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Ingestion and excretion of nitrogen by larvae of a cabbage armyworm: the effects of fertilizer application

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Running title: Nitrogen excretion by *Maestro brassicae* larvae

Abstract

15

1 Insect frass has significant impacts on decomposition and nitrogen (N) dynamics in soil. Although the frass contained N with various forms that may differently influence N dynamics in the decomposition process, it is poorly understood how the N form in the insect frass is influenced by host plant quality.

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2 This study examined the effects of application of fertilizer on leaf quality of *Brassica rapa* L. var. *perviridis* Bailey (Brassicaceae), and on the consumption, frass excretion, and frass quality of its insect pest, *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae), with a particular focus on dynamics of inorganic N.

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3 *Brassica rapa* increased total N concentration, and accumulated inorganic N (i.e., NO_3^- -N and NH_4^+ -N) into the leaves in response to application of fertilizer.

4 While amount of leaf consumption and excreted frass of *M. brassicae* was not affected by the levels of fertilization, frass quality was evidently influenced by host plant quality altered by fertilization. The frass contained high levels of total N, NO_3^- -N, and NH_4^+ -N under high fertilization. In particular, the larvae excreted much

30 more NH_4^+ -N than that ingested. We discussed on the relationship between host plant quality and insect frass quality, and suggested that the potential effects of such frass characteristics altered by the host plants on decomposition and nutrient dynamics.

Keywords Ammonium, frass, nitrate, nitrogen metabolism, nutritional ecology,

35 plant-insect interaction.

Introduction

There is increasing evidence that the consumption of living foliage by herbivorous insects has significant impacts on ecosystem processes (Belovsky & Slade, 2000; Hunter, 2001; Weisser & Siemann, 2004). Deposition of insect excrements (i.e., frass and honeydew) is one of the pathways affecting decomposition process and nutrient dynamics in soil (Hunter, 2001; Weisser & Siemann, 2004). Insect frass contains more labile carbon than does leaf litter (Lovett et al., 2002). Therefore, it can stimulate microbial growth in the soil (Frost & Hunter, 2004), which subsequently affects nitrogen (N) mineralization or immobilization (Lovett & Ruesink, 1995; Frost & Hunter, 2007). Thus, insect herbivores can play important role influencing N dynamics in soil, i.e., transforming organic N into inorganic N and vice versa, through frass decomposition process.

On the other hand, frass of herbivorous insects contains N with various forms (Cochran, 1985; O'Donnell, 2008). Proteins and amino acids are detected in insect frass, and they are regarded as excretion of unabsorbed, excessive amounts of proteins and

amino acids derived from the diet (Cochran, 1985). Uric acid (and related compounds such as allantoin and allantoic acid) is known as principal end-products of N metabolism in terrestrial insects (Craig, 1960; Cochran, 1985). Nitrogenous excretion

55 by uric acid will be an evolutionary consequence for water conservation in terrestrial animals; uric acid requires less water than ammonia and urea for excretion (Wright, 1995). Nevertheless, ammonia is commonly detected in the frass of terrestrial insects (Cochran, 1985; Lovett & Ruesink, 1995; Kuzhivelil & Mohamed, 1998; Lovett et al., 2002), and it was ca. 9-27 % of total N in the frass (Lovett & Ruesink, 1995; Kuzhivelil

60 & Mohamed, 1998). This indicates that plant organic N (proteins and amino acids) ingested by herbivorous insects is transformed to some extent into inorganic form during N metabolism of insects, prior to the decomposition of frass deposited to the soil.

Hence, understanding the effects of insect herbivores on N dynamics (mineralization and immobilization) through frass excretion will be incomplete without knowledge on

65 N form in the frass. In addition, composition of such N compounds in the frass of herbivorous insects is expected to vary as their diet quality, because N use efficiency of herbivorous insects is altered by amount of N in the host plants (Slansky & Feeny, 1977;

Simpson & Raubenheimer, 2001; Giertych et al., 2005). However, it is poorly understood the effects of plant quality on N form in the frass of herbivorous insects, which would potentially influence decomposition process and nutrient dynamics in soil.

The present study investigated the relationship between N status of the host plant and frass of the herbivorous insect, with a particular focus on total N and inorganic N concentration, in various fertilization levels. We examined the effects of application of fertilizer on leaf quality of a vegetable crop, *Brassica rapa* L. var. *perviridis* Bailey (Brassicaceae), and on the consumption, frass excretion, and frass quality of its insect pest, cabbage armyworm, *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae).

