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Differences in Heat Sensitivity between Japanese Honeybees and Hornets under High Carbon Dioxide and Humidity Conditions inside Bee Balls

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Upon capture in a bee ball (i.e., a dense cluster of Japanese honeybees forms in response to a predatory attack), an Asian giant hornet causes a rapid increase in temperature, carbon dioxide (CO_2), and humidity. Within five min after capture, the temperature reaches 46°C, and the CO_2 concentration reaches 4%. Relative humidity gradually rises to 90% or above in 3 to 4 min. The hornet dies within 10 min of its capture in the bee ball. To investigate the effect of temperature, CO_2, and humidity on hornet mortality, we determined the lethal temperature of hornets exposed for 10 min to different humidity and CO_2/O_2 (oxygen) levels. In expiratory air (3.7% CO_2), the lethal temperature was $\geq 2^{\circ}$ lower than that in normal air. The four hornet species used in this experiment died at 44–46°C under these conditions. Hornet death at low temperatures results from an increase in CO_2 level in bee balls. Japanese honeybees generate heat by intense respiration, as an overwintering strategy, which produces a high CO_2 and humidity environment and maintains a tighter bee ball. European honeybees are usually killed in the habitat of hornets. In contrast, Japanese honeybees kill hornets without sacrificing themselves by using heat and respiration by-products and forming tight bee balls.

Key words: Japanese honeybee, *Apis cerana japonica*, bee ball, lethal temperature, Asian giant hornet, *Vespa mandarinia*

INTRODUCTION

Asian giant hornet (*Vespa mandarinia*) is the main predator of the Japanese honeybee (*Apis cerana japonica*) in Japan; 10 or more people die every year because of the sting of this hornet. Giant hornets attack the nests of honeybees in groups of few to several tens of hornets, and can eradicate a colony within several hours to several days. In response to attack, Japanese honeybees capture hornets in a bee ball in an attempt to kill them (Tokuda, 1924). The hornet dies within a bee ball by "*futonmushi*," which is similar to a form of bullying, in which a person suffocates in a futon (blanket). The physiological mechanisms involved in hornet mortality are unclear. Ono et al. (1987) first reported the cause of hornet (*V. simillima*) mortality within a bee ball. They concluded that Japanese honeybees produce heat to kill hornets (*V. mandarinia* and *V. simillima*), as the temperature within a bee ball generated by honeybees is higher than the lethal temperature of hornets (Ono et al., 1987, 1995).

Sugahara and Sakamoto (2009) found that honeybees capture and kill giant hornets within 10 min of capture in a bee ball either in an open nest or near a closed hive. However, giant hornets do not die even when placed inside an incubator at 47°C for 10 min; this temperature is higher than the maximum temperature within a bee ball (45.9 ± 1.0°C). This contradictory finding suggests that heat alone does not kill a giant hornet. They later found that carbon dioxide (CO_2) concentration within a bee ball was 3.6 ± 0.2%, and the lethal temperature in expiratory air (3.7% CO_2) was 2°C lower than that in normal air (Sugahara and Sakamoto, 2009). They concluded that high CO_2 concentration and/or reduced oxygen (O_2) and heat are responsible for mortality of giant hornets in bee balls (Sugahara and Sakamoto, 2009).

When honeybees generate heat rapidly by consuming honey, the levels of CO_2 and water vapor seem to increase in a bee ball in which honeybees are closely packed together; however, no concrete data have been reported. In this study, we investigate how the microenvironment inside a bee ball changes and results in the death of hornets. In addition to *V. mandarinia*, we analyzed three other species commonly found in our area: *V. analis*, *V. simillima*, and *V. crabro*. These hornets capture honeybees going in and out of their nests. We thoroughly studied the survival/death of the trapped hornets, temperature within bee balls, and dif-

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ferences in heat sensitivity between predators and preys under high CO_2 and humidity conditions inside bee balls.

MATERIALS AND METHODS

Honeybees and hornets

We used Japanese honeybees of one- or two-year-old traditional closed hives and open nests as the test stage for bee ball formation. The open nest was prepared as follows: a swarm of honeybees was captured in Hirakata City and kept in a special hive box consisting of a fixed top board and removable bottom and lateral boards. We did not introduce hive boards. We hung the hive box under eaves. When the nest had developed sufficiently, we removed all bottom and lateral boards to create an open nest.

To determine mortality rate, we removed Japanese honeybee workers of a colony bred in our laboratory from a comb and placed them in a plastic container with a cover. We also removed European honeybee workers of a colony of Italian species, purchased from an apiary (Nonogaki Apiary, Ichinomiya in Aichi prefecture) and bred in our laboratory, from a comb and placed them inside a plastic container with a cover.

We collected the hornets *V. mandarinia* and *V. analis* while they flew in and out of their natural nests in Moriguchi City (34.75°N, 135.56°E) and Kameoka City (34.99°N, 135.55°E). We collected *V. simillima* while they attempted to capture honeybees (bred in our laboratory) and while they visited goldenrod (*Solidago altissima*) to prey on insects. We also collected *V. crabro* hornets that flew in and out of their nest, inside a tree hole next to the Yodo River in Moriguchi City. We anesthetized hornets and honeybees with CO_2 before the experiments.

