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Population density and diet type interactively affect individual growth of an omnivorous soil-dwelling insect (*Anomala cuprea*, Coleoptera: Scarabaeidae)

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Abstract

Density effects are a fundamental ecological question, but their impacts on the individual growth of insects are highly variable. Scarab larvae in soils often occur at high density, but density effects of their population are rarely reported. We examined how the density of the first instar larvae of the soil-dwelling omnivore *Anomala cuprea* Hope (Coleoptera: Scarabaeidae) affected their growth when fed two diet types in which carbon to nitrogen ratio (C:N ratio) differs. The C:N ratio was used as a parameter of diet quality in the context of ecological stoichiometry. The larvae were grown for 34 days at three population densities (one, three, or five larvae per cup) and fed two diet types (humus as a low C:N ratio diet or wood flakes as a high C:N ratio diet). An increase in population density reduced larval growth under the low C:N ratio diet, but it enhanced larval growth under the high C:N ratio diet. Larval mortality was always low, but it was observed only at a population density of three or five larvae. Compensatory growth, gut symbionts, and hormesis are discussed as possible mechanisms of these results. In nature, larvae occur at high density and the C:N ratio of their diets is low. Therefore, our results suggest that high population density will have positive effects under natural conditions.

Keywords C:N ratio | ecological stoichiometry | larval density | mortality

1. Introduction

The growth of arthropods depends on the balance between available diets and the essential elements of their development. Ecological stoichiometry is an elementary approach to evaluating the balance by measuring nutrient flows in a system (Sterner & Elser 2002). The carbon (C) to nitrogen (N) ratio (C:N ratio) is one of the most studied parameters of diet quality in the context of ecological stoichiometry because C and N are the first and second most abundant elements in the bodies of plants and arthropods, but the ratio is quite different between plants and arthropods (Sterner & Elser 2002, Schoonhoven et al. 2005, Hessen et al. 2013, Sardans et al. 2021). The differences in element compositions between plants and herbivores and/or decomposers determine the nutritional limitations imposed on the arthropods (Filipiak & Weiner 2017). Thus, a way to explain nutritional differences of diets for arthropods is the C:N ratio.

In response to growing interest in the ecosystem functions performed by soil macrofauna, scientists are also increasingly focusing their attention on diverse soil macrofauna although the most well-studied macrofauna is earthworm (Johnson & Rasmann 2015, Tsunoda & van

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Dam 2017, Gan & Wickings 2020, Mathieu et al. 2022). The feeding habits of scarab larvae in soils are interesting because they are able to feed upon diets with low C:N ratios, such as living plants, as well as diets with high C:N ratios, such as decay wood (Tsunoda et al. 2017, 2018). To successfully use high C:N ratio diets, scarab larvae may have some strategies. Larvae of *Popillia japonica* (Coleoptera: Scarabaeidae) show cannibalism, which can supply nutrition to promote their growth (Régnière et al. 1981). In contrast, there was no evidence of cannibalism and combat between grey-back canegrubs, *Dermolepida albohirtum* (Coleoptera: Scarabaeidae), or between *Phyllophaga hogei* larvae (Coleoptera: Scarabaeidae) (Logan & Kettle 2002, Ramírez-Corona & Morón-Ríos 2007). These suggest that scarab larvae also have adaptive mechanisms to high C:N ratio diets, such as fixing atmospheric nitrogen by symbiotic microbes (Johnson & Rasmann 2015, Douglas 2013, Franzini et al. 2016), or as seen in other organisms such as recycling nutrients from its uric acid in the cockroach (Patiño-Navarrete et al. 2014).

Density effects are one of fundamental ecological questions, and different densities of a population affect the nutritional acquisition of individuals (Begon et al. 1996, Sutherland et al. 2013). Increasing population density can either positively or negatively affect the individual performances. High density often causes negative effects through competition between individuals, which means increased population density can contribute to reduced individual nutrient acquisition. For example, the high population density of the cinnabar moth larvae, *Tyria jacobaeae* (Arctiidae), negatively affects larval survival when its diet is restricted (Crawley & Gillman 1989). However, crowded populations sometimes have positive effects on the individual performance; for example, some insects overwhelm defensive secretory canals in leaves because their collective feeding behavior minimizes the intake of defensive substances per capita (Dussourd & Denno 1991). Density effects may be important also for soil macrofauna because the two functions of habitat and diet are present in soils (Lavelle 2012). However, we are not aware of any other study where density effects of scarab larvae have been examined in different diet types.

