A preliminary assessment of the field diet and trophic position of the two-spotted cricket *Gryllus bimaculatus* using DNA metabarcoding and stable isotope analysis

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Abstract

Although *Gryllus bimaculatus* is widely used as a laboratory model organism, its ecology under natural conditions remains poorly understood. Here, we provide a preliminary assessment of the field diet and trophic ecology of wild *G. bimaculatus* using stable isotope analysis and gut-content DNA metabarcoding. Stable isotope analysis provided a preliminary estimate of the trophic position of *G. bimaculatus*, suggesting that it occupies an intermediate position between herbivores and carnivores. In contrast, gut-content DNA metabarcoding detected animal-derived material only rarely, while revealing a broad range of plant species. Plant-derived dietary taxa were dominated by Poaceae and Fabaceae. This apparent discrepancy may indicate that *G. bimaculatus* primarily relies on plant-derived resources, while consuming animal-derived material, such as insect carcasses, opportunistically when it becomes available. These findings provide baseline information on the natural diet of *G. bimaculatus* and may contribute to the development of laboratory diets that more closely reflect the species' natural nutritional resources, potentially improving the nutritional optimization of mass-rearing systems.

Keywords

Anthropogenic resources, gut contents, omnivory, opportunistic feeding, trophic ecology

Introduction

Crickets have been widely used as experimental animals in diverse fields, including physiology, developmental biology, neuroscience, ethology, ecology and evolutionary biology, because of their moderate body size, ease of rearing, and pronounced sexual dimorphism, especially in acoustic communication (Gerhardt and Huber 2003, Nakamura et al. 2022, Levy et al. 2024). Although the field ecology of crickets has received far less attention than laboratory studies, an increasing number of studies have examined mating behavior, sexual selection, and predator avoidance under natural conditions (e.g., Gray and Cade 1999, Fisher et al. 2015, 2016, Tregenza et al. 2022). In contrast, much less is known about their natural diet and trophic position. Crickets are generally considered omnivorous insects that feed on weed seeds, seedlings, and dead or living arthropods (Bidau 2014), but quantitative information on their diet in the wild remains limited.

The two-spotted cricket, *Gryllus bimaculatus* De Geer, 1773, has also been used as a model organism in a wide range of research fields (Horch et al. 2017). Despite the extensive accumulation of knowledge from laboratory studies, the ecology of this species in the wild remains poorly understood. Some field studies have examined mating behavior in *G. bimaculatus*, particularly in the context of sexual selection (e.g., Simmons 1988, Simmons and Zuk 1992, Gallego-Abenza and Wheatcroft 2025), but little is known about its natural diet and trophic position. Understanding these aspects of its field ecology is essential for interpreting how the traits of this species have evolved under natural conditions. Without such information, the biological relevance of laboratory findings is difficult to evaluate in an ecological context.

A recent *trnL* metabarcoding study of wild Iberian *G. bimaculatus* revealed that wild *G. bimaculatus* consumes a wide variety of herbaceous plants, particularly Asteraceae and Poaceae (Cruz et al. 2026). However, because that study targeted chloroplast DNA, it documented only the plant fraction of the diet and did not assess animal-derived dietary items or trophic position.

Gryllus bimaculatus has a broad geographical distribution, occurring across Africa, southern Europe, and large parts of Asia including Japan (Orthopterological Society of Japan 2006, Ferreira and Ferguson 2010). Here, we provide a preliminary case study of the field diet and trophic position of wild *G. bimaculatus* from Ishigaki Island in Japan by combining gut-content DNA metabarcoding and stable isotope analysis. A major strength of our approach is that we screened both plant- and animal-derived dietary DNA, allowing us to assess the contribution of animal-derived food to the diet. In addition, we used the nitrogen isotope values of the co-occurring herbivorous grasshopper *Aiolopus thalassinus tamulus* (Fabricius, 1798) as a reference to estimate the trophic position of *G. bimaculatus*. However, this study also has several limitations:

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stable isotope values of potential plant food sources were not measured, the sample size was small, and sampling was restricted to a single locality. Therefore, our findings should be regarded as a preliminary assessment that provides a basis for future, more extensive studies.

