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ORIGINAL PAPER

Elementary budget of stag beetle larvae associated with selective utilization of nitrogen in decaying wood

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Abstract

Wood degradation by insects plays important roles for the forest matter cycling. Since wood is deficient in nitrogen compared to the insect body, wood-feeding insects need to assimilate the nitrogen selectively and discard an excess carbon. Such a stoichiometric imbalance between food and body will cause high metabolic cost; therefore, wood-feeding insects may somehow alleviate the stoichiometric imbalance. Here, we investigated the carbon and nitrogen budgets of the larvae of stag beetle, *Dorcus rectus*, which feed on decaying wood. Assimilation efficiency of ingested wood was 22%, and those values based on the carbon and nitrogen were 27 and 45%, respectively, suggesting the selective digestion of nitrogen in wood. Element-based gross growth efficiency was much higher for nitrogen (45%) than for carbon (3%). As a result, the larvae released 24% of the ingested carbon as volatile, whereas almost no gaseous exchange was observed for nitrogen. Moreover, solubility-based elementary analysis revealed that the larvae mainly utilized alkaline-soluble-water-insoluble fraction of wood, which is rich in nitrogen. Actually, the midgut of the larvae was highly alkaline (pH 10.3). Stag beetle larvae are known to exhibit coprophagy, and here we also confirmed that alkaline-soluble-water-insoluble nitrogen increased again from fresh feces to old feces in the field. Stable isotope analysis suggested the utilization of aerial nitrogen by larvae; however, its actual contribution is still disputable. Those results suggest that *D. rectus* larvae selectively utilize alkaline-soluble nitrogenous substrates by using their highly alkaline midgut, and perhaps associate with microbes that enhance the nitrogen recycling in feces.

Keywords Decaying wood · Assimilation · Stoichiometric imbalance · Nitrogen fixation · Nitrogen recycling

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Introduction

Wood is the most abundant biomass in the terrestrial ecosystem, and its degradation represents the fundamental pathway of material cycling. Wood degradation is caused by both non-biological and biological processes. The non-biological process is mostly represented by leaching with water (Hafner and Groffman 2005), which is sometimes enhanced by corrosive substrates concentrated in wood (Blanchette et al. 1991a, b); however, those abiotic deteriorations are relatively slow. In the biological process of wood degradation, fungi and insects are considered as the key players in many forest ecosystems (Blanchette et al. 1990; Rose et al. 2001; Weslien et al. 2011; Stokland 2012). Wood-rotting fungi chemically decompose structural polymers such as cellulose, hemicelluloses, and lignin, which characterize physical properties of wood. Those chemical and physical modifications will encourage insects to utilize wood as food (Stokland 2012). Moreover, fungal mycelia accumulating in decaying wood also serve as food for some wood-feeding insects (Tanahashi et al. 2009;

Tanahashi and Kubota 2013; Mishima and Araya 2016; Mishima et al. 2016). Insects play a crucial role for the mechanical disintegration of wood by means of boring tunnels in wood and producing frass, which enhances further abiotic and biotic degradation (Ulyshen 2015). Insects also introduce microbial symbionts and/or wood decomposing fungi that help further degradation and digestion of wood (Beaver 1989; Kajimura 2000; Tanahashi et al. 2010; Toki et al. 2012; Jacobsen et al. 2017). Nutrient elements of wood are assimilated by insects and finally released when they emerge from the wood.

Nitrogen is one of the essential nutrient elements for all organisms and its elemental budget can explain a large part of the ecosystem dynamics. Although the nitrogen concentration is very low in wood, the wood-reserved nitrogen represents a large part of the total nitrogen in many forest ecosystems (Martin et al. 2014; Meriem et al. 2016; Rinne et al. 2017). Since the nitrogen is highly concentrated in insect bodies (Ulyshen 2015), insects are thought to represent important carriers of the nitrogen that will otherwise be confined within wood for a long time. However, since wood has a much higher carbon-to-nitrogen (C/N) ratio than the insect body, wood-feeding insects need to assimilate the nitrogen selectively and release an excess carbon by fecal excretion and/or respiration (Higashi et al. 1992). Such a stoichiometric imbalance between the food and body usually causes high metabolic cost (Elser and Hessen 2005). Therefore, wood-feeding insects may somehow alleviate the stoichiometric imbalance, by selective utilization of a highly nutritive part of wood (Filipiak and Weiner 2014), for example.

The Japanese lesser stag beetle, *Dorcus rectus* (Motschulsky) (Coleoptera: Lucanidae), is one of the dominant saproxylic insects in the lowland temperate forests in Japan (Kubota and Kubota 2004). *D. rectus* females deposit eggs on decaying wood infested with white-rot fungi (Araya 1993; Kubota and Kubota 2004). The larvae actively scrape the decaying wood by using their mandibles, whereas they often leave the scraped wood behind in the feeding tunnel, uneaten and pack it tightly, sometimes together with their fecal pellets (Fig. 1a, b). The larvae sometimes eat the wood-fecal remnant afterward (Kamata 2014; Araya 2006); therefore, this behavior is usually explained as an improvement of food quality (conditioning) and/or a reuse of their feces as food (coprophagy). Since the nitrogen is deficient in woody biomass, such behavior is probably aiming to the nitrogen recycling associating with microbial symbionts. Female stag beetles usually harbor the xylose-fermenting yeasts in their mycangium (Tanahashi et al. 2010, 2017; Tanahashi and Hawes 2016) and inoculate the yeasts on wood when deposit eggs. Nitrogen-fixing activity has also been reported in the guts of *D. rectus* larvae (Kuranouchi et al. 2006); however, the estimated value of nitrogen fixation is quite slow; thus, the ecological significance of the nitrogen fixation in stag beetles is needed to be reconsidered.

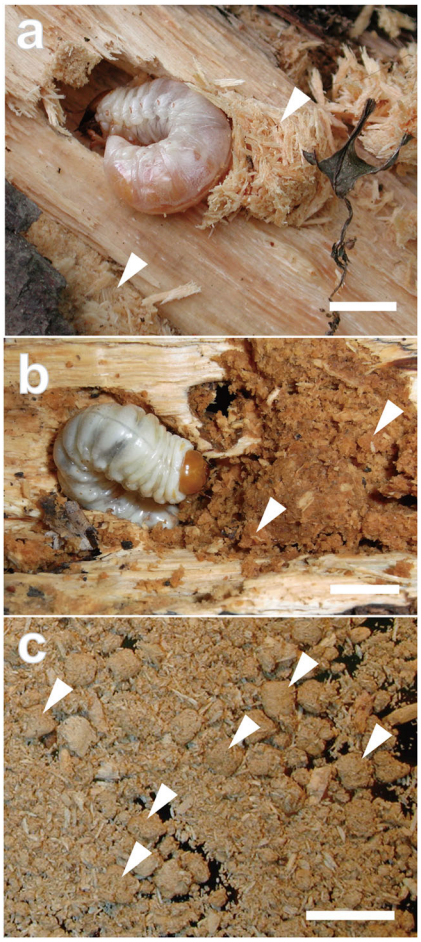

Fig. 1 *Dorcus rectus* third instar larvae. **a** Scraped wood (arrows) packed in the feeding tunnel. **b** Scraped wood mixed with fecal pellets. Arrows indicate the fecal pellets that slightly remained their original shape. **c** Fecal pellets when larvae were being fed with milled decaying wood in laboratory. Scale bars 10 mm

In this paper, we quantified the basic feeding performance and the material budget of *D. rectus* larvae that feed on decaying wood in the laboratory (Fig. 2). We also elucidated the detailed carbon and nitrogen budgets in relation to their water and alkaline solubility. The alkalinity of the larval guts was also measured. Finally, we investigated the possibility of nitrogen fixation in the field, by using the stable isotope analysis.

