**Effect of loach consumption on the reproduction of giant water bug *Kirkaldyia*
deyrolli: dietary selection, reproductive performance, and nutritional evaluation**

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Short running head: Prey of giant water bugs

Abstract

Rice fields provide major habitats for lentic aquatic insects including the giant water bug *Kirkaldyia* (= *Lethocerus*) *deyrolli* (Heteroptera: Belostomatidae) in Japan. Previous researchers have emphasized that conserving populations of the frogs, *Hyla japonicus* and *Rana nigromaculata*, is very important for preserving *K. deyrolli* because these frogs were found to be a major component of the diet of *K. deyrolli* adults. However, these previous studies were carried out in rice fields with no loaches, *Misgurnus anguillicaudatus*, which were probably been inhabited by loaches in the past. A series of field surveys and laboratory experiments were conducted to determine the dietary preference of *K. deyrolli* adults for loaches, frogs, and aquatic insects; the reproductive effects of consuming these foods; and their protein content. In the rice fields, *K. deyrolli* adults ate loaches when they were available. When the three prey species (frog: *R. nigromaculata*, loach: *M. anguillicaudatus*, and dragonfly: *Orthetrum albistylum speciosum*) were supplied in laboratory conditions, *K. deyrolli* adults ate more loaches than frogs or dragonflies. In addition, *K. deyrolli* adults provided with loaches or frogs laid more egg masses within the limited breeding season than when provided with dragonflies. The edible parts of the loaches' bodies were the largest of the three prey types. It is possible that *K. deyrolli* adults have been forced to eat frogs due to reductions in the population density of loaches because modern rice cultivation obstructs loach migration routes and reduces their spawning grounds.

Keywords: Alternative habitat; fish; Lethocerinae; loach-eater; *Misgurnus anguillicaudatus*

41 **Introduction**

42
43 Rice paddy ecosystem is a semi-natural freshwater ecosystem that has a long history
44 (several 1000 years), a wide geographical distribution (throughout Asia and other areas),
45 and is of great economic importance (half of the global population relies on rice as a
46 staple diet) (Settle et al. 1996). Rice fields have attracted concern regarding their
47 function as biodiversity conservation areas in recent years (Bignal and McCracken
48 1996; Elphick 2000; Kiritani 2000; Lawler 2001). Since many paddy fields have been
49 made via the modification of natural wetlands (Kiritani 2000; Lawler 2001), rice paddy
50 water systems are considered to be an important alternative habitat for species
51 originating in natural wetlands and are also known as alternative wetlands for many
52 aquatic animals (e.g., Fasola and Ruiz 1996; Maeda and Matsui 1999; Elphick 2000; but
53 see Lawler 2001; Machado and Maltchik 2010). Rice fields are an important breeding
54 habitat for aquatic insects (Saijo 2001) and fish (Katano et al. 2003; Yuma et al. 1998)
55 in Japan. However, the species diversity in these systems has been declining due to
56 recent land consolidation, the modification of traditional earth ditches to *U*-shaped
57 concrete ditches, and the use of agricultural chemicals such as insecticides and
58 herbicides in Japan (e.g., Fujioka and Lane 1997; Katano et al. 2003; Nishihara et al.
59 2006; Ichikawa 2008).

60 Improvements to rice fields and irrigation ditches that have been made over the
61 last three decades to reduce the workload of farmers may threaten fish diversity and
62 abundance (Katano et al. 2003; Fujimoto et al. 2008). These improvements include
63 covering the sides and bottoms of irrigation ditches with concrete, increasing the
64 difference in water level between rice fields and drainage ditches, and separating

irrigation ditches that supply and drain water. Mud loaches, *Misgurnus anguillicaudatus* (Cantor), one of the most common freshwater fish in Japan, use paddy fields and floodplains as spawning and nursery grounds (Saitoh et al. 1988; Tanaka 1999; Katano et al. 2003). A large number of loaches migrate to conventional paddy fields, which have no vertical gaps between the paddy fields and drainage ditches. However, a vertical gap between the water levels of the paddy field and the drainage ditch disturbs the upstream migration of loaches (Suzuki et al. 2001; Kano et al. 2010; Katayama et al. 2011), and hence, the loach population has rapidly decreased throughout the country. This species is now included in the *Red Data List* of species in 13 of 47 Japanese prefectures (Association of Wildlife Research & EnVision 2007). These reductions in loach population density may influence the diets of higher order predators such as carnivorous birds inhabiting rice fields (e.g., Narusue and Uchida 1993; Lane and Fujioka 1998).