Materials and methods

Sampling.—Six adult *G. bimaculatus* (5 females and 1 male) and eight adult grasshoppers *Aiolopus thalassinus tamulus* were collected from Ishigaki Island (Fig. 1; 24.33°N, 124.16°E, WGS 84) in Okinawa Prefecture in November 2024. Specimens were collected by searching the habitat on foot and capturing encountered individuals by hand. The crickets and grasshoppers were frozen within 5 h of collection and transported to the laboratory. The specimens were stored in a freezer at –20°C until processing. For DNA extraction, the specimens of *G. bimaculatus* were thawed and dissected, and the digestive tracts were removed. The dissected digestive tracts were refrozen and sent to a commercial laboratory for DNA metabarcoding. The remaining body tissues were refrozen and shipped in a frozen state to a commercial laboratory for stable isotope analysis. Gut-content DNA metabarcoding provides a short-term snapshot of recently ingested food items, whereas stable isotope analysis provides time-integrated information on assimilated dietary sources and trophic position.

Stable isotope analysis.—Stable isotope analyses were conducted by the Isotope Research Institute Inc. (Yokohama, Japan). Frozen samples were processed as follows. For stable isotope analysis, whole plant samples were homogenized. For insects, either the left or right hind leg was removed from each individual and homogenized. Carbon and nitrogen stable isotope ratios were measured using an elemental analyzer coupled to an isotope ratio mass spectrometer (DELTA V Advantage, Thermo Fisher Scientific) via a

Fig. 1. Photographs of the insects collected and the study site. **A.** Male field cricket, *Gryllus bimaculatus*; **B.** Grasshopper, *Aiolopus thalassinus tamulus*; **C.** Panoramic view of the park where the survey was conducted; **D.** Grassy area where the insects were actually collected (d).

Conflo interface. Stable isotope ratios are expressed in delta notation as parts per thousand (‰) relative to Vienna Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen.

The trophic position of *G. bimaculatus* was estimated using the nitrogen isotope ratio of *Aiolopus thalassinus tamulus* as a herbivorous insect baseline. Trophic position was calculated as TP = 2 + (δ^{15}N_{*G. bimaculatus*} – δ^{15}N_{*A. thalassinus tamulus*}) / 3.4. We used a trophic enrichment factor of 3.4‰ for δ^{15}N, a commonly used average value in trophic position estimates, following Post (2002). Because no plant isotope baseline was available, this estimate should be interpreted as an approximate estimate of trophic position relative to the co-occurring herbivorous grasshopper (Fig. 2).

DNA metabarcoding.—Gut-content DNA metabarcoding was performed by Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). The dissected digestive tract samples were freeze-dried using a VD-250R freeze dryer (TAITEC) and homogenized with a Multi-Beads Shocker at 1,500 rpm for 2 min. DNA was extracted from the homogenized samples using Lysis Solution F and Purification Solution (Nippon Gene), followed by purification with a Lab-Aid 824s DNA Extraction Kit (ZEESAN). Potential PCR inhibitors were removed using a DNeasy PowerClean Pro Cleanup Kit (Qiagen).

The concentration of the extracted DNA was measured using a Synergy LX microplate reader (Agilent Technologies) and the QuantiFluor dsDNA System (Promega). Sequencing libraries were prepared using a two-step tailed PCR protocol. Plant-derived food items were analyzed using gPlant primers targeting a chloroplast plant barcode region, whereas animal-derived food items were analyzed using primers targeting an approximately 380-bp fragment of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene. The *COI* forward primer was 1st-IntF (5'-GGWACWGGWTGAACWGTWTAYCCYCC-3'), based on the mlCOIintF primer described by Leray et al. (2013), and the reverse primer was 1st-HCOmR (5'-TAHACTTCNGGGTGKC-CRAARAATCA-3'), a degenerate HCO2198-derived primer originally described by Folmer et al. (1994). Illumina-compatible adapter/tail sequences were added to these primers for two-step tailed PCR library preparation.