Materials and methods

Experiment I: Basic feeding performances

Food preparation

A decaying willow (*Salix* sp.) trunk infested with a white-rot fungus *Trametes versicolor* was collected at Watarase flood control basin, Fujioka Town, Tochigi Prefecture on 7

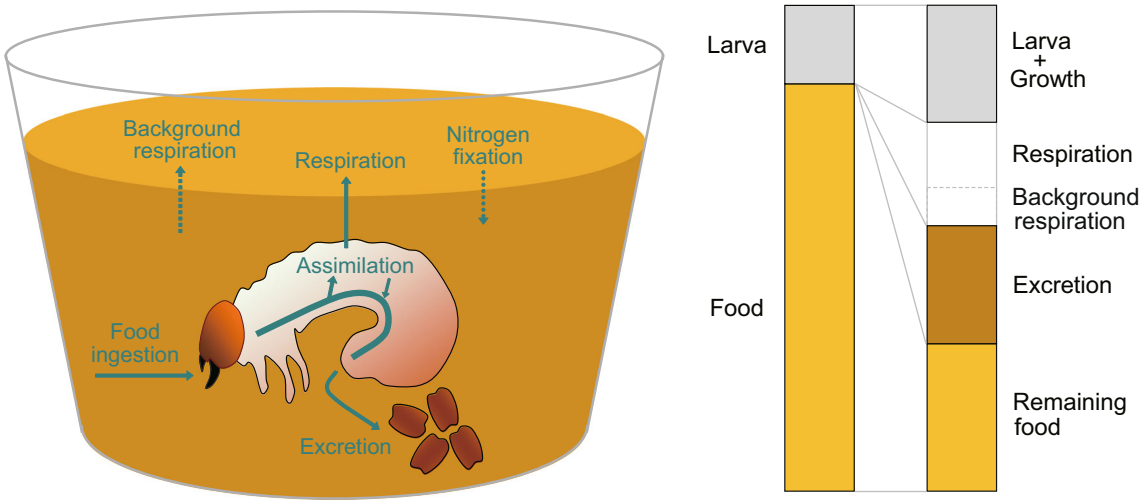

Fig. 2 An analytical scheme of the nutrient budget of *Dorcus rectus* larvae in the semi-open system

November 2004. After transferring to the laboratory, the decaying trunk was torn into small pieces. Because the decaying trunk was found to have some *D. rectus* larvae in the field, it might be in a good condition for the larvae of this species. A small wood piece (approximately, 3 g) was weighed and dried at 105 °C for 3 h, and the moisture content was determined to be 75.1% (based on the wet weight). For the preparation of larval diet, bark and poorly decaying part of the wood were carefully removed from the trunk and the remaining, fully decaying wood (approximately, 5 kg) was crushed into coarse particles by using the electric mill. The crushed wood was air-dried at the room temperature for 3 days and stored in − 20 °C until being used. This food stock was commonly used in Experiment I and II.

Rearing procedure

To prepare an experimental diet, 2000 mg of air-dried decaying wood and 5 ml of distilled water were added together to each 50-ml glass test tube (27.3 mm inner diameter) with a flat bottom and pressed to 3.5 cm above the bottom of the tube. Since the air-dried wood still contains some water, another set of 2000 mg air-dried decaying wood was further dried at 105 °C for 3 h to determine the absolute dry mass (= 1867 mg). Here we used relatively small amount of the food compared to the larval size, because we need to minimize the mass loss of the food that would possibly be caused by free-living microbes.

A decaying trunk that worn many fresh oviposition scars of *D. rectus* was collected on 30 September 2006 at the same field site as mentioned above. The trunk was placed in the ambient outdoor temperature for 2 months and a total of 12 second- or third instar larvae were recovered from the trunk (Fig. 3a). For initial habituation, the larvae were fed with the experimental diet at 25 °C for 7 days. After that, the larvae were weighed and put individually in the experimental diet and reared in the thermo-hygrostat chamber (25 °C, 100% R.H.) in the dark for 14 days. The larvae were recovered from the tubes and weighed again. Since the dry mass of the larvae at the beginning could not be determined directly, we converted the raw weight of the larvae into the dry mass by multiplying the literature value of the dry mass ratio of *D. rectus* third instar larvae (= 0.133) that had been reared on the same food material (Tanahashi and Togashi 2009). The residues in the tubes were hand-sorted into the larval feces or remaining diet (Fig. 1c), and dried again at 105 °C for 3 h to determine the accurate dry mass of each fraction. We repeated this experiment three times for each larvae (Fig. 3a). The first, second and third trials started on 31 December 2006, 13 January 2007 and 9 April 2007, respectively. Between the second and third trials, the larvae were kept at 4 °C. For a non-larval control, five experimental diets without larvae were incubated for 14 days and measured for the dry mass to determine the background aspiration by environmental microbes.

Calculation of feeding parameters

The analytical scheme of the feeding performance test was illustrated in Fig. 2. In this experiment, the amount of food ingestion (I) for each larva (i) was defined as a subtraction of the dry mass of remaining food (W_i) from that of the initial food supply (W_0);

$$I_i = W_0 - W_i$$

Since initial dry mass of the larva could never be determined, we used the literature value for the water content of *D. rectus* third instar larva (86.7%) reared in mostly the same condition (Tanahashi and Togashi 2009) to convert the fresh mass into the dry mass. To simplify the model, we assume that the larvae never re-ingest the feces. Excretion efficiency (rE), assimilation efficiency (rA) (also called approximate

