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COMPARATIVE STUDY OF THE COMPOSITION OF HORNET LARVAL SALIVA, ITS EFFECT ON BEHAVIOUR AND ROLE OF TROPHALLAXIS

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Abstract—1. The physiological and hydrolyzed amino acid compositions and carbohydrate contents of hornet larval saliva were analyzed in five species: *Vespa mandarinia*, *V. crabro*, *V. tropica*, *V. analis* and *V. xanthoptera*.

2. D-Glucose or trehalose was found in the larval saliva from five hornet species, but trehalose was not detected in *V. tropica*. D-Glucose contents (1.0–5.6 mg/ml) were higher than those of trehalose (1.2–9.0 mg/ml) except *V. xanthoptera*. The sum of D-glucose and trehalose was consistently 11–12% of the total carbohydrate.

3. The physiological amino acid compositions showed a common pattern among all five hornet species: the amounts of cystine and cysteine were very low or non-detectable; there were low contents of glutamic acid (0.5–3.0 mol %) and aspartic acid (0.10–0.15 mol %), against the contrarily high contents of proline, threonine and glycine; the molar ratios of essential amino acids (30–55 mol %) were similar.

4. The contents of the major physiological amino acids in fresh saliva fluctuated slightly among the species. Proline was abundant in *V. analis* (28.5 mol %), *V. xanthoptera* (21.7 mol %) and *V. mandarinia* (17.1 mol %), while threonine was dominant in *V. crabro* (10.4 mol %) and *V. tropica* (13.0 mol %). High amounts of glycine were found in *V. mandarinia* (18.2 mol %), *V. crabro* (12.1 mol %) and *V. tropica* (17.3 mol %).

5. The physiological amino acids accounted for over 76% of the total amount of hydrolyzed amino acids in larval saliva except in *V. mandarinia* (36%).

6. In experiments on the aggressive behaviour of *V. analis* and *V. xanthoptera*, the feeding of salival amino acid mixtures brought on a calmness in the worker hornets.

7. It is strongly suggested that the larval saliva is indispensable to hornet life, and also ecological or instinctive behaviour, especially the foundation of the social family, by virtue of the fact that the hornet has no stock foods.

INTRODUCTION

Hornets are social insects which populate big colonies nesting in large combs (Iwata, 1981). This giant insect is an exterminator of the insect food chain and preys on many kinds of insects to feed their meat-eating larva (Matsuura, 1973). Hornets generally eat fresh prey, and so do not commonly stock foods in their nests. Food acquisition is frequently performed by solo hunting trips to random food sources. Pellets made by the workers from insect prey are given to their larvae in exchange for larval saliva. Adult hornets eat only liquid foods; they can not eat solid foods due to the structural peculiarities of a short gut and very narrow esophagus through the coarctate waist. This trophallaxis between worker and larva is widely observed in many social wasps (Morimoto, 1960) as well as in bees, ants and termites (Wilson, 1971), but remains poorly characterized (Mashwitz, 1966). Whereas food-gathering habits are a source of reinforcement for many of the different instincts of social insects, the hornet colony lacks castes or divisions of labor (Matsuura, 1973), both of which are common in other social insects, together with ways of communication such as the honeybee dance (von Frich, 1967). The question arises as to why the hornet builds homes and forms a social family despite no social constraints on his life. The role of trophallaxis in hornet social structure has not been analyzed. From these points of view, we have studied the composition of larval saliva, which is at the center of trophallaxis, and effects of its composition on behaviour, a subject not previously analyzed in detail.

MATERIALS AND METHODS

Preparation of larval saliva

During ecological behavior observations, the nests of five hornet species, *V. mandarinia*, *V. crabro*, *V. analis*, *V. tropica* and *V. xanthoptera*, were collected, together with larvae, in the forests of central Japan. A few tens, to hundreds, of microliters of clear saliva secreted from the larval mouth at one time were obtained in capillary pipettes. The salival samples from each nest were combined and frozen quickly, then stored at −80°C until use.

Carbohydrate analysis of larval saliva

The total sugar content was determined by the phenol sulfuric acid colorimetric method (Koch *et al.*, 1956). The analysis of individual sugars was carried out by HPLC at 20°C on a Silica NH_2 column (diameter 4.6 × 250 mm) with elution by 80% acetonitrile at 60 kg/cm² and a flow rate of

1 ml/min. Peaks were detected using an RI detector. D-Rhamnose (19 min), D-xylose (21 min), D-fructose (26 min), D-glucose (31 min), D-galactose (36 min), maltose (60 min) and trehalose (64 min) were analyzed as standard sugars.