Materials and Methods

80 Culture of *B. rapa*

Brassica rapa plants (Rakuten, Takii Syubyo Co. Ltd., Kyoto, Japan) were individually grown in 500ml pots using nutrient-rich compost (Tanemaki-baido, Takii Syubyo Co. Ltd., Kyoto, Japan) as growth medium in a glass-shield greenhouse at 25°C under

natural light conditions. After seeding, plants were watered daily. The plants were

85 fertilized with liquid fertilizer (HYPONeX; N:P:K = 6:10:5, HYPONeX JAPAN Co. Ltd., Osaka, Japan). We used NPK fertilizer instead of N fertilizer, because our aim is to understand the effects of nitrogen addition on quality of host plant and insect frass without limitation of other nutrient elements, such as phosphorus. It is known that absorption and utilization of one nutrient element in plants and insects are affected by

90 amount of other nutrient elements (Sternner & Elser, 2002; Huberty & Denno, 2006). As our experimental treatments include relatively high fertilization levels (see below), only N fertilization may result in insufficient supply of other nutrient elements. Four fertilizer levels were established: (1) high: 30-fold dilution, (2) medium: 100-fold dilution, (3) low: 300-fold dilution, (4) none (control): water only. This fertilizer is

95 usually used with 500-fold dilution for culture of vegetables. Two weeks after seeding, when the plants reached the four-true-leaf stage, 50ml of fertilizer solution or 50ml of water were added to individual pots at one-week intervals. Plants were grown for 4 weeks, and then used for the analyses of leaf quality and feeding trials described below.

100 **Leaf quality of *B. rapa***

Leaf N analyses were conducted for randomly selected 12 individuals of *B. rapa* for each treatment. Two mature leaves without petiole were collected from each individual.

They were oven dried at 60°C for 72 h and ground to a fine powder. Leaf total N concentration was determined using an elemental analyzer (JM 1000CN, J-Science Co.

105 Ltd., Kyoto, Japan). Leaf nitrate-N (NO_3^- -N) and ammonium-N (NH_4^+ -N) were extracted using 1.5 mol/l KCl and concentrations were determined using a continuous flow analyzer (Integral Futura, Alliance Instruments, Frépillon, France).

Consumption, frass excretion, and frass quality of *M. brassicae*

110 *Mamestra brassicae* was from a laboratory population at the Center for Ecological Research, Kyoto University. Egg clusters were placed individually in petri dishes (9 cm in diameter) in an environmental chamber at 25°C with a 16L8D light cycle. The hatched larvae were reared together until the third instar, and thereafter five larvae were reared per petri dish. Prior to the feeding trials, larvae were provided with artificial diet
115 (Insecta LFS, Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan). Randomly selected

sixth (last) instar larvae were used for the feeding trials. It is known that most (60-80%) of the food consumption of immature stage occurs during the last instar larvae in Lepidoptera (Furuno, 1964; Scriber & Slansky, 1981). Hence, consumption and frass excretion during this larval period would be important on N dynamics from host plants

120 to insect frass. The larvae were kept for 12 h without diet before the feeding trials to excrete the frass of artificial diet origin. Each larva was placed in a petri dish (14.5 cm in diameter) with one or two mature, petiole-removed leaves of *B. rapa* from one each of the four fertilization treatments described above. The *B. rapa* leaves and *M. brassicae* larvae were weighed prior to the feeding trials. The larvae were reared for 48 h in the

125 environmental chamber, and then *B. rapa* leaves were removed and the larvae were kept for 12 h without diet to excrete the frass in the gut. Thereafter, larval frass excreted during the feeding trial was collected. *Brassica rapa* leaves, and frass were oven dried at 60°C for 72 h to determine dry weight. Twenty replicates were conducted for each treatment. Consumed leaf mass was determined as the difference in leaf dry mass

130 between the start and the end of the experiment. Dry mass of *B. rapa* leaves and *M. brassicae* larvae at the start of the experiment were estimated from their water contents,

which were measured using extra samples. The water contents were determined from the difference between fresh and dry mass which was measured after oven dried at 60°C for 72 h (n = 12 for *B. rapa* leaves for each treatment, and n = 15 for *M. brassicae* larvae).

For N analyses, frass was ground to a fine powder. Total and inorganic N in frass were determined by the same methods as for the analyses for *B. rapa* leaf N. Frass from 10 replicates for each treatment was used for total N analysis and that from another 10 replicates was used for inorganic N (i.e. NO_3^- -N and NH_4^+ -N) analysis, because one frass sample did not have sufficient mass to measure both total and inorganic N.

