



# A non-lethal sampling method for estimating the trophic position of an endangered giant water bug using stable isotope analysis

SHIN-YA OHBA,<sup>1</sup> JUN-ICHI TAKAHASHI<sup>2</sup> and NOBORU OKUDA<sup>1</sup> <sup>1</sup>Center for Ecological Research, Kyoto University, Otsu, Japan and <sup>2</sup>Faculty of Life Science, Kyoto Sangyo University, Kyoto, Japan

**Abstract.** 1. We propose a non-lethal sampling method involving stable isotope analysis for estimating the trophic position of the endangered giant water bug *Kirkaldyia* (= *Lethocerus*) *deyrolli* (Heteroptera: Belostomatidae) in the wild.

2. *Kirkaldyia deyrolli* individuals were collected and their  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values were measured. The  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of periphyton and particulate organic matter, the basal food sources in lentic ecosystems of rice fields, were also measured to estimate the trophic position of *K. deyrolli*. When individual isotopic signatures of the whole body were compared with those of their middle leg tarsus, we found strong correlations between them for both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ . To estimate their trophic position without killing individuals, we constructed a regression model incorporating their middle leg tarsus's isotopic signatures and their body size as explanatory variables. This non-lethal method revealed that *K. deyrolli* showed great individual variation in its  $\delta^{15}\text{N}$  which is a proxy of trophic position, ranging from 5.60‰ to 8.11‰.

3. To evaluate the negative effects of our non-lethal method on the fitness of *K. deyrolli*, we examined how the removal of the middle leg tarsus affected reproductive performance under laboratory conditions. A comparison between the manipulated and unmanipulated individuals revealed that the removal treatment did not have any negative effects on female clutch size or egg hatchability for males.

4. In conclusion, stable isotope analysis of the middle leg tarsus of *K. deyrolli* is useful for estimating its trophic position without lethal or any negative fitness effects.

**Key words.**  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , endangered species trophic niche, giant water bug, non-destructive method

## Introduction

The giant water bug *Kirkaldyia deyrolli* Vuillefroy (formerly *Lethocerus deyrolli*; see Perez Goodwyn, 2006) is a predacious aquatic insect that inhabits small ponds, marshes, and rice fields. Rice fields provide major habitats for lentic aquatic insects including the giant water bug *K. deyrolli* (Hashizume, 1994; Mukai *et al.*, 2005), and it is the top predator in rice field food webs, feeding on aquatic insects and vertebrates such as fish, anuran, and even reptiles (Hirai & Hidaka, 2002; Mori & Ohba, 2004; Ohba & Nakasuji, 2006; Hirai, 2007; Ohba *et al.*, 2008; Ohba, 2011a, 2012). In Japan, its wild populations have drasti-

cally declined during the last four decades, and it is now included on the *Red Data List* for local populations in 45 of 47 prefectures of Japan (Japan Environment Agency, 2000; Association of Wildlife Research & EnVision, 2007). Decreases in suitable aquatic habitats, as well as water pollution, urbanisation, and invasion of exotic crayfish have been found to contribute to the above-mentioned decline in *K. deyrolli* population sizes (e.g. Hirai & Hidaka, 2002; Ohba & Takagi, 2005; Ho *et al.*, 2009; Yoon *et al.*, 2010; Nagaba *et al.*, 2011; Ohba, 2011b).

Because the quantity and quality of prey animals are important factors determining a predator's life history and abundance (Lenski, 1984; Juliano, 1986), information on its food habits and trophic position is necessary for evaluation of its food availability and habitat quality, providing an opportunity to design conservation plans, especially for endangered species. A variety of techniques have been used to study the food habits of predacious insects, for instance, gut content analysis (Deding, 1988; Hicks,

Correspondence: Shin-ya Ohba, Center for Ecological Research, Kyoto University, 2-509-3, Hirano, Otsu 520-2113, Japan. E-mail: oobug@hotmail.com

1994), laboratory feeding experiments (Sugiyama *et al.*, 1996), direct observations in the field (Sota, 1985; Ohba & Nakasuji, 2006), food item identification using enzyme-linked immunological assays (Sunderland *et al.*, 1987), and DNA analysis (Zaidi *et al.*, 1999; King *et al.*, 2008).

For *K. deyrolli*, its food habits have often been investigated on the basis of field observations (Hirai & Hidaka, 2002; Mori & Ohba, 2004; Ohba & Nakasuji, 2006; Hirai, 2007; Ohba *et al.*, 2008). Although previous studies have reported on its predacious habits (Hirai & Hidaka, 2002; Mori & Ohba, 2004; Ohba & Nakasuji, 2006; Hirai, 2007; Ohba *et al.*, 2008; Ohba, 2011a, 2012), observational approaches can suffer from sampling bias because the prey handling time and consumption rate of *K. deyrolli* are markedly different between invertebrate and vertebrate prey (S. Ohba, unpubl. data).

