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Technical article

Development of a chemically defined diet for ants

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Abstract. A chemically defined diet is a useful tool for the study of nutritional physiology of organisms. We have developed such a diet for *Camponotus* carpenter ants to facilitate experiments on nutritional requirements of these ants. Worker colonies of *Camponotus floridanus* were fed with a chemically defined diet, containing all essential minerals, amino acids, vitamins, growth factors and sucrose in an agar matrix. After 13 weeks, neither the number of raised pupae, their dry weight, nor the mortality of workers in subcolonies fed with this diet differed significantly from control colonies fed with Bhatkar-Whitcomb-agar, in addition to cockroaches and diluted honey. Therefore, this diet is adequate for a normal brood production and a maximal growth rate of *C. floridanus* larvae, at least for a period of three months. This diet should be suitable for ants that are able to feed on agar-based food resources in general.

Keywords: Artificial diet, holidic diet, *Camponotus floridanus*, nutritional physiology.

Introduction

In recent years endosymbiotic bacteria as well as the gut microflora of insects have attracted more attention, especially in the light of nutritional upgrading by the microorganisms that enable the insect hosts to sustain on diets which lack nutrients that are essential for the hosts (Baumann, 2005). Nutritional interactions between hosts and their endosymbionts or gut microflora can be studied with different techniques. The most common approach is to generate aposymbiotic hosts by antibiotic treatment and then compare fitness between untreated host individuals and treated hosts. However, a major drawback in this technique is that most antibiotics have side effects that are hard to control, and thus the antibiotics themselves may lead to reduction in host fitness, e.g., damage to the intestine (Sauer, 2000) or the ultrastructure of mitochondria (Griffiths and Beck, 1974), or depression of the host protein synthesis (Douglas, 1988). An alternative or accompanying method is to provide the host with a chemically defined diet that allows omission of specific nutrients. Such artificial diets have been used successfully in other studies on the function of obligate insect endosymbionts like *Buchnera* in aphids (see Douglas, 1998, for review). Ants of the genus *Camponotus*, and related genera like *Polyrhachis* and *Echinopla*, comprise approximately 15% of all described ants species (Bolton, 1995) and every species from these three genera examined to date harbours the obligate intracellular endosymbiont *Blochmannia* (Sameshima et al., 1999; Sauer et al., 2000; Degnan et al., 2004; Feldhaar, unpubl. result for *Echinopla*). This gamma-proteobacterium resides in specialized bacteriocytes that are intercalated between the midgut cells (Blochmann, 1887). The genome sequences of two *Blochmannia* strains are known today, and the endosymbiont supposedly has a nutritional function (Gil et al., 2003; Degnan et al., 2005). However, experimental evidence is still lacking, and a chemically defined diet that can sustain the ant is therefore an important first step for such experiments. More information about nutritional demands of ants in general may also be gained from nutrient deletion experiments using artificial diets, as has been demonstrated with aphids, locusts and other insects (Dadd, 1985).

All artificial diets described so far for ants contained at least one chemically undefined raw natural material of animal origin (Smith, 1944; Bhatkar and Whitcomb, 1971; Keller et al., 1989; Vogt, 2003). From these diets, only limited information about the nutritive demands of ants can be obtained, since the undefined material may

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contain additional unknown nutrients. One very popular artificial diet often used in ant breeding consists of agar, whole egg, honey and vitamin-mineral capsules (Bhatkar and Whitcomb, 1971). Some studies demonstrated that this diet alone is not sufficient to provide maximal breeding success. When kept on Bhatkar-Whitcomb diet alone, several ant species cannibalised more larvae (Buschinger and Pfeifer, 1988) or raised fewer pupae than did colonies supplied with dead insects and sugar water or diluted honey (Porter, 1989; Alloway et al., 1991).

Most carpenter ant species have been traditionally reported to be omnivorous, utilizing honeydew from extrafloral nectaries or phloem-feeding insects, dead arthropods, exuvia, dying conspecifics or injured eggs, larvae and workers, vertebrate urine and possibly also fungal mycelia (Cannon, 1998; Davidson et al., 2004). Two recent comparative studies have used stable isotopes of carbon and nitrogen to assess the trophic levels at which ants feed. Both studies revealed that the genera *Camponotus* and *Polyrachis* have only slightly elevated levels of $\delta^{15}N$ in comparison to plants, pointing towards an important role of plant-derived resources in the nutrition of both species (Blüthgen et al., 2003; Davidson et al., 2003).

