



Contrasting effects of amino acid types on foraging behaviour, colony growth, and worker mortality in red ants and carpenter ants

Asim RENYARD, Kennedy HOVEN, Claire GOODING, Jonathan PETROV, Jaime M. CHALISSERY & Gerhard GRIES

Abstract

Foraging ants collect amino acids and proteins for developing larvae in their colony. Both essential amino acids (EAAs; some considered toxic to ants) and non-essential amino acids (non-EAAs) are important building blocks of proteins, but EAAs cannot be synthesized by animals and must be obtained from their diet. Whether ants specifically forage for EAAs, and how EAAs affect ant colony growth, has rarely been investigated. Using red ants, *Myrmica rubra*, and western carpenter ants, *Camponotus modoc*, as model species, we tested the hypotheses that (1) *M. rubra* and *C. modoc* colonies with brood preferentially forage for EAAs rather than non-EAAs; (2) *M. rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth; and (3) *M. rubra* workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs (which are considered less toxic). In laboratory choice experiments, colonies of *M. rubra* and *C. modoc* preferentially foraged for EAAs rather than non-EAAs. Colonies of *M. rubra* that consumed both EAAs and non-EAAs produced more larvae but not more workers and queens than colonies that consumed only EAAs or non-EAAs. In a mortality experiment, isolated *M. rubra* workers that consumed sucrose and EAAs died sooner than workers that consumed sucrose and non-EAAs, possibly because they could not feed EAAs to larvae. Our results indicate that EAAs on their own, while critically important, are insufficient for ant colony growth. However, EAAs as key macro-nutrients in combination with sucrose could be offered as a new bait for pest ant control.

Key words: Hymenoptera, Formicidae, eusociality, brood care, nutrition, macronutrient.

Received 15 April 2024; revision received 29 August 2024; accepted 30 September 2024

Subject Editor: Falko P. Drijfhout

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Introduction

Cooperative brood care is one of the defining features of eusocial insect societies including ants (WILSON 1971). Non-reproductive worker ants engage in nest construction, colony defense, grooming, and foraging to provide care for the colony's developing larvae and pupae (HÖLLDOBLER & WILSON 1990). The brood care by workers contributes to colony growth and produces reproductive queen ants that disperse and start new colonies (HÖLLDOBLER & WILSON 1990). While workers themselves do not reproduce, they provide allo-parental care, ultimately increasing their own fitness by the passing of shared genes through their queen (KORB & HEINZE 2008).

Worker ants sense, and behaviourally respond to, the needs of the colony's brood. They monitor the brood's well-being, keeping brood in suitable microclimates, removing pathogens, and providing adequate nutrition. Worker ants sense the brood's abiotic conditions, such as temperature (PORTER & TSCHINKEL 1993, ROCES & NUNEZ

1995, ANDERSON & MUNGER 2003, PENICK & TSCHINKEL 2008, KARLIK & al. 2016), humidity (POTTS & al. 1984, but see KARLIK & al. 2016), and CO₂ (RÖMER & al. 2018), and move brood to optimal growing conditions. Microbial pathogens on brood (KARLIK & al. 2016) prompt workers to engage in mechanical grooming and chemical secretions to remove or kill these microbes (UGELVIG & al. 2010, TRAGUST & al. 2013a, b). Lastly, the presence of brood shifts the behaviour of foragers, mobilizing them (PORTHA & al. 2002) to collect more food and more protein (CORNELIUS & GRACE 1997, DUSSUTOUR & SIMPSON 2008, 2009).

Workers collect the nutrients that the brood requires from nutritionally diverse food sources. Ant larvae signal hunger to ant nurses (CREEMERS & al. 2003, KAPTEIN & al. 2005, PEIGNIER & al. 2019) that, in turn, then solicit food from foragers (SORENSEN & VINSON 1981, SORENSEN & al. 1985). While worker ants require primarily carbohydrates (GROVER & al. 2007, COOK & al. 2010, DUSSUTOUR &

SIMPSON 2012, SHIK & SILVERMAN 2013, BAZAZI & al. 2016, ARGANDA & al. 2017, WITTMAN & al. 2018), developing larvae require additionally more proteins (PORTER 1989, EVANS & PIERCE 1995, FELDHAAR & al. 2007, GROVER & al. 2007, SHIK & SILVERMAN 2013). Whole proteins are obtained from prey and deceased insects, whereas free amino acids are obtained from sources such as plant nectar (BLÜTHGEN & al. 2004, GONZÁLEZ-TEUBER & HEIL 2009, SHENOY & al. 2012), aphid honeydew (BLÜTHGEN & al. 2004, WOODRING & al. 2004, SHAABAN & al. 2020), and insect hemolymph (KANOST 2009). Developing ant larvae metabolize proteins to amino acids, the building blocks of new proteins (CHAPMAN 2013, COHEN 2015). Generally, organisms incorporate 20 proteogenic (protein creating) amino acids in protein biosyntheses, and use non-proteogenic amino acids (e.g., γ -aminobutyric acid) for other functions such as signalling (CHAPMAN 2013, COHEN 2015). Some animals can synthesize non-essential amino acids (non-EAAs) but must obtain essential amino acids (EAAs) from their diet (CHAPMAN 2013, COHEN 2015). In insects, 8 - 10 amino acids are typically deemed essential (CHAPMAN 2013, COHEN 2015). Some ant taxa obtain both EAAs and non-EAAs through their gut microbiota that are capable of converting nitrogenous waste products to amino acids. For example, carpenter ants, *Camponotus* spp., and turtle ants, *Cephalotes* spp., harbour gut microbes that convert urea, or uric acid, to amino acids (FELDHAAR & al. 2007, HU & al. 2018). However, the effects of EAAs and non-EAAs on ant colony growth have yet to be rigorously investigated. Colonies of *Camponotus floridanus* that were provisioned with a diet lacking EAAs and containing antibiotics (to kill amino acid-synthesizing microbes) raised fewer pupae (FELDHAAR & al. 2007).