Species of the Belostomatidae subfamily Lethocerinae are cosmopolitan, and live in freshwater habitats such as ponds, lakes, and rivers (Cullen 1969; Perez Goodwyn 2006). The giant water bug, *Kirkaldyia deyrolli* Vuillefroy (formerly *Lethocerus deyrolli*; see Perez Goodwyn 2006), is distributed throughout Japan from central Honshu to the Ryukyu Islands, southeastern Asia, China, Taiwan and Korea. In Japan, rice fields provide major habitats for lentic aquatic insects including the giant water bug *K. deyrolli* (Hashizume 1994; Mukai et al. 2005). *Kirkaldyia deyrolli* are used as model animals in ethological studies because of their interesting behavior such as paternal care and sexual conflict (Ichikawa 1988; 1990, 1995; Ohba 2002; Ohba et al. 2006). Japanese populations of *K. deyrolli* have decreased sharply during the last four decades, and this species is now included in the *Red Data List* of species in 45 of 47

Japanese prefectures (Japan Environment Agency 2000; Association of Wildlife Research & EnVision 2007). Contributing factors such as decreases in suitable aquatic habitats and food resources, as well as water pollution and urbanization, have been investigated and verified in previous studies (e.g., Hirai and Hidaka 2002; Ohba and Takagi 2005; Ho et al. 2009; Yoon et al. 2010; Nagaba et al. 2011).

It is important for the conservation of *K. deyrolli* to reveal the best food for them during the reproductive season. Because the quantity and quality of prey animals are important factors determining a predator's life history and abundance (Lenski 1984; Juliano 1986), the information of the best food provide an opportunity to consider the environment and designing conservation projects for predatory species. Previous studies have reported that *K. deyrolli* mainly prey upon vertebrates including fish, snakes, turtles, and frogs in Japanese rice fields (Hirai and Hidaka 2002; Mori and Ohba 2004; Ohba and Nakasuji 2006; Hirai 2007; Ohba et al. 2008; Ohba 2011; Ohba 2012). Hirai and Hidaka (2002) and Hirai (2007) emphasized that the frog population is very important for the conservation of the *K. deyrolli* population because frogs are major constituents of the diet of *K. deyrolli* adults. However, their conclusion is not adequate because: 1) previous studies were carried out in rice fields with few loaches, which were probably inhabited by loaches in the past (Hirai and Hidaka 2002; Hirai 2007), and 2) these studies only described the prey present in the rice fields and did not evaluate the differences in performance of *K. deyrolli* such as egg production when consuming different prey animals or their nutritional content (Hirai and Hidaka 2002; Ohba and Nakasuji 2006; Hirai 2007).

Kirkaldyia deyrolli is sometimes called the “loach-eater insect” by the residents of certain areas of Japan (Hasizume 1994), perhaps indicating that loaches are better

food for *K. deyrolli* than frogs. It is possible that *K. deyrolli* currently mainly prey upon frogs in rice fields for two reasons: 1) *K. deyrolli* prefer frogs over loaches and 2) *K. deyrolli* must eat frogs as the population density of loaches has decreased because modern rice cultivation obstructs their migration route and has led to spawning ground loss. However, these possibilities have not been examined until now. Therefore, it is risky to conclude that frogs are the exclusive dominant prey for *K. deyrolli* when designing conservation projects.

Understanding the feeding habits of endangered species such as *K. deyrolli* in natural habitats is a fundamental step to developing a suitable environment for them, but it is often difficult to quantify them after environmental changes occurred. One possible approach is to compare the dietary components of endangered species in habitats with different prey levels and perform additional rearing experiments in laboratory conditions. In the present study, we carried out a field census and laboratory experiments to determine the dietary preference of *K. deyrolli* among loaches, frogs, and aquatic insects; the effects of consuming these foods on reproductive performance; and their nutritional reward. Finally, the conservation of *K. deyrolli* is discussed on the basis of our results.

Materials and Methods

Study animal

In central Japan, the giant water bug *K. deyrolli* is a univoltine species. Its reproductive period extends from late May to July (after overwintering), with the new generation

reaching adulthood between early July and September (Hashizume 1994; Hashizume and Numata 1997; Mukai et al. 2005). The egg masses of *K. deyrolli* are laid on the stems of plants above the water surface, not on the back of males as in many other members of the Belostomatinae (Smith 1997). The context of courtship, copulation, oviposition, and male-female interaction were described by Ichikawa (1989) in detail. Males supply the developing eggs with water and guard them against predators during the approximate 10 days of egg period (Ichikawa 1988, 1995; Ohba et al. 2006). Hatched nymphs moult five times, and adults emerge approximately 50 days later (Hashizume 1994). Finally, the adults disperse to their hibernation locations such as forest floors.

