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## RESEARCH

# Variation in C:N:S Stoichiometry and Nutrient Storage Related to Body Size in a Holometabolous Insect (*Curculio davidi*) (Coleoptera: Curculionidae) Larva

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**ABSTRACT.** Body size can be an important factor controlling consumer stoichiometry. In holometabolous insects, body size is typically associated with nutrient storage. Consumer stoichiometry is known to vary within species across a range of body sizes; however, the contribution of nutrient storage to this variation is not well understood. We used the fifth-instar larvae of the oak weevil (Coleoptera: *Curculio davidi* Fairmaire), which is characterized by a high capacity for nutrient storage, to investigate the effect of shifts in nutrient storage with body mass on variations in larva stoichiometry. Our results showed that weevil larvae with larger body mass had a lower carbon (C) content, reflecting decreases in the sequestration rate of C-rich lipids. Larger larvae had elevated concentrations of nitrogen (N), sulfur (S), and protein. The similar patterns of variation in elemental composition and macromolecule storage with body weight indicate that the shift in nutrient storage is the main factor causing the variation in larval stoichiometry with body weight. This finding was further supported by the low variation in residual larval biomass C, N, and S concentrations after lipid extraction. These results help decipher the physiological mechanism of stoichiometric regulation in growing organisms.

**Key Words:** body weight, *Curculio* larva, biological stoichiometry, nutrient storage, *Quercus* acorn

Body mass, an important indication of nutrient storage in consumers (Edgar 2006), has been considered an important factor of intraspecific variation in consumer stoichiometry (Elser et al. 1996, Hambäck et al. 2009). Previous studies have separately analyzed variation in nutrient storage (e.g., phosphorus storage and energy storage) (Sternler and Schwalbach 2001, Ventura and Catalan 2005, Sun et al. 2013) and body size on the intraspecific variation in consumer stoichiometry (Hambäck et al. 2009, El-Sabaawi et al. 2012). Generally, in adult organisms for a given species, nitrogen (N) and phosphorus (P) concentrations, and N:P display inverse relationships with body mass (Elser et al. 1996, Cross et al. 2003, Woods et al. 2004, Kay et al. 2006, Bertram et al. 2008, Schneider et al. 2010). Nevertheless, few studies have explored the relationships among the body size, nutrient storage, and variation in consumer stoichiometry.

Several factors probably influence the relationships between consumer stoichiometry and body size. Allometry in body organism N:P may be influenced by the variation in elemental composition of protoplasm and structural materials, by the allometric declines of growth rate with body weight (Elser et al. 1996) and by the variation of lipid and protein storage across body size (Thomas and Diwan 1990, Hurst and Conover 2003, Boswell et al. 2008). For instance, the variation in lipid and protein contents of differently sized larva is related to variation in carbon (C) and N, respectively (Sternler and Elser 2002), and larger consumers tend to have higher C:N and C:P with body mass due to increased storage of C-based energy (Sternler and Elser 2002, Woods et al. 2004). Holometabolous insect larvae, which have a high capacity to store nutrients, can adjust their levels of stored nutrients based on physiological demand (Arrese and Soulagés 2010). However, no study has investigated the macromolecular composition of this additional larval biomass and its contribution to overall body elemental content.

For holometabolous oak weevils (*Curculio davidi* Fairmaire), the larval stage is endoparasitic, completing their development through

five instars in a single host acorn (Cheng and Hsu 1956). The fifth-instar weevil larva contains many fat bodies, storing large nutrients for hibernation, metamorphosis, and future needs of adults. Because of differential nutrient storage, the body weight of fifth-instar larvae can vary greatly (Desouhant et al. 2000; Bonal and Muñoz 2008, 2009), providing a good opportunity to investigate the contribution of nutrient storage to the variation in consumer stoichiometry with the increase of body mass. Here, across a range of larval body mass, we hypothesize that the variation in nutrient storage of final-instar weevil larvae should be the main factor explaining variation in elemental content of these stage of weevil larvae. If this hypothesis is true, the variability of C, N, and S in residual larval biomass following nutrient extraction will be small compared with the variability of C, N, and S in total biomass. Specifically, as pre-diapause weevil larvae mass increases, lipids and protein concentrations should show similar patterns of variation with C and N (S) concentrations, respectively.