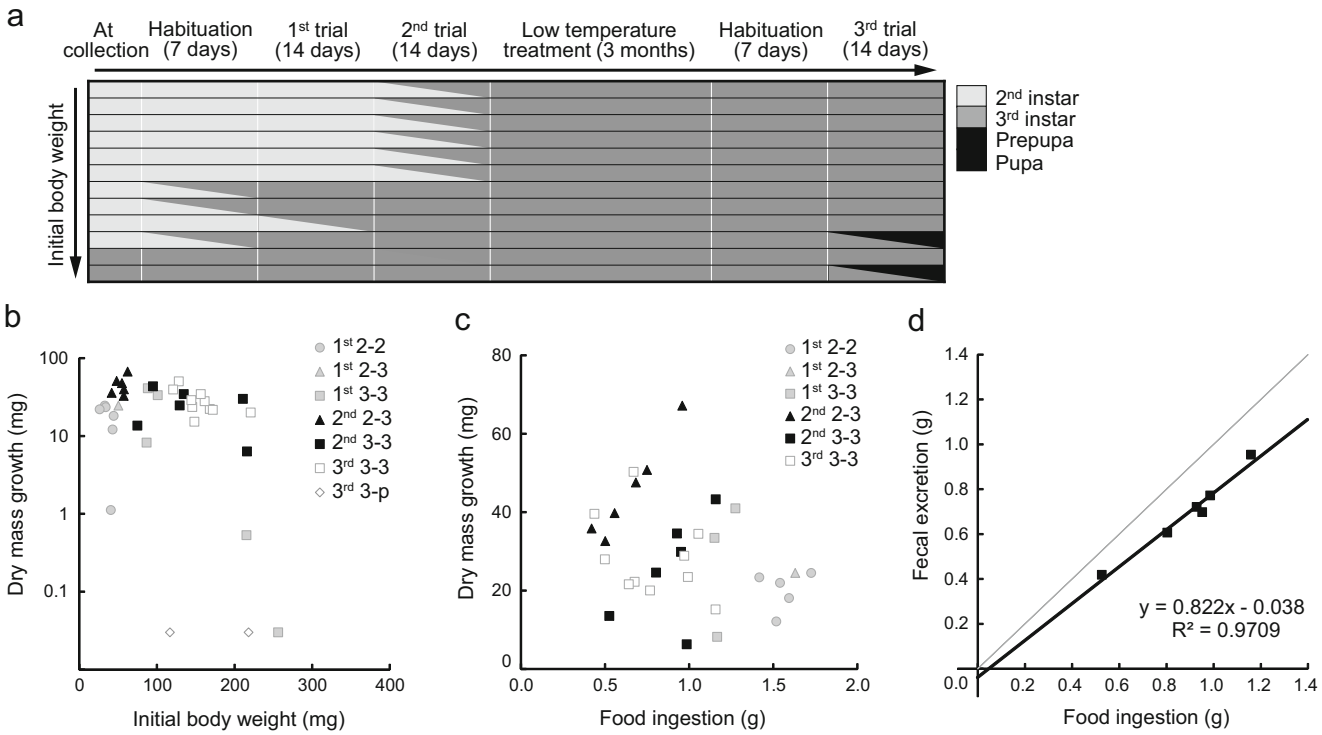

Fig. 3 Growth and assimilation performances of *Dorcus rectus* larvae reared on write-rotted *Salix* wood for 14 days. **a** Development of the larvae ($n = 12$) used in this study. Each column means the developmental stage of the larvae in each experimental period. A cell slashed into two colors means that the larva molted to the next developmental stage in that period. **b** The growth of the larvae plotted against the initial body weight (in dry mass, the same hereinafter). The label of the plots, for example, 2nd 2–3, means the larvae in the second experimental trial which developed from the second instar to the third instar (p indicates prepupa or pupa). The plots below 0.1 mg-growth indicate that they showed negative growth, thus were excluded from the analysis. **c** The growth of the larvae plotted against the food ingestion. **d** The fecal excretion plotted against the food ingestion, by using the third instar larvae in the second trial. The black line shows the linear regression line and the gray line indicates the line of $x = y$. **e** Changes of the assimilation efficiency in the three experimental trials. Bars show SD. Different letters above the column indicate significant difference by ANOVA followed by Bonferroni's correction ($p < 0.05$)

digestibility or AD) and gross growth efficiency (rG) were calculated by using the dry mass of excreted feces (E) and the dry mass-based growth of each larva (ΔM) as follows:

$$rE_i = E_i / I_i$$

$$rA_i = 1 - rE_i$$

$$rG_i = \Delta M_i / I_i$$

The total mass loss from the system (ΔS) was equivalent to the total respiration, which is supposed to comprise the inner-body respiration by larvae and the respiration by free-living aerobic microbes outside the body (Fig. 2). The latter, hereafter called environmental aspiration, was first supposed to be represented as a constant value (R_{env}) by using the total mass loss in the non-larval control. However, although green molds (they seemed to be the mycoparasitic and cellulolytic fungi, *Trichoderma* spp.) colonized naturally on all the wood diets of the non-larval control, no visible fungal colonies were observed in the wood diet harboring *D. rectus* larvae. Therefore, using the constant R_{env} in the larval treatment may lead to underestimation of the net larval respiration (R), and thus, we hereafter neglected the environmental aspiration in the calculation of the larval respiration (i.e. $R = \Delta S$). We also neglected the effect of nitrogen fixation on the total-mass based parameters, because of its very small values (0.25 μg fixed nitrogen per day per larva) that has been reported in *D. rectus* third instar larvae (Kuranouchi et al. 2006). Consequently, gross respiration efficiency (rR) of each larva was calculated as follows:

$$R_i = \Delta S_i = W_0 - (W_i + E_i + \Delta M_i)$$

$$rR_i = R_i / I_i$$

To test the effect of different experiment conditions on the larval performance, we performed a linear model fitting with the dry mass growth (ΔM) as the dependent variable and the number of the trials and the insect stages as the independent categorical variables. We also tested a linear model that explains the food excretion (I) by using the food excretion (E) and the aforementioned two categorical variables, in order to determine the coefficient of the food ingestion against the excretion. However, since the food ingestion significantly differed among the trials and larval stages, this coefficient value never represents the true excretion efficiency of any larval

stages. Therefore, we also calculated the feeding parameters independently for each larval stage for each trial. These analyses were performed with R software (http://www.r-project.org) version 2.12.2.

Experiment II: Carbon and nitrogen utilization

Rearing procedure

In the aforementioned experiment, we collected the larval feces every 14 days, because frequent food change might disturb the feeding behavior of larvae and thus lead to an improper estimation of the feeding parameters. However, although fecal pellets of wood-feeding insects are likely stable from the viewpoint of total mass, some chemical properties could have changed in the duration from excretion to sampling. Therefore, we conducted another experiment to determine the chemical properties of the larval feces accurately. To prepare the larval diet for this experiment, the same air-dried, crushed wood was moistened to 75% with distilled water and packed in polystyrene containers (6.5 cm diameter at bottom, 8 cm diameter at top, 7 cm deep) with a screw cap. Here we used larger amount of food material for each larva than in Experiment I, because we do not need to care about the environmental aspiration that will reduce the mass of food to some extent.

A total of 15 *Dorcus rectus* larvae were collected at the same field site as mentioned above on 7 November 2004. To habituate the larvae to the laboratory condition, the larvae were individually reared in the diet at 25 °C for the first 7 days, and six actively feeding larvae at the middle stage of the third instar were chosen for this experiment. The larvae were transferred to new diets every 3 days and the fecal pellets that kept the original shape (Fig. 1c) were manually collected from the old diet. The fecal pellets were quickly dried at 105 °C for 3 h, weighed, and stored in a desiccator to avoid further decomposition. Since daily yield of the fecal pellets varied among larvae, we stopped sampling for each larvae when the sum of the fecal pellets exceeded 600 mg in dry weight, or quitted the experiment after the sixth sampling in any cases. Fecal pellets from each larva were pooled and finally ground with mortar and pestle to pass through 0.5 mm mesh. For a food sample, approximately 3 g of moistened decaying wood (equivalent to 750 mg dry mass) was taken from a randomly chosen food container just before use, dried at 105 °C for 3 h, and ground as mentioned above. This food sampling was repeated three times.

Chemical analysis

Fecal samples ($n = 6$) and food samples ($n = 3$) were divided into three subsamples: the first 200 mg for the hot-water extraction, the second 200 mg for the hot-alkaline extraction and the rest 50–200 mg (depending on the total yield) for the non-extraction analysis.

For the hot water extraction (HWE), the first subsample and 10 ml of distilled water was added to a 50-ml glass test tube equipped with an air-cooling glass capillary. The test tube was placed in boiling water for 3 h, and the suspension was filtered through a glass-fiber filter (GA-100, 9 cm diameter; Advantec, Japan), the weight of which had been determined after heating treatment at 500 °C for 2 h. Residue on the filter paper was rinsed with 50 ml boiled distilled water and dried at 105 °C for 3 h. The net dry mass of the residue was weighed to the nearest 0.01 mg. The residue and the glass-fiber filter were then ground together with mortar and pestle and the carbon and nitrogen contents were determined using an elementary analyzer (CN Corder MT-700, Yanaco, Japan).

For the hot alkaline extraction (HAE), the second subsample and 10 ml of 1% (*w*/*w*) NaOH was added to the test tube and heated on boiling water for 1 h. The suspension was filtered on the glass-fiber filter, and the residue was rinsed with 20 ml boiled distilled water, 10 ml of 10% (*w*/*w*) acetic acid, and 20 ml boiled distilled water in that order. The residue was dried at 105 °C and the carbon and nitrogen contents were determined as described above.

The third subsample was directly subjected to the carbon and nitrogen analysis. As a result, the carbon and nitrogen contents of the water-soluble and alkaline-soluble fractions were calculated by subtracting the carbon and nitrogen values of the insoluble residues of HWE and HAE, respectively, from those of the non-extracted (third) subsample. Here we can assume that the alkaline-soluble fraction totally includes the water-soluble fraction, since the residue of HAE was once neutralized and then rinsed with plenty of hot water. Therefore, we defined the alkaline-soluble-water-insoluble fraction as a subtraction of the water-soluble fraction from the alkaline-soluble fraction. To compare the values between food and fecal samples, we tested whether the mean value of the food samples ($n = 3$; technical replicates on a homogenous food stock) was plotted within 95% confidential interval (CI) of the respective values of the fecal samples ($n = 6$; biological replicates).