Stable isotope analysis is a powerful tool to quantitatively evaluate animal diets because it reflects time-integrated information about its food habits (Fry, 2006). This technique relies on the fact that the stable carbon and nitrogen isotope ratios of a consumer are enriched by a given fractionation factor through the assimilation of prey. Generally, the carbon isotope ratio ( $\delta^{13}\text{C}$ ) is only changed by 0.8‰ as a result of a trophic interaction, and thus can be used to identify the basal carbon sources of consumers (DeNiro & Epstein, 1978; Fry & Sherr, 1984). On the other hand, the nitrogen isotope ratio ( $\delta^{15}\text{N}$ ) of consumers is enriched by, on average, 3.4‰ relative to that of their prey (Minagawa & Wada, 1984; Post, 2002). Thus, the  $\delta^{15}\text{N}$  is a good indicator for the trophic position of a focal consumer. Based on these principles of isotopic trophic enrichments, we can estimate the trophic position of consumers by measuring the  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of both basal food sources and consumers. Stable isotope analysis, which is based on metabolic physiology, can quantitatively estimate trophically assimilated materials, but is not suitable for identifying specific prey items, unless detailed dietary information is available. The effectiveness of stable isotope analysis is thus improved when it is used in combination with conventional dietary analysis (Vander Zanden *et al.*, 1997).

In spite of its utility and high analytical accuracy, this technique raises an ethical problem, especially if the study subjects are endangered species, because it entails the sacrifice of a member of the population being studied. Here, we propose a non-lethal method involving stable isotope analysis of a small body part for estimating the trophic position of *K. deyrolli*. For some vertebrates, it has been reported that the isotopic signatures of muscle tissues are highly correlated with those of other tissues at the individual position (Pinnegar & Polunin, 1999; Perga & Gerdeux, 2005). Taking advantage of stable isotope analysis, which requires only a small quantity of sample (usually less than 1 mg in dry weight), we may be able to predict the isotopic signatures of whole body tissues from those of a small portion of body parts without killing subject animals, while it is less likely that such a non-lethal sampling have negative effects on the subject's fitness. For example, the femur muscle of a large tree weta, *Hemideina thoracica*, was used for stable isotope analysis instead of the whole body (Wehi & Hicks, 2010).

To establish the non-lethal dietary analysis for endangered insect species, we conducted the stable isotope analysis for the

middle leg tarsus of *K. deyrolli*. However, it is possible that such body parts have important function-determining fitness consequences of insects. So, we examined how the removal of the middle leg tarsus affected the fitness components of *K. deyrolli* by measuring its reproductive performance under laboratory conditions.

## Materials and methods

### Stable isotope analysis

To establish the non-lethal dietary analysis for *K. deyrolli*, field samplings were conducted in the rice fields of western Hyogo, central Japan, from late May to early August in 2010. Belostomatidae, including *K. deyrolli*, are usually attracted to street lights in the night and are sometimes called 'electric light bugs' (Menke, 1979). This is a conservation issue because many *K. deyrolli* individuals become 'road-kill' whilst they are under a street light, and it is believed that such deaths are one of the primary factors in their recent population decline (Ohba & Takagi, 2005; Yoon *et al.*, 2010). Considering its endangered state, 15 individuals that died after being run over by a vehicle were collected from under streetlights. In addition, 10 individuals that died during the breeding experiment (see below) were also used in the analysis. The specimens were washed with distilled water, and their tarsus was clipped off from the left middle leg using dissecting scissors. Their whole body and tarsus tissue were preserved at  $-20\text{ }^{\circ}\text{C}$  separately for the stable isotope measurements.

To apply our non-lethal method to live *K. deyrolli* in nature, we collected 24 young adults with soft exoskeleton from five rice fields between early July and middle August, 2011. Because these individuals are estimated as being within a week of final instar moulting, it is assumed that their isotopic signatures almost reflect the lentic food web of rice fields where they lived the nymphal stage. To estimate their trophic position based on the stable isotope analysis, we also collected their three prey items, water bugs *Appasus japonicus* Vuillefroy ( $n = 3$ ), tadpoles *Hyla japonica* Günther ( $n = 4$ ) and dragonfly nymphs *Sympetrum* spp. ( $n = 3$ ), as well as two basal food sources, particulate organic matter (POM) and periphyton, embedded within the lentic food web of the rice fields. *Kirkaldyia deyrolli* and its three prey items were captured with D-frame net (3 mm mesh, 0.28 m wide). After cutting middle leg tarsus of *K. deyrolli* young adults using the dissecting scissors, we released them carefully at their capture sites. The POM and periphyton were collected from five locations of the study rice fields three times during the study period (early and late July, and middle August). The POM was filtrated on a Whatman GF/F glass-fibre filter after size-screening by a 20  $\mu\text{m}$  mesh net. We scraped periphyton off stones or any hard substrata in the rice fields using a toothbrush. For the POM and periphyton, we pooled their sub-samples from five different locations into one sample to take an average in each sampling period, considering spatial heterogeneity of their isotopic signatures. All samples, including animals and basal food sources, were preserved at  $-20\text{ }^{\circ}\text{C}$  for the following stable isotope analysis.

Prior to the stable isotope analysis, the frozen samples were dried at 60 °C for 24 h. Thereafter, the dry weight of *K. deyrolli* was recorded as an index of body size. For insects and vertebrates, their whole body and muscle tissues were used for the stable isotope analysis respectively. The animal samples were pulverised and immersed in 2:1 chloroform-methanol solution for 24 h to remove lipids (Bligh & Dyer, 1959). All dry samples were wrapped in tin capsules for combustion. Then, their carbon and nitrogen stable isotope ratios were measured using continuous-flow isotope ratio mass spectrometers (Delta V Plus; Thermo Fisher Scientific Inc., Waltham, MA, USA) with Flash ES (Thermo Fisher Scientific Inc.) and Delta S (Finnigan MAT, Germany) with EA1108 (Fisons, Italy), at the Center for Ecological Research, Kyoto University. The isotopic notations of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) are expressed as per mil deviation from the relevant standards (Pee Dee belemnite limestone carbonate for  $\delta^{13}\text{C}$  and atmospheric nitrogen for  $\delta^{15}\text{N}$ ), as defined by the following equation:

$$\delta^{15}\text{N} \text{ or } \delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000(\text{‰})$$

where  $R$  denotes  $^{15}\text{N}/^{14}\text{N}$  or  $^{13}\text{C}/^{12}\text{C}$ . We also used working standards (CERKU-01, 02, 03, 04), which were provided by the Cooperative Research Facilities of the Center for Ecological Research, Kyoto University (Tayasu *et al.*, 2011). The analytical precision, which was defined as the standard deviation of the measurements of the working standards, was 0.1‰ for  $\delta^{13}\text{C}$  and 0.2‰ for  $\delta^{15}\text{N}$  in all runs.

#### Fitness consequences of tarsus removal

In this study, laboratory experiments were conducted to evaluate the effects of clipping off the middle leg tarsus on reproductive performance as the primary fitness components of *K. deyrolli* from 7 June to 7 August, 2010. In nature, it is difficult to measure the survival rate and prey capturing ability of *K. deyrolli* because of its small population size and broad home range. Clutch size, the number of eggs in an egg mass, was recorded to measure female fecundity. Egg hatchability was recorded to measure male parental ability because it depends on male brooding behaviour (Ichikawa, 1988).

*Kirkaldyia deyrolli* adults (12 males and 10 females) were collected from the same area as the sampling sites from late May to early June 2010. All bugs were transferred to our laboratory and maintained individually in plastic cups (10 cm diameter, 10 cm high, and 5 cm water depth). The captive conditions were set so that they were the same as those of the *K. deyrolli*'s natural habitat ( $24.6 \pm 0.21$  °C). All bugs were fed daily with live *Carassius* fish in an *ad libitum* manner until the start of the experiment. The leftovers were immediately removed from the plastic cup. To evaluate the effects of clipping off the middle leg tarsus on female and male fitness, the tarsi of 12 individuals were surgically clipped off from the left middle leg using dissecting scissors (hereafter referred to as the manipulated: six males and six females). As controls, 10 individuals were captured but their tarsi were not removed (hereafter referred to as the unmanipulated: six males and four females).

When a female had a greenish and bellied abdomen, indicating egg maturation, they were introduced to a breeding aquarium (300L × 175W × 210H mm). The aquarium was supplied with dechlorinated tap water and a 10 mm-thick layer of river gravel. Two sticks (20 mm diameter, 150 mm height) were served as oviposition substrata, and a manipulated or unmanipulated male was randomly introduced into the breeding aquarium. After 24 h, the female was returned to their plastic cage, irrespective of whether they had laid an egg mass. This procedure was repeated until all the females had laid an egg mass. The egg mass and its attending male were transported to another plastic cage (10 cm diameter, 20 cm high, and 5 cm water depth) until the egg mass hatched. Egg hatchability was recorded at 12 h after the first egg had hatched. The experimental subjects were released at their capture sites after the breeding experiment but those that accidentally died were preserved at -20 °C prior to the stable isotope analysis.

#### Data analysis

Assuming that young adults of *K. deyrolli* are embedded within the rice field food web starting from two basal food sources, POM and periphyton, their trophic level was estimated using the following equation:

$$\alpha = (\delta^{13}\text{C}_{\text{bug}} - \delta^{13}\text{C}_{\text{periphyton}} - 0.1\text{TL}) / (\delta^{13}\text{C}_{\text{POM}} - \delta^{13}\text{C}_{\text{periphyton}})$$

$$\text{TP} = 1 + \{\delta^{15}\text{N}_{\text{bug}} - [\alpha \cdot \delta^{15}\text{N}_{\text{POM}} + (1 - \alpha) \cdot \delta^{15}\text{N}_{\text{periphyton}}]\} / 2.6$$

where  $\delta^{13}\text{C}_{\text{bug}}$  is the  $\delta^{13}\text{C}$  of *K. deyrolli*'s whole body estimated from that of its middle leg tarsus, and  $\delta^{13}\text{C}_{\text{periphyton}}$  and  $\delta^{13}\text{C}_{\text{POM}}$  the mean  $\delta^{13}\text{C}$  of the periphyton and POM samples respectively. The notation of  $\delta^{15}\text{N}$  is the same as that of  $\delta^{13}\text{C}$ . TP is the trophic position of an individual *K. deyrolli*. As trophic enrichment factors for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , we used the literature value reported for predacious water strider with similar feeding ecology: 0.1‰ for  $\delta^{13}\text{C}$  and 2.6‰ for  $\delta^{15}\text{N}$  (Jardine *et al.*, 2008).

We used the multiple regression model in R statistical package (version 2.12.1; R Development Core Team 2011) to estimate  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of *K. deyrolli*'s whole body (WB) from those of its middle leg tarsus, considering any body size- and sex-specific effects on their stable isotope signatures. As body size and sex are highly correlated with each other, two models were separately constructed to remove multicollinearity, as follows.

$$\text{Model (A)} \quad \delta R_{\text{WB}} = \beta_0 + \beta_1 \cdot \delta R_{\text{TS}}$$

$$\text{Model (B)} \quad \delta R_{\text{WB}} = \beta_0 + \beta_1 \cdot \delta R_{\text{TS}} + \beta_2 \cdot \text{size}$$

$$\text{Model (C)} \quad \delta R_{\text{WB}} = \beta_0 + \beta_1 \cdot \delta R_{\text{TS}} + \beta_3 \cdot \text{sex}$$

Here  $\delta R_{\text{WB}}$  and  $\delta R_{\text{TS}}$  represent  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  for the whole body and middle leg tarsus of *K. deyrolli*, respectively, and  $\beta_n$  the standardised coefficient. We selected the best-fit one out of these three models with a stepwise method on the basis of the Akaike's information criterion.