Formation of bee balls, measurement of temperature inside the bee balls, and mortality rates of trapped hornets

We anesthetized a hornet before attaching it to the tip of a thermometer probe using scotch tape. Five minutes later, when the hornet revived from the anesthesia, we placed the tip of the thermometer probe (with the hornet fixed to it) against the honeybee nest. Immediately, honeybees formed a bee ball on the surface of the comb. The temperature inside the bee ball (around the hornet) was recorded using a digital thermometer (Yokogawa Model 2455) coupled with a digital video recorder (SONY DCR, TR V20). After 10 min, we removed the bee ball (attached to the probe) from the nest, and dispersed the remaining bees around the trapped hornet by spraying a repellent (Skin Guard, Johnson Co., LTD). The survival/death of trapped hornets was determined in 30 min according to the following criteria: (1) alive, presence of a response to contact stimulation and movement, and (2) dead, protrusion of the sting, no response to contact stimulation, and no movement.

Measuring CO_2 concentration inside bee balls

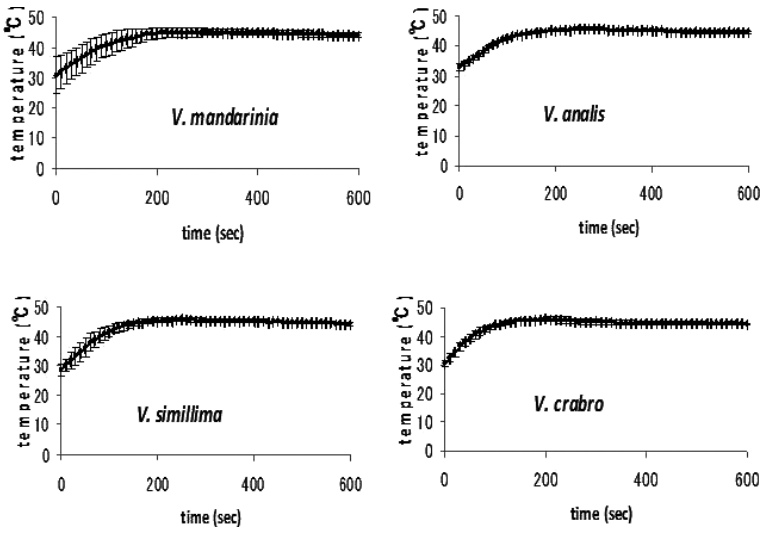
We measured CO_2 within bee balls using two methods. In the first method, a portable gas detector (COSMOS XP-3140) measured the CO_2 concentration. Because the gas inlet was large (internal diameter (id), 5 mm), two *V. mandarinia* were fixed to the inlet tip (Sugahara and Sakamoto, 2009). Thereafter, the inlet tip was touched against the open nest and the nest in the closed hive. Experimental honeybees formed a slightly larger bee ball (ca. 6 cm in diameter) during this procedure than during the temperature experiment (indicated above). We recorded the CO_2 concentration within the bee ball (at a flow rate of 250 ml/min) by using a digital video recorder.

Because the CO_2 levels may decrease during continuous sampling of gas from bee balls, we deployed a low-volume gas sampling method. We used a GASTEC (GV-100S) with a detector tube (2H), designed to suck 100 ml of gas in 5 min from the air to measure the CO_2 concentration based on a change in the coloration of the detector tube. Because the detector tube has a smooth glass surface that prevents the formation of a compact bee ball, a plastic short straw was attached to the tip of the detector tube to fix 2–4 *V. mandarinia* and facilitate bee ball formation (Fig. 2A). We measured CO_2 concentrations in bee balls of an open nest and a closed hive.

Measurement of humidity inside bee balls

We simultaneously measured humidity and temperature within bee balls with a digital hygrometer (SK-110TRH II, TYOE3 SATO). The hygrometer displays "Hi" when the relative humidity exceeds 98.0%. At 35–50°C, the hygrometer has ± 5% measurement error. The probe is so thick (8 mm, id) that it cannot be set at the core of a bee ball at the tip of the probe with one or two *V. mandarinia*. Thus, we fastened three *V. mandarinia* hornets with a rubber band to the probe to facilitate the formation of bee balls, which were larger than the abovementioned cases (Fig. 3C).

