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Heat production by balling in the Japanese honeybee, *Apis cerana japonica* as a defensive behavior against the hornet, *Vespa simillima xanthoptera* (Hymenoptera: Vespidae)

M. Ono, I. Okada and M. Sasaki

Laboratory of Entomology, Faculty of Agriculture, Tamagawa University, Machida, Tokyo 194 (Japan), 24 February 1987

Summary. As an effective counterattack strategy against predacious hornets, especially *Vespa simillima xanthoptera,* workers of *Apis cerana japonica* showed a distinct balling reaction, usually involving 180–300 bees. This produced heat for as long as 20 min, giving rise to temperatures inside the ball higher than 46 °C, which is lethal to the hornet but not to the bees.
Key words. Heat production by balling; defensive behavior; *Apis cerana japonica;* the Japanese honeybee.

The hornet, *Vespa simillima xanthoptera* Cameron (Hymenoptera: Vespidae) is a major natural enemy of the Japanese honeybee, *Apis cerana japonica* Radoszkowski (Hymenoptera: Apidae). From July to October, the worker hornets appear in the vicinity of the hive (or nest in the natural habitat) entrance of the honeybee, and hunt the honeybees to feed to their larvae. However, the predators are sometimes captured by the honeybees and killed by engulfing or balling (fig. 1).

Thirty-six ballings were observed at hive entrances from August to September in 1984, 1985 and 1986 at Machida-shi, Tokyo, Japan. In 20 of them, the temperature inside the ball was monitored by using a thermometer (Model R116, Takara Kogyo Co. Ltd.) fitted with 3 micro-thermistors (O.D. 1 mm, Model TZL-64). The micro-thermistors were placed: 1) inside the ball; 2) in the center of the honeybee colony; and 3) beside the hive (recording the ambient temperature). An example of the results is shown in figure 2. As soon as the balling started, the temperature inside the ball increased rapidly and reached more than 46 °C within the first 4 min. After the temperature had been maintained for about 20 min, the temperature slowly fell until it was the same as that of the central part of colony (ca 34 °C). After that, the temperature of the ball rapidly dropped to the ambient level again. The average maximum internal ball temperature was 46.1 °C (range 45.2 °C to 47.0 °C) in the 20 balls observed.

The behavioral sequence was also observed. Within 15 s after the counterattack by the first guard bee, many others simultaneously rushed onto the captured hornet. As a result, the hornet was engulfed by some 250 worker honeybees (ca 180–300). The number of bees balling the hornet was usually constant for more than 30 min, and then gradually decreased. When the number of balling bees was reduced to about 10, the dead hornet was visible together with some bitten honeybees. Guard bees did not pay strong attention to the dead hornet, and some of them removed it by carrying it in their mandibles. The average duration of the whole sequence was 60.7 min (range 40.1 min to 108.5 min). We observed that all the hornets were killed without exception. No honeybee sting was found in the hornet corpses and the hornet wings were sometimes curled by the produced heat (fig. 1D). The honeybees in the ball did not sting even if it was put on the palm of the hand (fig. 3).

Although a similar balling reaction has also been observed in introduced European honeybees (*A. mellifera* L.), the workers readily use their stings against the hornet during balling. Because of their sting autotomy, 2 or 3 stings usually remain in the intersegmental membrane of the dead hornet. The average maximum temperature inside the ball was 42.8 °C, which is significantly lower than that of the native Japanese honeybee. This appears to be related to the less frequent use of the sting by the Japanese honeybee in defensive behavior than in the European honeybee[1,2].

To verify the above-mentioned observations, upper lethal temperatures for both the Japanese honeybee and the hornet were compared in the laboratory. Fifty honeybees were collected from the actual ball and the same number of hornets were collected from those visiting the apiary. Individuals were put in 100-ml flasks. The internal flask temperature was raised at a rate of 2.7 °C/min by immersion in a constant-temperature water bath. The results showed that the upper lethal temperature for the Japanese honeybee was 48–50 °C, but that for the hornet was 45–47 °C. This result indicates that the Japanese honeybee can kill the hornet at the temperature produced by balling, without stinging.

Evidence of defense by heat production in poikilothermal animals has not been found previously and it is very interesting from the ecological and evolutionary points of view in insect societies.