To examine whether the clipping off treatment had negative effects on the clutch size and egg hatchability of *K. deyrolli*, the data were analysed with a generalised linear mixed model

(GLMM) using the glmmML package in R with a Poisson distribution for clutch size and a binomial distribution for egg hatchability. The treatment was incorporated into our model as a fixed effect, and each individual was fitted as a random effect because these parameters were repeatedly measured for the same individuals. Body length was not significantly different between manipulated and unmanipulated individuals in females (manipulated:  $57.25 \pm 0.52$  mm,  $n = 6$  unmanipulated:  $55.01 \pm 1.43$  mm,  $n = 6$ ;  $F_{1,10} = 2.16$ ,  $P = 0.172$ , one-way ANOVA) and males (manipulated:  $64.76 \pm 0.74$  mm,  $n = 4$ , unmanipulated:  $64.72 \pm 1.80$  mm,  $n = 4$ ;  $F_{1,8} = 0.0003$ ,  $P = 0.986$ ).

All statistical tests were conducted using the R statistical package (version 2.12.1; R Development Core Team 2011). For descriptive purposes, means  $\pm$  SE are given, except for egg hatchability, which is expressed as the median  $\pm$  quartile range.

## Results and discussion

In order to estimate isotopic signatures of *K. deyrolli*'s whole body, we constructed the best-fit model (Tables 1 and 2). Although isotopic signatures of middle leg tarsus accounted for a large part of variation in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of *K. deyrolli*'s whole body (Figure 1), the body size was incorporated as additional explanatory variable into the best-fit model, as follows:

$$\delta^{15}\text{N}_{\text{WB}} = 1.090 + 0.810 \cdot \delta^{15}\text{N}_{\text{TS}} + 0.523 \cdot \text{size} (R^2 = 0.77, P < 0.001)$$

$$\delta^{13}\text{C}_{\text{WB}} = -2.853 + 0.912 \cdot \delta^{13}\text{C}_{\text{TS}} + 0.586 \cdot \text{size} (R^2 = 0.69, P < 0.001)$$

Although we did not evaluate the turnover rate of the whole body and middle leg tarsus tissues in *K. deyrolli* in the present study, it has been reported for another aquatic predacious bug (water strider *Aquarius remigis*) whose turnover rate is 1.5 days for carbon and 7.8 days for nitrogen (Jardine *et al.*, 2008). These rates are much higher than that of another terrestrial insect (21 days for adult ladybird beetles, *Harmonia variegata*; Ostrom *et al.*, 1997). In the case of our study, however, we used exoskeleton tissues of leg tarsus which usually have a slower turnover rate compared to reproductive organs and fat tissues (Gratton & Forbes, 2006), implying that the leg tarsus tissue of *K. deyrolli* would integrate isotopic information on its feeding habits for longer time relative to that of *A. remigis*. In our model, the body size showed a positive function of the whole body isotopic signa-

**Table 1.** Selected models explaining the variation in their body tissues the isotopic signatures of *Kirkaldyia deyrolli*.

Response variable	Explanatory variable	AIC
$\delta^{15}\text{N}_{\text{WB}}$	$\delta^{15}\text{N}_{\text{TS}}$	49.96
	$\delta^{15}\text{N}_{\text{TS}} + \text{size}$	45.97
$\delta^{13}\text{C}_{\text{WB}}$	$\delta^{15}\text{N}_{\text{TS}} + \text{sex}$	50.95
	$\delta^{13}\text{C}_{\text{TS}}$	58.25
	$\delta^{13}\text{C}_{\text{TS}} + \text{size}$	55.00
	$\delta^{13}\text{C}_{\text{TS}} + \text{sex}$	55.98

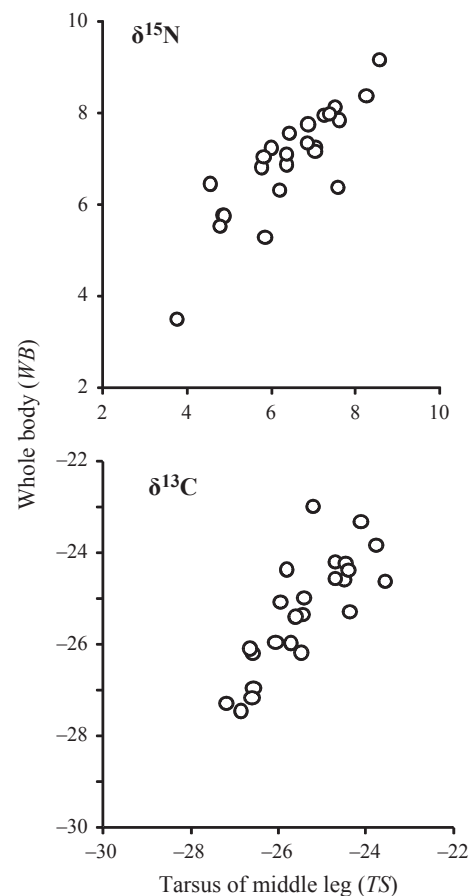
AIC, Akaike's information criterion.