A



B

Species	n	Max. temp., °C	Time for max. temp., sec	Final temp., °C
V. mandarinia	15	45.9 ± 0.9	292 ± 72	44.4 ± 0.8
V. analis	4	46.1 ± 0.4	275 ± 13	44.7 ± 0.4
V. simillima	4	45.9 ± 0.4	255 ± 74	44.1 ± 0.4
V. crabro	5	46.1 ± 0.7	184 ± 32	44.3 ± 0.4

Fig. 1. Measurement of temperature inside bee balls. The average maximum temperatures (max. temp.) were as follows: 45.9°C for *Vespa mandarinia*, 46.1°C for *V. analis*, 45.9°C for *V. simillima*, and 46.1°C for *V. crabro*. The temperature reached its maximum after 5 min and was subsequently maintained until 10 min (2-phase temperature). From the time-temperature curves in Fig. 1A, we selected three parameters to characterize the curves and expressed the parameters as numbers (Fig. 1B). We compared four means for each of the parameters by using the pairwise *t*-test function of the statistical package R. This function performs the two sample *t*-test between every pair of groups among more than 2 groups with adjustment of the *p* value by the Holm correction method for the multiple comparison testing. For the time of max. temp., we observed a difference between *V. mandarinia* and *V. crabro* (P = 0.015), but no differences between any other pair of species. There were also no significant differences in the max. temp. and the final temp. between any pair of the four species.

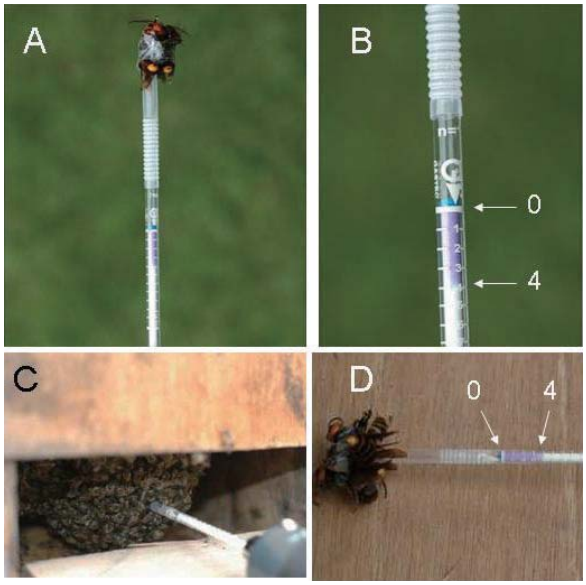


Fig. 2. Measurement of carbon dioxide (CO_2) inside bee balls from open nests and closed hives. Measurements obtained with GASTEC are shown. After fixing a straw at the tip of the detector tube, 2 *V. mandarinia* individuals were attached with a Scotch tape **(A)**. Five minutes after the formation of the bee ball on an open nest, 100 ml of gas was aspirated from the bee ball. The change in the color of the detector tube indicates the presence of 4% CO_2 **(B)**. Four *V. mandarinia* individuals were fixed and a bee ball was formed in a closed hive **(C)**. By the aspiration of 100 ml gas after 5 min, a change in the color of the detector tube indicates the presence of 4% CO_2 **(D)**.

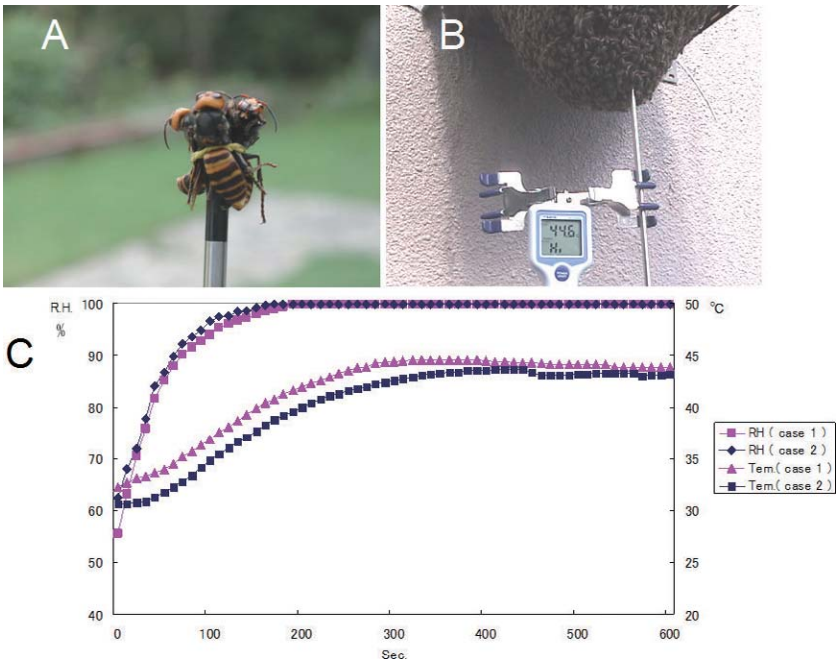


Fig. 3. Measurement of the relative humidity and temperature inside bee balls. When three *V. mandarinia* individuals, which were fixed at the tip of a probe with rubber band **(A)**, were placed on the surface of the cluster of honeybees of an open nest, a bump-like bee ball was formed **(B)**. The time-relative humidity and the time-temperature curves for two cases **(C)** showed that the humidity and temperature reached their maximum after 3 and 5 min, respectively.