To investigate the exact nutritional demands of ants, a completely synthetic artificial diet (also called holidic diet) would provide the advantage that the chemical composition and concentration of every single component is exactly known. An establishment of such a diet for carpenter ants is the aim of this study. Since ants show the highest metabolic rate during larval development and during the metamorphosis to the imago (Wheeler and Buck, 1992), the suitability of an artificial diet for ants can easily be assessed by the number and weight of pupae raised by ant workers after a substantial time of feeding, in comparison with control groups fed with standard diets.

Table 1. Nutrient composition of the holidic artificial diet. All amounts are based on a final volume of 100 ml.

0.12 mg	$CuCl$		
0.92 mg	$FeCl_3$		
0.22 mg	$MnCl_2$		
1.00 mg	$NaCl$		
0.40 mg	$ZnCl_2$		
Amino acids:		**Salts:**	
100 mg	Alanine	250 mg	KH_2PO_4
100 mg	Arginine	242 mg	$MgSO_4$
100 mg	Asparagine		
100 mg	Aspartic Acid	**Vitamins:**	
100 mg	Cysteine	10 mg	p-Aminobenzoic Acid
100 mg	Glutamic Acid	100 mg	Ascorbic Acid
100 mg	Glutamine	0.1 mg	Biotin
100 mg	Glycine	5 mg	Calcium Pantothenate
100 mg	Histidine	2.5 mg	Cholesteryl Stearate
100 mg	Isoleucine	1 mg	Folic Acid
100 mg	Leucine	10 mg	Nicotinic Acid
100 mg	Lysine	2.5 mg	Pyridoxin Hydrochloride
100 mg	Methionine	5 mg	Riboflavin
100 mg	Phenylalanine	2.5 mg	Thiamine Hydrochloride
100 mg	Proline	25 mg	beta-Carotene
100 mg	Serine		
100 mg	Threonine	**Growth factors:**	
100 mg	Tryptophane	50 mg	meso-Inositol
100 mg	Tyrosine	50 mg	Cholin Chloride
100 mg	Valine	**Carbohydrates:**	
100 mg	gamma-Amino Butyric Acid	1 mg	Agar (Kobe I)
		20 mg	Sucrose

Materials and methods

Design of the holidic artificial diet: WHY

A holidic diet was designed by modification of several artificial diets for aphids (Dadd, 1967; Dadd and Krieger, 1968; Ehrhardt, 1968a,b; Akey and Beck, 1971, 1972): Trace elements, salts, amino acids, vitamins, growth factors (choline and inositol), cholesterol and sucrose (see Table 1) were mixed with double distilled water and agar and then frozen.

Potassium dihydrogene phosphate was included as a dietary source of phosphorus, since its growth enhancing effects on larval development have been demonstrated in a number of insect genera (McFarlane, 1991; Perkins et al., 2004).

Insects, as well as all other organisms, require a continuous supply of trace minerals which are involved as structural components or enzyme co-factors in many cellular reactions (for review see Cohen, 2004). Iron, calcium, zinc, manganese and copper were added to the diet, since aphids fed with an artificial diet lacking these trace metals could not reproduce more than 2–3 generations (Dadd, 1967; Ehrhardt, 1968a; Akey and Beck, 1972). The trace metals were provided in the form of their chloride cations and not as metal chelates, since more than 46 continuous generations of aphids have been reared with diets containing chloride salts, but no more than three generations could be reared with diets where the cations were chelated with EDTA and other agents (Akey and Beck, 1971, 1972).

Larval development of insects also requires several vitamins of the B group (thiamin, riboflavin, nicotinic acid, folic acid, pantothenic acid and pyridoxin). However, some of these are required at extremely low concentrations and can be transmitted with the germline to the next generation, so many subsequent generations on a vitamin deficient diet are necessary to find evidence for their essentiality (Dadd, 1985). P-amino benzoic acid, which is an essential nutrient for some bacteria and is sometimes called vitamin Bx, has been often included in insect artificial diets (Dadd, 1960 for locusts; Akey and Beck, 1971 for pea aphids). However, it remains unknown whether it is required by the host insect itself, or possibly by bacteria like endosymbionts or specific gut microflora. P-amino benzoic acid is required by bacteria for the synthesis of folic acid (Madigan et al., 2001).

