were subjected once (for 101 s) to a recording of one bout of 40 vibrations (A) 36 (B), 11.5 (C) or 5 h (D) before the mean hatching time of eggs kept separately, the controls. The recordings of vibrations used are shown in Fig. 7A. *n* = 12. Variance (S^2) is shown for each treatment. Different letters in each panel indicate a significant difference in the mean hatching time at the 5% level using Tukey's multiple-comparison test. Red arrowheads indicate the time when subjected to recordings of one bout of vibrations.

ments (*F*-test; *P* = 0.07; Fig. 7D). However, the variance recorded in these treatments was significantly smaller than that recorded for the controls kept separately (*F*-test; $P < 0.05$ each), which indicates that eggs kept separately hatch synchronously when they were subjected to recording of a large bout of vibrations shortly before hatching.

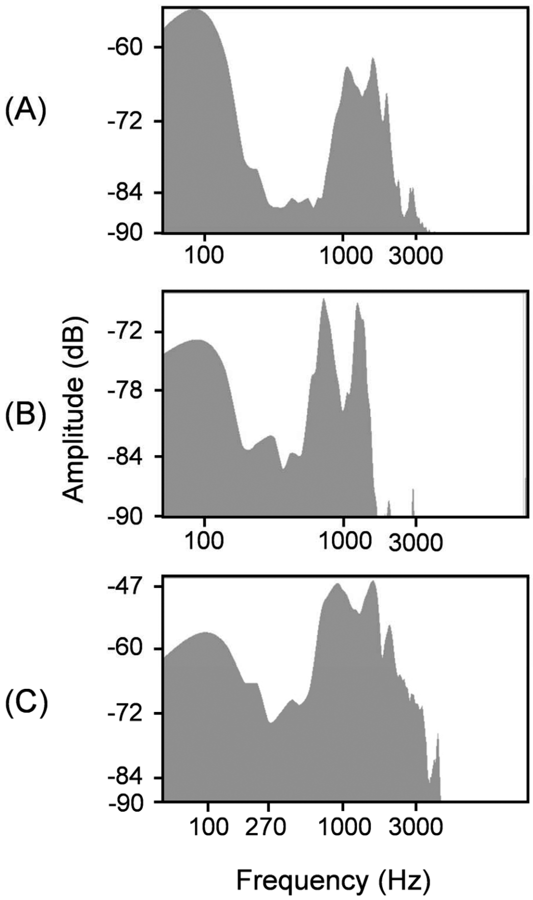

Fig. 8. Spectral analysis of the vibrations emitted by eggs of the migratory locust kept singly. The data analysed were obtained by subjecting the raw data to the noise reduction function in Audacity; 0 dB on these graphs is the maximum value recorded by this system. (A) one bout consisting of 14 vibrations; (B) one bout consisting of four vibrations; (C) one bout consisting of 31 vibrations. The spectra shown include only those for particular vibrations. There were two major peaks, at about 100 Hz and 1.5 kHz.

Spectrographic analysis of the specific vibrations

Three examples of a spectrographic analysis of vibrations are presented in Fig. 8. The patterns obtained from 3 bouts consisting of 4, 14 and 31 vibrations recorded from different eggs looked similar; there were two major peaks located at frequencies of about 100Hz and 1.5kHz. Seven other bouts of vibrations recorded from single eggs of different pods had similar patterns (data not shown).

To determine which of the frequencies was important in controlling the hatching time of locust eggs, the spectrogram of a bout consisting of 31 vibrations recorded from an egg (shown in Fig. 8C) was arbitrarily divided into two parts of < and ≥ 270 Hz, (the lowest point between the two major peaks; Fig. 9A, B). These were then separately played back to eggs kept separately 2 days before they hatched. The lower of the two frequencies did not significantly affect the hatching time of the eggs (Fig. 9C; Tukey's multiple-comparison test, $P > 0.05$) and its variance (*F*-test; df = 11, 11; $P = 0.17$) compared with those of untreated single control eggs, whereas the high frequency caused the eggs to hatch significantly earlier than the untreated single control eggs (Fig. 9D; Tukey's multiple-comparison test, $P < 0.05$)