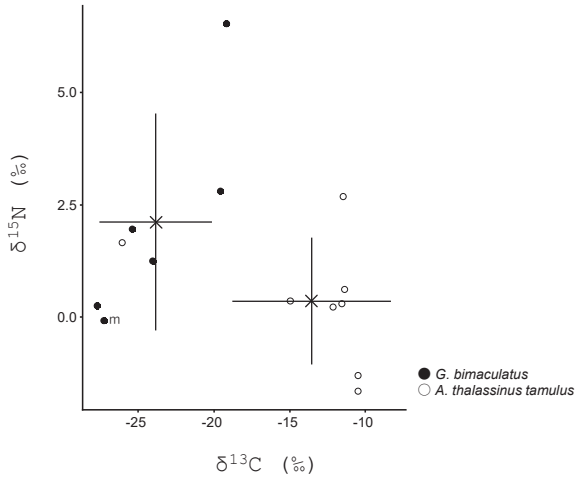

Fig. 2. Carbon (δ^{13}C) and nitrogen (δ^{15}N) stable isotope ratios of *Gryllus bimaculatus* and Aiolopus thalassinus tamulus. Crosses indicate mean values, and horizontal and vertical error bars represent ± SD. The letter "m" indicates the single male *G. bimaculatus* individual.

To reduce amplification of host-derived DNA, *G. bimaculatus*-specific blocking primer, Futahoshikorogi_COI_block (5'-CAC-CTCTATCAACAGGAATTGCTCATGCAGG-3'-3SpC3), was included in the *COI* PCR. This blocking primer was independently designed by Bioengineering Lab. Co., Ltd. to compete with the forward primer over a 4-bp overlapping region. The 3'-terminal C3 spacer was used to prevent extension of the blocking primer during PCR. The first-PCR products, including the adapter/tail sequences, were approximately 440 bp in length.

The amplified libraries were purified using VAHTS DNA Clean Beads (Vazyme), quantified using the QuantiFluor dsDNA System, and assessed using a Fragment Analyzer with the dsDNA 915 Reagent Kit (Agilent Technologies). Paired-end sequencing was performed using an Illumina sequencing platform with 2 × 300 bp reads.

Raw sequence reads were processed separately for the *COI* and gPlant datasets. Reads with exact matches to the primer sequences at their 5' ends were extracted using the fastx_barcode_splitter tool in FASTX-Toolkit v.0.0.14. Primer sequences were trimmed using fastx_trimmer, and low-quality reads with quality scores below 20 were removed using sickle v.1.33. Reads shorter than 40 bp and their paired reads were discarded. Paired-end reads were merged using FLASH v.1.2.11 with a minimum overlap of 10 bp. Chimeric and noisy sequences were removed using the DADA2 plugin in Qiime2 v.2024.10, and representative sequences and ASV tables were generated. Representative sequences were taxonomically assigned using BLASTN v2.16.0 against the NCBI nucleotide database. For the *COI* dataset, sequences assigned to *G. bimaculatus* were regarded as host-derived DNA and excluded before dietary interpretation.

When multiple candidate plant species were suggested by DNA metabarcoding, we primarily assigned the taxon to the species known to occur on Ishigaki Island and most consistent with the sequence data, with reference to distribution records in the National Museum of Nature and Science's Science Museum Net database (https://science-net.kahaku.go.jp/) and the regional floras of Hatusima (1975) and Ohashi et al. (2015, 2016a, b, 2017a, b). Plant common names in Table 2 were assigned primarily based on the USDA Plants Database (https://plants.sc.egov.usda.gov).

Results

The results of stable isotope analysis of *G. bimaculatus* and *A. thalassinus tamulus* are shown in Fig. 1. *G. bimaculatus* exhibited higher δ15N values than *A. thalassinus tamulus* (Fig. 1), corresponding to an estimated trophic position of 2.52. However, the difference in δ15N values between the two species was not statistically significant (cricket: 2.11 ± 2.41 (mean ± SD), hopper: 0.35 ± 1.41, $t_{7.54} = 1.59$, $p = 0.15$, Welch's t test). δ13C values were significantly higher in *A. thalassinus tamulus* than in *G. bimaculatus* (cricket: -23.84 ± 3.72, hopper: -13.56 ± 5.24, $t_{11.99} = 4.29$, $p < 0.01$, Welch's t test).