## Materials and Methods

**Study Areas and Sampling.** The study site is located at central area of Dabieshan Mountain (31° 35' N, 116° 08' E) in Anhui province in central China with a transit of warm temperate to subtropical climate. The mean annual temperature and precipitation are 15.6°C and 1,336 mm, respectively. The zonal vegetation is deciduous broadleaf forests with oriental oak as dominant tree species. We chose the one plot midway up the sunny slope of a mountain, with the elevation of 659 m. The sample stand was natural secondary forests and aged at 56 yr. In the stand, the mean tree height and diameter at breast height were 18.5 m and 29.8 cm, respectively.

In the middle of October 2009, we sampled acorns from the ground during the peak of acorn production. In the laboratory, the acorns were stored about 1 wk in room temperature, allowing the larvae emerge from acorns. Larvae were collected within 24 h of emergence. The weevil larvae were cleaned with distilled water and blotted dry by super

absorbent paper. We used these samples to carry out two experiments to explore the cause of body size-related variation in weevil larva elemental composition.

In the first experiment, we measured the elements and nutrient storage separately. The samples were divided into two groups. The one group was oven-dried at 50°C for 1 wk to constant weight, stored in silica gel bag, and kept cool (4°C) until processing for C, N, and S analyses. The second group was immediately frozen at -80°C for later analysis of lipid and soluble protein.

In the second experiment, the samples were sorted to different classes according to the fresh mass (Table A1), and for one class, we chose 8–10 similar body mass larvae as one sample for measuring elements and macromolecules simultaneously, because individual larvae are too small for simultaneous analyses.

**Chemical Analysis.** In the first experiment, we chose 20 larvae across a range of different body sizes for C, N, and S analysis. Before analysis, the individual larvae were weighed to nearest 0.001 mg and were placed inside each vial with three small magnetic stir bars. The vials were capped and then vortexed for 1 min to completely homogenize the sample. The concentrations of C, N, and S ( $\text{mg g}^{-1}$ ) were assayed by using an elemental analyzer (Vario ELIII). See the detailed methods in the Sun et al. (2012).

Similarly, we chose 40 larvae with different body sizes for lipid ( $N=20$ ) and protein analysis ( $N=20$ ). Each larva was weighed to the nearest 0.001 mg, and then homogenized with six small magnetic stir bars and phosphate-buffered saline (PBS) (for protein) or 2%  $\text{Na}_2\text{SO}_4$  (for lipids) in vial. Protein concentrations were determined with the Bradford method after extraction on PBS (Nestel et al. 2003). Lipids were extracted from individual weevils with a chloroform-methanol separation method. Quantification of the storage substance was carried out using the colorimetric techniques developed for fly analysis as modified by Nestel et al. (2003).

In the second experiment, we created a homogenized sample for each size class by grinding larvae in a vial. A part of fresh sample was used to measure protein and lipid. Another part of fresh sample was weighed and oven-dried at 50°C, then weighed again for calculating the water content. Lipids were removed by chloroform-methanol, and C, N, and S concentrations in residual larval biomass were measured.

**Statistical Analysis.** Linear regression was employed to fit the relationships of weevil larva stoichiometry and nutrient storage (lipid and protein) with body size. The same analysis was also used for analyzing the relationships among elements. Finally, we used hierarchical partitioning to explore the contribution of nutrient storage and body mass to variation in larva stoichiometry (Heikkinen et al. 2004). Hierarchical partitioning was conducted using the “hier.part package” version 0.5–1 (Nally and Walsh 2004), as a part of the R statistical package. All analyses were conducted R 2.2.1 (R Development Core Team 2005).

## Results

**Stoichiometric Traits and Nutrient Storage of Weevil Larvae.** The C, N, and S concentrations of homogenized acorns (C, 43.70%, N, 0.73%, and S, 0.17%) were low compared with larval C, N, and S concentrations, respectively (Fig. A1, Table 1). There was substantial variation in elemental concentrations among the individuals. The ranges of concentrations were 58.75–64.43% for C, 3.56–6.30% for N, and 0.24–0.59% for S, respectively (Table 1). Additionally, N and S concentrations were positively correlated and both decreased with C (Fig. A2). C and N showed positive relationships with lipid and protein, respectively (Fig. A2). The coefficient of variation (CV) of C, N, and S in residual larval biomass following lipid and protein extraction was low relative to their respective CV in total biomass (Fig. 1).