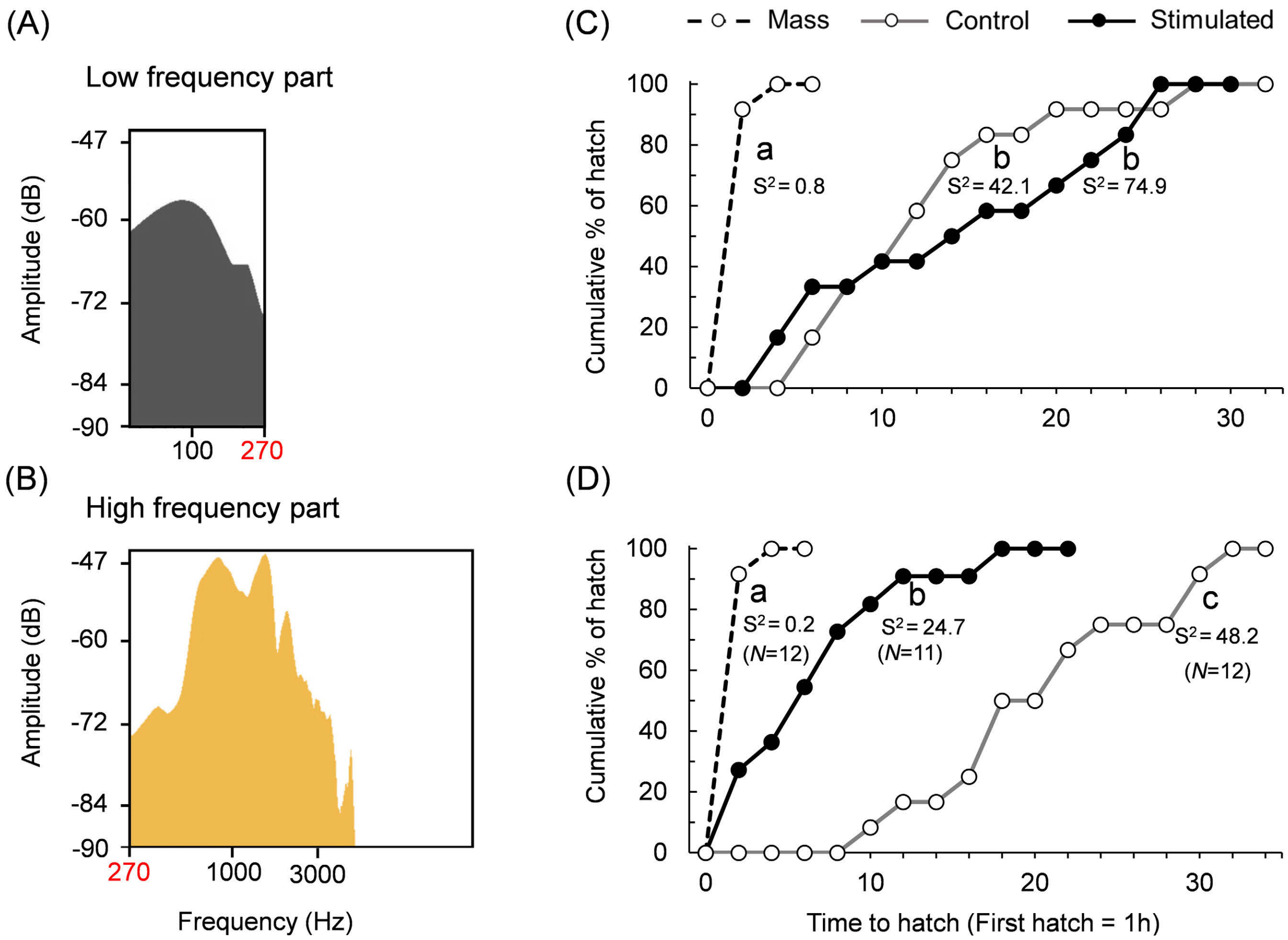

Fig. 9. Effects of recordings of the low- and high- spectrum ranges of the vibrations emitted by an egg of the migratory locust on the hatching times of eggs kept separately. The low- and high-frequency parts in Fig. 8C were divided at 270 Hz (A, B) and played back separately to 12-day-old eggs kept separately continuously until they hatched. C – results of the playback of the low-frequency part. D – results of the playback of the high-frequency part. Different letters in each panel indicate a significant difference in the mean hatching times at the 5% level using Tukey's multiple-comparison test. $n = 12$ with the exception of the eggs used in (D). Variance (S^2) is shown for each treatment.

with similar variances (*F*-test; df = 11, 11; $P = 0.15$), but significantly later than the untreated mass of eggs (Tukey's multiple-comparison test, $P < 0.05$).