In this study, we examined the density effects of first instar larvae of the soil-dwelling omnivore *Anomala cuprea* Hope (Coleoptera: Scarabaeidae) under two diet types of different C:N ratio. The larvae of *A. cuprea* feed on roots of various herbaceous species as well as plant litter and soil organic matter (Tsunoda et al. 2017, 2018). The larvae are agricultural pests of crops such as sweet potato and turnip (Okuno et al. 1978, Sakai & Fujioka 2007). The low C:N ratio diet used in this study had a C:N ratio of 50:1 (Appendix A), of which C:N ratio is the same as the C:N ratio of common litter such as grass hay although the C:N ratio of legume litters is about 10:1 (Brust 2019). On the other hand, the high C:N ratio diet used in this study had a C:N ratio of 150:1, so it was used as a food with a significantly high C:N ratio (Appendix A). The C:N ratios of these diets differ three times, therefore we referred to as low C:N and high C:N ratio diets for convenience.

We hypothesized that the effects of increased population density of *A. cuprea* on mortality and individual growth depend on C:N ratio of their diets. We predicted that more individuals would die under a high C:N ratio diet under the low density conditions due to malnutrition. If the density is high, cannibalism occurs under a high C:N ratio diet, thus, the coefficient of variation (CV) of the larval fresh weight in a cup is larger than that under a low C:N ratio diet because some individuals grow better. Under a low C:N ratio diet, we predict that growth is better in low density conditions because competition occurs in high density conditions.

2. Materials and methods

2.1 Study species

The larvae of *A. cuprea* were prepared from eggs laid in humus (commercial horticulture humus mainly composed of broad leaves, 'Kanjuku-fuyodo', Hirota-shoten Co., Ltd., Tochigi, Japan) in the laboratory of Tokyo Metropolitan University by adult *A. cuprea* females collected from a floodplain of the Tama River (35°38'N, 139°23'E) in July 2015. In this experiment, the first instar larvae were used because the small body size could be affected by differences in diet types.

2.2 Growth experiment

The first instar larvae were grown in a two-way factorial fully randomized design with eight replications in a growth chamber (Koitotron, Koito Industries, Ltd., Kanagawa, Japan) with a 14-h day/10-h night photoperiod at 23°C. The two factors were population density and diet type. The population density treatment had three levels: one, three, or five larvae per cup. The diet type treatment had two levels: humus (the abovementioned commercial horticulture humus; sieved through 6-mm mesh) as the low C:N ratio diet or wood flakes ('Kunugi-daioh' containing mainly *Quercus acutissima*; Mitani Co., Ltd., Ibaraki, Japan) as the high C:N ratio diet. In each diet type, eight larvae were totally used in the one larvae per cup conditions, 24 larvae in the three larvae per cup conditions, and 40 larvae in the five larvae per cup conditions. Therefore, 144 larvae were used in total during this study.

The larvae were weighed and added at the specified density to a 200-mL plastic cup (9-cm diameter and 12-cm depth) with 150 mL of low or high C:N ratio diet. Tap water was added daily to maintain moisture, but no additional diet was provided. The larval survival and fresh weight were recorded at 12, 20, 27, and 34 days after the experiment began. There was a 12-day interval between setting up the experiment and the first measurement, as the larvae were considered to be particularly small and susceptible to shock. Thereafter, measurements were taken every week or so. Even if no dead larva was found, we recorded mortality if the number of living larvae decreased. We calculated mean fresh weight of individual larvae per cup at each time point because we could not distinguish each individual before and after the experiment. The CV of the larval fresh weight in a cup was calculated for the three- and five-larvae density conditions. The CV was used as an index of size variance in a cup. Total C and N of the diets before and after the experiment were determined based on four randomly chosen replicates using an elemental analyzer (CN coder MT-700, Yanaco, Kyoto, Japan).

2.3 Statistical analysis

The effects of the treatments on larval mortality rate were analyzed by using a generalized linear mixed model (GLMM), assuming a binomial error distribution with a logit-link function. In this model, the response variable was the numbers of dead and survived larvae in each cup, and the explanatory variables were the population density, diet type, and their interaction. The random effect was a cup. The log-transformed evaluated day was treated as an offset term in the model.