In the aforementioned calculation, content (percent) values of each fraction in the feces were determined based on the total mass of the excreted feces, which already suffered some loss due to larval assimilation. Therefore, to clarify the utilization of each fraction, we corrected the fraction values of the feces by multiplying the mean value of the excretion efficiency (rE_{mean}) determined in Experiment I. Finally, we also calculated the carbon and nitrogen budgets through the passage of larval gut, by integrating the data from Experiments I and II and using the equations mentioned above.

Alkalinity in larval gut

Three other *D. rectus* third instar larvae that were not used for the feeding experiment were chosen after the habituation period, then anesthetized in carbon dioxide gas and dissected on

ice. To determine the pH of the larval gut, each 20 μl fluid of the midgut and the anterior half of the hindgut was taken by using a plastic pipette tip, placed on paraffin film (Parafilm, Bemis Company, Inc) and touched with a flat-head pH probe (Horiba, Japan). The measurement was repeated three times for each individual for each part of the gut, and the median value of the three measurements was recorded.

Experiment III: Chemical analyses on field-collected materials

Changes in carbon and nitrogen properties in larval tunnel

We also analyzed the water- and alkaline solubility of the wood that had been eaten by *D. rectus* larvae in the field. A uniformly white-rotted branch of *Celtis sinensis* (approximately 20 cm diameter and 50 cm length) that harbored one *D. rectus* third instar larva was collected in Tama City, Tokyo, Japan on 23 February 2005. The dead branch was split carefully in the laboratory and four different samples (A–D) were obtained from the branch: (A) decaying wood that did not contact with feeding tunnels of the larvae, (B) coarse woody particles (so called, frass) made by the larvae, still not being eaten, (C) presumably fresh fecal pellets that were keeping the original shape, and (D) old fecal pellets which had been crushed and packed in the larval tunnel. Each sample was dried at 105 °C for 3 h, then the carbon and nitrogen contents of HAE and HWE residues were determined as described in Experiment II.

Nitrogen fixation in larval tunnel

To test the possibility of nitrogen fixation in larval tunnels in the field, we conducted the stable isotope analysis on the decaying wood that had been eaten by larvae. Three white-rotted, dead branches of *Salix* sp. that harbored a third instar larva of *D. rectus* were collected from different standing trees at Narito, Kaizu City, Gifu, Japan on 24 Nov. 2005. Then, three different samples (A–C) as mentioned above and a larva (X) were taken from each branch. They were dried at 60 °C for 3 days and smashed up using a bead cell disrupter (MS-100, Tomy, Japan). After that, the samples were examined for the total carbon and nitrogen contents, followed by the stable isotope analysis for the carbon (^{13}C) and the nitrogen (^{15}N) as described by Ikeda et al. (2007). Isotope ratios were expressed in parts per thousand (‰) relative to the international standard: $\delta^{13}C$, $\delta^{15}N = (R_{sample} - R_{standard}) / R_{standard} \times 1000$, where $R = {}^{13}C / {}^{12}C$ or ${}^{15}N / {}^{14}N$. Pee Dee belemnite and atmospheric nitrogen were used as the carbon and nitrogen isotope standards, respectively. Since $\delta^{15}N$ value generally increases from prey to predator due to isotopic discrimination (Δ_{dig}), $\delta^{15}N$ values of *D. rectus* larvae ($\delta^{15}N_{insect}$) would be higher than in the decaying wood they eat ($\delta^{15}N_{wood}$). However, if the larvae utilize atmospheric nitrogen, $\delta^{15}N$ of the larvae would shift toward the near-zero negative value, which is described as the isotopic discrimination by nitrogen-fixing organisms (Δ_{fix}). Using those parameters, the fraction of nitrogen derived from the atmosphere ($\%N_{dfa}$) can be estimated by the formula of $\%N_{dfa} = (\delta^{15}N_{wood} + \Delta_{dig} - \delta^{15}N_{insect}) / (\delta^{15}N_{wood} + \Delta_{dig} - \Delta_{fix}) \times 100$, as described in Tayasu et al. (1994). However, since we do not know actual values of Δ_{dig} and Δ_{fix} for *D. rectus* larvae, we tentatively fixed those unknown parameters as $\Delta_{dig} = 3.4$ (Tayasu et al. 1994) and $\Delta_{fix} = -1.0$, then fluctuated them within the possible ranges of $1.4 \le \Delta_{dig} \le 3.4$ (Minagawa and Wada 1984; Perkins et al. 2014) and $-2.0 \le \Delta_{fix} \le 0$ (Tayasu et al. 1994). A paired one-way ANOVA was performed to compare the difference in the total content and isotope composition of each element among wood/fecal samples (A–C), followed by the multiple-comparison test with Bonferroni correction. Additionally, a paired *t* test was applied to compare $\delta^{13}C$ and $\delta^{15}N$ values between the food (A) and the larvae (X). All the statistical analyses were performed with R 2.12.2.

Results

Feeding performance

Throughout the three trials in Experiment I, all the second instar larvae molted to the third instar and three larvae developed to prepupal or pupal stages (Fig. 3a). Since matured third instar larvae are known to exude large amount of moisture before pupation (Fremlin and Tanahashi 2015), we excluded the data of the specimen which pupated, prepupated or showed negative growth within a certain trial (Fig. 3b). When the dry-mass growth (ΔM_i) is plotted against the food ingestion (I_i), the plots of the second trial showed a significant positive linear correlation within each developmental stage ($p < 0.001$ for 2nd 2–3 and $p < 0.01$ for 2nd 3–3 in Fig. 3c) whereas no significant correlation was observed in the first or third trials, implying that the feeding performance has once converged due to the habituation effect, but diverged again after the long low-temperature treatment. The linear model also showed that both the number of the trial and the larval stage significantly affected the gross growth efficiency (rG) (Suppl Table S1). Background respiration (R_{env}) in the non-larval control was 0.076 ± 0.009 g (mean ± SD, $n = 5$); however, it was neglected as mentioned previously.

The coefficient of the food ingestion against the excretion determined by the linear model was 0.921 (Suppl Table S2). However, the food ingestion significantly differed among the trials and larval stages; therefore, we calculated the feeding parameters independently for each larval stage for each trial, as shown in Suppl Table S3. Since the larvae in the middle third instar were used in Experiment II, we hereafter focused on the results from the third instar larvae of the second trial in

Experiment I, in which the similar larval condition was achieved (Fig. 3a). Using this data subset, the mean excretion efficiency (rE_{mean}) based on the dry mass was determined as 0.779 (Table 1).

Carbon and nitrogen utilization

In Experiment II, all the larvae fed actively on decaying wood and excreted 32.1 ± 7.5 mg dry mass of feces per day (mean ± SD, $n = 6$). The carbon and nitrogen contents of the decaying wood were 53.2 and 0.52%, respectively. The carbon content was decreased slightly in fecal pellets (CI 47.9–51.3) whereas the nitrogen content largely decreased (CI 0.33–0.40) (Table 2). As a result, C/N ratio was significantly higher in feces than in decaying wood.