Statistical analysis

All comparisons were tested by one-way ANOVA with the Tukey-Kramer HSD test ($P < 0.05$). Percentage data were arcsine-square root transformed prior to analysis. The excretion efficiency for consumed biomass, total N, NO_3^- -N, and NH_4^+ -N was shown by regression plots with treatment averages, and the relationships between ingested and excreted mass were tested by regression analysis. All analyses were conducted using

JMP version 6 (SAS Institute Japan, Tokyo, Japan).

150 Results

Leaf quality of *B. rapa*

The water content of *B. rapa* leaves was not affected by the fertilization level (ANOVA; $F_{3,47} = 1.31$, $P = 0.28$), and the mean percentage of leaf water was 87.4 ± 0.3 % (SE).

155 Fertilization significantly affected total N, NO_3^- -N, and NH_4^+ -N concentration in leaves (ANOVA: $F_{3,47} = 19.3$, $P < 0.0001$ for total N; $F_{3,47} = 13.3$, $P < 0.0001$ for NO_3^- -N; $F_{3,47} = 16.6$, $P < 0.0001$ for NH_4^+ -N; Fig. 1a-c). Total N concentration was highest in the high fertilization treatment, followed by the medium fertilization treatment. Plants in the low and no fertilization treatments had the lowest percentages of total N, and there was no significant difference between these two treatments (Fig. 1a). NO_3^- -N concentration was highest in the high fertilization treatment followed by the medium fertilization treatment. NO_3^- -N concentration did not differ between the low and no fertilization treatments (Fig. 1b). Plants in the high fertilization treatment had a significantly higher percentage of

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$\text{NH}_4^+\text{-N}$, compared to other three treatments. $\text{NH}_4^+\text{-N}$ concentration did not significantly
165 differ among the medium, low, and no fertilization treatments (Fig. 1c).

Consumption, excretion, and frass quality of *M. brassicae*

The larval dry mass at the start of the experiment did not differ significantly among the
four treatments (ANOVA: $F_{3,79} = 1.05$, $P = 0.38$, overall mean \pm SE = 86.1 ± 1.0 mg).

170 The larvae consumed at most 70 % of biomass of the leaves provided for the feeding
trials, and this indicates that food shortage did not occur during the experiment. The dry
mass of *B. rapa* leaves consumed by *M. brassicae* did not significantly differ among the
treatments (ANOVA: $F_{3,79} = 0.79$, $P = 0.50$; Fig. 2a). Amount of frass excreted by *M.*
brassicae did not differ among the treatments (ANOVA: $F_{3,79} = 1.85$, $P = 0.14$; Fig. 2b).

175 There was no relationship between ingested and excreted mass (Fig. 2c), and on average,
 $50.2 \pm 3.4\%$ of ingested food was excreted as frass.

Total N concentration in frass differed significantly among the treatments ($F_{3,39} =$
 14.2 , $P < 0.0001$; Fig. 3a). Frass excreted by the larvae in the high fertilization
treatment had the highest level of N, followed by that from larvae in the medium

180 fertilization treatment. Frass N in the no fertilization treatment was the lowest. NO_3^- -N
and NH_4^+ -N concentrations in frass were also affected by the fertilization treatment
(ANOVA: $F_{3,39} = 6.16$, $P = 0.0017$ for NO_3^- -N; $F_{3,39} = 10.2$, $P < 0.0001$ for NH_4^+ -N; Fig.
3b and c). Frass in the high fertilization treatment had the highest levels of NO_3^- -N and
 NH_4^+ -N, and frass in the low and no fertilization treatments had the lowest levels.

185 Excreted mass of N and NO_3^- -N increased linearly in response to ingested each of
them (Fig. 4a and b). Overall, $54.6 \pm 3.8 \%$ and $60.4 \pm 4.4 \%$ of the ingested N and
 NO_3^- -N was excreted as frass, respectively. While there was no significant relationship
between ingested and excreted NH_4^+ -N, the larvae excreted more NH_4^+ -N than ingested
one (Fig. 4c). On the other hand, there was significant relationship between ingested N
190 and excreted NH_4^+ -N, and excreted NH_4^+ -N increased in response to ingested N (Fig.
4d).