As most ants obtain EAAs from their diet, it follows that they are able to distinguish between EAAs and non-EAAs. Generally, many ants prefer nutrient solutions containing amino acids (LANZA & KRAUSS 1984, BLÜTHGEN & FIEDLER 2004, GONZÁLEZ-TEUBER & HEIL 2009), particularly at higher concentration (LANZA 1991, LANZA & al. 1993, GONZÁLEZ-TEUBER & HEIL 2009), but among these studies there is none reporting consistent preference for specific amino acids. Some but not all ants preferentially feed on EAAs (SHENOY & al. 2012, CSATA & al. 2020). Argentine ants, *Linepithema humile*, prefer EAAs to non-EAAs for consumption, and when deprived of a single EAA preferentially forage for that EAA (CSATA & al. 2020). Interestingly, *L. humile* workers consuming the EAAs methionine, threonine, and phenylalanine had a shorter lifespan than workers consuming other proteogenic amino acids (ARGANDA & al. 2017), suggesting that these three EAAs could also be toxic to other ant species.

To broaden our study on amino acid foraging of ants, we selected two species that prefer different macro-nutrients (RENYARD & al. 2024a): nitrogenous urea and essential amino acids (*Camponotus modoc*) and sucrose (*Myrmica rubra*). We worked exclusively with *M. rubra* for testing Hypotheses 2 and 3 (see below), because they

can easily be collected in the field and maintained in small laboratory colonies. *Camponotus modoc* is native to the west coast of North America, excavating nests in the wood of conifers (HANSEN & KLOTZ 2005). *Myrmica rubra* is an aggressive, stinging, and soil-nesting ant (WETTERER & RADCHENKO 2010) that is native to Eurasia but has invaded coastal areas of eastern and western North America (WETTERER & RADCHENKO 2010). *Camponotus modoc* colonies typically have 3000 - 25,000 workers and a single queen (AKRE & al. 1994), whereas *M. rubra* colonies have 400 - 1900 workers and 3 - 13 queens (GRZEŚ & al. 2018).

We tested three hypotheses: (1) colonies of *Myrmica rubra* and *Camponotus modoc* preferentially forage for EAAs rather than non-EAAs; (2) *M. rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth; and (3) *M. rubra* workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs.

Material and methods

Maintenance of ant colonies

Colonies of *Camponotus modoc* (containing workers, brood, and a queen) were identified by taxonomic key (see HANSEN & AKRE 1985), collected, and maintained as previously reported (RENYARD & al. 2019). Briefly, six nests were excised from conifer logs and stumps, and placed in large plastic bins (64 × 79 × 117 cm) that were kept in an outdoor undercover area, where they experienced natural temperature and light cycles. Bins were connected to glass containers via barbed plumbing fixtures and Tygon® tubing (Courbevoie, France). Ants were provisioned with 20% (w / v) sugar water, cockroaches, meal worms, and apples ad libitum.

Colonies of *Myrmica rubra* (containing workers, brood, and one or more queens) were identified by taxonomic key (RADCHENKO & ELMES 2010), collected, and maintained similar to previous reports (HOEFELE & al. 2021, RENYARD & al. 2021). Six *M. rubra* nests were excavated at Inter River Park (North Vancouver, BC, Canada) and placed, together with nesting soil, in glass containers (26 × 21 × 40.6 cm) or plastic bins (41 × 29 × 24 cm) kept indoors at 25 °C and at a 16:8 light:dark cycle. Nests were sprayed with water several times per week, and ants were fed the same diet as *Camponotus modoc* (see above).

Preparation of liquid nutrient solutions – general descriptions

Liquid nutrient solutions (Tabs. 1 and 2 for nutrient concentrations and compositions, respectively) were prepared by weighing dry ingredients (TR 204 scale; Denver Instrument Company, CO, USA), and then mixing them in distilled water. Aliquots (1 mL) of these solutions were pipetted into 1.5-mL Eppendorf tubes (Thermo Fisher Scientific, Waltham, MA, USA) which were stored in a freezer (-4 °C) until needed.

For testing Hypothesis 1 (H1), that colonies with brood preferentially forage for EAAs rather than non-EAAs,

Tab. 1: Hypotheses (H) tested and experiments and replicates (n) run, with colonies of *Camponotus modoc* and *Myrmica rubra*. ^aBlend compositions and chemical suppliers of nutrients in Tab. 2; ^bEAA = essential amino acids; ^cnon-EAA = non-essential amino acids; ^dPercentages are expressed as weight by volume (w / v).

Exp. #	Stimuli ^a tested	Species tested (n)
<i>(H1) Colonies with brood preferentially forage for EAAs^b rather than non-EAAs^c</i>		
1–2	EAAs + non-EAAs (1.05% ^d) vs non-EAAs (0.5%) vs Water	<i>C. modoc</i> (6); <i>M. rubra</i> (5)
3–4	EAAs + non-EAAs (1.05%) vs EAAs (0.55%) vs Water	<i>C. modoc</i> (5); <i>M. rubra</i> (6)
<i>(H2) Colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth</i>		
5	EAAs + non-EAAs (2.1%) vs EAAs (1.1%) vs non-EAAs (1.0%)	<i>M. rubra</i> (10)
<i>(H3) Workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs</i>		
6	Sucrose (5.55%) vs Sucrose (4.55%) + EAAs (1%) vs Sucrose (4.55%) + non-EAAs (1%)	<i>M. rubra</i> (10)

Tab. 2: List of macro-nutrients (sucrose; essential amino acids, EAAs; non-essential amino acids, non-EAAs) micro-nutrients (salts, vitamins, others), and their chemical purities, suppliers, and chemical abstract service (CAS) numbers, used in test stimuli. ^a“Fraction of total” denotes the proportion of a chemical in a nutrient group; ^bSA = Sigma Aldrich, Burlington, MA, USA; AC = Anachemia Canada Inc., Lachine, QC, Canada; MI = Millipore, Burlington, MA, USA; OW = Oakwood Products, Inc., Estill, SC, USA; FI = Fisher Scientific International, Inc., Pittsburgh, PA, USA; AA = Alfa Aesar, Ward Hill, MA, USA; CL Caledon Laboratories Ltd., Georgetown, ON, Canada; BS = Bio-Serv, Flemington, NJ, USA; ^cAmino acids listed as in FELDHAAR & al. (2007).