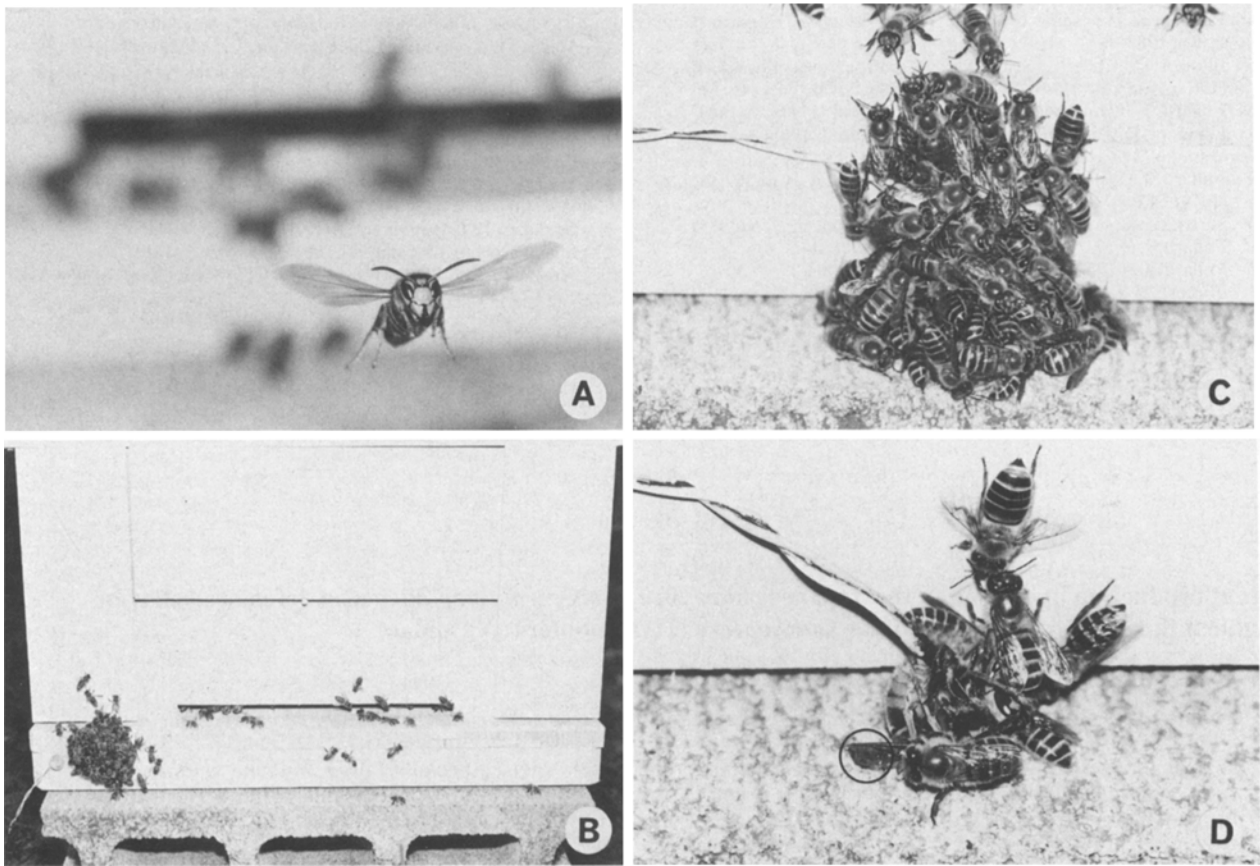

Figure 1. Behavioral sequence of balling in the Japanese honeybee *(Apis cerana japonica)*. *A* Hornet hunting in front of hive entrance, *B* Hornet captured by worker honeybees at hive entrance, *C* Ball of approximately 250 worker honeybees with internal temperature monitored by micro-thermistor, *D* Dead hornet with curled wings (indicated by circle).

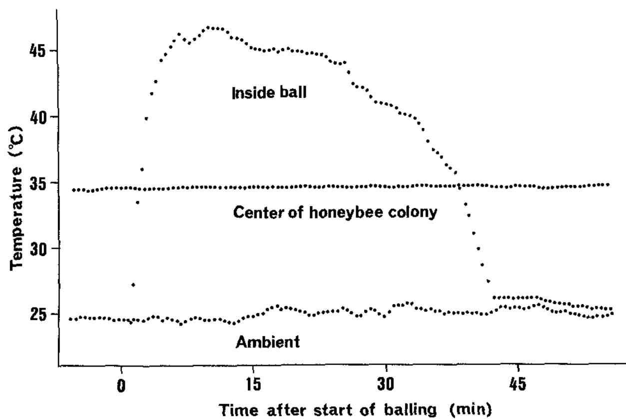

Figure 2. A typical example of the temperature change monitored from inside the ball of *Apis cerana japonica*.