Discussion

195 **Accumulation of inorganic N in *B. rapa* leaves**

The present study clearly showed that *B. rapa* not only increased total N, but also accumulated inorganic N (i.e., NO_3^- -N and NH_4^+ -N) into the leaves in response to the application of fertilizer. The process of N assimilation in plants has been well documented, and summarized by several authors (e.g. Huppe & Turpin, 1994; Crawford, 200 1995). In brief, plants can use both nitrate and ammonia as N resource. While ammonia is directly utilized for synthesis of amino acids, nitrate is first reduced to nitrite by nitrate reductase. Nitrite is then reduced to ammonia by nitrite reductase. Thereafter, ammonia is fixed into glutamate to produce glutamine by the action of glutamine synthetase. When the plants were provided with excess nitrate, the nitrate is stored in 205 vacuoles by regulation of nitrate reduction process (Martinoia et al., 1981). The high level of accumulation of nitrate in *B. rapa* leaves observed in the high fertilization treatment indicates that the fertilization level in this study was excessive beyond the level of nitrate that *B. rapa* is able to assimilate. Yorifuji et al. (2005) reported that *B. rapa* plants in Japanese markets have on average 4,060 ppm nitrate (n=197, range: 210 128-9,460 ppm) in fresh weight. This indicates that many of *B. rapa* in Japanese

markets contained higher level of nitrate than the maximum level in vegetables (4,500 ppm), which was established by European Commission Regulation (European Commission, 2005). However, *B. rapa* having high nitrate is being circulated because there is no regulation about nitrate in vegetables in Japan. When the NO_3^- -N concentration observed in the present study was converted to the nitrate concentration in fresh weight, the highest concentration was 5,337 ppm for the leaves in the high fertilization treatment. This value is within a range of the concentration of *B. rapa* in Japanese markets. Hence, the fertilization level in the present study appears to be within the range of the fertilization level used in *B. rapa* culture in Japan. In addition to the nitrate accumulation, our results showed that ammonium was also accumulated in the leaves under high fertilization, although the concentration was lower than that of nitrate. Because the fertilizer used in the present study contained both nitrate and ammonium, the fertilization level in this study would be also excessive beyond the levels of ammonium that *B. rapa* is able to synthesize glutamine.

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Consumption, excretion, and frass quality of *M. brassicae*

While a number of studies have dealt with the effects of host plant quality on feeding behaviour, nutrient utilization, and growth of herbivorous insects (e.g. Slansky & Feeny, 1977; Fischer & Fiedler, 2000; Simpson & Raubenheimer, 2001; Giertych et al., 2005; 230 Chen et al., 2007; Hwang et al., 2008; Staley et al., 2009), the effects of host plant quality on the frass quality, in particular inorganic N, have received less attention. However, N forms in the frass would be important to understand the roles of insect herbivores on N dynamics at ecosystem level through the frass excretion.

The present study showed that the *B. rapa* leaf quality altered by fertilization did 235 not affect the amount of leaf consumption and excreted frass of *M. brassicae* larvae. One reason why the plant quality did not affect the feeding and excretion behaviour of the insect would be a short time period for the feeding trials. While *M. brassicae* larva takes approximately one month until pupation at our rearing condition (H. Kagata, personal observation), the feeding trial was conducted only for 48h with last instar 240 larvae. Therefore, our experimental design would not be sufficient to detect the effects of host plant quality on such behaviour.

On the other hand, the present study clearly demonstrated that quality of *M. brassicae* frass was influenced by host plant quality, and that the frass had high levels of total N, NO_3^- -N, and NH_4^+ -N as a result of the altered quality of the host plants under high fertilization. Inorganic N in the frass mostly comprised ammonium form, and it was from 9 % (in no fertilization) to 27 % (in high fertilization) of total N in the frass (see Fig. 3). The rest would be organic N such as amino acids and proteins, which were unabsorbed in the insect gut, and uric acid and related compounds as end-products of N metabolism (Cochran, 1985; Lovett et al., 2002). NO_3^- -N in the frass is likely to be diet origin, rather than N metabolism product of the herbivorous insect, because the amount of excreted NO_3^- -N was less than that ingested, and it was well explained by amount of ingested NO_3^- -N. In addition, there is no report, in our knowledge, that nitrate was produced as subsequence of N metabolism in herbivorous insects. In contrast, NH_4^+ -N in the frass would originate from N metabolism by the insect, rather than diet origin. The amount of excreted NH_4^+ -N was as from 5- to 17-fold greater as that ingested (see Fig. 4c), and amount of excreted NH_4^+ -N as the frass was explained by amount of ingested N rather than by ingested NH_4^+ -N (see Fig. 4c and d). These indicate that high

levels of N in the host plants would promote the N metabolism, and subsequently accelerate ammonium excretion of the herbivorous insect.