Nutrients	Chemicals	Fraction of total ^a	Supplier ^b	% Purity	CAS
Sucrose	D-sucrose	1.00	SA	≥99	57-50-1
non-EAAs ^c	L-asparagine	0.1	SA	≥99	5794-13-8
	L-aspartic acid	0.1	SA	≥98	56-84-8
	L-cysteine	0.1	SA	≥97	52-90-4
	L-glutamine	0.1	SA	≥99	56-85-9
	L-glycine	0.1	SA	≥98	56-40-6
	L-lysine	0.1	SA	≥98.5	56-87-1
	L-proline	0.1	SA	≥99	147-85-3
	L-serine	0.1	SA	≥99	56-45-1
	L-tyrosine	0.1	AC	≥98	60-18-4
	γ-amino butyric acid	0.1	SA	≥99	56-12-2
EAAs	L-glutamic acid	0.091	SA	99	56-86-0
	L-alanine	0.091	SA	≥98	56-41-7
	L-isoleucine	0.091	MI	≥98	73-32-5
	L-leucine	0.091	SA	97	61-90-5
	L-valine	0.091	SA	≥98	72-18-4
	L-tryptophan	0.091	SA	≥98	73-22-3
	L-arginine	0.091	SA	≥98	74-79-3
	L-histidine	0.091	SA	≥99	71-00-1
	L-threonine	0.091	SA	≥98	72-19-5
	L-methionine	0.091	SA	≥98	63-68-3
	L-phenylalanine	0.091	SA	99	63-91-2
Salts	CuCl ₂	0.0002	SA	97	7447-39-4
	FeCl ₃	0.0019	OW	98	7705-08-0
	MnCl ₂	0.0004	SA	≥99	7773-01-5

Nutrients	Chemicals	Fraction of total ^a	Supplier ^b	% Purity	CAS
Salts	NaCl	0.0020	FI	≥99	7647-14-5
	ZnCl ₂	0.0008	AA	≥99	7646-85-7
	KH ₂ PO ₄	0.5054	SA	≥99	7778-77-0
	MgSO ₄	0.4892	CL	≥99	7487-88-9
Vitamins/MISC	<i>p</i> -amino benzoic acid	0.0424	SA	≥99	150-13-0
	ascorbic acid	0.4235	BS	≥97	50-81-7
	biotin	0.0004	SA	≥99	58-85-5
	calcium D-pantothenate	0.0212	SA	≥98	137-08-6
	folic acid	0.0042	SA	≥97	59-30-3
	nicotinic acid	0.0424	SA	≥98	59-67-6
	pyridoxin hydrochloride	0.0106	SA	≥98	58-56-0
	riboflavin	0.0212	SA	≥98	83-88-5
	thiamine	0.0106	SA	≥99	67-03-8
	<i>meso</i> -inositol	0.2118	SA	≥99	87-89-8
	choline chloride	0.2118	SA	≥99	67-48-1

amino acids were deemed either EAAs or non-EAAs as listed in FELDHAAR & al. (2007). For testing H2, that colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth, the diet was prepared as described in STRAKA & FELDHAAR (2007), but some unavailable ingredients were omitted (Tab. 2). For testing H3, that worker ants feeding on EAAs die sooner than worker ants feeding on non-EAAs, EAAs or non-EAAs were combined with sucrose, a preferred sugar of *Myrmica rubra* (RENYARD & al. 2021). For testing H1 and H2, either EAAs or non-EAAs were omitted from the binary blend of EAAs and non-EAAs without adjusting the concentration of test stimuli. This experimental design allowed us to isolate the effect of presence, or absence, of amino acid type on the ants' preferential response without altering the proportions of each individual amino acid, which ants are likely to sense (CSATA & al. 2020). For testing H3, the total concentration of all test stimuli was kept equal to isolate the effect of macro nutrient composition, rather than nutrient concentration, on the ants' survival.

Prior to experiments, Eppendorf tubes were removed from the freezer to thaw and then vortexed to dissolve all solutes. Then, a 1-cm long piece of cotton dental wick (Richmond Dental & Medical, Charlotte, NC, USA) was inserted into each tube, thus allowing ants to ingest the liquid without spillage.

Specific experiments

(H1) *Myrmica rubra* and *Camponotus modoc* colonies with brood preferentially forage for EAAs rather than non-EAAs (Exps. 1 - 4): All experiments followed an established protocol (RENYARD & al. 2021) with slight modifications. Prior to bioassays, *Camponotus modoc* colonies (n = 6) were deprived of 20% (w/v) sugar water as well as of cockroaches and apples for 24 h and

4 h, respectively (the maximum time ants could endure without these foods before they attempted to chew their way out of containers). Bioassays were run in plexiglass containers (50.5 × 30.5 × 33 cm; Fig. 1A) covered by a lid with mesh holes to allow ventilation. To prevent ant escape, the upper inner container walls were coated with an equal mix of Vaseline (Unilever, London, UK) and paraffin oil (Anachemia, Lachine, QC, CA). For each experiment, a set of tubes was prepared for nutrient consumption by ants and another set of tubes for monitoring passive water evaporation ("evaporation controls"). All Eppendorf tubes were weighed prior to bioassays. Eppendorf tubes were taped, with positions randomly assigned and spaced equidistantly in an arc, to the arena bottom, 22 cm away from the container entrance hole. Corresponding evaporation control tubes were taped to a plexiglass platform suspended from the container lid. To initiate a replicate, tubes were uncapped and each container was connected via Tygon® (diameter 2.54 cm) and barbed plumbing connectors (diameter 2.54 cm) to a *C. modoc* housing bin, allowing ants to freely forage in the container. Bioassay replicates were run for 4 h but were terminated sooner if ants had completely consumed the test solution of any one tube. At the end of each replicate, tubes were reweighed to determine consumption by ants and the amount of water evaporation. Bioassay containers were cleaned with hexane and ethanol (70%), and plumbing fixtures and Tygon® tubing were washed with soapy water.