**Table 2.** Results of the multiple regression analysis in order to estimate the isotopic signatures of *Kirkaldyia deyrolli*.

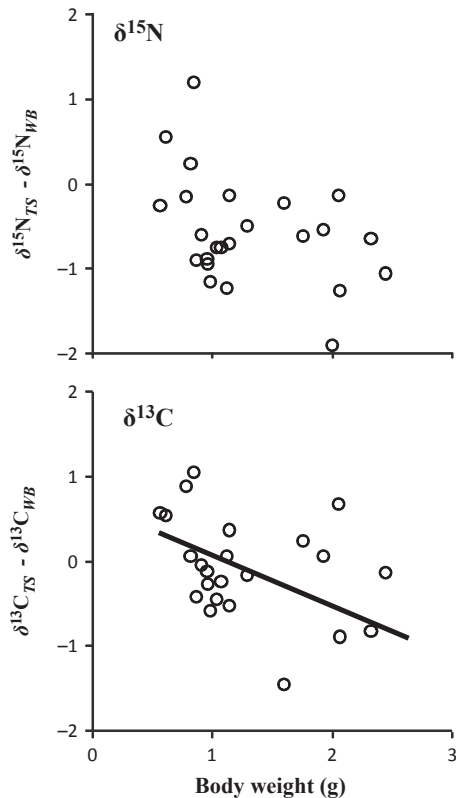
Response variable	Explanatory variable	Parameter estimate	SE	<i>t</i>	<i>P</i>
$\delta^{15}\text{N}_{\text{WB}}$	Intercept	1.090	0.661	1.648	0.114
	$\delta^{15}\text{N}_{\text{TS}}$	<b>0.810</b>	<b>0.098</b>	<b>8.197</b>	<b>&lt;0.001</b>
	<b>Size</b>	<b>0.523</b>	<b>0.215</b>	<b>2.441</b>	<b>0.024</b>
$\delta^{13}\text{C}_{\text{WB}}$	Intercept	-2.853	3.624	-0.787	0.440
	$\delta^{13}\text{C}_{\text{TS}}$	<b>0.912</b>	<b>0.140</b>	<b>6.511</b>	<b>&lt;0.001</b>
	<b>Size</b>	<b>0.586</b>	<b>0.259</b>	<b>2.265</b>	<b>0.034</b>

Bold type factors are significant ( $P < 0.05$ ).

tures in *K. deyrolli*. This result means that isotopic difference between the whole body and the middle leg tarsus diminishes with the increase in body size. Although it is reported that such an ontogenetic isotopic deviation of the focal tissue shows a non-linear pattern for juvenile but not adult fish partly due to their metamorphosis (Schielke & Post, 2010), we confirmed that the isotopic deviation diminished linearly with the increasing body size for adult *K. deyrolli* (Fig. 2), which validates the assumption of linearity in our model.

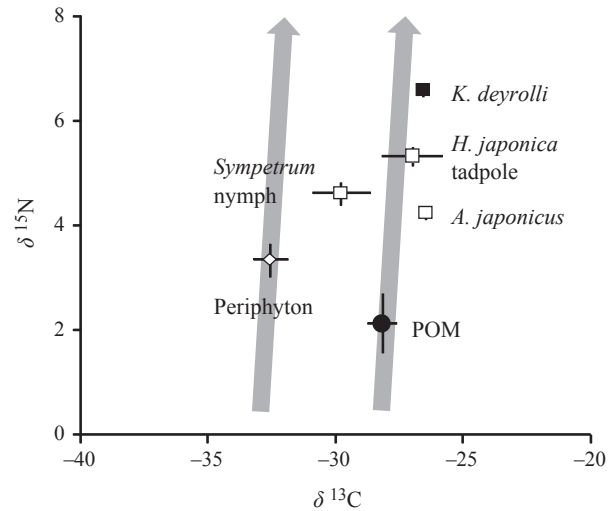


**Fig. 1.** Relationships between the  $\delta^{15}\text{N}$  (upper) or  $\delta^{13}\text{C}$  (lower) values of the whole body (WB) and the tarsus of the middle leg (TS) in *Kirkaldyia deyrolli*.



**Fig. 2.** Relationships between the body weight and the differences in tarsus of the middle leg (TS)–whole body (WB) isotope values.  $\delta^{15}\text{N}$  (upper),  $y = -0.4609x + 0.0457$ ,  $R^2 = 0.161$ ,  $P = 0.052$ ,  $y = -0.5623x + 0.5818$ ,  $R^2 = 0.184$ ,  $P = 0.036$ .