Regarding the total substrates, alkaline-insoluble fraction of the diet (59.8%) significantly increased in feces (CI 63.5–66.8), whereas water-insoluble fraction (88.1%) showed a slight increase (CI 88.5–90.7); resulting in decreased percent mass of alkaline-soluble-water-insoluble substrates in feces (Fig. 4a). Similar patterns were observed when focusing on the carbon content (Fig. 4b), because the carbon mass usually represents the total mass of the natural organic matter. For the nitrogen content, however, alkaline-insoluble nitrogen represented 0.111% mass in decaying wood and it did not change in feces (CI 0.106–0.116) whereas water-insoluble nitrogen (0.388% in decaying wood) significantly decreased in feces (CI 0.267–0.299) (Fig. 4c), suggesting that both water-soluble nitrogen and alkaline-soluble-water-insoluble nitrogen decreased by larval feeding. Moreover, the net budgets of each fractions were estimated by using the mean excretion ratio ($rE_{mean} = 0.779$) of third instar larvae. When this value was multiplied to the data from the feces, all fractions were revealed to have been consumed to some extent from the decaying wood to the feces (Fig. 4d–f). Of those, the alkaline-soluble-water-insoluble nitrogen largely decreased, indicating that this fraction was selectively assimilated by larvae (Fig. 4f). Solubility-based calculation of the elemental budgets revealed that nearly half of the carbon and nitrogen assimilated by larvae were derived from the alkaline-soluble-water-insoluble fraction (Table 3).

We also estimated the element-based material budgets, by combining the results from the two experiments. Here we assumed that the carbon and nitrogen contents of the feces and the larval bodies are virtually consistent between the two experiments, if only using the larvae in the same developmental stage. Hence, we performed the budget calculations on the third instar larvae of the second trial in Experiment I (Table 1). In this calculation, the gross assimilation efficiency of the nitrogen (= 0.454) was higher than that of the carbon (= 0.274), suggesting the selective utilization of the nitrogen-rich fraction. The gross growth efficiency was also much higher in the nitrogen (= 0.454) than in the carbon (= 0.025). Subtraction of the growth from the assimilation indicates that the larvae released 24.9% of ingested carbon as volatile, whereas the nitrogen budget came out to be virtually zero. Suppl Tables S4–S5 also show the carbon and nitrogen budgets for every trial and larval stage; however, we should note that those calculations were based on

Table 1 Assimilation, growth and respiration efficiencies of *Dorcus rectus* third instar larvae in laboratory

Variables		Calculation based on		
		Total dry mass	Carbon*	Nitrogen*
Initial food supply	W_0	1.867 g	0.993 g	9.67 mg
Remaining food	W	0.975 g	0.519 g	5.05 mg
Excreted feces	E	0.695 g	0.345 g	2.52 mg
Ingested food	I	0.892 g	0.474 g	4.62 mg
Larval growth[a]	ΔM	0.025 g	0.012 g	2.12 mg
Mass loss from system[b]	ΔS	0.171 g	0.118 g	−0.02 mg
Excretion efficiency	rE	0.779	0.726	0.546
Assimilation efficiency	rA	0.221	0.274	0.454
Gross growth efficiency	rG	0.028	0.025	0.454
Gross respiration efficiency[b]	rR	0.193	0.249	0.000

The larvae were reared on white-rotted *Salix* wood for 14 days. The table shows the mean values of each variable determined for each larva in the second experimental trial ($n = 6$). See also Suppl Tables S3–S5 for the standard errors and the results from the other trials

*Element-based calculations were done by using the carbon and nitrogen contents of wood, feces (see Table 2) and larva (46.4 and 8.35%, respectively) (Tanahashi and Togashi 2009)

[a] Wet mass growth was converted into dry mass by using the water content value of living *D. rectus* third instar larvae (86.7%) (Tanahashi and Togashi 2009)

[b] They include any gaseous exchanges (release of CO_2, CH_4, NH_4, NO_x and other volatile molecules as well as N_2 fixation) other than water

Table 2 Changes of carbon and nitrogen properties in the decaying wood eaten by *Dorcus rectus* third instar larvae in laboratory

Materials	*n*	Extraction method	Residual rate (%)	Carbon in residue (%)	Nitrogen in residue (%)	C/N
Wood	3[a]	None	100	53.18 ± 0.08	0.518 ± 0.000	103 ± 0
		HWE	88.14 ± 0.05	53.21 ± 0.07	0.440 ± 0.000	121 ± 0
				(46.91 ± 0.04)	(0.388 ± 0.001)	
		HAE	59.78 ± 0.56	51.64 ± 0.06	0.186 ± 0.003	278 ± 5
				(30.79 ± 0.28)	(0.111 ± 0.003)	
Feces	6[b]	None	100	49.61 ± 1.49	0.363 ± 0.031	138 ± 9
		HWE	89.61 ± 0.95	49.82 ± 0.97	0.316 ± 0.015	158 ± 8
				(44.65 ± 1.26)	(0.283 ± 0.014)	
		HAE	65.13 ± 1.43	50.22 ± 0.83	0.170 ± 0.007	296 ± 13
				(32.71 ± 0.99)	(0.111 ± 0.004)	

The larvae were reared on white-rotted *Salix* wood and the fecal pellets were recovered every 3 days. In the extraction method, none indicates that the samples were directly supplied for the carbon and nitrogen analysis. HWE and HAE represent hot water extraction and hot alkaline (1% NaOH) extraction, respectively, followed by the elemental analysis using those extraction residues. Percent values in the parentheses are those based on the total mass before extraction

[a] Repeated measurements on a homogeneous sample (technical replicate)

[b] Samples from different individuals (biological replicate)

somewhat improper assumption that the carbon and nitrogen contents of the body and the feces are still consistent between different developmental stages.

Alkalinity of larval gut

The midgut fluid of the *D. rectus* larvae was highly alkaline (pH 10.3 ± 0.1) (mean ± SD, $n = 3$), whereas the fluid of anterior hindgut was nearly neutral (pH 7.3 ± 0.5).

Changes in carbon and nitrogen properties in larval tunnel

In the decaying *Celtis sinensis* branch, the alkaline-insoluble fraction represents 72.6–75.7% of the total mass (Fig. 5a) and the values were somewhat higher than those of the decaying *Salix* wood used in the Experiment I and II (Table 2). Although the total carbon content was almost constant (Fig. 5b); the total nitrogen

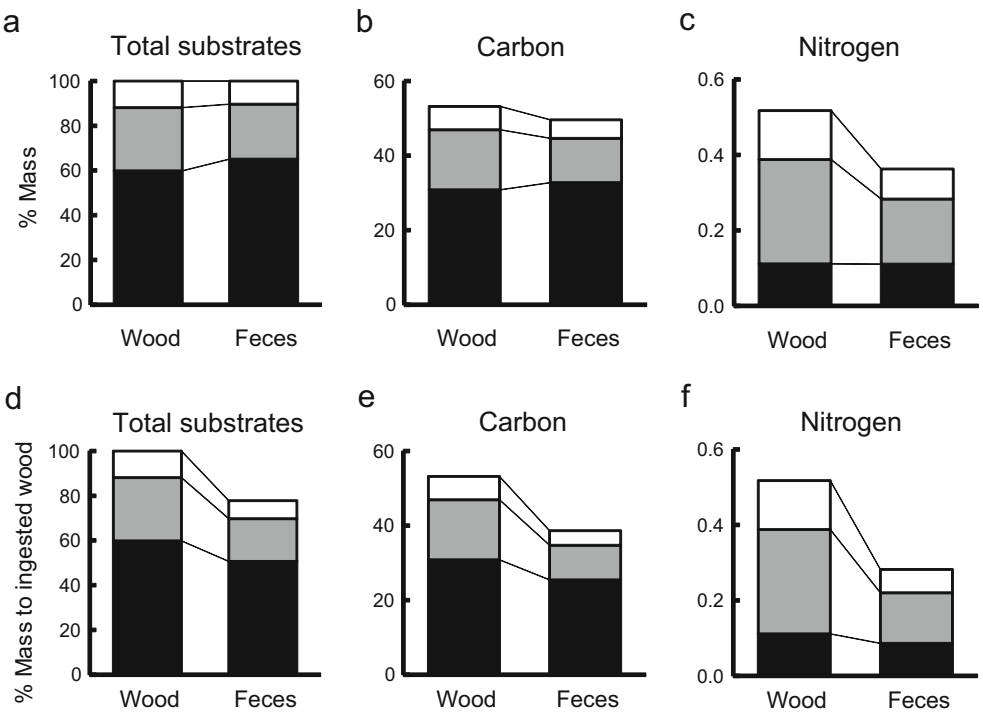

Fig. 4 The carbon and nitrogen utilization by *Dorcus rectus* third instar larvae based on the solubility. Upper figures (**a**–**c**) show the percent mass changes of **a** total substrates, **b** carbon, and **c** nitrogen from wood diet to feces, where white columns indicate water-soluble fraction, gray columns indicate alkaline-soluble-water-insoluble fraction, and black columns indicate alkaline-insoluble fraction. In this experiment, the larvae ($n = 6$) were fed with white-rotted willow wood and the feces were sampled every 3 days to prevent microbial degradation. Lower figures (**d**–**f**) also show the same data sets, but the percent values of the fecal samples were corrected by using the excretion ratio, rE_{mean} (see Table 1)