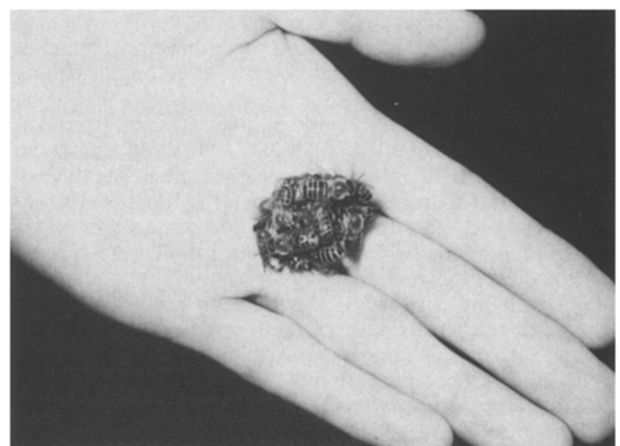

Figure 3. Ball on palm of hand; no stinging behavior was observed.

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0014-4754/87/091008-02$1.50 + 0.20/0
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Terminal acetylation in pheromone biosynthesis by *Mamestra brassicae* L. (Lepidoptera: Noctuidae)[1]

H. J. Bestmann, M. Herrig and A. B. Attygalle

Institut für Organische Chemie der Universität Erlangen-Nürnberg, Henkestr. 42, D-8520 Erlangen (Federal Republic of Germany), 2 March 1987

Summary. The alcohols found in the pheromone gland of *Mamestra brassicae* act as the immediate precursors of pheromone acetates. The esterase responsible for the terminal step in sex pheromone biosynthesis shows a low degree of substrate specificity. A number of saturated, unsaturated, primary and secondary alcohols applied to the gland were converted to their corresponding acetates.

Key words. Pheromone biosynthesis; *Mamestra brassicae;* nonspecific acetylation; solid sample injection technique.

A solid sample injection technique, in conjunction with capillary gas chromatography, has been employed recently in the analysis of excised pheromone glands of some species of Lepidoptera. Analyses by this method of glands that contain alkenyl acetates as sex attractants always resulted in the detection of corresponding alkenols as well, in small but significant quantities. For example, the glands of *Mamestra brassicae*[2] showed the presence of (*Z*)-11-hexadecenol, the alcohol corresponding to its major pheromone component (*Z*)-11-hexadecenyl acetate ((*Z*)-11-HDA). Similar results have been obtained with *Ostrinia nubilalis*[2], *Cryptophlebia leucotreta*[3] and *Spodoptera sunia*[4]. These pests utilize (*Z*)-11-tetradecenyl acetate, (*Z*)- and (*E*)-8-dodecenyl acetate, and (*Z*)-9-tetradecenyl acetate as the major components in their respective pheromone blends. The analysis by a closed-loop stripping system[5] of the volatiles emitted by the pheromone glands of *M. brassicae* did not result in the detection of (*Z*)-11-hexadecenol[6]. It should be noted that this alcohol is reported to be an inhibitor for this species[7]. Furthermore, a specific cell in the sensilla of male *M. brassicae* antennae responding to (*Z*)-11-hexadecenol has been found by single-cell-recording (electrosensillogram) experiments[8,9].

In a recent paper, Teal and Tumlinson[10] reported that *Heliothis* species use the corresponding alcohols as biosynthetic precursors of aldehydes. The alcohol was not emitted into the air by this species.

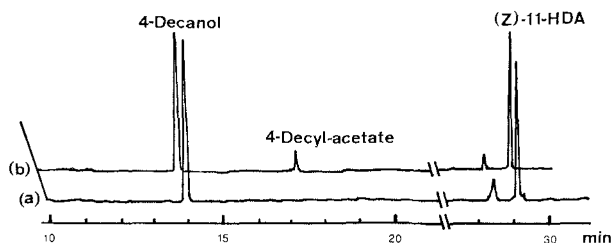
The present study was undertaken in order to verify whether the alcohols found in the sex pheromone gland of *M. brassicae* are simply artefacts of the analytical procedure or represent genuine glandular components that may act as biosynthetic precursors of the acetates.