The effects of treatments on gain in larval fresh weight were analysed by a linear mixed model (LMM) assuming a gaussian error distribution with a canonical-link function. In this model, the response variable was the difference between the fresh weight of the evaluated day and the initial fresh weight was treated as the response variable. The explanatory variables were population density, diet type, and the interaction between them. The random effect was a cup. The log-transformed evaluated day was treated as an offset term in the model.

The CV of larval fresh weight in a cup was analyzed by a LMM assuming a gaussian error distribution with a canonical-link function. In this model, the response variable was the difference between CV of larval fresh weight on the recorded day and initial CV of the larval fresh weight, and the explanatory variables were population density, diet type, and their interaction. The random effect was a cup. The log-transformed evaluated day was treated as an offset term in the model.

The percentages of total C and total N after the experiment were arcsine-transformed and analyzed by two-way analysis of variance (ANOVA). The percentage was treated as the response variable, and the explanatory variables were population density, diet type, and their interaction.

All analyses were performed with the statistical software R version 3.5.1 (R Core Team 2018). The larval fresh weight and CV of larval fresh weight were log-transformed if the homogeneity of variance and/or the normality were not satisfied by the Bartlett test and the Shapiro–Wilks test, respectively. If a larva was not recovered from a cup, the cup was treated as a missing value. The lme4 package (Bates et al. 2015) was used to calculate LMMs and GLMM using maximum-likelihood estimation. To determine the effects of the fixed factors, we used likelihood-ratio tests and the chi-squared test statistic using Anova function in the car package (Fox & Weisberg 2011).

3. Results

3.1 Larval mortality rate

At the end of the experiment, out of eight individuals, no mortality was recorded in both diet types in the one individual per cup conditions. In the three individuals per cup conditions, out of 24 individuals, only one died in the low C:N ratio diet and five in the high C:N ratio diet. In the five individuals per cup conditions, out of 40 individuals, 11 died in the low C:N ratio diet and six in the high C:N ratio diet. The mortality rate of the larvae marginally significant among the population density conditions (Fig. 1, Tab. 1). Higher population densities tended to increase mortality rate. Diet type did not affect mortality (Tab. 1).

3.2 Gain in larval fresh weight and CV of larval fresh weight

The mean gain in larval fresh weight was significantly affected by the diet types and the diet type × population density interaction (Fig. 2, Tab. 2). The larvae fed upon the low C:N ratio diet generally grew larger than those fed upon the high C:N ratio diet, but larval growth did not differ between diets at the highest density. At the end of the experiment, larval growth was better on the high C:N ratio diet than on the low C:N diet in five larvae per cup conditions (Fig. 2). The CV of larval fresh weight was not significantly different depending on the two experimental factors (Fig. 3, Tab. 3).

3.3 Total C and total N of each diet

Treatments affected the total C and total N concentrations of diets after the experiment. The total C concentration of the N-rich diet was again significantly lower than that of the N-poor diet (Tab. 4); in all three density treatments, the total C in the N-rich diet showed a greater decline from the baseline value than that of the N-poor diet (Fig. 4b). The total N concentration of the N-rich diet was again significantly higher than that of the N-poor diet (Tab. 4).

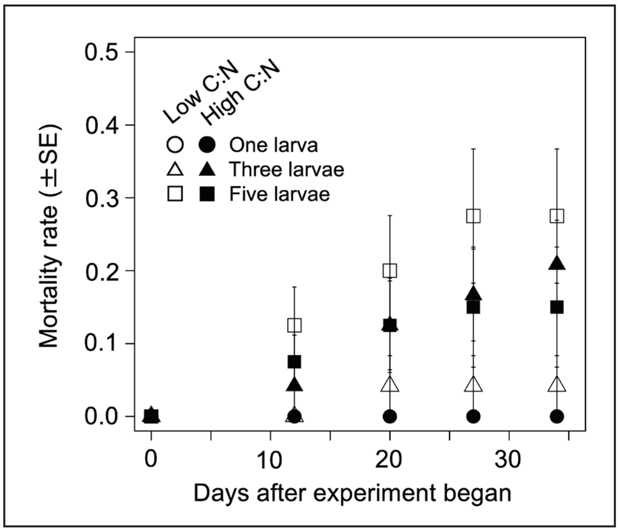

Figure 1. Effects of diet type and population density on larval mortality rate. Error bars indicate standard error. See Tab. 1 for details of statistical results.