Amino acid analysis of larval saliva

Twenty microliters of the larval saliva were mixed with 1 ml of 0.2 N Li–citrate buffer, pH 2.2, and the physiological amino acids were detected by our previous method using *o*-phthalaldehyde and ninhydrin (Abe *et al.*, 1989). The same amount of saliva was hydrolyzed in 5.8 N HCl at 115°C for 24 hr, and the composition was analyzed by the same procedure as for physiological amino acid detection. The standard amino acids used for analysis were as follows: taurine (Tau), phosphoethanolamine (P-EtAm), aspartic acid (Asp), threonine (Thr), serine (Ser), asparagine (Asn), glutamic acid (Glu), glutamine (Gln), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), cystine (Cys), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), β-alanine (β-Ala), γ-aminobutyric acid (GABA), ethanolamine (EtAm), ammonia (NH_3), ornithine (Orn), lysine (Lys), tryptophan (Trp), histidine (His), 1-methylhistidine (1-MeHis), 3-methylhistidine (3-MeHis) and arginine (Arg).

Assay of aggressiveness

Salival amino acid mixtures containing 0.3% glucose and 0.9% trehalose, the composition depending on the species, and 40% sucrose solution were prepared as food for hornets. Ten *V. analis* workers and ten *V. xanthoptera* workers, collected in the field by CO_2 anesthetization, were reared with each liquid food *ad libitum* in a natural light–dark cycle for 5 days at 20°C. In the case of *V. analis*, hornets from different nests were mixed in a small cage (9 × 12 × 18 cm) and observed for aggressive behaviour, such as furious biting, trembling of the alae and attacking by mandible. By a similar method, the aggressive behaviour between *V. analis* and *V. xanthoptera* was also observed.

RESULTS

Contents of carbohydrates

Saliva was simply secreted from the larval salivary gland by stimulation of the mandible, labrum or head. The amount of saliva depended on both nutritional conditions and sizes of the larva. Workers, especially newborn adults, had a taste for the saliva, and sometimes transferred from mouth to mouth. In the saliva, D-rhamnose, D-xylose, D-fructose, D-galactose and maltose were not found in any species. D-Glucose or trehalose, which is mainly used as an energy source for flights (Beenakkers *et al.*, 1985), was found in the larval saliva from five species, as shown in Table 1. The sum of D-glucose and trehalose was consistently 11–12% of the total carbohydrate. Compared to other species, the low content of both sugars in *V. mandarinia* might reflect their behavioral willingness to swarm on sap, and the fact that they inhabit oak (*Quercus acutissima*) forests where sap is available as a nutritional supplement (Abe, 1985). The comparatively low sugar content in *V. tropica* saliva may arise from the fact that this species collects honey of the sorrel vine (*Cayratia japonica*) or the sap of oak tree (Matsuura, 1973).

Composition of physiological amino acids in larval saliva

The physiological amino acids, when taken orally, are good and effective nutrients for adult hornets, since hornets are liquid-food consumers with a simplified gut and an open capillary circulation system. The physiological amino acids in the larval saliva of *V. mandarinia*, *V. crabro*, *V. tropica*, *V. analis* and *V. xanthoptera* ranged between 24 and 97 μmol/ml (Table 2) and accounted for over 76% of the total amino acids except in *V. mandarinia* (36%) (Table 1). The composition in the five species showed a common pattern (Fig. 1). Significant features of the compositions included the very low content of aspartic and glutamic acids, with a contrastingly high content of proline, glycine and threonine. Other minor amino acids detected were taurine, phosphoethanolamine, asparagine, cystine, methionine, β-alanine, GABA, ethanolamine, ornithine, 1-methylhistidine and 3-methylhistidine.

The low content of aspartic acid (0.10–0.15 mol %) and glutamic acid (0.5–3.0 mol %), both transmitters

Table 1. Sugar and amino acid contents of hornet larval saliva

Species	Total carbohydrate (mg/ml)	D-Glucose (mg/ml)	Trehalose (mg/ml)	% of D-Glu and Tre†
V. mandarinia	19.3	1.00	1.18	11.3
V. crabro	41.2	4.00	1.18	12.6
V. tropica	35.5	3.75	N.D.*	10.6
V. analis	69.8	5.63	2.35	11.5
V. xanthoptera	98.5	3.08	9.02	12.3