260 Thus, we concluded that N in the host plant largely influenced insect frass quality, especially ammonium concentration, by enhancing the metabolic process in the herbivorous insect. Such frass characteristics altered by the host plants would differently influence decomposition and nutrient dynamics in soil. In general, N is one of the factors that control decomposition rate, and the substrates with high N are more rapidly
265 decomposed (Hättenschwiler et al., 2005). Nitrate and ammonium in the frass may also have impacts on nutrient dynamics, because they are the form that plants can directly utilize without time lag necessary for N mineralization in usual decomposition process. In addition, it is known that the frass quality differed depending on insect species, even when they fed on same host plant (Madritch et al., 2007). Therefore, effects of insect
270 frass on decomposition and nutrient dynamics may be more variable than we expected from the frass decomposition experiments using a single plant-insect interaction (e.g. Lovett & Ruesink, 1995; Christenson et al., 2002; Frost & Hunter, 2004, 2007). Further studies to clarify the effects of insect frass quality on the decomposition and nutrient

dynamics will contribute to generate a general picture of how herbivores can influence
275 ecosystem processes.

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Figure Legends

365 Fig. 1: (a) total N concentration, (b) NO_3^- -N concentration, and (c) NH_4^+ -N
 concentration of the *B. rapa* leaves at different fertilization levels. Means \pm SE are
 presented. Different letters indicate a significant difference ($P < 0.05$).

Fig. 2: (a) Mass consumption, (b) frass mass excreted by *M. brassicae* during the 48 h
 feeding trials. Means \pm SE are presented. ns indicates no significant difference (P
 370 > 0.05). (c) efficiency of frass excretion. Efficiency of frass excretion was shown
 by the relationship between ingested and excreted mass. Each point represents the
 treatment average with SE. Dotted line shows $Y = X$. Area above the line
 represents that excreted mass is more than ingested mass, and vice versa.

Fig. 3: (a) total N concentration, (b) NO_3^- -N concentration, and (c) NH_4^+ -N
 375 concentration in the frass of *M. brassicae*. Means \pm SE are presented. Different
 letters indicate a significant difference ($P < 0.05$).

Fig. 4: (a) efficiency of total N excretion, (b) efficiency of NO_3^- -N excretion, and (c)
 efficiency of NH_4^+ -N excretion, and (d) relationship between ingested N and

380 excreted $\text{NH}_4^+\text{-N}$. Efficiency of excretion was shown by the relationship between
ingested and excreted mass. Each point represents the treatment average with SE.
Dotted line shows $Y = X$. Area above the line represents that excreted mass is more
than ingested mass, and vice versa.