All bioassays with *Myrmica rubra* colonies (n = 6; food-deprived 24 h) were run in their nesting containers (Fig. 1B). Prior to each bioassay replicate, all Eppendorf tubes were weighed, and tubes with nutrients for consumption by ants were randomly assigned to positions on the edge of a jar lid (diameter 15 cm), whereas corresponding evaporation control tubes were taped, inacces-

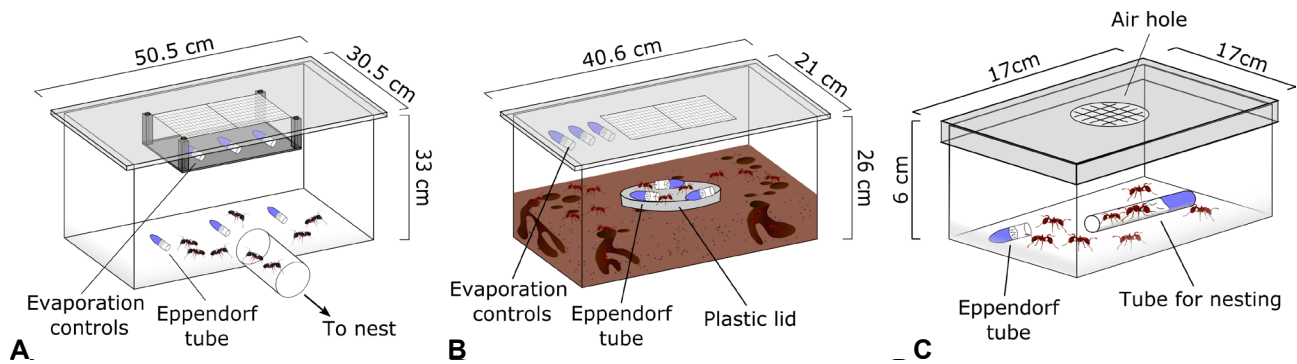


Fig. 1: Illustrations of experimental designs used to test effects of essential and non-essential amino acids on ant colony feeding preferences and reproductive fitness. **(A, B)** Experimental designs used to bioassay consumption of aqueous amino acid blends by carpenter ants, *Camponotus modoc*, and red ants, *Myrmica rubra*. Amino acid blends were presented in 1.5-mL Eppendorf tubes plugged with a piece of cotton wick to allow blend consumption by ants without spillage. For each *C. modoc* bioassay **(A)**, Eppendorf tubes were presented in a container connected via Tygon tubing (Courbevoie, France) to the ants' nest; for each *M. rubra* bioassay **(B)**, Eppendorf tubes were arranged on a plastic lid which was placed directly into the ants' nesting container. Evaporation control tubes were placed on a platform beneath the container lid **(A)** or taped directly to the underside of the container lid **(B)**. **(C)** Design used for testing the effect of essential and non-essential amino acids on *M. rubra* colony growth and worker mortality; small colonies or groups of workers were placed in Tupperware containers fitted with a test tube for nesting and an Eppendorf tube containing a nutrient blend. Note: drawings are not to scale.

sible to ants, to the underside of container lids. Bioassay replicates were initiated by uncapping all Eppendorf tubes and placing the jar lids with Eppendorf tubes on the soil surface inside bioassay containers. Bioassays were run for 6 h but were terminated sooner if ants had consumed the entire nutrient solution in an Eppendorf tube. Tubes were capped and reweighed after replicates to determine nutrient consumption by ants. Jar lids were cleaned with soapy water between experiments.

All experiments with colonies of *Camponotus modoc* and *Myrmica rubra* were run on warm, sunny days with observable ant activity. Bioassay durations for *C. modoc* and *M. rubra* colonies were based on ant colony size (HANSEN & AKRE 1985, WETTERER & RADCHENKO 2010) and preliminary experiments that determined the time needed to obtain measurable consumption responses. Colonies of *C. modoc* were bioassayed on a single day between 12:00 and 16:00, and *M. rubra* colonies were bioassayed on two days between 10:00 and 16:00. Each ant colony was given at least 36 h between experiments.

To test H1, colonies in Experiments 1 (*Camponotus modoc*) and 2 (*Myrmica rubra*) were offered three choices (Tab. 1): (1) 11 EAAs + 10 non-EAAs (1.05% total w/v) in water; (2) 10 non-EAAs (0.5%) in water; and (3) a water control. Additionally, colonies in Experiments 3 (*C. modoc*) and 4 (*M. rubra*) were offered: (1) 11 EAAs + 10 non-EAAs (1.05%) in water; (2) 11 EAAs (0.55%) in water; and (3) a water control.

(H2) *Myrmica rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth (Exp. 5): To test H2, *Myrmica rubra* colonies were collected at Inter River Park and sorted into 30 small lab colonies, each consisting of 30 workers and two queens housed in small plastic containers

(17 × 17 × 6 cm) with a mesh-covered hole in the lid for air exchange (Fig. 1C). Each container was fitted with a 10-mL test tube (13 × 100 mm) filled halfway with water and plugged with a cotton ball to provide a humid environment. Colonies were provisioned with one of three types of nutrient blends. All blends contained sucrose, salts, and vitamins (as in STRAKA & FELDHAAR 2007; Tabs. 1 and 2) but differed in amino acid composition: (1) 11 EAAs + 10 non-EAAs (2.1%); (2) 10 non-EAAs (1.0%); and (3) 11 EAAs (1.1%). All blends were provided in Eppendorf tubes and replaced twice per week. After 16 weeks – when colony growth had plateaued – each colony was frozen, and larvae, worker ants, and queen ants were counted.