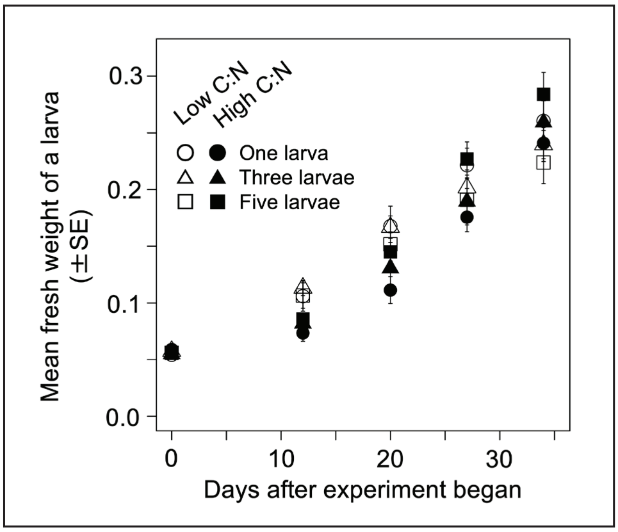

Figure 2. Effects of diet type and population density on mean fresh weight of a larva. Error bars indicate standard error. See Tab. 2 for details of statistical results.

4. Discussion

If the density is high, we predicted that cannibalism occurs under a high C:N ratio diet, thus, the CV of the larval fresh weight in a cup is larger than that under a low C:N ratio diet because some individuals grow better. However, the diet type did not affect mortality (Fig. 1). Moreover, the CV of the larvae in a cup also was not affected by the diet type (Fig. 3, Tab. 3). These results suggest that the first instar larvae of *A. cuprea* do not actively show cannibalism and/or combat with each other, as well as the previous studies on *D. albohirtum* and *P. hogei* (Logan & Kettle 2002, Ramírez-Corona & Morón-Ríos 2007). It should be noticed, however, the cannibalism reported by Régnière et al. (1981) occurred between third instar larvae of the Japanese beetle, thus, the instar differences may affect the nutritional requirement of the insects.

As we predicted, growth was generally better in low density conditions than in high density conditions under a low C:N ratio diet (Fig. 2). However, the prediction that more individuals die under a high C:N ratio diet even under the low density conditions due to malnutrition

Table 1. Effects of population density (PD) and diet type (DT) on larval mortality rate. The binomial error distribution was used in the generalized linear mixed model.

	df	χ2	P
PD	2	5,807	0,055
DT	1	0,000	1,000
PD × DT	2	4,446	0,108

Table 2. Effects of population density (PD) and diet type (DT) on increase in larval fresh weight. Bold letters indicate significant differences at P < 0.05.

	df	χ2	P
PD	2	0,571	0,752
DT	1	16,113	< 0.001
PD × DT	2	8,473	0,014

Table 3. Effects of population density (PD) and diet type (DT) on the coefficient of variation (CV) of larval fresh weight.

	df	χ2	P
PD	1	0,062	0,803
DT	1	0,003	0,959
PD × DT	1	0,132	0,716

was not supported. The N concentration in diet after the experiment increased under all conditions (Fig. 4), which suggest that *A. cuprea* larvae could fix atmospheric nitrogen by microbes in their gut, as known from other scarab species (Johnson & Rasmann 2015, Douglas 2013, Franzini et al. 2016). This mechanism might allow the larvae to survive on a high C:N ratio diet. During the later of the experiment, we observed the compensatory growth under a high C:N ratio diet (Fig. 2). Compensatory growth is a period of accelerated growth (Steinberg, 2018). The larvae used in this experiment was laid in the low C:N ratio diet as egg, thus, all of the larvae would feed upon the low C:N ratio diet before this experiment. The high C:N ratio diet would cause short-term food deprivation, which might be a reason why the larva showed the compensatory growth under the high C:N ratio diet.

Although it is not certain how symbionts are obtained by the larvae of *A. cuprea*, one possibility is coprophagy (e.g., Nalepa et al. 2001, Engel & Moran 2013). In cockroaches, fecal bacteria are responsible for their host aggregation, and cockroaches are thought to acquire gut microbiota via coprophagy (Wada-Katsumata et al. 2015). Coprophagy would explain why, under the high C:N ratio diet, the larvae grew larger with the increase of population density because the volume of faeces increases with population density. Indeed, the CV was larger under the three-larvae condition than under the five-larvae condition (Fig. 3), which indicates that high population density equalizes individual sizes. Another possibility is a hormesis-like effect, which is characterized by a biphasic or triphasic response to exposure to increasing amounts of a substance (Filipiak & Bednarska 2021). Some substances in the diets might have hormesis-like effects. A further experiment is needed to clarify these issues in future.