Measurement of lethal temperatures

To determine the 50% lethal temperature (LT_{50}), we used an incubator (Takasaki Scientific Instruments CORP, TXY-9R-3F and EYELA LTI-601SD) and measured temperature-induced mortality rates of hornets. In the case of normal air, we maintained hornets in a 100-ml plastic container with a cover of punched holes to adjust the air to the set temperature of the incubator. We placed the container in the incubator for 10 min. In the case of human expiratory air or a gas mixture (A or B) (Japan AIR GASES), the hornet was kept in an aluminum-coated plastic bag (340 × 240 mm, 4-l volume, Lamizip AL-24 Seinichi K.K.) filled with human expiratory air or gas mixture (A or B) and equipped with a small battery-powered fan for stirring the air. The bag was preheated for 30 min to adjust the air to the set temperature of the incubator and placed in the incubator for 10 min. Gas mixture A contained 5% CO_2, 15% O_2, and 80% N_2 that simulated human expiratory air. Gas mixture B contained 5% CO_2, 20% O_2, and 75% N_2 that compensated for the reduced O_2 level of gas mixture A. After 10 min, we evaluated the condition (alive/dead) of the hornets.

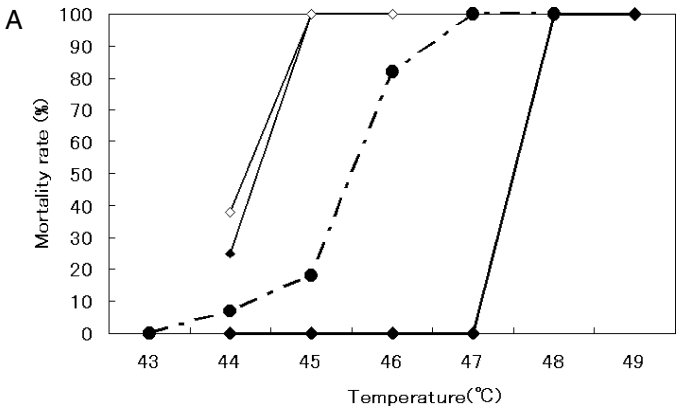
The lethal temperature 50% (LT_{50})of honeybees in normal air was determined using a small plastic container (20-ml volume) with small holes. In the case of human expiratory air or gas mixtures, we placed honeybees directly into a plastic bag equipped with a small battery-powered fan for stirring the air and preheated for 30 min to adjust the air to the set temperature of the incubator. After 10 min of incubation, the condition (alive/dead) of the honeybees was examined.

Setting of humidity to measure the lethal temperature

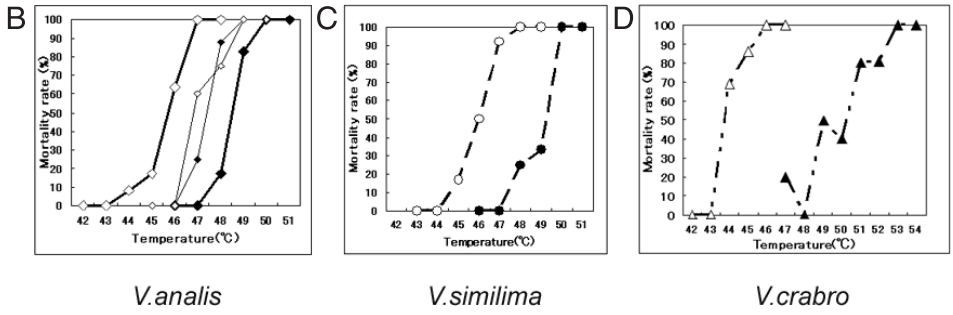
To measure the lethal temperature in normal air, we placed a small water tank inside the incubator to prevent unusually low relative humidity. Because the survival of bees at high temperatures depends on the duration of exposure and relative humidity (Free et al., 1962), we used medium relative humidity conditions to determine lethal temperatures. We measured humidity in the incubator with a simple hygrometer (HYGROMETER Sinwa). When we raised the temperature during the measurement, the relative humidity remained between 30% and 45%. To determine the lethal temperature in expiratory air, we measured the humidity in an aluminum-coated plastic bag (containing expiratory air) with a digital hygrometer (SK-110TRH II, TYOE3 SATO). When the expiratory air is just released, its relative humidity is nearly 100%, but this declines with increase in temperature. For example, the average humidity of expiratory air in the plastic bag was 53% at 46°C, which is similar to the level established for normal air. We mixed gas mixtures A and B at a low humidity and stored in a cylinder. When we raised the temperature of the gas mixtures to 46°C, the relative humidity dropped to 13%. To create environments with 90% or higher relative humidity at 46°C, which produces a humid environment in the bee balls, we placed wet paper towels in the aluminum-coated plastic bag of gas mixtures A and B for the measurement of the lethal temperature for *V. mandarinia*.