(H3) *Myrmica rubra* workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs (Exp. 6): To test H3, worker mortality was tracked for 84 days in response to consumption of specific macro-nutrients (Tab. 1): (1) aqueous sucrose alone (5.55% w/v), (2) aqueous sucrose (4.55%) + 11 EAAs (1%), and (3) aqueous sucrose (4.55%) + 10 non-EAAs (1%). Colonies were field collected, sorted, and housed as described (see H2), but each colony contained only 30 workers without queens and brood. Each nutrient solution was provided in Eppendorf tubes and replaced twice per week. Every week, dead workers in each colony were counted.

Statistical analysis

For Experiments 1 - 4, consumption of nutrient solutions by ants was calculated by subtracting the weight loss in evaporation controls from the weight loss of nutrient test solutions. In some replicates with little ant colony activity, nutrient test solutions had a slightly negative value

following weight loss subtraction due to water evaporation. As there could not be “negative feeding” on a nutrient solution by a colony, these values were considered to be a zero. Non-responding colonies were filtered by including only nests which had at least one positive consumption value. To account for differences in ant colony activity, proportions were analysed rather than absolute amounts. To calculate proportional consumption, the consumption value of a given treatment (nutrient solution) was divided by the total amount of consumption by a colony. Data of all experiments were analysed using generalized linear models (GLM) and generalized linear mixed models (GLMM). A standard transformation was used to restrict the data between the interval 0 and 1, and a beta distributed GLMM was fit to the data (SMITHSON & VERKUILEN 2006). Proportional consumption was fit as the response variable and treatment as the predictor variable, with ant colony as a random intercept. In Experiment 5, the number of larvae was modelled using a GLM with a negative binomial distribution, and the number of queens and workers was modelled using a Conway-Maxwell Poisson distribution with treatment as the predictor variable. For Experiment 6, the proportion of live ants was fit as a binomial GLMM with a treatment by day interaction, and colony as a random intercept. For all models, the significance ($\alpha = 0.05$) of each predictor was assessed using a likelihood ratio test (LRT), and Tukey adjusted pairwise comparisons between means (Exps. 1 - 5).

R (v. 4.2.2) and R studio (v. 2022.07.1+554) were used to analyse data and produce graphics (R CORE TEAM 2022). Data were processed using the “tidyverse” packages (WICKHAM & al. 2019) and the “plyr” package (WICKHAM 2011). The “glmmTMB” package (BROOKS & al. 2019) was used to fit models, and the “DHARMA” package was used to inspect their fit (HARTIG 2022). Estimated marginal means and 95% confidence intervals were obtained using the “emmeans” package (LENTH 2023). The “ggplot2” package (WICKHAM 2016) was used to create graphs, and Inkscape was used for final figure assembly (v. 1.0.2). Data and code can be accessed at RENYARD & al. (2024c).

Results

(H1) *Myrmica rubra* and *Camponotus modoc* colonies with brood preferentially forage for EAAs rather than non-EAAs (Exps. 1 - 4)

In Experiments 1 and 2, the composition of amino acid blends (11 EAAs + 10 non-EAAs or 10 non-EAAs only) significantly affected consumption by *Camponotus modoc* (LRT: $\chi^2 = 21.59$, d.f. = 2, $p < 0.0001$) and *Myrmica rubra* (LRT: $\chi^2 = 28.28$, d.f. = 2, $p < 0.0001$). Ants consumed blends containing both EAAs and non-EAAs significantly more than blends containing only non-EAAs, which were ingested as little as the water control (Tukey adjusted p-value < 0.05 ; Fig. 2; Tab. S1, as digital supplementary material to this article, at the journal's web pages).

In Experiments 3 and 4, the composition of amino acid blends (11 EAAs + 10 non-EAAs or 11 EAAs only) again significantly affected consumption by *Camponotus modoc* (LRT: $\chi^2 = 23.68$, d.f. = 2, $p < 0.0001$) but not by *Myrmica rubra* (LRT: $\chi^2 = 5.6$, d.f. = 2, $p = 0.06$). Colonies of *C. modoc* consumed the blend of EAAs and non-EAAs only slightly more (but statistically significant) than the blend of EAAs, both blends being consumed more than water (Tukey adjusted p-value < 0.05 ; Fig. 2; Tab. S1). In contrast, colonies of *M. rubra* consumed the blend of EAAs and non-EAAs as much as the blend of EAAs, and they ingested both blends numerically (but not statistically) more than the water control (Tukey adjusted p-value > 0.05 ; Fig. 2; Tab. S1).