I. Field survey

Field surveys were conducted at two paddy field study sites (sites A and B) in western Hyogo, western Japan, from 2 June to 30 July 2010. The earth ditches had been replaced with *U*-shaped concrete drains at site A due to farmland consolidation. There were vertical gaps of more than 10 cm between the water levels of the rice fields and the drainage ditch at site A. Therefore, it is expected that there were few loaches in the rice fields at site A, whereas the earth ditches at site B contained a relatively high density of loaches.

Five rice fields at each study site (site A: 136, 142, 136, 164, and 158 m²; site B: 86, 82, 144, 90, and 52 m²) were set up as census plots to investigate the diet of *K. deyrolli* and the frequencies of *K. deyrolli* and potential prey. The rice fields were surrounded by a ridge covered with weeds, which acted as a small convenient footpath that reduced site disturbance between adjoining rice fields. The water temperature in the

ditches ranged from 18–30°C during the study period. Before rice is planted, the rice fields are ploughed and irrigated, and then the muddy bottoms of the rice fields are leveled off. Subsequently, the rice fields are filled with water to a depth of 5–15 cm. The water in all of the rice fields at the two study sites was maintained from mid-May to the end of July (irrigation period). In early July, the water was drained from the field, and the rice field draining continued for a few weeks, leading to the ground being exposed to the sun (drainage period). Nevertheless, the ditch water remained at a depth of 3–5 cm, even during the drainage period.

Sampling of *K. deyrolli* and its potential prey animals

To measure the number of *K. deyrolli* and prey animals in the ditches, we conducted censuses at intervals of 7–10 days (a total of 9 times at both study sites during the study period). We counted *K. deyrolli*, frogs, loaches, and aquatic insects carefully as we walked on the footpath adjoining rice fields. The census included each ditch and the surrounding area (within 100 cm from the edge of the water site) and was performed at night from 2000 to 0100 h. Censuses were also conducted along the ridges around the rice fields. Censuses were performed by visual observation of *K. deyrolli* using a flashlight (11000 lux). Primarily a nocturnal animal, *K. deyrolli* ambushes its prey at the water surface after sunset. It is much easier to observe *K. deyrolli* at night than in the daytime (S. Ohba, unpublished data). The flashlight illumination did not interfere with the foraging behavior of *K. deyrolli* because they do not stop feeding and ambushing their prey, even in strong light (S. Ohba, unpublished data). During the direct observation, the observer maintained a constant distance from the water surface (0.5 m)

and a constant pace (10 m/min walking speed). Sampling was not carried out during periods of rain to maintain sampling consistency. We caught *K. deyrolli* using a 500- μ m mesh dipnet (0.15 $\times$ 0.10-m mouth opening) and recorded their development stage (adults or nymphs). After their capture and recording, the *K. deyrolli* were carefully released at their point of capture. At each study site, we added the total number of *K. deyrolli* from all ditches.

Two-way multiple analysis of variance (MANOVA) was used with “site” and “sampling date” as independent variables and density (n/m^2) of each aquatic animal (frogs, loaches, aquatic insects, and *K. deyrolli* adults) as dependent variables to compare the species composition during the study periods. Separate two-way ANOVAs on the density of each aquatic animal were used after a significant difference was found by the two-way MANOVA. The five rice fields within each study site were nested within site A or B in this MANOVA and ANOVA model. $\log_{10}(x + 1)$ transformations for exact values were made in order to standardize variances and improve normality to satisfy the assumptions of the ANOVA model.

Diet of *K. deyrolli*

To investigate the diet of the *K. deyrolli* in the ditches, we conducted censuses at intervals of 1-5 days (a total of 24 times at both study sites during the study period). When we found *K. deyrolli* holding prey with their raptorial legs, we carefully observed whether the proboscis had been inserted into the prey. Prey into which the proboscis had been inserted were recorded as dietary components and were preserved in 80% ethanol prior to their identification. From the preliminary survey, the dietary components were

classified into four prey categories: frogs (*Hyla japonica*, *Rana nigromaculata*, *Rana limnocharis*, *Rana rugosa*, and *Rhacophorus schlegelii*), loaches (*Misgurnus anguillicaudatus*), aquatic insects (Odonata nymphs, aquatic Heteroptera, aquatic Coleoptera), and other prey (tadpoles, river crabs, and terrestrial insects). To compare the diet of *K. deyrolli* between the study sites, the four dominant prey categories (loaches, frogs, aquatic insects, and others) were compared using χ^2 -contingency tests.