Statistics

We performed two types of statistical analysis, one for the results presented in Fig. 1B, and the other for the results presented in Figs. 4, 5. The first statistical analysis involved a comparison of time-temperature curves among the four species of hornet. To interpret the



		LT_{50}			°C	43	44	45	46	47	48	49
V. mandarinia	normal air	47.5	◆	n			4	4	5	6	6	6
	human expiratory air	45.4	●	n		4	14	17	17	5	5	
	gas mixture A (R.H. > 90% at 46°C)	44.1	◆	n			8	8	8			
	gas mixture B (R.H. > 90% at 46°C)	44	◇	n			8	8	8			



		LT_{50}			°C	42	43	44	45	46	47	48	49	50	51	52	53	54
V. analis	normal air	48.5	◆	n					4	4	12	12	8	4				
	human expiratory air	45.6	◇	n		4	4	12	12	11	4	4						
	gas mixture A (R.H. 13% at 46°C)	47.4	◆	n						4	8	8	4	4				
	gas mixture B (R.H. 13% at 46°C)	47.1	◇	n					4	6	10	8	4	4				
V. simillima	normal air	49	●	n						5	8	8	12	8	4			
	human expiratory air	45.9	○	n			4	4	12	12	12	4	4					
V. crabro	normal air	49.8	▲	n							10	11	20	15	15	16	3	4
	human expiratory air	43.8	△	n		7	10	13	14	11	4							

Fig. 4. Temperature-induced mortality rates (%) of hornets. Results of *V. mandarinia* **(A)**: normal air, thick solid line; human expiratory air, thick 1-dot chain line; gas mixtures A and B, a thin solid line. The calculated LT_{50} and tested numbers are show in the table of a bottom. Results of *V. analis* **(B)**: normal air and human expiratory air, thick solid line; gas mixture A and B, thin solid line. Results of *V. simillima* **(C)**: normal air and human expiratory air, thick dotted line. Results of *V. crabro* **(D)**: normal air and human expiratory air, thick 2-dot chain line. The calculated 50% lethal temperature (LT_{50}) values and tested numbers are shown.

results, we chose three meaningful parameters to characterize the curves: maximum temperature, the point at which the maximum temperature was reached for the first time, and the temperature at the final timepoint. We calculated the sample mean and standard deviation (SD) for each of these three values and for each of the 4 hornet species. For each of the values, we compared four means by using the pairwise *t*-test, which is a multiple comparison method for means. The second statistical analysis involved comparing of the mortality rate between the two air conditions. We compared these air conditions in two different ways. One involved a comparison between normal and human expiratory air, and the other involved a comparison between gas mixtures A and B. We used a dichotomous (dead or alive) logistic regression analysis with 1 covariate (temperature) and 1 factor (2 air conditions). Through this analysis, we calculated the LT_{50} (Figs. 4, 5) and estimated the odds ratios between the two air conditions (Table 2).

We used the statistical package R version 2.9.2, and we chose a P value of < 0.05 to define the statistical significance (R Development Core Team, 2009). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