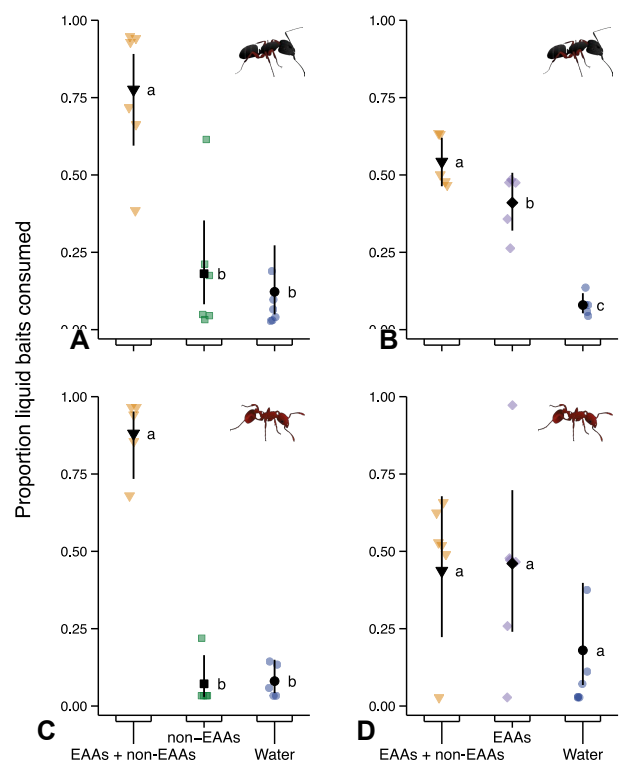


Fig. 2: Proportional consumption of aqueous amino acid baits by colonies of *Camponotus modoc* (A, relative effects of non-essential amino acids, non-EAAs, $n = 6$; B, relative effects of essential amino acids, EAAs, $n = 5$) and *Myrmica rubra* (C, relative effects of non-EAAs, $n = 5$; D, relative effects of EAAs, $n = 6$). Colonies were offered aqueous baits of 11 EAAs (0.55% w / v), 10 non-EAAs (0.5% w / v), and both 11 EAAs and 10 non-EAAs (1.05% w / v) as well as a water control. Each coloured symbol represents the result of an individual replicate, whereas black symbols and whiskers are modelled estimated marginal means and 95% confidence intervals from a generalized linear mixed model. Treatment was a significant predictor of proportional consumption in all experiments except for Experiment 4. In each subpanel, different letters assigned to proportional consumptions of amino acid baits denote statistically significant differences in Tukey adjusted pairwise comparisons (Tab. S1).

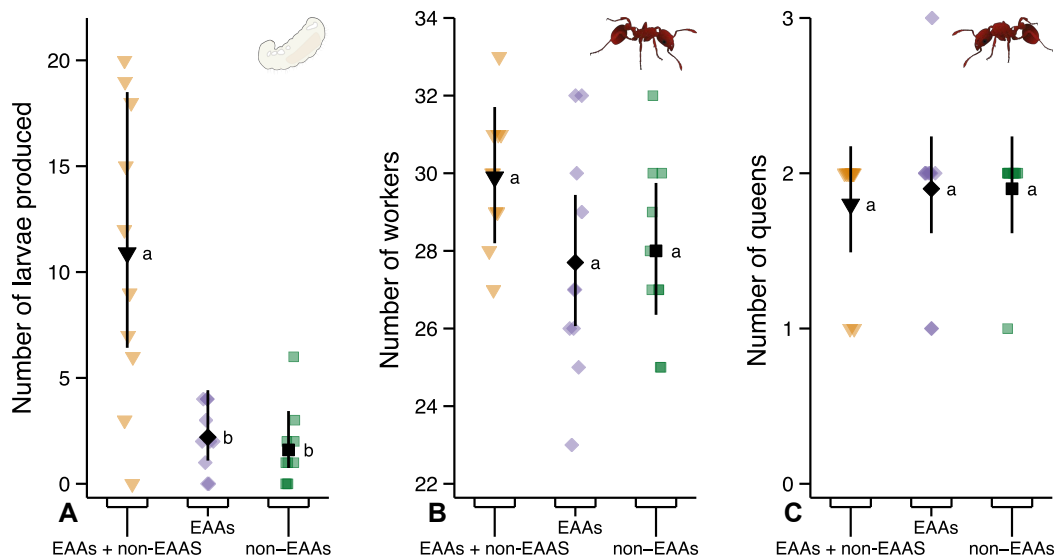


Fig. 3: Production of larvae (A), worker ants (B), and queen ants (C) by *Myrmica rubra* colonies after a 16-week rearing experiment. Colonies were reared on synthetic diets containing 11 essential amino acids (EAAs, 1.1% w / v; Tab.2), 10 non-essential amino acids (non-EAAs, 1.0% w / v; Tab.2), and both 11 EAAs and 10 non-EAAs (2.1% w / v; Tab.2). Each coloured symbol represents the results of a single colony (n = 10), whereas black symbols and whiskers are modelled estimated marginal means and 95% confidence intervals from a generalized linear mixed model. Treatment was a significant predictor for the number of larvae (likelihood ratio test: $p < 0.05$) but not for the number of worker and queen ants produced. In each subpanel, different letters assigned to numbers of larvae, worker ants, and queen ants produced in response to amino acid bait composition denote statistically significant differences in Tukey adjusted pairwise comparisons (Tab. S2).

(H2) *Myrmica rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth (Exp. 5)

The composition of the amino acid blend [(1) 11 EAAs + 10 non-EAAs; (2) 11 EAAs; or (3) 10 non-EAAs] significantly affected the number of ant larvae (LRT: $\chi^2 = 21.48$, d.f. = 2, $p < 0.0001$) but not the number of worker ants (LRT: $\chi^2 = 5.04$, d.f. = 2, $p = 0.08$) and queen ants (LRT: $\chi^2 = 0.36$, d.f. = 2, $p = 0.83$) present in colonies. Colonies provisioned with both EAAs and non-EAAs produced more larvae than colonies provisioned with either EAAs or non-EAAs, with the latter two colonies having similarly few larval offspring (Tukey adjusted p value < 0.05 ; Fig. 3; Tab. S2).

(H3) *Myrmica rubra* workers feeding on sucrose and EAAs die sooner than workers feeding on sucrose and non-EAAs (Exp. 6)

Nutrient blend [(1) sucrose + 11 EAAs; (2) sucrose + 10 non-EAAs; (3) sucrose only] was a significant predictor of worker mortality, day in experiment, and interaction between mortality and day (mortality: $\chi^2 = 19.13$, d. f = 4, $p = 0.0007$; day: $\chi^2 = 2269.4$, d.f. = 3, $p < 0.0001$; interaction: $\chi^2 = 9.29$, d.f. = 2, $p = 0.01$; Fig. 4). The negative interaction term between “day” and “EAAs” and the positive interaction term between “non-EAAs” and “day” indicate that over time worker ants consuming EAAs died the fastest, followed by workers consuming sucrose and sucrose plus non-EAAs (RENYARD & al. 2024c).