The frequency of occurrence of animal- and plant-derived food items identified by DNA metabarcoding is summarized in Tables 1, 2, respectively. Animal-derived food items were detected infrequently, although sequences assigned to domestic fowl *Gallus gallus* (Linnaeus, 1758) and wild boar or domestic pig *Sus scrofa* Linnaeus, 1758, were identified (Table 1). A total of 15 plant taxa were detected, including four species of Fabaceae and six species of Poaceae (Table 2). Sequences assigned to common wheat *Triticum aestivum* Linnaeus, 1753, a species not recorded at the study site, were also detected (Table 2).

Table 1. Frequency of occurrence of animal-derived dietary items detected by DNA metabarcoding targeting the *COI* region in the gut contents of *Gryllus bimaculatus*.

Scientific name	Common name	Family	Frequency of occurrence*
Allopeas gracile	Land snail	Achatinidae	16.7% (1)
Gallus gallus	Domestic fowl	Phasianidae	33.3% (0)
***Prenolepis* sp.**	Ant	Formicidae	16.7% (0)
Sus scrofa	Wild boar or domestic pig	Suidae	33.3% (0)

*Values in parentheses indicate the presence or absence of each taxon in the single male individual sampled.

Discussion

Stable isotope analysis showed that *G. bimaculatus* tended to have higher $\delta^{15}N$ values than the herbivorous grasshopper *A. thalassinus tamulus*. Assuming that *A. thalassinus tamulus* represents a herbivorous primary consumer, the trophic position of *G. bimaculatus* was estimated to be approximately 2.5. The estimated trophic position is similar to that of katydids *Gampsocleis mikado* Burr, 1899 (Chikaraishi et al. 2014). However, because *A. thalassinus tamulus* may not be an exclusively herbivorous baseline, this estimate may be conservative and could underestimate the actual trophic position of *G. bimaculatus*.

In contrast, gut-content DNA metabarcoding detected a broad range of plant taxa, with particularly high frequencies of occurrence for Poaceae and Fabaceae, whereas animal-derived DNA was detected only infrequently. This apparent contrast between the stable isotope and metabarcoding results may reflect occasional or opportunistic consumption of animal material by *G. bimaculatus*. For example, wild individuals may consume animal-derived resources only when easily accessible items, such as insect carcasses or vulnerable arthropods, are encountered. Occasional consumption of nitrogen-rich animal material could elevate $\delta^{15}N$ values despite the rare detection of animal-derived DNA in gut contents. Alternatively, the two methods reflect different temporal windows: Gut-content DNA metabarcoding mainly captures recently ingested material, whereas stable isotope values integrate assimilated dietary resources over a longer period.

The higher $\delta^{13}C$ values of *A. thalassinus tamulus* compared with *G. bimaculatus* are consistent with greater reliance on C4 plants by the grasshopper. Differences in $\delta^{13}C$ among the plants are determined by isotopic fractionation of carbon during photosynthesis (Quinby et al. 2020). Consequently, C3 plants generally have lower $\delta^{13}C$ values than C4 plants, and these differences are largely reflected in the tissues of herbivorous insects. Together with the DNA metabarcoding results, these findings indicate differences in plant resource use between the two species, with *A. thalassinus tamulus* primarily associated with C4 plants and *G. bimaculatus* exploiting a broader range of plant resources, predominantly C3 plants. This pattern is consistent with previous DNA metabarcoding studies showing that both *G. bimaculatus* and *Locusta migratoria* (Linnaeus, 1758) consume a wide diversity of plant species (Cruz et al. 2026).

Gut-content DNA metabarcoding also detected sequences assigned to *Sus scrofa* and domestic fowl. Although DNA metabarcoding could not distinguish between wild boar and domestic pig for sequences assigned to *Sus scrofa*, these sequences may have originated from anthropogenic food resources, such as discarded food waste or pet food provided for free-ranging cats. Similarly, some plant taxa detected in the gut contents, such as maize and wheat, may also reflect human-derived food resources rather than naturally occurring vegetation. This interpretation is plausible because the crickets were collected in a park where garbage bins were present and free-ranging cats were frequently observed. Thus, at least some of the dietary DNA detected in this study may reflect the use of anthropogenic resources in a human-modified habitat. Crickets commonly occur in human-modified environments, such as parks, gardens and agricultural fields (Bidau 2014). The broad and opportunistic diet of *G. bimaculatus* may facilitate its use of such habitats by allowing individuals to exploit a wide range of naturally occurring and anthropogenic food resources. Although the present data do not allow us to determine whether the detected vertebrate or crop-derived DNA was directly ingested as food, these results suggest that anthropogenic resources could contribute to the diet of wild *G. bimaculatus* in urban or peri-urban environments.