Table 3 Carbon and nitrogen assimilated by *Dorcus rectus* third instar larvae. Values were shown in %dry mass when a larva ingests 100 (%) of decaying wood

Fraction	Ingested wood				Assimilated substrates			
	Total mass	C	N	C/N	Total mass	C	N	C/N
Water-soluble	11.9	6.3	0.13	48	3.8	2.4	0.07	35
Alkaline-soluble-water-insoluble	28.4	16.1	0.28	58	9.3	6.8	0.14	48
Alkaline-insoluble	59.8	30.8	0.11	277	9.0	5.3	0.02	214
Total	100.0	53.2	0.52	103	22.1	14.5	0.23	62

content apparently increased from 0.25% (A: decaying wood) to 0.31% (D: old fecal pellets) and the alkaline-soluble-water-insoluble nitrogen (from 0.13 to 0.18%) was mostly responsible for this change (Fig. 5c).

Nitrogen fixation in larval tunnel

In the stable isotope analysis of the decaying branches of *Salix* sp., the total carbon content did not change (Fig. 6a) while the total nitrogen content significantly increased from $0.17 \pm 0.02\%$ (A: decaying wood) to $0.32 \pm 0.00\%$ (C: fresh fecal pellets) (mean ± SD, $n = 3$), along with the passage thorough the larval gut (Fig. 6b). However, no significant changes were observed in $\delta^{13}C$ and $\delta^{15}N$ values among three wood-fecal samples (A–C) ($p = 0.33$ and $p = 0.41$, respectively) (Fig. 6c). The larvae (X) tended to show greater $\delta^{15}N$ values ($\delta^{15}N_{insect} = 2.52 \pm 0.59$) than their food ($\delta^{15}N_{wood} = 1.13 \pm 0.53$) ($p = 0.06$) (Fig. 6c). The fraction of atmospheric nitrogen ($\%N_{dfa}$) was estimated to be $36 \pm 11\%$ under the fixed parameters of $\Delta_{dig} = 3.4$ and $\Delta_{fix} = -1.0$. However, when we changed the both two parameters, $\%N_{dfa}$ was largely fluctuated from zero (or large minus value) to 42%.

Discussion

Elementary budget in stag beetle larvae

In the first experiment, we elucidate the basic feeding performance of *D. rectus* larvae. On the basis of our calculation (Table 1), when a third instar larva ingests 100 mg (dry mass) of decaying wood, it would assimilate 22.1 mg of the food and excrete 77.9 mg feces. The assimilation efficiency, which is also referred to as approximate digestibility (AD), is not far from the values of xylophagous and cambiophagous insects (around 30%) found in the literatures (Haack and Slansky 1987; Walczyńska 2007). Out of the assimilated materials, the larva uses 2.8 mg for its growth and releases the rest 19.3 mg by respiration.

However, such a simple dry-mass-based calculation sometimes leads to improper estimation of the net material budget, because water molecules are often accompanying with biochemical reactions in metabolic pathways. Moreover, dry-mass-based calculation gives no information about utilization of each element by the larvae. Actually, element-based calculation clearly revealed that the nitrogen is more effectively or selectively assimilated than the carbon (Table 1). Since nitrogen is deficient in woody biomass and thought to be the primary factor to limit the growth of *D. rectus* larvae (Tanahashi

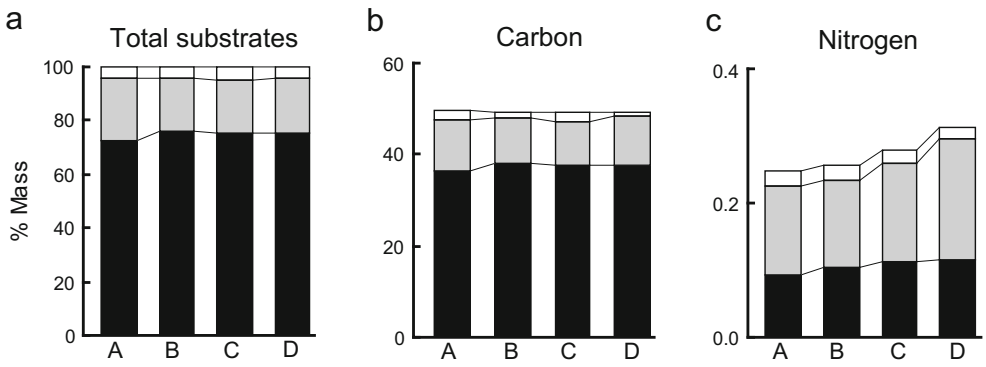

Fig. 5 Changes of the carbon and nitrogen contents and their solubility in a field-collected decaying log of *Celtis sinensis*, along with possible time course of the feeding activity of a *Dorcus rectus* larva ($n = 1$). A–D represent woody samples in different status: (A) decaying wood that did not contact with feeding tunnel of the larva, (B) coarse woody particles made by the larva, still not being eaten, (C) presumably fresh fecal pellets that were keeping the original shape, and (D) old fecal pellets which had been crushed and packed in the larval tunnel. White columns indicate water-soluble fraction, gray columns indicate alkaline-soluble-water-insoluble fraction, and black columns indicate alkaline-insoluble fraction

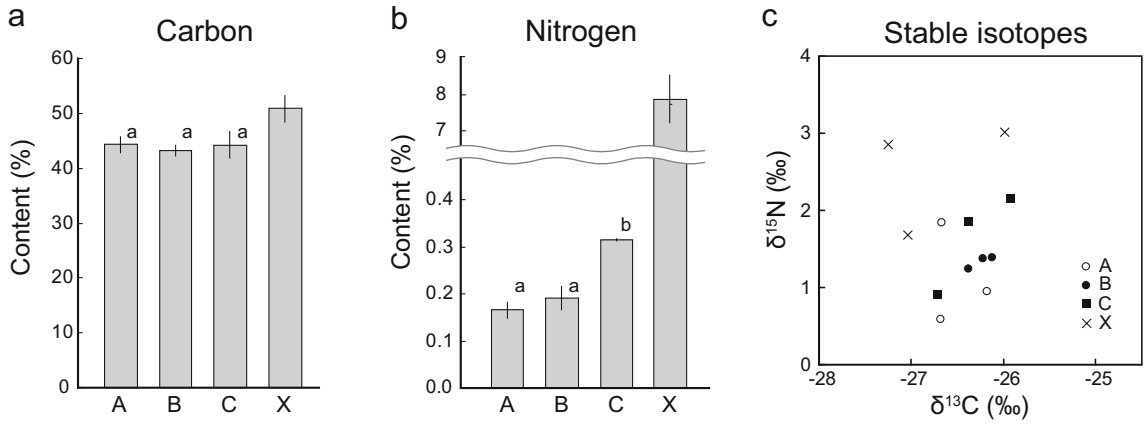

Fig. 6 Changes in the carbon and nitrogen contents and the ratios of the stable isotopes of field-collected decaying log of *Salix* sp., along with possible time course of the feeding activity of *Dorcus rectus* larvae ($n = 3$). A–C and X represent different samples: (A) decaying wood that did not contact with feeding tunnels of the larvae, (B) coarse woody particles made by the larvae, still not being eaten, (C) presumably fresh fecal pellets that were keeping the original shape, and (X) the larvae. Bar graphs (a–b) show the mean and the standard deviation of the carbon and the nitrogen contents ($n = 3$ for each category). Different letters above the columns indicate significant difference ($p < 0.05$) using ANOVA followed by Bonferroni's correction. Data of the larvae (X) was not included in this statistic analysis. In the scatter plot (c), $\delta^{13}N$ values of each individual sample are plotted against the $\delta^{13}C$ values