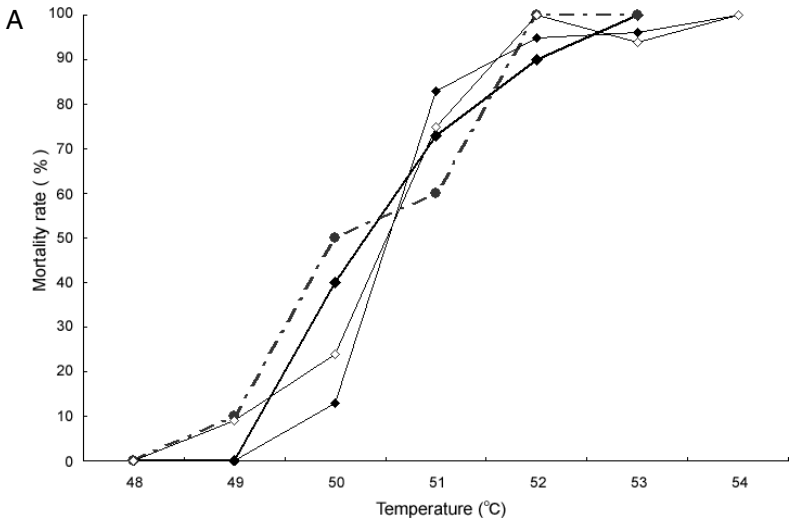
RESULTS

Mortality inside the bee ball

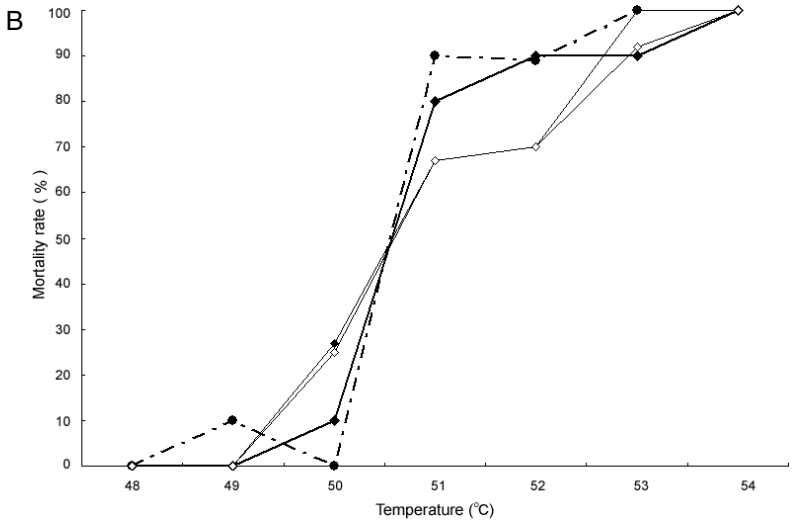
Mortality rates were determined 10 min after the hornets were captured within the bee balls (Table 1). All workers of *V. mandarinia*, *V. analis*, and *V. crabro* died when captured in a bee ball for 10 min (Table 1). Five of eight *V. simillima* workers were barely alive. As natural bee balls are normally maintained for 20 min or longer (Ono et al., 1987, 1995), it is likely that most *V. simillima* die within the bee ball in natural conditions. The queens of the three hornet species died in 10 min, and there was no difference in the mortality rates between castes, despite the large differences in their body sizes. We did not find any sting apparatus of honeybees in any of the hornets captured in the bee balls.

Temperature inside the bee ball

Figure 1 illustrates the temperature changes inside the bee balls (for 10 min). The mean maximum temperatures in the bee balls formed by honeybees were as follows: 45.9°C for *V. mandarinia*, 46.1°C for *V. analis*, 45.9°C for *V. simillima*, and 46.1°C for *V. crabro*. These temperatures showed little change despite the wide variation in the body lengths of the workers (maximum 4 cm for *V. mandarinia* and 2.4 cm for *V. simillima*) (Matsuura, 1995). The graph showed little change for the four hornet species. Five minutes later, the temperature within the bee balls reached the maximum. Subsequently, the temperature was maintained (2-phase temperature). The size of a bee ball (6 cm for *V. mandarinia*



		LT_{50}		°C	48	49	50	51	52	53	54
A. c. japonica	normal air	50.5	◆ n		10	10	10	15	10	10	
	human expiratory air	50.4	● n		11	10	10	15	10	10	
	gas mixture A (R.H. 13% at 46°C)	50.6	◆ n		8	10	15	18	24	23	5
	gas mixture B (R.H. 13% at 46°C)	50.5	◇ n		8	11	17	16	24	17	6



		LT_{50}		°C	48	49	50	51	52	53	54
A. mellifera	normal air	50.8	◆ n		10	10	10	10	10	10	10
	human expiratory air	50.6	● n		10	10	10	10	9	11	
	gas mixture A (R.H. 13% at 46°C)	50.8	◆ n		12	12	15	33	30	12	6
	gas mixture B (R.H. 13% at 46°C)	50.9	◇ n		12	12	24	24	23	12	6

Fig. 5. Temperature-induced mortality rates (%) of honeybees. Results of *A. cerana japonica* **(A)**: normal air, thick solid line; human expiratory air, thick 1-dot chain line; gas mixture A and B, thin solid line. The calculated LT_{50} values and tested numbers are shown at the bottom of the table. Results of *A. mellifera* **(B)**: normal air, thick solid line; human expiratory air, thick 1-dot chain line; gas mixture A and B, thin solid line. The calculated LT_{50} values and tested numbers are at the bottom of the table.

and 4.5 cm for *V. simillima*) increased with the increase in the body length of the hornet.

CO_2 concentration inside the bee ball

As reported previously (Sugahara and Sakamoto, 2009), the CO_2 concentration measured by the continuous aspiration gas detector was 3.6 ± 0.2%. By the detector tube method with a single aspiration, we found that the CO_2 concentration in the bee ball for *V. mandarinia* was 4.0% in both the open nest and the closed hive (Fig. 2).

Humidity inside the bee ball

Bee balls around *V. mandarinia* were created on the probe of a hygrometer to measure the relative humidity (Fig. 3). The relative humidity rose gradually, and the maximal humidity was recorded after 3 min from the capture of *V. mandarinia* hornets in the bee ball. Due to the dead space created by the three hornets around the air inlet of the probe, the maximum temperature may not rise as high as the case of the temperature measurement. The relative humidity after the maximal level was reached probably exceeded 90%.

Lethal temperature

The LT_{50} of the four hornet and two honeybee species held for 10 min in normal air, human expiratory air, and gas mixtures were measured as shown in Figs. 4 and 5. From Fig. 4A, LT_{50} values of *V. mandarinia* were 47.5°C in normal air and 45.4°C in human expiratory air. Therefore, the LT_{50} in expiratory air dropped by 2.1°C compared with that in normal air. Furthermore, the LT_{50} in gas mixture A or B decreased by 1.3–1.4°C compared to the LT_{50} in expiratory air. The values of relative humidity of gas mixture A and B were 90% or higher at 46°C, and therefore, they were similar to the relative humidity in the bee balls. We observed no differences between the LT_{50} values in gas mixtures A and B. As shown in Fig. 4B, *V. analis* and *V. mandarinia* had similar LT_{50} values in normal air and expiratory air. LT_{50} values in the gas mixtures A and B were 47.4°C and 47.1°C, respectively, and higher than those in expiratory air, owing to low humidity (13%). The insufficient O_2 concentration in gas mixture A had no effect on the LT_{50} value. *Vespa simillima* showed high LT_{50} values in normal air and expiratory air, compared to the LT_{50} values of the other three species. Although the cause is not clear, in the case of *V. crabro*, LT_{50} was highest in normal air, and lowest in expiratory air; that is, *V. crabro* showed the highest sensitivity to CO_2 concentration.