In a natural population of *K. deyrolli*, we detected great individual variation in the stable isotopic signatures of young adult whole body estimated from those of their middle leg tarsus ( $\delta^{15}\text{N}$ :  $6.6 \pm 0.63\text{‰}$ , min. =  $5.60\text{‰}$ , max. =  $8.11\text{‰}$ ;  $\delta^{13}\text{C}$ :  $-26.6 \pm 0.20\text{‰}$ , min. =  $-28.7\text{‰}$ , max. =  $-24.4\text{‰}$ ; Fig. 3). As young adults with soft exoskeleton were estimated as being within a week of final instar moulting, their isotopic signatures would reflect the lentic food web of rice fields where they lived the nymphal stage. Assuming that they relied on the lentic food web entirely, we estimated their trophic level as 2.3–3.3 ( $2.7 \pm 0.05$ ,  $n = 24$ ). When considering that *K. deyrolli* is a top predator in the rice field ecosystems (Hirai & Hidaka, 2002; Ohba & Nakasuji, 2006; Ohba *et al.*, 2008), it seems that the estimated values of trophic level were lower than expected. One of the possible reasons is that the trophic enrichment factor ( $\delta^{15}\text{N} = 2.6\text{‰}$ ) reported for a water strider, *A. remigis*, was not appropriate for this species. In our study, difference of  $\delta^{15}\text{N}$  between *K. deyrolli* and its three major prey items was  $2.4\text{‰}$  for *A. japonicus*,  $1.1\text{‰}$  for *H. japonica* tadpole and  $2.0\text{‰}$  for *Sympetrum* nymph, with the average of  $1.8\text{‰}$ . This value was lower than the reported value of  $2.6\text{‰}$  but similar to the literature mean for carnivorous invertebrates (1.62: Vanderklift & Ponsard, 2003). Rearing experiments to measure the trophic enrichment factor are necessary for exactly estimating the trophic position of *K. deyrolli*.



**Fig. 3.** The C–N map of *Kirkaldyia deyrolli*, its major prey items, and basal sources in the rice fields. Arrows represent hypothetical trophic pathways starting from each primary producer [left: periphyton; right: particulate organic matter (POM)].

Irrespective of the exact trophic position estimation, the great individual variation in their stable isotopic signatures might reflect intra-specific variation in prey preference or foraging area. In many aquatic consumers, it is well known that the body size is a proxy of trophic position because it is a general rule of aquatic food webs in that smaller organisms are eaten by larger ones (Jennings *et al.*, 2007). In *K. deyrolli*, its  $\delta^{15}\text{N}$  was positively correlated with its body size (Table 2), suggesting that larger nymphs can consume prey with a higher trophic position, as also shown in other predacious insects (Sasakawa *et al.*, 2010). Although our regression model of  $\delta^{13}\text{C}$  also suggested that *K. deyrolli* has a size-specific niche variation in relation to trophic pathways, detailed mechanisms for this pattern remain unknown. In combination with field observations of individual feeding habits, the stable isotope analysis provides useful information on the feeding ecology and trophic position of *K. deyrolli*, which will help to conserve its wild populations.

We finally confirmed whether the clipping off treatment had any negative effects on the reproductive performance of *K. deyrolli*. The breeding experiment revealed that there was no significant difference between the manipulated and unmanipulated individuals with regard to clutch size (manipulated:  $80.86 \pm 4.61$  eggs,  $n = 15$ , unmanipulated:  $91.38 \pm 3.45$  eggs,  $n = 8$ ; d.f. = 20,  $z = 0.0112$ ,  $P = 0.991$ , GLMM) or egg hatchability (manipulated:  $97.22 \pm 0.04\%$ ,  $n = 12$ , unmanipulated:  $97.80 \pm 0.02\%$ ,  $n = 10$ ; d.f. = 19,  $z = 0.0029$ ,  $P = 0.998$ ). A few studies have examined the effects of non-lethal sampling method on behaviour or survival in winged insects such as Lepidoptera (Vila *et al.*, 2009; Hamm *et al.*, 2010; Kosciński *et al.*, 2011), Hymenoptera (Holehouse *et al.*, 2003; Chaline *et al.*, 2004), and Odonata (Fincke & Hadrys, 2001). Although our results do not completely rule out the possibility that such treatments decreased other fitness components such as feeding success and

survival rate, the middle leg tarsus is less likely to be essential for perching and climbing vegetation above the water surface in order to care their egg, suggesting that the fitness consequences of our treatment were negligible.

In conclusion, the present study showed that the stable isotopic signatures of middle leg tarsus tissue can act as a good proxy for those of the whole body in *K. deyrolli*. Our breeding experiments also demonstrated that the removal of the middle leg tarsus had no significant negative effects on the reproductive performance of *K. deyrolli*. Using this method, we can estimate the trophic position of *K. deyrolli* in nature without doing them any harm as well as killing bugs. Our non-lethal sampling method is useful and ethically promising for understanding the feeding ecology of *K. deyrolli* and its ecological role in the rice field ecosystems. The method is broadly applicable to animal conservation studies, especially those involving endangered insect species.

### Acknowledgements

We thank J. Shibata, N.F. Ishikawa, and Y. Sakai for their helpful advice regarding the stable isotope analysis, Y. Inatani for his help with the sample collection, and N. Sonoda and Y. Ohba for their kind assistance during this study. The present study was conducted under the guidance of Cooperative Research Facilities (Isotope Ratio Mass Spectrometer) of Center for Ecological Research, Kyoto University. This study was supported by a Grant-in-Aid for JSPS Fellows (22-4 to S. Ohba); Grants-in-Aid from the Japan Society for the Promotion of Science (no. 20370009 and 23657019 to N. Okuda); The Environment Research and Technology Development Fund (D-1102) of the Ministry of the Environment, Japan; and the Global COE Program A06 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to S. Ohba and N. Okuda).