M. brassicae females used in these studies were kept under a reversed (15:9 h) light:dark cycle at 25 °C. 2–3-day-old insects were anesthetized with carbon dioxide and the intersegmental membrane between the segments 8 and 9 was treated with 5 µl of an aqueous solution of (13,13,14,14,15,15,16,16,16-D_9)(*Z*)-11-hexadecenol (200 µg/ml) containing TWEEN 60 (= polyoxyethylene sorbitane monostearate) as detergent. After 10 min, the membrane between the 8th and 9th abdominal segments was excised, cleaned thoroughly from inner tissues, and sealed in a soda glass capillary[2]. Subsequent GC and GC-MS analyses revealed the presence of (D_9)(*Z*)-11-hexadecenyl acetate (GC: Packard United Technologies 438A, fused silica capillary column (FSCC) SP2340, 25 m × 0.22 mm; GC-MS: Finnigan 3200 E with Data System 6000, FSCC SE54, 25 m × 0.22 mm). The column, used for the GC analyses, was able to resolve the deutero acetate peaks from unlabeled ones; the labeled pheromone shows a sligthly shorter retention time than that of the unlabeled acetate. Even the glandular membranes excised prior to the application of the labeled alcohol could bring about the conversion of alcohol to acetate. From these results, it was deduced that the alcohol is the biogenetic precursor of the acetate. Furthermore, it can be assumed that the enzyme responsible for this reaction must be bound to the surface of the cuticle, since the penetration of the aqueous alcohol through the hydrophobic cuticle is unlikely within the short period of reaction time allowed. The fact that (*Z*)-11-hexadecenol produced in the gland is not released into the surrounding air also suggests that the precursor is transported or diffused from the interior to the cuticle for subsequent acetylation.

To determine the degree of substrate specificity of the enzyme involved in the acetylation, an unsaturated alcohol, (*Z*)-7-dodecenol, was applied topically to the gland as described before. After a 5-min reaction time the formation of (*Z*)-7-dodecenyl acetate, a compound not found in the gland of *Mamestra brassicae*, was observed. Decyl acetate was used as an internal standard to monitor the rate of formation of acetates. Several glands were cut into two equal parts and both parts treated with (*Z*)-7-dodecenol. After 2 min, one part of each gland was shock-frozen by cooling to −78 °C, and the corresponding second part of the gland was allowed to react for either 3, 5 or 10 min. The amount of (*Z*)-7-dodecenyl acetate formed in the 3-, 5- and 10-min samples, was determined by gas chromatography, and the relative reaction yields were calculated by comparing the values with those of corresponding shock-frozen samples in order to rule out the differences in biosynthetic activity between individual glands. A significant increase of the acetate content over time was observed.

Similar experiments were carried out with saturated primary and secondary alcohols, such as dodecan-1-ol, decan-2-ol, decan-4-ol, hexadecan-2-ol and benzyl alcohol. The generation of the corresponding acetates was observed in all cases except benzyl alcohol. The conversion even of secondary alcohols into their acetates is particularly remarkable. Clearly, the esterase responsible for the final step of pheromone biosynthesis in *M. brassicae* shows a low degree of substrate specificity.

In order to determine whether the acetylation is a genuine enzymatic reaction, a few glands (N = 5) were cut into two halves and one half of each gland was heated to denature the enzymes. After treating each glandular part with 4-decanol, a subsequent GC-MS analysis revealed that only the unheated halves were able to perform the acetylation. This confirmed that the reaction is enzymatic; no observable non-enzymatic trans-esterification was taking place (fig.).



Gas chromatograms of volatiles, after applying 4-decanol on (a) heat-treated, (b) intact glands. The glands were incubated for 5 min at 26°C and chromatographed, by a solid sample injection technique, on a 25 m × 0.22 mm fused silica capillary column coated with SE-54. The oven was programmed from 60 to 260 °C at a rate of 6 °C/min.

Finally, the bioacetylation of alcohols showed no dependence on the light:dark cycle to which the insects were subjected. Even the glands sampled during the 'non-calling' time were able to carry out the alcohol-acetate transformation.

We wish to thank the Deutsche Forschungsgemeinschaft for financial support and the Alexander von Humboldt Stiftung for a fellowship to A.B.A.

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0014-4754/87/091033-02$1.50 + 0.20/0
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Separation of canavanine and canaline by high performance liquid chromatography

J.N.A. van Balgooy

Department of Neurobiochemistry, Beckman Research Institute of the City of Hope, Duarte (California 91010, USA),
16 December 1986

Summary. The plant amino acid canavanine and its hydrolytic product canaline were successfully separated and identified by Reverse Phase High Performance Liquid Chromatograph (RP-HPLC). This procedure was used to demonstrate the arginase-mediated cleavage of canavanine to yield canaline and urea, and the subsequent formation of a Schiff's base complex between canaline and pyridoxal phosphate. Both aforementioned reactions were demonstrated by RP-HPLC.
Key words. Canavanine; canaline; alfalfa; fava; high performance liquid chromatography; arginase.