Total amino acids (mg/ml)‡	Physiological amino acids (mg/ml)	Physiological amino acids (%)§	Proline (mg/ml)	Proline (%)‖	ω-amino acids¶ (μmol/ml)	Body weight** of worker (mg)
25.44	9.58	37.7	1.56	16.3	15.17	1365 ± 285
12.58	11.73	93.2	0.68	5.8	12.98	931 ± 365
6.44	6.99	108.5	0.33	4.7	10.18	1325 ± 187
7.00	6.08	86.9	1.69	27.8	5.96	600 ± 80
3.29	2.76	83.9	0.58	21.0	3.08	355 ± 53

*Not detectable.
†% in total carbohydrate.
‡Calculated from acid hydrolysate of the saliva.
§Physiological amino acids divided by total amino acids.
‖% of physiological amino acids.
¶Totals of Tau, Gly, β-Ala and GABA.
**Averages of 403 workers for *V. mandarinia*, 301 for *V. crabro*, 84 for *V. tropica*, 109 for *V. analis* and 296 for *V. xanthoptera* were calculated as to mean ± SD.

in the excitatory neuromuscular junction of insects (Usherwood *et al.*, 1968), and the high content of ω-amino acids (11.6–19.2 mol %) such as taurine, glycine, β-alanine and GABA, transmitters of the inhibitory nerve (Curtis *et al.*, 1965), may produce calm behaviour through the suppression of the excitatory nerves and the activation of inhibitory nerves. Interestingly, the content of ω-amino acids in the saliva of different species is inversely related to their combative nature on hunting or sap collecting (Table 1). In fact, the feeding order on the sap of oak trees is observed as follows: *V. mandarinia*, *V. crabro*, *V. analis*, *V. tropica* and *V. xanthoptera* are orderly dominant (Matsuura, 1969). *V. mandarinia* is really aggressive since he preys often on other *Vespa* species in late autumn. *V. crabro* preys often on Cicadae which are over 10 times larger than the hunter (Matsuura, 1973). *V. tropica* also attacks the nests of wasps to feed the larvae (Sakagami *et al.*, 1957). Thus, the aggressive behaviour of the hunter will be reduced in the nest where trophallaxis is peformed. Calmness is very important to an ordered social life under high hornet density conditions inside the nest, especially for the longevity of the founding female (our observation, in preparation). The low concentration of ω-amino acids in *V. xanthoptera* (3.1 μmol/ml) compared with the other hornet species (15.2 μmol/ml for *V. mandarinia*, 13.8 for *V. crabro*, 10.2 for *V. tropica*, 6.0 for *V. analis*) probably causes the tumultuous wandering nature on the envelope of the nest, even at night, as is found in combative nests. Finally, the life-span of a founding female could be reduced to less than 1 year by accidents relating to aggression; this rate was 45.9% for *V. xanthoptera*, 27.0% for *V. analis* and 22.3% for *V. mandarinia* (our observation, in preparation).

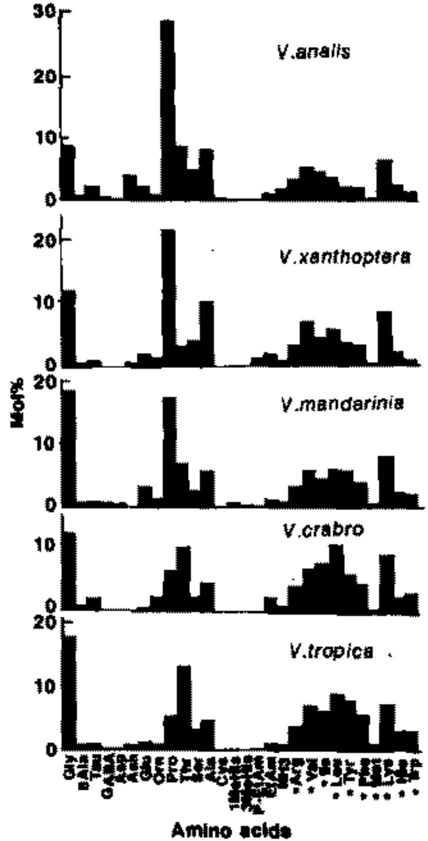

Fig. 1. Molar ratio of amino acids contained in hornet larval saliva. Twenty-eight amino acids as shown in the figure were determined in the larval saliva of five species. * Is essential amino acids.