Fig. 1

385

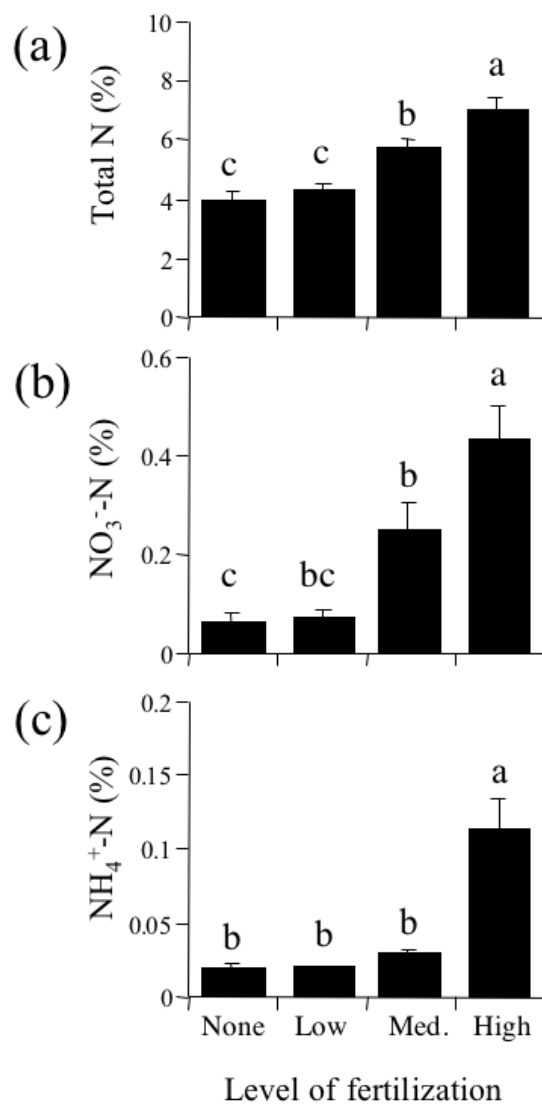
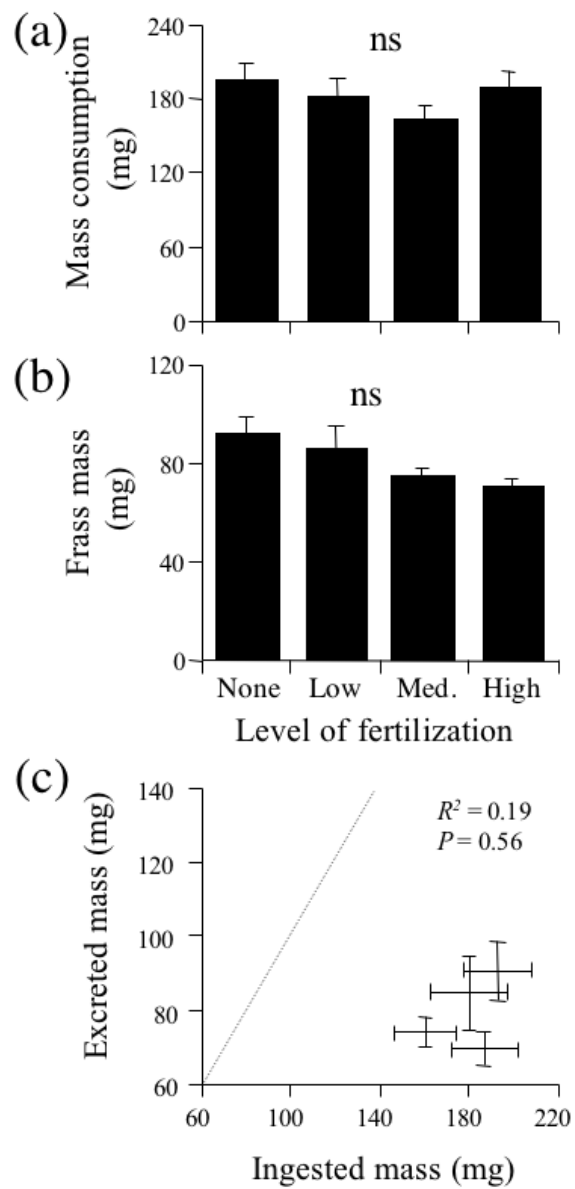


Fig. 2



390 Fig. 3

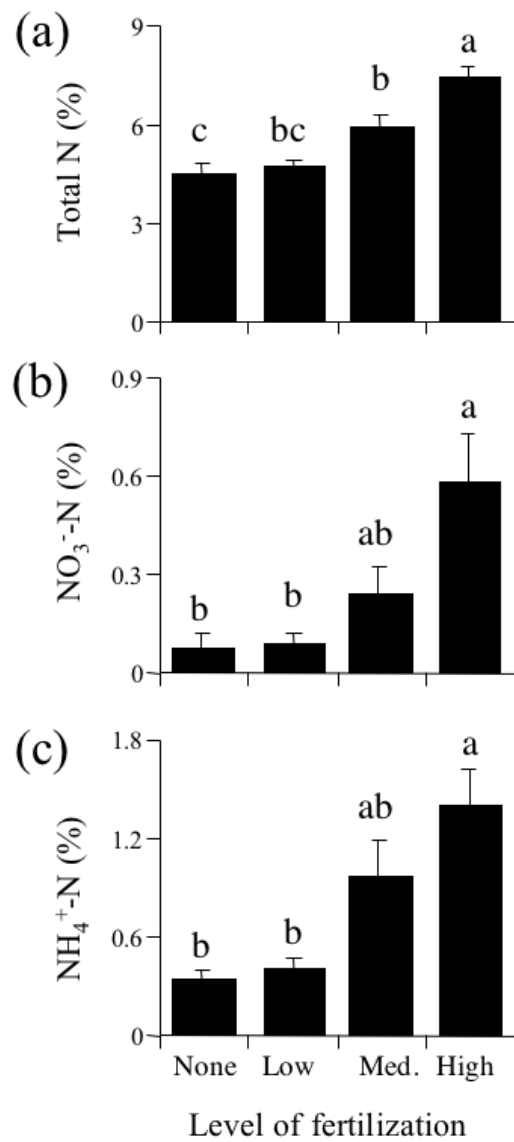


Fig. 4