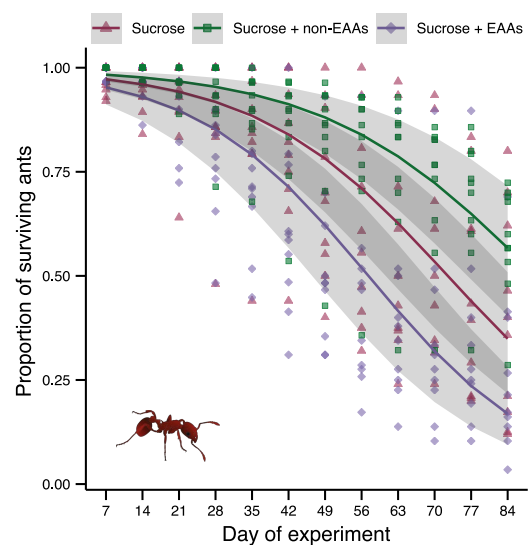


Fig. 4: Proportional survival of 30-worker groups of *Myrmica rubra* (n = 10) when fed aqueous solutions of sucrose only (5.55% w / v), sucrose (4.55%) plus 11 essential amino acids (EAAs, 1%; Tab. 2), and sucrose (4.55%) plus 10 non-essential amino acids (non-EAAs, 1%; Tab. 2). Coloured symbols represent the proportion of surviving workers in individual replicates. Lines and shaded regions are back transformed model predictions and 95% confidence intervals from a binomial generalized linear mixed model with a logit link function. The type of nutrient solution, day of experiment, and interaction between nutrient solution and day were all significant predictors in the model (likelihood ratio test: $p < 0.05$).

Discussion

Our data support the hypotheses that *Myrmica rubra* and *Camponotus modoc* colonies with brood preferentially forage for EAAs rather than non-EAAs, and that *M. rubra* workers feeding on EAAs die sooner than workers feeding on non-EAAs. However, our data do not support the hypothesis that *M. rubra* colonies provisioned with EAAs, instead of non-EAAs, have greater brood production and colony growth. Below, we elaborate on these results.

There is increasing evidence that some ant taxa preferentially forage for EAAs rather than non-EAAs (SHENOY & al. 2012, CSATA & al. 2020, this study). In our study, the presence of EAAs stimulated feeding by both *Camponotus modoc* and *Myrmica rubra*, whereas non-EAAs were as unappealing as water. For *C. modoc*, but not *M. rubra*, a blend of both EAAs and non-EAAs was more appealing than the EAA-only blend, revealing a contributing effect of non-EAAs to the overall blend “appeal”. That the equivalent effect was not observed with *M. rubra* colonies may be due, in part, to their highly variable feeding responses. To test Hypothesis 1, that colonies of *M. rubra* and *C. modoc* preferentially forage for EAAs rather than non-EAAs, we contemplated multiple experimental designs. First, we considered to test EAAs versus non-EAAs head-to-head at identical total concentration, bearing in mind that concentration matters and affects preferential nutrient consumption by ants (e.g., HOJO & al. 2008, WADA & al. 2001). However, with dissimilar numbers of EAAs (11) and non-EAAs (10) in each group, the relative proportion of each amino acid in both groups would have differed. More importantly, EAAs and non-EAAs co-occur in at least some nutrient sources (CHAPMAN 2013, KANOST 2009), and both types of amino acids may be needed to prompt significant consumption by ants. It was for this reason that we opted to assess the relative importance of amino acid types by testing the blend of EAAs and non-EAAs versus either EAAs or non-EAAs. Also, this subtractive type of design (BYERS 1992) has been proven effective in many studies (e.g., RENYARD & al. 2020) for revealing the relative contribution of blend constituents for the behavioural responses of ants. Here, the subtractive method revealed that EAAs are more “appealing” to ants than non-EAAs (Fig. 2). Even though the EAAs were tested at a concentration only half that of the binary blend of EAAs and non-EAAs, they were nearly as appealing to ants as the binary blend, supporting the conclusion that it is the EAAs in the blend that drive the ants’ preferential response. Preferential selection of EAAs, compared to concurrently offered non-EAAs, was also reported in studies with *Linepithema humile* (CSATA & al. 2020) as well as *Technomyrmex albipes* and *Polyrachis dives* (STAAB & al. 2016) but not with *Myrmicaria brunnea* (SHENOY & al. 2012). Our findings and those reported for *L. humile* indicate that ants retain their EAA preference even when, experimentally, they have been deprived of all amino acids and proteins. This selective consumption of EAAs is perplexing, considering that a lack of either EAAs or non-EAAs resulted in smaller broods (Fig. 3). An explanation may lie in the scarceness of EAAs in the ants’

food sources and the difficulty to obtain them. In contrast to non-EAAs, EAAs are deemed not to be abundantly and reliably present in ant food sources such as extra-floral and floral nectar and aphid honeydew (BLÜTHGEN & al. 2004, WOODRING & al. 2004, SHENOY & al. 2012, SHAABAN & al. 2020, but see GONZÁLEZ-TEUBER & HEIL 2009). Sensory perception of certain amino acids may also be affected by the co-occurrence of other nutrients such as sugars (WADA & al. 2001, HOJO & al. 2008). Although proteins of insect prey generally contain both EAAs and non-EAAs (CHAPMAN 2013), and insect hemolymph contains all 20 amino acids (KANOST 2009), *C. modoc* foragers rarely return to the nest with insect prey (TILLES & WOOD 1986), which would result in limited supply of EAAs for nestmates and brood. Selective consumption of EAAs by *C. modoc* and *M. rubra* may ultimately be motivated by the scarceness of these amino acids in the regular food sources of these ants.