Ascorbic acid was included since it is essential for development and ecdysis in many herbivorous insect taxa (Dadd, 1985). Also beta carotene was mixed into the diet, since it is required by all insects studied so far for normal eye development, pigmentation and light orientated behaviour (Reinecke, 1985; Nation, 2002).

Table 2. Breeding success in number of pupae raised per subcolony, pupal weight and worker mortality of the worker subcolonies of *Camponotus floridanus* after 13 weeks of feeding with a holidic artificial diet or the control diet (Bhatkar-Whitcomb diet, diluted honey and cockroaches), respectively. The mean and the standard deviation per treatment are shown. The dry weight of the raised pupae [mg] is averaged over five pupae per subcolony.

		Colony							mean	SD
		#1	#2	#3	#4	#5	#6	#7		
Number of raised pupae	holidic diet	60	53	81	51	66	97	70	68	16
	control diet	43	29	53	66	15	99	91	57	31
Dry weight [mg] of raised pupae	holidic diet	2.484	2.199	2.332	2.299	2.293	1.561	1.916	2.155	0.314
	control diet	2.030	2.268	2.694	2.064	1.898	1.554	2.024	2.076	0.348
Number of dead workers	holidic diet	127	97	89	64	81	112	31	86	32
	control diet	78	115	63	55	81	48	52	70	23

Steroids have to be included in an artificial diet for ants, since insects cannot synthesise steroids but require them in larval development. The steroid of choice was cholesterol since it achieved the highest breeding success in honeybees, when various steroids were tested (Herbert et al., 1980).

Cholin and inositol are two essential components of membrane phospholipids which cannot be synthesised by insects; furthermore, inositol may act as a feeding stimulant in ants, since inositol solutions were 10% more attractive to *Solenopsis* workers than was the water control (Vander Meer et al., 1995). Sucrose, the most attractive feeding stimulant, preferred by most tested ant species over other sugars (Vander Meer et al., 1995; Cannon, 1998; Barbani, 2003; Blüthgen and Fiedler, 2004), was used in the concentration of 20% (w/v) which has been reported to evoke the highest consumption rate in *Camponotus pennsylvanicus* (Cannon, 1998).

The nutrients were incorporated into an agar gel which keeps the nutrients dissolved, prevents the food from desiccation and allows the workers to stand on the food without drowning.

Preparation of the holidic artificial diet: HOW

The trace metals, amino acids, and salts (Table 1) were dissolved in 50 ml distilled water. The water soluble vitamins, growth factors and cholesteryl stearate were dissolved in 10 ml double distilled water, and united with the first volume, and the pH of the solution was adjusted with potassium hydroxide (KOH) to 7.5. The beta-carotene was then suspended in the solution by vigorous mixing. Meanwhile, the sucrose and Agar-Agar (Kobe I, Applichem) were dissolved in 40 ml distilled water and heated until the solution reached the boiling point. To prevent any heat degradation of vitamins (Cohen, 2004), this carbohydrate solution was first chilled down to 40 °C and then united with the other mixture. After vigorous mixing, the solution was poured into Petri dishes. After polymerisation of the agar matrix, the gel was stored at −20 °C.

Preparation of the control diet

The modified Bhatkar-Whitcomb diet was prepared as follows: 250 ml tap water were boiled with 5 g Agar-Agar (Kobe I, Applichem). Another volume of 250 ml was stirred with two raw eggs and 250 g honey and mixed with the agar solution after cooling. The mixture was poured into a flat dish and stored at 4 °C (modified after Bhatkar and Whitcomb, 1971). This agar-based diet was always supplemented by freshly killed cockroaches (*Nauphoeta cinerea*) and 50% honeywater (w/v) *ad libitum*.

Ants

Founding queens of *C. floridanus* were collected in Tarpon Springs, western Florida, by A. Endler in 2003 and kept in a climatic chamber with a constant temperature of 25 °C, and a regular 12 h day-night regime, until they raised their own colonies with 1000–10000 workers. These mother colonies were fed twice a week with the control diet described above.