Proline is metabolized by insects to produce energy for flight (Beenakkers *et al.*, 1985). It is interesting to note that the content of salival proline was very high in *V. analis* (28.5 mol %) and *V. xanthoptera* (21.7 mol %), both of which built mid-air nests consisting of closed envelopes on trees or structures in open spaces, and whose newborn adults were usually able to fly within one day of emergence. Those species with proline- and/or sugar-rich saliva, *V. analis*, *V. xanthoptera* and *V. mandarinia* (Table 1), had a wider hunting area than the other species. A high proline content might therefore be advantageous for the flight behaviour of mid-air-dwelling species. On the other hand, the salival proline content of *V. mandarinia* (17.1 mol %) was similar to that of glycine (18.2 mol %), while the proline contents in *V. tropica* (5.1 mol %) and *V. crabro* (6.4 mol %) were much lower than those of glycine (17.3 and 13.0 mol %, respectively) and threonine (12.1 and 10.4 mol %, respectively). These species generally nested in tree caves or in structures on a surface or underground. Their newborn adults, for a couple of days after emergence, had soft and incomplete alae, and were unable to fly. Also of interest is the correspondence between proline content and flight capacity, that is, *V. analis*, *V. xanthoptera* and *V. mandarinia* had a wider hunting range than *V. crabro* and *V. tropica*.

Fair amounts of isoleucine (4.3–7.5 mol %) and leucine (3.1–10.5 mol %), which are metabolized in muscle for energy sources, were found in the saliva (Fig. 1, Table 2). Tryptophan (1.2–3.4 mol %), a precursor of serotonin found in venom (Harbermann, 1968), and histidine (2.5–3.2 mol %), which is decarboxylated to histamine, another major component of venom (Ishay *et al.*, 1974), were also detected. The composition of essential amino acids in the saliva (29.4–55.0 mol %) showed similar patterns among all five species (Fig. 1). These amino acids are probably needed for the synthesis of venom, since peptides and proteins are the main venom components, such as physiological amino acids (Abe *et al.*, 1989), serotonin (Harbermann, 1968), histamine (Ishay *et al.*, 1974), vespakinine (Yashuhara *et al.*, 1977), mastoparan (Hirai *et al.*, 1978), mandaratoxin (Abe *et al.*, 1982) and phospholipase A_2 (Takasaki *et al.*, 1989).

Total amino acid composition of larval saliva

The amino acid content hydrolyzed larval saliva was extremely high in *V. mandarinia* (221.5 μmol/ml), one-half as high in *V. crabro* (121.1 μmoles/ml), one-fourth in *V. analis* and *V. tropica* (68.8 and 58.8 μmol/ml, respectively), and one-eighth in *V. xanthoptera* (31.8 μmol/ml) (Table 3). Glutamic acid content in the hydrolyzed saliva was higher than in fresh saliva as shown in Tables 2 and 3. This suggests the presence of peptides or proteins containing glutamic acid. However, both the physiological and hydrolyzed amino acid compositions in *V. tropica* were very similar when the decomposition of threonine, serine and tryptophan, during acid hydrolysis, was considered (Tables 2 and 3). This indicates that the saliva consists mainly of amino acids, not proteins or peptides. This may be helpful for digestion and absorption by the simplified alimentary tract of hornets. Conversely, the hydrolysate of *V. mandarinia* saliva was found to contain large amounts of aspartic acid and glutamic acid despite very low concentrations of these amino acids in fresh saliva (Table 2). Glycine, valine, isoleucine, leucine, lysine, histidine and arginine levels were also increased in the hydrolysate. In fact, the sum of the amino acids in the hydrolyzed saliva was about 3 times higher than in fresh saliva (Table 1). This suggests that the saliva of *V. mandarinia* is rich in peptides or proteins compared with other species. The analytical data also demonstrate that this saliva is protein-rich; the ratio total amino acids to total carbohydrates is 1.32 for *V. mandarinia*, 0.31 for *V. crabro*, 0.18 for *V. tropica*, 0.10 for *V. analis* and 0.033 for *V. xanthoptera*. Interestingly, the increase in these ratios was correlated to body weight (Table 1) and the order of ecological dominance. Trophallaxis is preserved by a mechanism of self-reinforcement backed by the eating of fresh foods high in amino acids and proteins.

Effects of salival amino acid mixtures on hornet aggressiveness

It is a general instinct of hornets that individuals from different colonies fight with one another. It was often observed in the field that *V. mandarinia* workers fight violently at the hunting place until one of them dies. When *V. analis* workers collected from different nests were combined and raised together, the hornets showed intimidating behaviour such as furious biting of the mandible, trembling of the alae and attack by mandible. When workers from different nests were reared on a sugar solution or on a salival amino acid mixture containing 0.3% glucose and 0.9% trehalose for 5 days and then placed together in a cage, however, they did not show any intimidating behaviour. When *V. analis* workers reared on the sugar solution were brought together with *V. xanthoptera* workers also reared on the sugar solution or on salival amino acids, the *V. analis* workers exhibited the furious biting, trembling of alae, and attack by mandible against the *V. xanthoptera* workers. On the other hand, *V. analis* workers reared on the salival mixture did not show any intimidating behaviour against *V. xanthoptera* workers reared on either food. This finding suggests that the salival amino acid mixture affects the calmness of workers. This would be caused by ω-amino acids or a deficiency of glutamic acid and aspartic acid in the saliva.