Contrary to our prediction, EAAs and non-EAAs – tested head-to-head at nearly identical concentration – contributed equally to the number of brood in *Myrmica rubra* colonies (Fig. 3). That the binary blend of EAAs and non-EAAs had a stronger effect on brood production than either the EAAs or the non-EAAs on their own (Fig. 3) could be due to the presence of both amino acid types in the binary blend and / or to the higher concentration of the binary blend. The correct explanation could be ascertained by testing the binary blend, the EAAs, and the non-EAAs at identical concentration. Even though insects can synthesize non-EAAs, a large deficiency of non-EAAs may impair development and growth (CHAPMAN 2013). Lack of even a single EAA in an insect’s diet can greatly reduce developmental growth (HOUSE 1961, CHAPMAN 2013). Although necessary for insect growth, EAAs on their own are commonly insufficient, and specific non-EAAs are needed to improve growth. In honey bees, *Apis mellifera*, EAAs as dietary constituents improve brood development but not overall colony growth (HENDRIKSMA & al. 2019). Larvae of *Culex pipiens* mosquitoes developed poorly on a diet containing just the 12 EAAs, but diets containing all amino acids or just the non-EAAs glycine or serine equally improved larval development (DADD 1978). Similarly, caterpillars of the silkworm, *Bombyx mori*, developed better on a diet also containing non-EAAs (ITO & ARAI 1967). That the gut endosymbionts of *Cephalotes* and *Camponotus* ants provide their ant hosts with both EAAs and non-EAAs (FELDHAAAR & al. 2007, HU & al. 2018) exemplifies the physiological importance of either amino acid type.

Proteins and amino acids are vital for ant colony growth (PORTER 1989, EVANS & PIERCE 1995, FELDHAAAR & al. 2007) but are toxic to worker ants (COOK & al. 2010, DUSSUTOUR & SIMPSON 2012, BAZAZI & al. 2016, ARGANDA & al. 2017, this study). Particularly detrimental to worker ant survival are the EAAs methionine, threonine, and phenylalanine and the non-EAA serine (ARGANDA & al. 2017). These detrimental effects could be due to elevated levels of amino acid metabolites which can be toxic to animals (WU 2021). In our study (Fig. 4), ants consuming sucrose

and EAAs died sooner than ants consuming sucrose and non-EAAs, with the latter group of ants living longer than ants consuming only sucrose. These data imply that non-EAAs help maintain vital physiological functions in ants. The toxic effects of amino acids or proteins on workers, as shown in our study and others (COOK & al. 2010, DUS-SUTOUR & SIMPSON 2012, ARGANDA & al. 2017), may be attributable to the experimental design which restricted the ants' diet to these macro-nutrients over a relatively long time scale (~ 5 - 100 days). This explanation seems plausible because the deleterious effects of amino acids or proteins on worker ants were ameliorated or not observed when workers could pass these amino acids to brood via trophallaxis (DUS-SUTOUR & SIMPSON 2009, ARGANDA & al. 2017) and when colonies were offered choices between nutritionally diverse food sources (BAZAZI & al. 2016). Our interpretation that brood provisioning motivates preferential EAA consumption by worker ants is supported by distinct morphological and physiological characteristics of ants. Their narrow petiole physically restricts protein movement through the digestive tract (HÖLLDOBLER & WILSON 1990), and their low protease activity (PETRALIA & al. 1980, ERTHAL & al. 2007) slows protein breakdown.

With the physical and metabolic inability of worker ants to process proteins, their quest for EAAs is likely motivated by brood provisioning. Although both *Camponotus modoc* and *Myrmica rubra* colonies preferentially foraged for EAAs, non-EAAs were still needed to increase the number of brood in *M. rubra* colonies. It follows that preferential foraging for EAAs is likely driven by their relative scarceness in the ants' food sources and the ants' inability to synthesize EAAs. As the presence of brood motivates protein-foraging by ants (CORNELIUS & GRACE 1997, DUS-SUTOUR & SIMPSON 2008, 2009), a possible follow-up experiment could investigate how the presence or absence of brood modulates foraging for EAAs and non-EAAs. We noticed pupae throughout the experiment, but at week 16 there were very few pupae present. Comparable numbers of worker ants at weeks 0 and 16 (Fig. 3) – the onset and end of the experiment, respectively – imply that pupation and mortality rates were similar. As larva counts did not correlate with ant worker counts, it follows that nutrient consumptions by ants in feeding trials do not necessarily reflect the colonies' needs for macro- and micro-nutrients and that effects of dietary constituents on ant colony functioning and growth must be investigated in proper context and long-term studies.

Lastly, our findings have significant implications for control of (invasive) pest ants. We have previously shown that ant baits containing both apples (carbohydrates) and mealworms (proteins, amino acids) elicit stronger foraging responses by *Myrmica rubra* colonies than either apples or mealworms alone (HOEFELE & al. 2021). We have also argued that key carbohydrates and amino acids should be identified so that they can be incorporated in ant baits with extended shelf life (HOEFELE & al. 2021). We made progress toward this goal by combining sucrose – a preferred sugar for both *Camponotus modoc* and *M. rubra* (REN-

YARD & al. 2021) and for other ants (CORNELIUS & al. 1996, BLÜTHGEN & FIEDLER 2004, ZHOU & al. 2015, RENYARD & al. 2021) – with EAAs (RENYARD & al. 2024a). The blend of sucrose and EAAs was consumed by multiple species of ants and prompted consistent feeding over their active foraging season (RENYARD & al. 2024a). A bait containing sucrose, EAAs, and the lethal agent boric acid reduced field populations of invasive *M. rubra* (RENYARD & al. 2024b).

Acknowledgements

We thank Michael Gudmundson for field assistance in locating and collecting carpenter ant nests, Heike Feldhaar for personal communications on the compositions of essential and non-essential amino acid blends, Grady Ott for generously donating plastic bins for housing carpenter ants, Regine Gries for technical support, Adam Blake for advice on statistics and graphics, colleagues at the Stats-Beerz group for advice on data analysis, Robert Higgins for assistance in ant identification, Jenelle Breen, Kenza Zobaidi, Ranjot Bhandal, April Lin, and Jasper Li for help with ant care, Sharon Oliver for comments, and three anonymous reviewers and Subject Editor Falko Drijfhout for constructive comments.

Declaration on use of generative artificial intelligence tools

The authors declare that they did not utilize generative artificial intelligence tools in any part of the composition of this manuscript.

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