Feeding experiments

From each mother colony (N = 7), two separate worker subcolonies were prepared. One was fed with the holidic artificial diet for 13 weeks, and the other with the control diet. All seven pairs of experimental and control subcolonies comprised 150 minor workers and 30 first-instar larvae. Twenty queen eggs were added to prevent egg-laying by the workers themselves (Endler et al., 2004). Every third week, new first-instar larvae and queen eggs were added to the subcolonies. Thus, until the 13th week, every subcolony had received 80 eggs and 120 larvae altogether.

The subcolonies were fed twice a week. Every subcolony received a thawed 0.5 cm^3 cube of the artificial diet, or the control diet, respectively. The remains of the last food were removed. The ant subcolonies were provided with tap water *ad libitum*.

To measure the colony growth and worker mortality, all raised pupae and dead workers were counted once a week and then removed to prevent cannibalism. In the 13th week of the experiment, five of the last pupae per subcolony were dried for three days at 40 °C and 10% humidity and then weighed on a UMT5 Comparator (Mettler) scale. Larvae that were still present in the subcolonies after the 13-week period were left in the subcolonies to determine whether they would hatch after pupation and melanise.

Results

After 13 weeks of feeding, all worker subcolonies had raised considerable numbers of pupae from the provided larvae (see Table 2). In the subcolonies fed with the holidic artificial diet, the number of pupae raised did not differ significantly from the subcolonies fed with Bhatkar-Whitcomb diet, cockroaches and honey water (Wilcoxon matched-pair-test, $N = 7$, $Z = 1.183$, $p = 0.237$). Also the dry weight of brood which had pupated in the last week of the experiment did not differ significantly between the two feeding treatments (Wilcoxon matched-pair-test, $N = 7$, $Z = 0.676$, $p = 0.499$).

The worker mortality was slightly higher in the subcolonies fed with the holidic diet, but did not differ significantly from the control groups (Wilcoxon matched-pair-test, $N = 7$, $Z = 1.153$, $p = 0.249$). After the 13th week, the remaining larvae pupated, hatched and developed to normal workers in both treatments. Additionally, in six queenright colonies of *C. floridanus* kept on the artificial diet over a period of four months, eggs were laid continuously by queens, and neither larval development nor worker mortality differed from control colonies (Berthold and Feldhaar, unpubl. results).

Discussion

Number and weight of pupae raised did not differ significantly between the subcolonies fed the holidic diet and those fed the modified Bhatkar-Whitcomb diet, cockroaches and honey-water. Thus, the chemically defined diet is sufficient to guarantee a high brood production and a normal growth rate of the larvae at least for a period of three months. The chemically defined artificial diet also seems to be suitable for whole colonies, since egg laying by queens did not cease over a period of four months. In contrast, brood production in *Camponotus floridanus* colonies fed with Bhatkar-Whitcomb agar and honey-water only but no cockroaches or other additional sources of protein, decreased considerably after three months (A. Endler, pers. comm.).

During the preparation of the diet, it was observed that the amino acids did not completely dissolve in water, probably because of the limited solubility of tyrosine. To avoid this problem in further experiments, the total amount of tyrosine in the diet could be reduced to its maximal solubility (0.38 g/l at 20 °C) and compensated by raising the total amount of phenylalanine. In most of the insects examined, tyrosine is dispensable and can be completely derived by hydroxylation of phenylalanine (Dadd, 1985). Alternatively, the missing tyrosine could be compensated by addition of beta-alanyltyrosine, as used in the synthetic aphid diet by Febvay et al. (1999).

In this diet, gamma-amino butyric acid was included because of its phagostimulatory effect in many insect orders (Mullin et al., 1994). However, this effect has not yet been demonstrated in ants, so this substance may be dispensible.

In further feeding experiments, using deletion of single nutrients or changes in the ratio of nutrients and subsequent monitoring of the worker survival, breeding success and larval growth rate, it will be possible to clarify the nutritional demands of carpenter ants and related genera, as well as a possible nutritive role of their endosymbiont *Blochmannia*. Additionally, experiments need to be done on the optimal composition of the chemically defined diet in relation to the colony composition, e.g. a colony with higher worker to brood ratio may require more carbohydrates and less protein.

This is the first successful establishment of a completely synthetic artificial diet in an ant species. Because all chemical components of this diet are exactly known, and since their concentrations can be manipulated, it provides the ant physiologist with a powerful new tool. The exact knowledge of all components will allow new insights into the importance of specific nutritive components for ants. This diet should be suitable for all ant species that are able to feed on food with the consistency of the Bhatkar-Whitcomb-agar.

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