and Togashi 2009; Tanahashi et al. 2010) and other wood-feeding insects (Mattson 1980; Nalepa 1994; Jönsson et al. 2004), selective utilization of nitrogen totally makes sense for wood-feeding insects to optimize their growth performance. Moreover, our calculation shows that the nitrogen loss from the whole system is nearly zero, suggesting that there is no income or outcome of nitrogen beyond this semi-open system. Slightly minus value of the nitrogen respiration efficiency would mean the possibility of nitrogen fixation to some extent; however, the amount of nitrogen fixation is supposed to be very small or almost negligible, as also shown in the previous study (Kuranouchi et al. 2006). Consequently, *D. rectus* larvae have possibly been associated with the selection pressures to improve the assimilation efficiency of nitrogen and minimize the gaseous nitrogen release.

Carbon assimilation efficiency still keeps a higher value than that based on the total dry mass, implying that water is newly produced along with the metabolic process (in other words, ingested food molecules contain the source of H_2O). Here we can explain this phenomenon by using a hypothetical, non-respiratory organism: the organism ingests 180 g (1 mol) of glucose ($C_6H_{12}O_6$), metabolizes 90 g (0.5 mol) of ingested glucose and finally gains 36 g dry mass by producing 3 mol of pure carbon as well as 54 g of metabolic water; thus, the carbon-based assimilation efficiency (= 0.5) shows much higher than the dry-mass-based value (= 0.2). Some desert insects are known to produce such metabolic water by oxidizing hydrocarbons and/or carbohydrates to improve their desiccation tolerance (Frank 1988; Waterson et al. 2014). Although stag beetle females usually prefer to oviposit on decaying wood with relatively high moisture content, the environment sometimes suffers severe desiccation and the larvae can survive within the dry wood for over a year (personal observation by MT and KK). Wood contains vast amount of carbohydrates that will be the source of water molecule. Therefore, assimilation of excess amount of carbohydrates in *D. rectus* larvae may not always be a cost, but also sometimes beneficial for the desiccation tolerance by means of producing the metabolic water.

Budget calculation based on stoichiometric homeostasis

Here we can perform another theoretical calculation on the gross respiration efficiency and the gross growth efficiency on the basis of stoichiometric homeostasis, only using the measured values of the carbon and nitrogen contents of food and fecal pellets, with the given, presumably constant values of the assimilation (excretion) efficiency and the carbon and nitrogen contents of the target insect. When a larva feeds 100 mg of decaying wood, the food contains 53.2 mg carbon and 0.52 mg nitrogen. The larva excretes 77.9 mg (= $100 \times rE_{mean}$) feces, which contains 38.6 mg (49.6%) carbon and 0.28 mg (0.36%) nitrogen. As a result, the larva is supposed to assimilate 14.6 mg carbon and 0.24 mg nitrogen. The C/N ratio of the assimilated fraction is 61, which is lower than in food ($C/N = 103$), but still much higher than the C/N ratio of *D. rectus* third instar larvae ($C/N = 5.6 \pm 0.3$) that was reared under the similar condition (Tanahashi and Togashi 2009). Here we can assume that the C/N ratio of larval body is nearly constant (= 5.6) on the basis of stoichiometric homeostasis; therefore, the excess of assimilated carbon must be discharged as volatiles. Supposing that the gaseous nitrogen budget is negligible as discussed above, only 1.3 mg (= 0.28 × 5.6) carbon (equivalent to 2.9 mg of larval dry mass) can be utilized for growth and the excess of 13.3 mg carbon (equivalent to

24.9 mg decaying wood) must be released by respiration. Consequently, the gross growth efficiency (= growth/ingestion) and the gross respiration efficiency (= respiration/ingestion) are calculated as 0.029 and 0.249, respectively. Interestingly, those theoretical calculations are not so far from the experimental measurements (Table 1). For this calculation, we do not need a direct measurement of the body mass of the target specimen. Therefore, our theoretical calculation will be quite useful when researchers are not allowed to disturb the target specimen, as is often the case of strictly protected species, such as the long-armed chafer, *Cheirotonus jambar* (Coleoptera: Scarabaeidae). We should note that this calculation is based on the assumption that the aerial nitrogen budget is virtually zero. Therefore, if the level of the nitrogen fixation is turned out to be non-negligible, we would have to add a correction term of the nitrogen fixation.

Selective utilization of nitrogen

Wood consists largely of structural polymers such as cellulose, hemicellulose and lignin, which comprise almost 90% of total mass (Haack and Slansky 1987). Hemicellulose is a general term that refers to any of polysaccharides other than cellulose and pectin in the plant cell wall. It usually represents 30–40% of the fresh wood dry mass and can be separated by alkaline-soluble fraction of wood (Reid 1997). Most white-rotting fungi decompose cellulose, hemicellulose and lignin at virtually the same ratio and quickly utilize the digested materials, therefore, the alkaline solubility of white-rot wood is nearly consistent during the decay process (Kirk 1973). On the other hand, the amount of water-soluble fraction in fresh wood is known to be highly variable depending on the tree species, ages, seasons and the sampling parts (sapwood or heartwood), and typically, ranging 1–5% in Norway spruce wood (Gäumann 1928; Roffael 2016).

Here we also elucidated that *D. rectus* larvae mainly consumed alkaline-soluble substrates of decaying wood. Although we did not investigate the molecular species of each extractive fraction, it is supposed that the hot-water extract (HWE) contains pectin, xyloglucan and arabinogalactan (Kochumalayil and Berglund 2014) as well as small nutritional molecules such as mono- and oligo saccharides, amino acids and peptides, whereas the hot-alkaline extract (HAE) mainly consists of hemicelluloses and their derivatives, and some large peptides conjugating with the lignocellulosic matrices. Although the water-soluble fraction is limited in the decaying wood, it has lowest C/N ratio (Table 3) and can be utilized quickly and effectively by the larvae. Here we should note that the water-soluble fraction of the feces are likely to contain secondary products by the larvae (i.e., digestive enzymes and urinary excretion), which could have been originated from an insoluble fraction of the food. Therefore, simple subtraction of the water-soluble fraction between food and feces, as shown in Table 3, may result in an under-valuation of the actual amount of water-soluble materials utilized by the larvae.

The alkaline-soluble-water-insoluble fraction represents 28.4% mass of the decaying wood diet, and its C/N ratio is still much lower than that of the alkaline-insoluble substrates. Even though being decaying wood, this fraction is supposed to consist mainly of hemicelluloses, since water and alkaline solubility is virtually constant during white-rot process (Kirk 1973). Hemicelluloses do not possess nitrogen atom in their molecules; however, the alkaline-soluble-water-insoluble fraction represents 53.4% of the total nitrogen of the decaying wood, and also represents approximately 60% of the assimilated nitrogen (Table 3). That suggests that digestion of nitrogenous substrates that may be conjugating with lignocellulosic matrices is essential for the nutrition of *D. rectus* larvae.

The extreme difference in C/N ratio between food and body causes stoichiometric imbalance and the influences are thought to be strong selective pressure for herbivores to alleviate these impacts (Elser and Hessen 2005). *D. rectus* larvae may alleviate the stoichiometric imbalance by selective utilization of alkaline-soluble fractions in decaying wood.