### References

- Association of Wildlife Research & EnVision (2007) *Search System of Japanese Red Data*. <<http://www.jpnrdb.com/index.html>> 16th May 2011.
- Bligh, E. & Dyer, W. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, **37**, 911–917.
- Chaline, N., Ratnieks, F.L.W., Raine, N.E., Badcock, N.S. & Burke, T. (2004) Non-lethal sampling of honey bee, *Apis mellifera*, DNA using wing tips. *Apidologie*, **35**, 311–318.
- Deding, J. (1988) Gut content analysis of diving beetles (Coleoptera: Dytiscidae). *Natura Jutlandica*, **22**, 177–184.
- DeNiro, M.J. & Epstein, S. (1978) Influence of diet on the distribution of carbon isotopes in animals. *Geochimica et Cosmochimica Acta*, **42**, 495–506.
- Fincke, O.M. & Hadrys, H. (2001) Unpredictable offspring survivorship in the damselfly, *Megaloprepus coerulatus*, shapes parental behavior, constrains sexual selection, and challenges traditional fitness estimates. *Evolution*, **55**, 762–772.
- Fry, B. (2006) *Stable Isotope Ecology*. Springer, New York, New York.
- Fry, B. & Sherr, E.B. (1984)  $\delta^{13}\text{C}$  measurements as indicators of carbon flow in marine and freshwater ecosystems. *Marine Science*, **27**, 13–47.
- Gratton, C. & Forbes, A.E. (2006) Changes in  $\delta^{13}\text{C}$  stable isotopes in multiple tissues of insect predators fed isotopically distinct prey. *Oecologia*, **147**, 615–624.
- Hamm, C.A., Aggarwal, D. & Landis, D.A. (2010) Evaluating the impact of non-lethal DNA sampling on two butterflies, *Vanessa cardui* and *Satyrodes eurydice*. *Journal of Insect Conservation*, **14**, 11–18.
- Hashizume, H. (1994) *All About the Giant Water Bug – Wishes for Symbiosis with Aquatic Animals*. Tombo Publishing Co, Osaka, Japan (in Japanese).
- Hicks, B.J. (1994) Foregut contents of adult *Ilybius* Erichson (Coleoptera: Dytiscidae) from Newfoundland. *Coleopterists Bulletin*, **48**, 199–200.
- Hirai, T. (2007) Diet composition of the endangered giant water bug *Lethocerus deyrolli* (Hemiptera: Belostomatidae) in the rice fields of Japan: Which is the most important prey item among frogs, fish, and aquatic insects? *Entomological Science*, **10**, 333–336.
- Hirai, T. & Hidaka, K. (2002) Anuran-dependent predation by the giant water bug, *Lethocerus deyrollei* (Hemiptera: Belostomatidae), in rice fields of Japan. *Ecological Research*, **17**, 655–661.
- Ho, C., Kim, H. & Kim, J.G. (2009) Landscape analysis of the effects of artificial lighting around wetland habitats on the giant water bug *Lethocerus deyrollei* in Jeju Island. *Journal of Ecology and Field Biology*, **32**, 83–86.
- Holehouse, K., Hammond, R. & Bourke, A. (2003) Non-lethal sampling of DNA from bumble bees for conservation genetics. *Insectes Sociaux*, **50**, 277–285.
- Ichikawa, N. (1988) Male brooding behaviour of the giant water bug *Lethocerus deyrollei* Vuillefroy (Hemiptera: Belostomatidae). *Journal of Ethology*, **6**, 121–127.
- Japan Environment Agency (2000) *Threatened Wildlife of Japan. Red Data Book*, 2nd edn. Environment Agency of Japan, Tokyo, Japan (in Japanese).
- Jardine, T.D., Kidd, K.A., Polhemus, J.T. & Cunjak, R.A. (2008) An elemental and stable isotope assessment of water strider feeding ecology and lipid dynamics: synthesis of laboratory and field studies. *Freshwater Biology*, **53**, 2192–2205.
- Jennings, S., de Oliveira, J.A.A. & Warr, K.J. (2007) Measurement of body size and abundance in tests of macroecological and food web theory. *Journal of Animal Ecology*, **76**, 72–82.
- Juliano, S. (1986) Food limitation of reproduction and survival for populations of *Brachinus* (Coleoptera: Carabidae). *Ecology*, **67**, 1036–1045.
- King, R.A., Read, D.S., Traugott, M. & Symondson, W.O.C. (2008) Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular Ecology*, **17**, 947–963.
- Koscinski, D., Crawford, L.A., Keller, H.A. & Keyghobadi, N. (2011) Effects of different methods of non-lethal tissue sampling on butterflies. *Ecological Entomology*, **36**, 301–308.
- Lenski, R.E. (1984) Food limitation and competition: a field experiment with two *Carabus* species. *The Journal of Animal Ecology*, **53**, 203–216.
- Menke, A.S. (1979). Family Belostomatidae. *The Semiaquatic and Aquatic Hemiptera of California (Heteroptera: Hemiptera)* (ed. by A.S. Menke), pp. 76–86, Bulletin of the California Insect Survey, Berkeley, Los Angeles, California.