The pharmacological and physiological properties of the plant amino acid canavanine and its hydrolytic product canaline have been the subject of intense research[1-5], since both have toxic effects in a great range of organisms. Canavanine interferes with arginine metabolism[6], inhibits protein synthesis[7,8], blocks RNA and DNA synthesis[9] and affects the immune system[10]. While the toxic effect of canaline appears to be mainly the inhibition of pyridoxal phosphate-dependent enzyme reactions[11,12], these biochemical events are also of great interest nutritionally since canavanine is found in several edible legumes[13,14]. The isolation and identification of the aforementioned amino acids have usually been performed using a cation exchange resin, such as Dowex-50, followed by thin layer chromatography or electrophoresis[15]. Quantitation of canavanine has routinely been performed with a colorimetric method, utilizing pentacyanoammonium ferrate[16] or fluorometrically with phenanthrenequinone[17], while canaline has been estimated by the rather unspecific Jaffe reaction[18], after extensive sample clean-up or indirectly by conversion to 2-amino-4-ureidooxybutyric acid[19]. RP-HPLC offers a simple and direct solution to the detection, identification and quantitation of canavanine and canaline.

Materials and methods. L-Canavanine, L-canaline, L-arginine, amino acids for standards and tripotassium citrate were purchased from Sigma Chemical Co. (St. Louis, MO 63178 USA). Solvents for HPLC were obtained from Alltech Associates (Los Altos, CA 94022, USA).

Pre-o-phthaldialdehyde (OPT) derivatization followed by RP-HPLC has been used extensively for the detection and quantitation of α-amino acids[20,21]. The method employed in this investigation is basically that of Burbach et al.[21] with the following modifications: Potassium citrate (0.05 M) was substituted for 0.1 M sodium citrate and the gradient (mobile phase) scheme based on A: 90% 0.05 M potassium citrate-5% dioxane-5% iso-propanol (v/v) and B: 85% methanol. The gradient was changed to 100% A, 0% B (initial) then concave 10 min to 50% A-50% B followed by a 4-min isocratic run of 50% A-50% B, then concave 12 min to 15% A-85% B, followed by 2 min isocratic at 15% A-85% B. An Alltech Econosphere C-18 (250 × 4.6 mm) reverse phase column (Alltech Associates, Los Altos, Ca 94022, USA) in conjunction with a Waters HPLC system with dual pumps, a data module (Waters Associates, Milford, MA 01757, USA) and a Schoeffel Model 970FS (Schoeffel Instruments, Westwood, N.J. 07675, USA) were used for chromatography and analysis. Samples containing protein were deproteinized with 6% perchloric acid and neutralized with 5 M potassium carbonate.

Experimental design. Hydrolysis of canavanine was demonstrated by incubating 20 mM L-canavanine (Sigma No. C9758) in 10 mM Tris buffer, pH 9.3, with a dilute Mn^{++}-activated rat liver homogenate (particle free) for 0 (A), 15 (B) and 30 (C) min at 37°C (fig. 1). A pH of 9.3 was chosen, because it is the optimum pH for mammalian liver arginase. This pH also facilitates the detection of minor canavanine hydrolysis; for instance, at pH 9.3 it was found that homogenates of rat heart, brain, and intestine hydrolyzed easily detectable amounts of L-canavanine; these three homogenates had activities of respectively 1/70th, 1/25th, and 1/4th of that of liver. In contrast, at pH 7.2, the hydrolysis of L-canavaline was not discernible in the homogenates of rat heart, brain, and intestine.

When L-canavanine or L-canaline was added to fava bean homogenate approximately 98% was recovered by HPLC analysis. Addition of the same amino acids to pooled mouse serum (Sigma Chemical Co.) resulted in a recovery of 95% for canavanine and only 90% for canaline (mean of five assays).

Formation of a canaline pyridoxal phosphate complex is illustrated in figure 2. When a mixture of α-amino acids plus L-canavanine and L-canaline were preincubated with an aldehyde prior to OPT derivatization the quantity of canaline (peak 8) was reduced.

To satisfy the need for detection of canavanine and canaline in plant material, two different leguminous seeds, fava *(Vicia faba)* and alfalfa *(Medicago sativa)* were obtained from a