Mechanism for emitting vibrations

After examining the film of locust embryos emitting vibrations, we observed that an embryo that was emitting relatively small vibrations 20 h before hatching was moving the posterior part of its abdomen (Fig. S3). The movement of the abdomen was more vigorous and clearer in embryos emitting larger vibrations shortly before hatching (Fig. S4); the movement and sound transformed from the vibrations coincided perfectly with one another. In the present study, however, no attempt was made to determine exactly how the low and high frequency vibrations were produced by the embryo.

DISCUSSION

For synchronous hatching, the embryos in a mass of eggs must control their hatching times unless they rely on other signals such as those provided by a parent (Ohba, 2002; Mukai et al., 2012). In the migratory locust, the hatching of an egg pod is completed within 0.5 to 2.5 h (Chen 1999; Nishide et al., 2017b). However, the eggs that are individually separated hatch later and over a longer period than those in a pod (Tanaka, 2017). Similar phenomena are reported for several other species including true bugs (Kiritani, 1964; Endo & Numata, 2017) and another locust, *S. gregaria* (Nishide & Tanaka, 2016). However, the mechanisms underlying synchronous hatching are unexplored in insects.

The eggs of the giant water bug, *Kirkaldyia deyrolli,* hatch in response to water splashed onto them by the male parent and that the rupture of the membrane enveloping a nymph produces sound or vibration (Ohba, 2002). Based on the hatching patterns of eggs in an egg mass, he suggested that this sound or vibration produced at hatching might serve as a trigger for the simultaneous hatching of the other individuals in the same egg mass. A similar phenomenon is reported in another species of bug, *Halyomorpha halys*, in which a recording of the vibrations associated with the eggs cracking stimulated eggs to hatch (Endo et al., 2019). Isolated pharate-larvae of the cockroach, *Periplaneta americana*, hatch early when gently stroked with the tips of forceps and it is suggested that tactile stimulation is involved in the synchronous hatching of the many larvae in an ootheca (Provine, 1976, 1977).

Previously we reported that the hatching of the eggs of the migratory locust is advanced or delayed depending on whether they are kept together with an older or younger egg, respectively (Tanaka, 2017). This control is mediated via vibrational stimuli emitted by the embryos (Tanaka et al., 2018), although the exact stimuli have not been identified. Here, we report that eggs of migratory locusts emit vibrations and the number of vibrations per bout increased significantly toward hatching time. Each bout of vibrations varied in its amplitude; the mean of the largest five amplitudes measured was small at 10 h before hatching and increased significantly just prior to hatching. Our playback experiments revealed that the vibrations recorded during the last 2.5 h before hatching caused eggs to hatch significantly earlier than single control eggs, which indicates that the vibrations emitted shortly before hatching induce hatching. Similar effects are reported when eggs kept separately are subjected to the vibrations associated with music or the presence of a hatchling for about 2 days before hatching (Tanaka et al., 2018), which in both cases advanced the time of hatching. Here, we found that vibrations recorded 8 to 10 h before hatching significantly delayed the hatching of eggs kept separately compared to those that were not so treated or those kept in a mass, which indicates that vibrations emitted long before hatching may function to delay hatching by influencing the time taken to enter the standby stage. These results indicate that eggs of migratory locust might control hatching time by means of age-dependent differences in the vibrations they emit, although the characteristics of the vibrations that control hatching time are yet to be demonstrated.

In the experiments testing the effects of vibrations on the hatching of the eggs of migratory locusts, the variance in the times to hatching of the eggs subjected to recordings of the vibrations was greater than that recorded for the eggs in a mass and as large as that for eggs kept in isolation and subjected to recordings of the vibrations, which indicates that the treated eggs did not hatch synchronously. This was because only some of the eggs responded to the vibrations by hatching. This result might be explained by the two-step hypothesis for synchronous hatching in this locust (Tanaka et al., 2018). Embryos enter a standby stage as a result of vibrations emitted by neighbouring embryos and hatch when neighbouring embryos emit strong vibrations. In the experiments designed to test this hypothesis, separated eggs hatched synchronously (with significantly reduced variance in hatching times) only when one bout of vibrations occurred shortly before hatching, by which time most embryos were in the standby stage (Fig. 7D).