Lipids were the main source of reserves in larvae, making up about a third of the wet weight, with an average concentration of 30.49%, and

**Table 1. Statistics of nutrient concentrations (dry mass), molar ratios, and macromolecule (wet mass) (%) in larvae**

Elements/ratios	Mean	Min	Max	SE	<i>n</i>
C	61.81	58.75	64.43	0.19	20
N	4.84	3.56	6.30	0.19	20
S	0.33	0.24	0.59	0.02	20
C:N	15.37	20.88	10.97	2.95	20
C:S	531.92	702.98	272.21	112.09	20
S:N	0.03	0.03	0.02	0.00	20
Macromolecule					
Lipid	30.49	17.49	52.61	2.34	19
Protein	5.62	3.72	8.41	0.24	24

varied from 17.49 to 52.61%, greater than soluble protein (5.62%) in larvae (Table 1).

**Variation in Weevil Larva Stoichiometry and Nutrient Storage With Body Weight.** The C concentration in weevil larva was negatively associated with body weight, whereas N and S were positively associated with body weight (Figs. 2 and 3). The relationships between larval C:N and C:S and body weight were negative, and the larvae S:N did not change with body weight (Fig. 2). The body weight depend-variable patterns observed for C and N concentrations, to some extent, can be also found for lipid and protein (Figs. 2–4).

Based on hierarchical partitioning (Table 2), the variations in C and N with body size were partly explained by lipid and protein storage, whereas the S was partly explained by protein storage.

## Discussion

Consistent with our hypothesis, the N, S, and protein concentrations in weevil larval bodies increased with body mass, whereas C and lipid decreased. These results implied that shifts in nutrient storage are the main causes for variation in weevil larva stoichiometry. This finding was further supported by our observation that the variation in larval C, N, and S decreased following lipid extraction.

**Nutrient Storage and Related Stoichiometric Traits in Weevil Larvae.** One intriguing phenomenon was that larval C concentration reached 61.81% of dry weight, higher than acorns (43.71%) (Fig. A1). This phenomenon was also found in metamorphosis copepodite (with C concentration of 60.00%) prior to the adult stage (Villar-Argaiz et al. 2002). Larval N (4.84%) and S (0.33%) concentrations were low compared with N (8.52%) and S (0.46%) concentrations in residual larval biomass after nutrient extraction, respectively, and were also low relative to other adult insects N (*Orphulella pelidna*, 9.27% and *Manduca sexta*, 11.87%) (Fagan et al. 2002), and nymphs S (*Schistocerca americana*, 0.65%) (Boswell et al. 2008), respectively, reflecting the dilution of N and S due to nutrient storage. Our result suggests that insects that undergo metamorphosis store nutrients before reaching their adult stage, resulting in a deviating stoichiometry with high C, and low N and S.

The lipid concentration in our study organism (30.49%, wet mass) was higher than the reports of Lease and Wolf (2011), which showed that the larval holometabolous Coleoptera lipid concentration was 16.03% (dry mass). We also observed high protein concentrations in weevil larvae (about 112  $\text{mg g}^{-1}$  of the dry weight), and even higher protein concentrations have been measured in *Heliothis virescens* pupae (up to 170  $\text{mg g}^{-1}$  of the dry weight) (Telang et al. 2002). Storage proteins are known to play important roles in insect molting (Singh and Brown 1957) and reproduction (Wilson and Hill 1989; Telfer and Kunkel 1991; Pan and Telfer 2001; Burmester, 2001, 2002; Telang et al. 2002).

**Coupled Variation Pattern in Weevil Larva Stoichiometry and Nutrient Storage With Body Weight.** The existing literatures indicate linear (isometric) or hypermetric scaling relationships of lipids in adult insects with body mass (Hurst and Conover 2003, Boswell et al. 2008).