Function of highly alkaline gut

We also confirmed that the midgut of *D. rectus* larvae is highly alkaline (pH 10.3), which is nearly the same pH level as previously shown by Mishima and Araya (2016). Alkaline midgut is also common in humus-feeding and root-feeding Scarabaeoidea larvae; for example, larvae of a humus-feeding scarab beetle *Pachnoda ephippiata* (Gerstaecker) (Coleoptera: Scarabaeidae) have a midgut with maximum pH of 10.2 (Lemke et al. 2003), and that of a pasture pest *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae) reaches 10.9 (Biggs and McGregor 1996). We hypothesized the four general functions of highly alkaline midgut in humus and decaying wood-feeding insects: (1) decrystallization and swelling of structural polymers of wood or plant cell walls, (2) chemical hydrolysis of those polymers, (3) achieving optimum pH for the digestive enzymes, and (4) extraction of alkaline-soluble nitrogenous compounds conjugated to those polymers. Actually, some studies indicated that of optimal cellulose digestion is achieved in high alkaline condition (Helmy 1993). However, most of the polysaccharide-digestive enzymes found in the guts of *D. rectus* larvae attain optimal performance at neutral pH (Mishima and Araya 2016). That suggests that the high alkalinity of midgut is unlikely to associate with effective digestion of wood cellulose and hemicelluloses, and that it may rather be useful to extract nitrogenous compounds from lignocellulosic matrices. *D. rectus* larvae are thought to utilize fungal biomass in decaying wood (Tanahashi et al. 2010; Mishima and Araya 2016) and they need the water-insoluble fraction of fungal

mycelium as essential nutrients (Tanahashi and Kubota 2013). Decaying wood contains large amount of fungal biomass, which sometimes represents more than 30% of decaying wood (Jones and Worrall 1995). Therefore, highly alkaline midgut of *D. rectus* may be associating with extraction and digestion of the water-insoluble fraction of fungal biomass in decaying wood.

Herbaceous lepidopteran larvae are also known to have highly alkaline midgut and the biochemical mechanism of the alkalinity has been well documented. In lepidopteran larvae, potassium transportation from hemolymph to gut cavity and subsequently generated potassium carbonate (K_2CO_3) are thought to be the prime factor of alkalinity in midgut fluid (Dow 1992). The alkalinity of the midgut fluid in herbaceous lepidopteran larvae is thought to reduce tannin/protein binding and eliminate the inhibition activity of tannins against digestive enzymes (Martin and Martin 1983), and the pH value changes depending on the host plant species and the time after feeding (Schultz and Lechowicz 1986). Wood lignin is also a kind of tannins and it is known to inhibit cellulase activity by predominantly binding both the enzyme itself and the enzyme-binding site of cellulose (Vermaas et al. 2015); therefore, *D. rectus* larvae would also benefit from such a counter-tannin activity by possessing a high alkaline midgut.

Microbial activities in feeding tunnels

Our laboratory experiment clearly revealed that the nitrogen level significantly decreased through the passage of larval gut while the carbon shows virtually no change, resulting in increasing C/N ratio of the fecal pellets. That result is quite reasonable because the larvae are likely to utilize nitrogen selectively; however, it is controversial to most previous studies where the C/N ratio decreased from wood to feces (Araya 2006; Kuranouchi et al. 2012), and our field samples also showed the opposite tendencies (Figs. 5 and 6). Since stag beetles are associated with xylose-fermenting yeasts (Tanahashi et al. 2010, 2017; Hawes 2013) as well as some aerobic bacteria (Miyashita et al. 2015), and the larval hindgut contains large number of living yeasts (Tanahashi and Hawes 2016), the microbes will continuously consume polysaccharides as energy resource in fecal pellets, resulting in reduced C/N ratio in feces depending on the time duration after excretion.

D. rectus larvae are possibly associating with some nitrogen fixing microbes, because acetylene reduction activity has been reported in *D. rectus* larvae (Kuranouchi et al. 2006) and sequences of the microbial nitrogen-fixing gene (*nifH*) are constantly detected from the larval gut (unpublished data by MT). Our stable isotope analysis also shows that the fraction of nitrogen derived from atmosphere ($\%N_{dfa}$) of field-collected *D. rectus* larvae is about 20–40% of total nitrogen; however, this estimation is mostly depending on the two undetermined parameters for isotopic discrimination, Δ_{dig} and Δ_{fix}. Actually, Δ_{dig} is known to be largely varied among different species and ecosystems (Tayasu et al. 1994; Perkins et al. 2014) and we can obtain any $\%N_{dfa}$ estimation within the range of 0–40% with variable Δ_{dig} ($1.4 \leq \Delta_{dig} \leq 3.4$). In the case of wood-feeding termites, $\delta^{15}N$ values are usually lower in insect body than wood ($\Delta_{fix} < 0 < \delta^{15}N_{insect} < \delta^{15}N_{wood}$) and such $\delta^{15}N$ decrease cannot be explained without a dilution effect by atmospheric nitrogen (Tayasu et al. 1994), therefore, undetermined Δ_{dig} rarely affect the qualitative conclusion. However, in this study, $\delta^{15}N_{insect}$ is always larger than $\delta^{15}N_{wood}$ and here we cannot conclude that the larvae actually utilize atmospheric nitrogen.

Our laboratory experiment revealed that *D. rectus* larvae selectively utilize the alkaline-soluble-water-insoluble nitrogen in decaying wood, possibly by using highly alkaline midgut. However, in the field observation, the level of alkaline-soluble-water-insoluble nitrogen seems to recover in the old feces packed in the larval tunnel, suggesting that the microbial activities in the feces may play a key role to convert the nitrogen wastes into nutritive substrates that larvae can utilize again. From this point of view, *D. rectus* larvae are likely to utilize their feeding tunnel as an "external gut system," where different microbial activities can be expected than in the internal gut. Such nitrogen recycling by microbial symbionts and reingestion of feces by wood-feeding insects would also affect the material dynamics inside decaying wood, and thus, should be investigated in future studies.

Ecological significance of stag beetles on wood degradation

In temperate and tropical forests, termites are often said to play a dominant role in wood consumption and/or mechanical fragmentation (Buxton 1981; Schuurman 2005; Ulyshen et al. 2014). However, although the occurrence of stag beetle larvae is not so frequently as termites, their biomass level in specific decaying wood is still comparable to the wood-feeding termites. For example, we actually collected more than ten *D. rectus* larvae from a decaying trunk in this study (see Experiments I and II) and the total body mass could exceed 10 g/trunk in wet weight, which is comparable to the biomass-based abundance of termites in the tropical forests ($10–10^4$ mg/m^2) (Dahlsjö et al. 2014). Therefore, stag beetle larvae can also be responsible for the forest mater cycling, where they turn a low nutritious food source into protein-rich insects that form an important food source of a large part of the animal biodiversity of forests, and consequently, become the basis of several trophic levels that depend on them. Moreover, *D. rectus* larvae are often dominant in partially dead trunks and branches on living trees of the open forests in Japan (Kubota and Kubota 2004), where only few other saproxylic insects are found (personal observation by MT and KK).

Mäkipää et al. (2017) suggested that the nitrogen transportation from soil to wood at the late stages of decay may further improve the activities of wood-rotting fungi. However, since the soil nitrogen is unlikely to reach such arboreal dead wood, the nitrogen fixation and the nitrogen recycling associated with insect-microbe symbiosis are of much more significance in the arboreal habitat. Female stag beetles possess highly developed mycangia that harbor the specific symbiotic yeasts (Tanahashi et al. 2010, 2017) as well as a large variety of culturable and unculturable bacteria (Miyashita et al. 2015; unpublished data by MT) and introduce them into the arboreal environment. Consequently, stag beetles and their microbial symbionts may represent another key players for wood decomposition and matter cycling in the forest ecosystem.

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