There is no doubt that synchronous hatching is brought about by the embryos of migratory locust interacting with one another. However, it is unknown whether embryo-embryo communication exists, because this phenomenon can also be explained by a "passive" hypothesis by which the embryo simply responds to age-dependent changes in the vibrations emitted by another embryo in the same mass. Although both single eggs and eggs in a mass emitted vibrations, no attempt was made to determine the presence of embryo-embryo communication in the present study.

Spectrographic analysis revealed that the vibrations produced by embryos of migratory locusts consisted of two major peaks (Fig. 8). Our playback experiments showed that the high-frequency range with a peak of about 1.5 kHz had a significant positive effect on hatching time, whereas the low-frequency range of about 100 Hz had no significant effect.

CONCLUSIONS

Our study using video and vibration recordings revealed that the embryos of the migratory locust move the posterior part of their abdomens in a characteristic way when the vibrations are emitted. The amplitude and number of vibrations were small at about 10 h before hatching and increased significantly as hatching time approached. We detected the earliest vibration 31 h before hatching (data not shown). However, eggs laid up to about 40 h apart by different female locusts can modify their hatching times for synchronous hatching when put together at least 2 days before hatching (Tanaka, 2017). Therefore, our findings indicate that the vibrations recorded possibly control synchronous hatching in this locust, but that other factors, such as smaller vibrational signals that might be detected only with a more sophisticated recording device, are likely to be involved in this phenomenon. The migratory locust may serve as an ideal species in which to identify such specific signals, because its eggs and egg masses are relatively large and easily available, and most importantly separated eggs either show an advance or delay in hatching time, depending on the age of the egg they are paired with (Tanaka, 2017).

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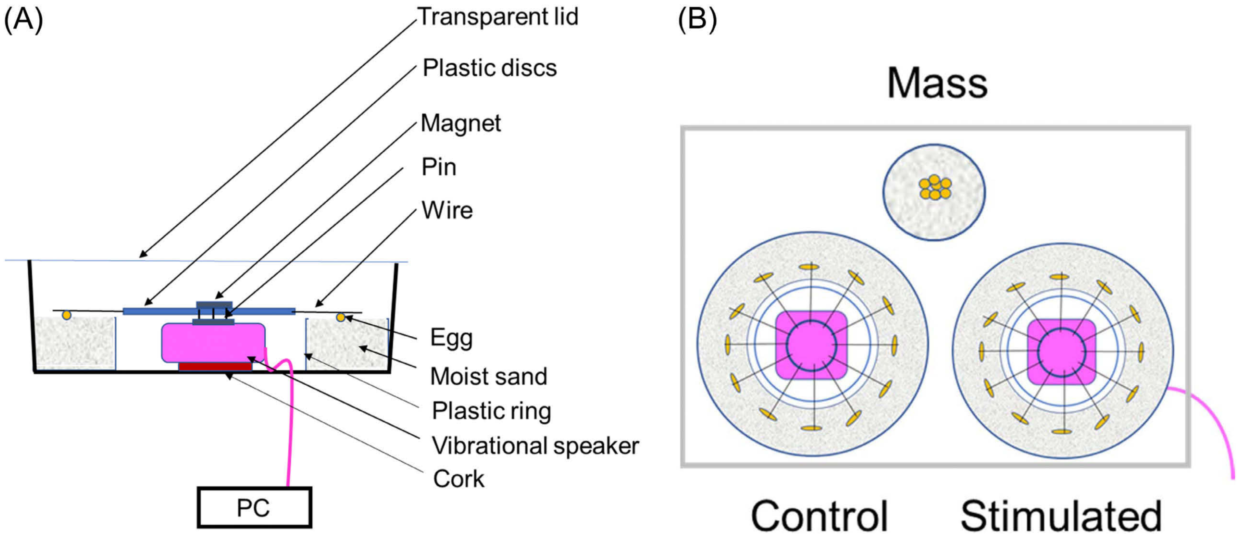

Fig. S1. Apparatus used to test the effect of the recordings of vibrations on hatching. The apparatus used was basically similar to that described previously (Tanaka et al., 2018) except for the use of a vibrational speaker (A). Twelve eggs of the migratory locust were placed separately on a sand mount and each connected by a wire sandwiched between plastic discs that were placed on a vibrational speaker connected to a PC (right, B). Another 12 eggs were similarly placed on another sand mount but not subjected to recordings of vibrations, the control (left, B), and another 12 eggs were placed as a mass in a sand pit in a Petri dish (top, B). All eggs came from the same pod.