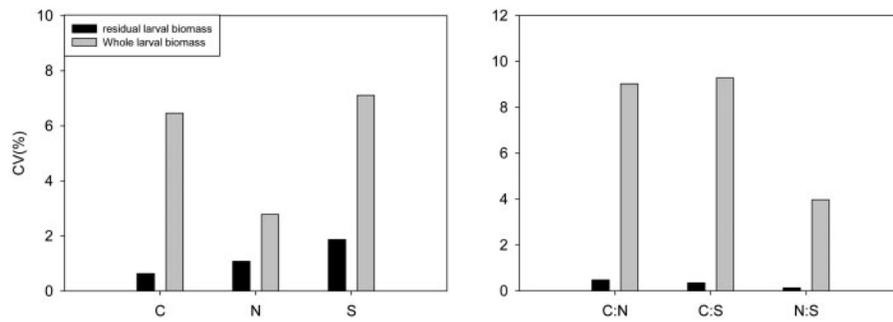


Fig. 1. The CV of C, N, S, and their ratios in residual larval biomass following lipid extraction and total larval biomass.

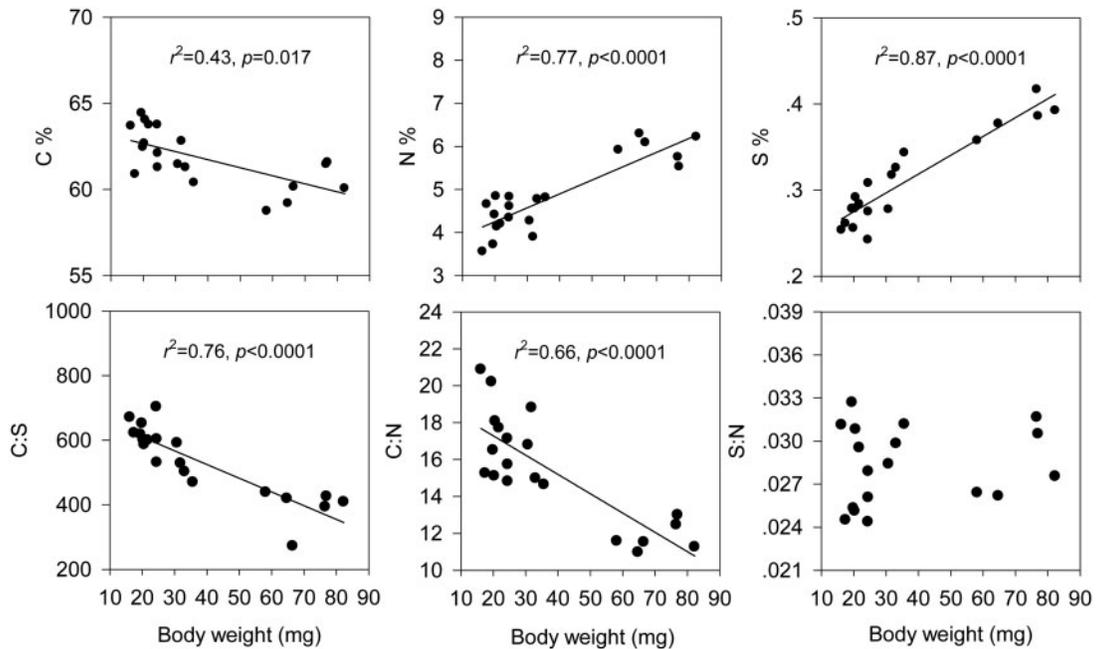


Fig. 2. C, N, and P concentrations and their ratios in weevil larva with body weight (dry mass).

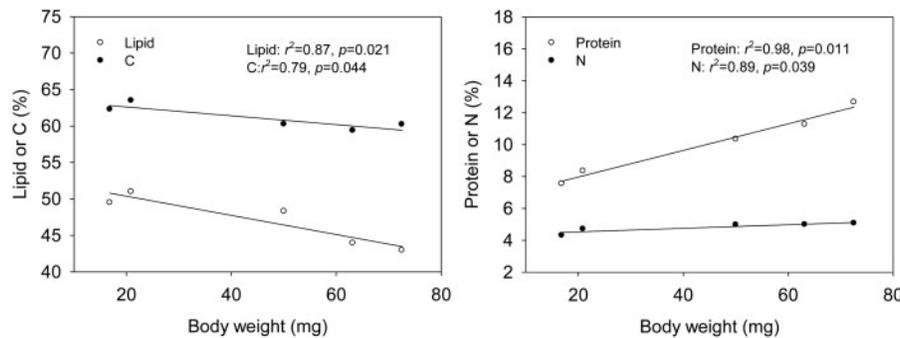


Fig. 3. Macromolecule (lipid and protein) and element composition (C and N) in weevil larva along body weight (dry mass).