- Minagawa, M. & Wada, E. (1984) Stepwise enrichment of  $^{15}\text{N}$  along food chains: further evidence and the relation between  $\delta^{15}\text{N}$  and animal age. *Geochimica et Cosmochimica Acta*, **48**, 1135–1140.
- Mori, A. & Ohba, S. (2004) Field observations of predation on snakes by the giant water bug. *Bulletin of the Herpetological Society of Japan*, **2004**, 78–81 (in Japanese).
- Mukai, Y., Baba, N. & Ishii, M. (2005) The water system of traditional rice paddies as an important habitat of the giant water bug, *Lethocerus deyrollei* (Heteroptera: Belostomatidae). *Journal of Insect Conservation*, **9**, 121–129.
- Nagaba, Y., Tufail, M., Inui, H. & Takeda, M. (2011) Hormonal regulation and effects of four environmental pollutants on vitellogenin gene transcription in the giant water bug, *Lethocerus deyrollei* (Hemiptera: Belostomatidae). *Journal of Insect Conservation*, **15**, 421–431.
- Ohba, S. (2011a) Field observation of predation on a turtle by a giant water bug. *Entomological Science*, **14**, 364–365.
- Ohba, S. (2011b) Impact of the invasive crayfish *Procambarus clarkii* on the giant water bug *Kirkaldyia deyrolli* (Hemiptera) in rice ecosystems. *Japanese Journal of Environmental Entomology and Zoology*, **22**, 93–98.
- Ohba, S. (2012) Field observation of predation on a Japanese mamushi, *Gloydius blomhoffii*, by a giant water bug, *Kirkaldyia deyrolli*. *Japanese Journal of Entomology (New Series)*, **15**, 92–93 (in Japanese with English Abstract).
- Ohba, S., Miyasaka, H. & Nakasuji, F. (2008) The role of amphibian prey in the diet and growth of giant water bug nymphs in Japanese rice fields. *Population Ecology*, **50**, 9–16.
- Ohba, S. & Nakasuji, F. (2006) Dietary items of predacious aquatic bugs (Nepoidea: Heteroptera) in Japanese wetlands. *Limnology*, **7**, 41–43.
- Ohba, S. & Takagi, H. (2005) Food shortage affects flight migration of the giant water bug *Lethocerus deyrolli* in the prewintering season. *Limnology*, **6**, 85–90.
- Ostrom, P.H., Colunga-Garcia, M. & Gage, S.H. (1997) Establishing pathways of energy flow for insect predators using stable isotope ratios: field and laboratory evidence. *Oecologia*, **109**, 108–113.
- Perez Goodwyn, P. (2006) Taxonomic revision of the subfamily Lethocerinae Lauck & Menke (Heteroptera: Belostomatidae). *Stuttgarter Beiträge zur Naturkunde, Serie A (Biologie)*, **695**, 1–71.
- Perga, M. & Gerdeaux, D. (2005) 'Are fish what they eat' all year round? *Oecologia*, **144**, 598–606.
- Pinnegar, J. & Polunin, N. (1999) Differential fractionation of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  among fish tissues: implications for the study of trophic interactions. *Functional Ecology*, **13**, 225–231.
- Post, D.M. (2002) Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology*, **83**, 703–718.
- R Development Core Team (2011) *R: a Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Sasakawa, K., Ikeda, H. & Kubota, T. (2010) Feeding ecology of granivorous carabid larvae: a stable isotope analysis. *Journal of Applied Entomology*, **134**, 116–122.
- Schielke, E.G. & Post, D.M. (2010) Size matters: comparing stable isotope ratios of tissue plugs and whole organisms. *Limnology and Oceanography: Methods*, **8**, 348–351.
- Sota, T. (1985) Activity patterns, diets and interspecific interactions of coexisting spring and autumn breeding carabids: *Carabus yaconinus* and *Leptocarabus kumagaii* (Coleoptera, Carabidae). *Ecological Entomology*, **10**, 315–324.
- Sugiyama, A., Takagi, M. & Maruyama, K. (1996) A laboratory experiment of the predation by possible predators on *Culex tritaeniorhynchus* larvae. *Tropical Medicine*, **38**, 7–12.
- Sunderland, K., Crook, N., Stacey, D. & Fuller, B. (1987) A study of feeding by polyphagous predators on cereal aphids using ELISA and gut dissection. *Journal of Applied Ecology*, **24**, 907–933.
- Tayasu, I., Hirasawa, R., Ogawa, N.O., Ohkouchi, N. & Yamada, K. (2011) New organic reference materials for carbon- and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology. *Limnology*, **12**, 261–266.
- Vander Zanden, M.J., Cabana, G. & Rasmussen, J.B. (1997) Comparing trophic position of freshwater fish calculated using stable nitrogen isotope ratios ( $\delta^{15}\text{N}$ ) and literature dietary data. *Canadian Journal of Fisheries and Aquatic Sciences*, **54**, 1142–1158.
- Vanderklift, M.A. & Ponsard, S. (2003) Sources of variation in consumer-diet  $\delta^{15}\text{N}$  enrichment: a meta-analysis. *Oecologia*, **136**, 169–182.
- Vila, M., Auger-Rozenberg, M.A., Goussard, F. & Lopez-Vaamonde, C. (2009) Effect of non-lethal sampling on life-history traits of the protected moth *Graellsia isabelae* (Lepidoptera: Saturniidae). *Ecological Entomology*, **34**, 356–362.
- Wehi, P.M. & Hicks, B.J. (2010) Isotopic fractionation in a large herbivorous insect, the Auckland tree weta. *Journal of Insect Physiology*, **56**, 1877–1882.
- Yoon, T., Kim, D., Kim, S., Jo, S. & Bae, Y. (2010) Light-attraction flight of the giant water bug, *Lethocerus deyrolli* (Hemiptera: Belostomatidae), an endangered wetland insect in east Asia. *Aquatic Insects*, **32**, 195–203.
- Zaidi, R.H., Jaal, Z., Hawkes, N.J., Hemingway, J. & Symondson, W.O.C. (1999) Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? *Molecular Ecology*, **8**, 2081–2087.

Accepted 5 February 2012

First published online 21 March 2012

Editor/associate editor: Yoshitaka Tsubaki