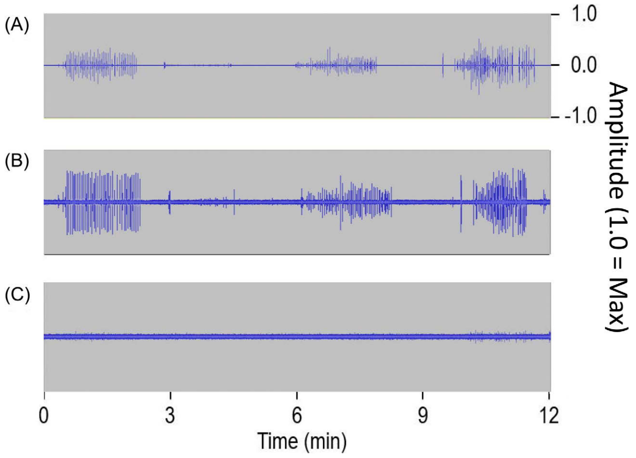

Fig. S2. Reproducibility of the vibrations emitted by an egg of the migratory locust gauged by eye. (A) Original pattern of vibrations recorded using a PC; (B) pattern of vibrations transmitted along the wire of the playback apparatus when (A) was played back; (C) background noise recorded as in (B), except that the recording pin did not touch the wire. The system used appeared to play back the original vibrations fairly faithfully.

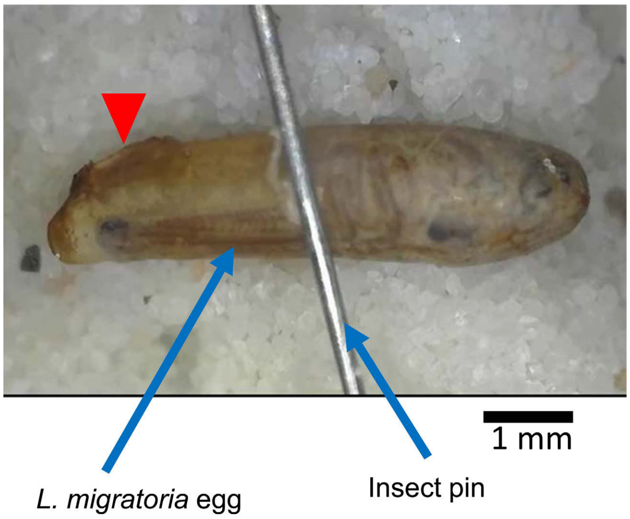

Fig. S4. Movie (click here) showing an embryo of the migratory locust emitting vibrations approximately 7 min before hatching. Vibrations were synchronized with the movement of the end of the abdomen of the embryo (arrow).

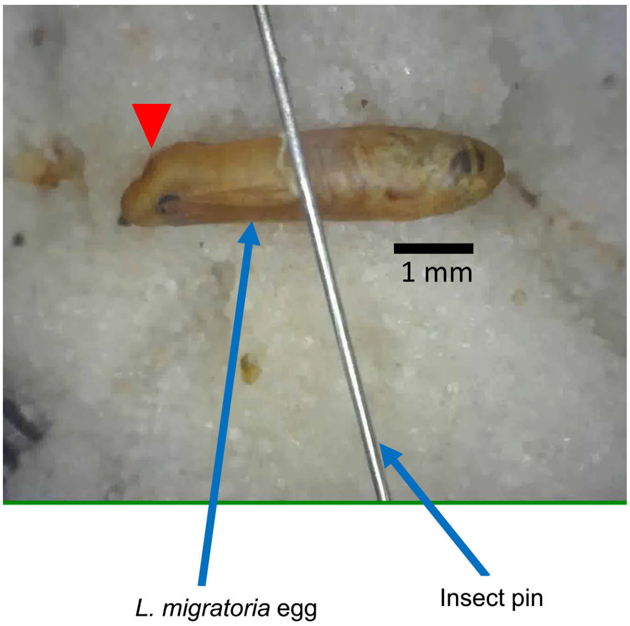

Fig. S3. Movie (click here) showing an embryo of the migratory locust emitting vibrations approximately 20 h before hatching. Vibrations were synchronized with the movement of the end of the abdomen of the embryo (arrow).