In this study, the C and lipid concentrations in weevil larva rather had a negative relationship with body mass. Just one previous study that we are aware of suggested a positive relationship between protein concentrations and body mass (Thomas and Diwan 1990), agreeing with the results observed in the current analysis. For adult insects, N concentration decreased with body size, whereas in this study, N and S in weevil larva showed positive variation patterns with body size, together with relatively constant S:N, reflecting an increased concentration of S-protein with body mass.

In this study, the variation of C, N, and S concentrations in residual larval biomass after lipid extraction was small and low relative to the variation of C, N, and S concentrations in total biomass. These results imply that the body mass-depend variability of C, N, and S concentrations may be caused by variation in nutrient storage across the spectrum of larval body mass. This explanation was supported by the result of the positive relationships between C and lipid, and N and protein. The hierarchical partitioning also showed that the variations in C, N, and S concentrations were partly explained by the shifts in lipid and protein

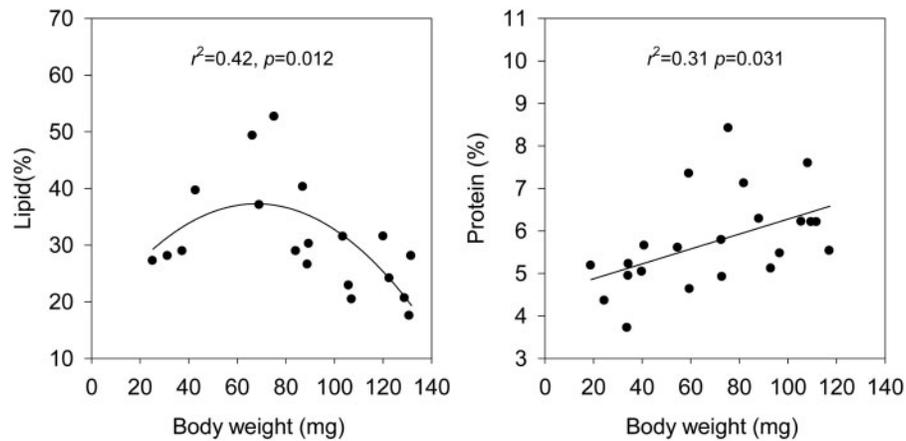


Fig. 4. Macromolecule (lipid and protein) concentration in weevil larva along body weight (wet mass).

Table 2. Results of hierarchical partitioning for the effect of nutrient storage and body size on the weevil larva C, N, and S concentration

Elements	Full model ( $r^2$ )	Contribution of the individual predictor (%) factor		
		Body mass	Lipid	Protein
C	0.99	42.17	24.95	32.87
N	0.96	36.62	25.73	37.64
S	0.73	47.10	6.56	46.34

storage. Here, the variation in weevil larva stoichiometry across body weight may be a production of the allometric scaling relationship between nutrient storage and body weight.

**Possible Ecological Consequences of the Variation in Weevil Larva Stoichiometry.** From an adaptive standpoint, the patterns of stoichiometric variation with body mass may be needed to fulfill the nutrients requirements of diapause, potentially increasing their fitness. According to our results and some published literatures (Tamaru et al. 1996, Wheeler et al. 2000, Rivero et al. 2001, O'Brien et al. 2002, Telfer and Pan 2003, Calvo and Molina 2005), we speculate that the high C:N in larval storage implies that holometabolous insects retain more lipid for survival. For the low C:N and C:S in larval storage, reflecting consumer sequestered more sulfur protein for metamorphosis and reproduction. Therefore, the internal nutrient reserves accumulated prior to diapause are critical for the weevil survival and reproduction, potentially playing an important role in weevil population dynamics. Gaining a better understanding of how consumer stoichiometry affects their fitness may be a fruitful area of future study.