As shown in Fig. 5A, B, Japanese and European honeybees showed almost the same LT_{50} values (50–51°C) in expiratory air, normal

Table 1. Mortality rates (MRs) of four species of hornet captured inside bee balls for 10 min. No sting apparatus was found in the hornets. The bee ball size increased according to the size of hornets. The results for *V. mandarinia* were obtained from open nests (24) and closed hives (7). The results of other hornets were obtained from open nests.

Species	Caste	MR	n	Notes
V.mandarinia	worker	100%	31	Died with the sting protruded
	queen	100%	3	Died with the sting protruded
V.analis	worker	100%	11	Died with the sting protruded
	queen	100%	1	Died with the sting protruded
V.simillima	worker	38%	8	Dying and died with time
V.crabro	worker	100%	5	Died with the sting protruded
	queen	100%	3	Died with the sting protruded

Table 2. Logistic regression analyses. Dichotomous (dead or alive) logistic regression analyses with one covariate (temperature) and one factor (two air conditions) for each species of hornets was performed. Table 2-1 shows the odds ratios of the human expiratory air relative to normal air. There were significant differences between the human expiratory air and normal air for *V. mandarinia*, *V. analis*, *V. simillima*, and *V. crabro*; however, there were no differences for *A. cerana japonica* and *A. mellifera*. Table 2-2 shows the odds ratios of gas mixture B relative to A. There were no significant differences between these air conditions for any of the treated species.

Species	p-values	Odds ratio	95% CI of odds ratio	
			Lower	Upper
V. mandarinia	< 0.0001	598	24.8	14400
V. analis	< 0.0001	1040	38.4	28000
V. simillima	< 0.0001	687	38	12400
V. crabro	< 0.0001	578	71.6	4660
A. cerana japonica	0.786	1.16	0.394	3.42
A. mellifera	0.537	1.49	0.419	5.31

Species	p-values	Odds ratio	95 % CI of odds ratio	
			Lower	Upper
V. mandarinia	0.592	1.8	0.21	15.4
V. analis	0.414	1.93	0.4	9.26
A. cerana japonica	0.646	1.26	0.473	3.34
A. mellifera	0.778	0.907	0.458	1.8

air, and the two gas mixtures (with a relative humidity of 13%). We observed no difference between the LT_{50} values in gas mixtures A and B.

DISCUSSION

Japanese honeybees generate heat to kill hornets (*V. simillima*, *V. mandarinia*) within a bee ball (Ono et al., 1987, 1995). Sugahara and Sakamoto (2009) compared the LT_{50} values measured in normal air (0.04%) and human expiratory air (3.7% CO_2), and showed that the LT_{50} value decreased by 2°C in expiratory air. Based on those results, it was reported that an increased CO_2 concentration (or reduced O_2 concentration), as well as heat, caused the death of hornets (Sugahara and Sakamoto, 2009). In this study, we determined the LT_{50} values of *V. mandarinia* in normal air and expiratory air by using a larger number of hornets. Moreover, to analyze the influence of O_2 level, we compared the LT_{50} values in the two gas mixtures containing O_2 concentrations of 15% and 20%. Death of *V. mandarinia* in the bee ball was not caused by O_2 deficiency, as the lethal temperature did not change even when O_2 was supplied to compensate for the reduced amount of O_2 owing to increased CO_2. From the results for *V. mandarinia*, when the relative humidity of the gas mixture was increased to 90% or above to simulate the humidity conditions within a bee ball, the LT_{50} was lower in the gas mixture than in expiratory air. Many insects tolerate high temperatures through evaporative cooling. When this mechanism does not work well at high humidity, the lethal temperature decreases (Wigglesworth, 1972; Prange, 1996). Therefore, we believe that *V. mandarinia* died because they could not lower their body temperatures by evaporative cooling. These results suggest that the cause of *V. mandarinia* mortality within bee balls is a decrease in lethal temperature due to high humidity and CO_2 concentration. We think that honeybees kill *V. mandarinia* in a short time by metabolizing honey to generate heat and air at 90% or higher relative humidity and 4% CO_2 inside a tight bee ball.