In conclusion, population density and diet type interactively affected individual growth of first instar larvae of the soil-dwelling omnivore *A. cuprea*. The first instar larvae of *A. cuprea* can grow on both low and high C:N ratio diets, probably because symbiotic microbes fix atmospheric nitrogen. In natural environments, scarab larvae inhabit soil and litter at relatively high densities. In addition, the nutrition of their diets, such as plant roots and litter are usually lower than that of living leaves. Increased population density enhanced individual growth under the high C:N ratio diet, suggesting positive effects of dense populations in natural environments. More studies of *A. cuprea* to the evaluate other possible

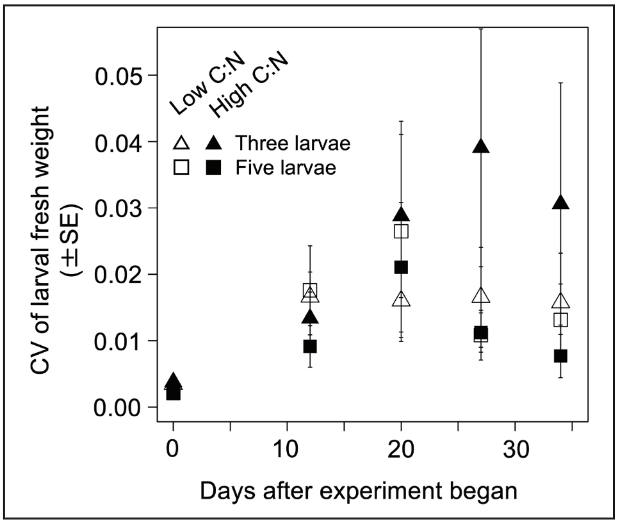

Figure 3. Temporal changes in the coefficient of variation (CV) of larval fresh weight at two population densities and with two diets. Error bars indicate standard error. See Tab. 3 for details of statistical results.

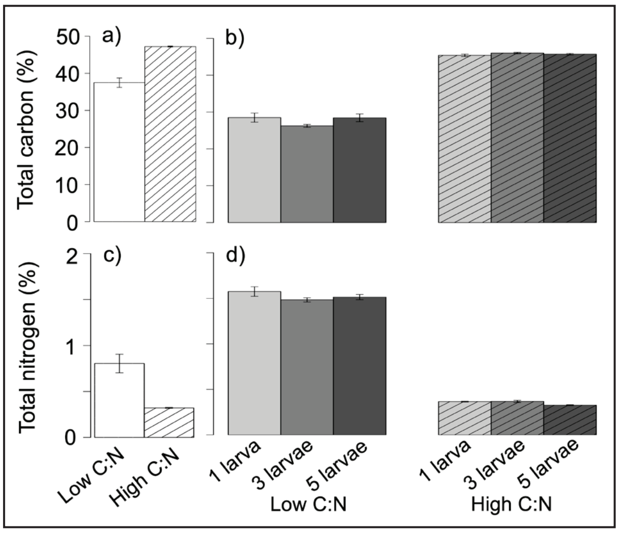

Figure 4. (a) total carbon (%) in the diet before the experiment and (b) after the experiment, and (c) total nitrogen (%) in the diet before the experiment and (d) after the experiment in the two diets and three population densities.

Table 4. Analyses of variance to test the effects of population density (PD), diet type (DT), and their interactions on total carbon (C) and nitrogen (N) after the experiment. Bold letters indicate significant differences at P < 0.05.

		C			N		
	df	**SS**	**F**	**P**	**SS**	**F**	**P**
PD	2	4,05	1,176	0,332	0,010	2,279	0,133
DT	1	2159,04	1252,163	**< 0.001**	7,807	3705,813	**< 0.001**
PD × DT	2	10,43	3,025	0,075	0,009	2,162	0,146

mechanisms with different larval instars are necessary to improve our understanding of this agricultural pest.

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7. Authors' contributions

T.T. designed and carried out the experiments, analyzed data and wrote the first draft of this manuscript. All authors contributed to writing this article.

8. Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A

The carbon (C) and nitrogen (N) ratio of each diet before the experiment. The C:N ratio of the high C:N ratio diet was significantly higher than that of the low C:N ratio diet (*t*-test, $P < 0.001$).