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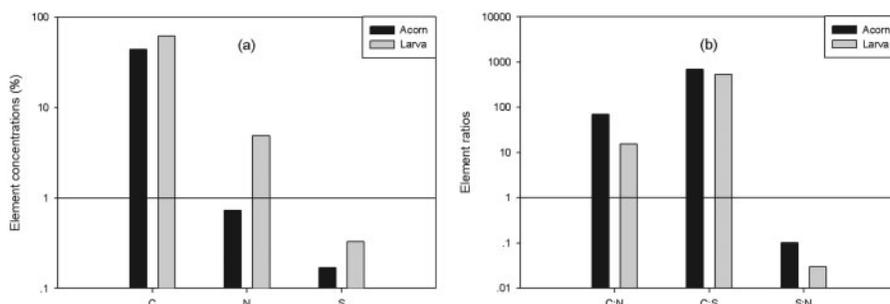
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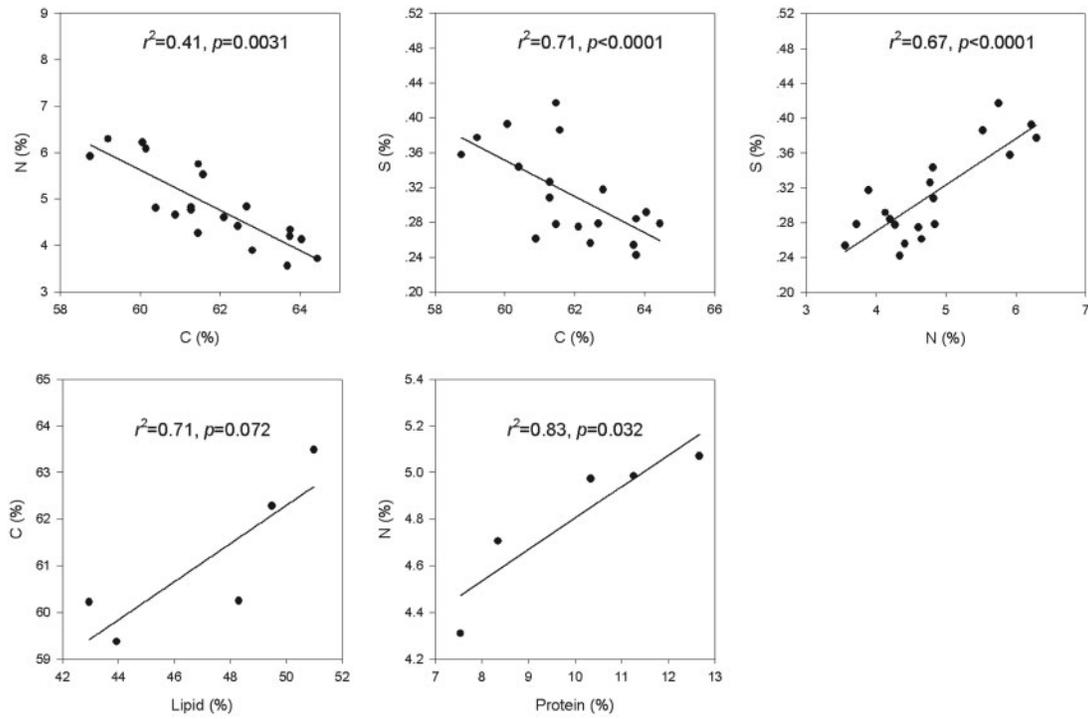
## Appendix

**Table A1. Summary of body mass (dry weight) (mg), nutrient substances, and element concentrations (%)**

Body mass	Lipid	C	Protein	N
16.9	49.5	62.3	7.5	4.3
20.9	51.0	63.5	8.3	4.7
50.0	48.3	60.3	10.3	5.0
63.2	43.9	59.4	11.3	5.0
72.6	43.0	60.2	12.7	5.1



**Fig. A1.** The C, N, and S concentrations and their ratios in acorn and larva.



**Fig. A2.** The relationships among elements and nutrient storage.