The results shown in Fig 4B show that the LT_{50} values of the other three species in expiratory air were $\geq 2°$ lower than those in normal air, similar to what was observed in the case of *V. mandarinia*. *Vespa analis* was easier to capture than was *V. mandarinia,* so we used *V. analis* to estimate the effect of O_2. The LT_{50} values of *V. analis* were measured in low-humidity (13%) gas mixtures A and B to determine the actual effect of insufficient O_2 concentration alone by separation of the effect of humidity. We found that a lack of O_2 in bee balls was not responsible for the death of *V. analis*. We think the reason that the LT_{50} values of *V. analis* in gas mixture A and B (relative humidity of 13% at 46°C) were higher than those in the expiratory air was the evaporative cooling, which probably worked more effectively in the low humid gas mixtures than in the expiratory air. Consequently, the LT_{50} values of *V. analis* in the gas mixtures were higher than those in the expiratory air.

Surprisingly, the LT_{50} values of *A. cerana japonica* in normal air, expiratory air, and gas mixture A and B were nearly the same as shown in Fig. 5A. Interestingly, these data were nearly identical to those of *A. mellifera* as shown in Fig 5B. Overwintering as a colony without hibernation is commonly observed in these honeybees. During winter, honeybees must create heat and maintain a high temperature (ca. 32°C) within the bee balls, and this means they produce a high CO_2 and humidity environment at the same time. Bee ball formation and the by-products of respiration, especially high CO_2 as a greenhouse gas, might be favorable for maintaining the temperature. Hornets are cold-blooded animals, and only the queen hibernates during the winter; thus, hornets do not need a mechanism to generate heat during the winter. Consequently, they do not rapidly acclimate to the high CO_2 and humidity conditions within bee balls. This might explain why honeybees and hornets differ in sensitivity to heat in bee balls.

In the book entitled “Hot-Blooded Insects,” Heinrich (1979, 1993) describes changes in the body temperature of European honeybees (*A. mellifera*) during their daily activities. As he reports, body temperature of *A. mellifera* changes as follows: 36°C when leaving a hive, 30°C when returning to the hive, and 31°C when visiting a flower. When

swarming honeybees took off to search for a place to build a new hive, their body temperature increased to 36°C (Heinrich, 1981). The body temperatures during daily activities of Japanese honeybees were as follows: 41°C when leaving a hive, 40°C when returning to the hive, and 34°C when visiting a flower (Sugahara, 2005). The body temperature of swarming honeybees at take off was 41°C (Sugahara, 2003). The body temperature of European honeybees was 3–10°C lower than that of *A. cerana japonica* in all situations. The maximum temperatures within bee balls generated by European honeybees were 42.8°C (Ono et al., 1987) and 44.1°C (Ken et al., 2005). The maximum temperatures within Japanese honeybee balls, formed in the presence of the 4 hornet species (analyzed in this study) were comparable at 46°C. This temperature is 2°C to 3°C higher than that within European honeybee balls. This suggests that the temperature within bee balls formed by Japanese honeybees is set at 46C, and that of European honeybees, at 43–44°C, or that these are the maximum temperatures that each honeybee can achieve. In an environment where predatory hornets coexist, Japanese honeybees have obtained the ability to achieve temperatures that are slightly higher than the temperatures achieved by European honeybees, and these high temperatures may lead to the death of the predator captured in the bee ball.

LT_{50} values in normal air differed between species, in the following descending order, *V. crabro*, *V. simillima*, *V. analis*, and *V. mandarinia*. *Vespa crabro* builds a nest in a closed space (e.g., a tree hollow). Direct sunlight may increase the temperature in the nest. *Vespa simillima* builds a nest in the shade, such as at the edge of eaves. The afternoon sun seems to increase the temperature in the nests. *Vespa analis* builds a nest in shrubbery. This nest is in the shade all day. *Vespa mandarinia* builds a nest below the ground, and the temperature of this nest does not rise (Matsuura, 1995). The LT_{50} values, therefore, seem to reflect the temperatures of the environments in which these hornets build their nests.

The CO_2 concentration in hives may reach 6% (Buhler et al., 1983). Moreover, honeybees are sensitive to CO_2 and regulate the concentration of this gas in the hive by wing fanning. Interestingly, a reduced O_2 concentration initiates no action of honeybees to control the CO_2 concentration (Seeley, 1974; Dietlein, 1985; Nicolas and Sillans, 1989). Honeybee mortality due to high temperature correlates with high relative humidity (Free and Spencer-Booth, 1962). In this study, the relative humidity in expiratory air (approximately 53%) was so low that it could not kill honeybees. Therefore, the honeybees did not die within a bee ball at 46°C.

Apis cerana generates heat to kill *V. velutina* (Ken et al., 2005; Abrol, 2006) and *V. magnifica* (Abrol, 2006). Furthermore, *A. nuluensis* generates heat to kill *V. multimaculata* (Koeniger et al., 1996). However, the time involved in capturing and killing a hornet within a bee ball of these two honeybee species has not been determined. The time to lethality probably shortens as the lethal temperature increases (Schmidt-Nielsen, 1997). Further studies on the time required for heat-induced killing and the effects of exposure time on lethal temperature will increase our understanding of the causes of hornet mortality.

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