DISCUSSION

All social insects have manners indispensable to the maintenance of the social life, such as caste, homing instinct, queenly control and communication. It is well-known that the social organization of ants and termites involves differentiated caste systems (Wilson, 1971). Honeybees can communicate with each other as to the direction and distance of food sources (von Frich, 1967). However, it is not clear whether hornets communicate information about food sources to other members of the colony (our observation). Moreover, the hornet social organization does not involve a well-developed caste system or social communication despite their large colonies (Matsuura, 1973). If the founding female dies by accident, the colony is kept together by a few workers as alternative females (our observation). The social roles of the founding female are therefore not performed as rigidly as in other social insects. On the other hand, the meat-eating hornets occupy a final position in the insect food chain, so that the strongest individuals are free from the hard struggle for existence and restraint by others. Nevertheless, hornets do exist in social units, and how social binding of the individuals in a colony arises is not fully known. In our study, it was suggested that trophallaxis, based on larval saliva, controls hornet social life because a deficiency of saliva or a lack of trophallaxis brings on the death of individuals and decomposition of the colony. Saliva must thus contain essential nutrients for hornet life and trophallaxis. The question arises, therefore, as to what compounds in the saliva have those functions. There are many interesting nutritional aspects of saliva that are completely different from other insect fluids (Woodring, 1985). For example, the high content of proline influences flight behaviour; i.e. hornets whose saliva has a high proline content nest in mid-air and have a wide hunting range, while those whose saliva is low in proline live underground and hunt over a narrow area. While these salival amino acid mixtures produce a low blood lactate level in the reinforced exercising mouse (Abe *et al.*, 1989, 1990), they might prevent fatigue during flight. The essential amino acids in saliva would be used for venom synthesis. ω-Amino acids may produce calmness inside the nest. The amount of amino acids in the saliva of each species correlates with body size and weight, and also with the position of a species in the ecological order, which, from high to low, is as follows: *V. mandarinia*, *V. crabro*, *V. tropica*, *V. analis* and *V. xanthoptera* (Matsuura, 1973). These findings suggest that the mechanism of trophallaxis may have developed in social wasps more favorably than in other social or non-social insects. The evolutionary development of aculeate wasps may have resulted from the fact that the stronger species consume the better food, which makes a strongly-built body. All of the findings also suggest that the amino acids in the saliva are totally functional foods which control hornet behaviours or instincts. The most important role of saliva is to act as a water supply for workers. As stocked honey provides water to honeybee workers, the larvae may be indispensable as water suppliers in the severely hot and dry environments prevailing from late July to early September, or the expansion of habitat. The larvae also play the indispensable role as stock foods similar to honey and pollen in honeybee. This nutritionally complete food might be an attractant for the worker; in other words, hornets may hunt in order to get saliva and the nest may simply represent another food source apart from the hunting place. The evolutionary acquirement of trophallaxis has enabled hornets to maintain a social colony in the absence of a founding female. This is undoubtedly important for species conservation. However, because of this acquirement, hornets may have lost the instinct for swarming or removal, except to very close sites in cases of *V. xanthoptera* and *V. crabro*.

The range of amino acid concentrations in hornet larval saliva (24–98 μmol/ml) is similar to that in other insect larvae and adult plasma (18–111 μmol/ml), such as Odonata, Orthoptera, Hemiptera, Coleoptera, Lepidoptera, Diptera and Hymenoptera (Woodring, 1985). However, the compositions of glutamic and aspartic acids are rich in the plasma, but contrastively poor in saliva. Glucose and trehalose are the major sugars in the saliva. The concentration of salival glucose (1.0–5.6 mg/ml) is similar to that in the plasma of the above insects, except *Apis* in Hymenoptera (20 mg/ml) (Woodring, 1985). The amounts of salival trehalose (0–9.0 mg/ml) are a little higher than in the plasma. The ratio of trehalose to glucose in the saliva is greater than 1 in many hornets, the same as in the plasma of other insects (Woodring, 1985). The concentrations of these compounds in both saliva and plasma are similar. This isotonic property of saliva would be advantageous for intestinal absorption. The analytical data suggest that the saliva in trophallaxis is physically essential for hornet life and also plays a crucial role in the control of ecological and instinctive behaviours.

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