Although artificial diets such as cat food, rabbit food, rodent food and ornamental fish food are commonly used for laboratory rearing of *G. bimaculatus*, our results suggest that caution

Table 2. Frequency of occurrence of plant-derived dietary items detected by DNA metabarcoding targeting a chloroplast barcode region in the gut contents of *Gryllus bimaculatus*.

Scientific name	Common name	Family	Frequency of occurrence*
Alysicarpus vaginalis	Alyce clover	Fabaceae	83.3% (0)
Bothriochloa ischaemum	Yellow bluestem	Poaceae	50.0% (0)
Capillipedium parviflorum	Scented-top grass	Poaceae	50.0% (0)
Desmanthus illinoensis	Illinois bundleflower	Fabaceae	50.0% (1)
Digitaria violascens	Violet crabgrass	Poaceae	16.7% (0)
Euphorbia hirta	Pillpod sandmat	Euphorbiaceae	50.0% (0)
Glycine max	Soybean	Fabaceae	83.3% (1)
Livistona humilis	Sand Palm	Arecaceae	16.7% (1)
Medicago lupulina	Black medick	Fabaceae	16.7% (0)
Oenothera flava	Yellow evening primrose	Onagraceae	16.7% (0)
Oryza rufipogon	Brownbeard rice	Poaceae	50.0% (1)
Oxalis pes-caprae	Bermuda buttercup	Oxalidaceae	16.7% (0)
Phyla nodiflora	Turkey tangle frogfruit	Verbenaceae	16.7% (0)
Triticum aestivum	Common wheat	Poaceae	83.3% (1)
Zea mays	Corn	Poaceae	66.7% (1)

* Values in parentheses indicate the presence or absence of each taxon in the single male individual sampled.

is needed when selecting diets for laboratory experiments. Because wild individuals appear to rely mainly on plant-derived resources, with only occasional use of animal-derived material, animal-based diets may not fully reflect the nutritional environment experienced by this species in nature. This view is also consistent with previous studies on other cricket species showing that herbivore-oriented diets can be more suitable for growth and survival than cat food-only diets (Judge et al. 2010, Ogita et al. 2021). For some experimental purposes, incorporating plant-based or herbivore-oriented food items may therefore provide a more ecologically relevant rearing condition. More broadly, our study highlights the importance of understanding the field ecology of laboratory model organisms when interpreting the biological significance of laboratory results.

Because this study was based on a small sample from a single locality, we could not evaluate dietary variation between sexes or among developmental stages. Future studies should examine wild populations across multiple habitats and multiple time points and incorporate comparisons among sexes and life stages. Combining gut-content DNA metabarcoding with vegetation surveys would also make it possible to distinguish selective feeding from opportunistic feeding on locally abundant plants. Future vegetation surveys should also consider microhabitat variation within the park, as crickets may preferentially feed on plants occurring in specific vegetation strata or micro-environments.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

All experimental procedures in this study were conducted in accordance with the guidelines for the use of animals in research in Kagoshima University.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.

Regarding the use of AI in the preparation of this manuscript, the authors declare the following: GPT

Used for: Language, style and writing

The authors used ChatGPT (OpenAI) to improve the English language and readability of the manuscript.

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Author contributions

T. K. conceived the study, collected and analysed the data, interpreted the results, and wrote the manuscript. M. K. confirmed the occurrence of plant species at the study site and identified the plant samples. Both authors reviewed and approved the final manuscript.

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Data availability

The gut-content DNA metabarcoding data for Gryllus bimaculatus have been deposited in the DDBJ Sequence Read Archive under BioProject accession number PRJDB19985. Other data available within Supplementary materials.

Supplementary material 1

Author: Takashi Kuriwada, Motohiro Kawanishi

Data type: txt

Explanation note: Raw $\delta^{13}C$ and $\delta^{15}N$ values for all plant and insect samples used in the stable isotope analysis, including *Gryllus bimaculatus* and *Aiolopus thalassinus tamulus*.

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