II. Laboratory experiments

Kirkaldyia deyrolli adults (10 males and 14 females, 55.6 ± 2.45 , 63.4 ± 2.45 mm in body length, mean $\pm$ S.D.) were collected from western Hyogo, central Japan, in late May 2008. All bugs were returned to the laboratory and maintained individually in plastic cups (10 cm diameter, 10 cm high, and 5 cm water depth), and kept at 29.0°C under a 16:8 (L:D) light- dark cycle until testing. All bugs were fed *ad libitum* with tadpoles of *Rana nigromaculata*, *Hyla japonica*, and/or *Rhacophorus schlegelii* on a daily basis until the beginning of each experiment. Dead prey were immediately removed from the plastic cup.

Selectivity of *K. deyrolli*

To examine the selectivity of *K. deyrolli* when three prey types were supplied, an experiment was conducted in an aquarium (550 $\times$ 400 mm, 345 mm height) from June to July 2008. River gravel was laid on the bottom of the aquarium in a 10 mm thick layer, and dechlorinated tap water was added over the gravel surface to a depth of 50

mm. A land territory (20 × 400 mm) was established at the edge of the aquarium by thick layer of river gravel (60 mm), and two sticks (20 mm diameter, 150 mm height) were erected in the river gravel of the water area as a perching site for *K. deyrolli* and the prey animals.

We selected three prey types, loaches (*Misgurnus anguillicaudatus*), frogs (*R. nigromaculata*), and dragonfly nymphs (*Orthetrum albistylum speciosum*), as representative dominant food resources in the fields in which *K. deyrolli* are distributed (Ohba and Nakasuji 2006; Ohba et al. 2008). In previous studies, *Hyla japonica* and *Rana nigromaculata* were found to be the dominant prey animals of *K. deyrolli* in rice fields (Hirai and Hidaka 2002; Hirai 2007) but *H. japonica* quickly perch on the wall or lid using their suctorial fingers so *K. deyrolli* cannot capture *H. japonica* in an aquarium. Therefore, we used *R. nigromaculata*, which have no suctorial fingers, in this experiment. Just before the beginning of the experiment, all prey animals were collected from the rice fields. The *K. deyrolli* females were fasted for 24 h before the experiment. Female adults were used for the experiment because males prey upon few prey animals in the reproductive season (S. Ohba unpublished data).

Two loaches (30-50 mm in body length), two frogs (30-35 mm in Snout-to-vent-length (SVL)), two dragonfly nymphs (ca. 20 mm in body length), and a female *K. deyrolli* adult were introduced into the aquarium. These prey size are based on the natural size during the reproductive season of *K. deyrolli*. Half an hour before the beginning of the experiment, the prey animals were added to the aquarium for acclimation. The number of carcasses (= fed on by *K. deyrolli*) of each prey type was counted at 24 h after the beginning of the experiment. Eight replicates were performed. For the number of prey types consumed (selectivity of *K. deyrolli*), Scheffe's test was

performed to assess the differences among the three prey types when significant effects were detected in the separate one-way ANOVA.

Effect of prey animal consumption on *K. deyrolli* reproduction

To examine the effects of consuming different prey on the egg production of *K. deyrolli* female adults, experiments were conducted for each of the three prey treatments; i.e., loaches, frogs, and dragonfly nymphs, from 4 June to 30 July 2008. The loach and frog treatment used *M. anguillicaudatus* (30-50 mm in body length) and *H. japonica* (30-35 mm in SVL), respectively. In the dragonfly nymph treatment, several species of dragonfly nymph (Libellulidae: *Orthetrum albistylum speciosum* Uhler, *Sympetrum frequens* Selys, *S. infuscatum* Selys, Aeshnidae: *Planaeschna milnei* Selys, and *Anax parthenope julius* Brauer; 20–30 mm in body length) were used because we found it difficult to collect many nymphs of a single species (*O. albistylum speciosum*). The prey density in each treatment was maintained at a constant level (four individuals per plastic cage for the loach and frog treatments, and ten individuals per plastic cage for the dragonfly nymph treatment). The prey density levels were set to supply enough food for *K. deyrolli* reproduction, confirmed by not all of the prey having been attacked by the *K. deyrolli* females within 24 h. We counted the prey animals daily to maintain the same prey density throughout the experiment. All females were individually transferred to a plastic cage (10 cm diameter × 10 cm height) on 4 June. The females laid egg masses repeatedly during the reproductive season (Hashizume 1994). Before the beginning of the experiment, all females were paired with males in order to lay their first egg mass which had already produced in their ovaries. To prevent the water quality deteriorating

drastically, all of the plastic cages were placed in a large aquarium (635 × 439 mm × 226 mm) kept at 29.3 C° water temperature under a 16L:8D light cycle. The aquarium was filled with water to a depth of 15 cm and aerated with an air pump and air stone (Ohba 2008).

The number of prey animals consumed in each plastic cage was recorded for each prey treatment every day. When females had greenish-bellied abdomens due to egg production, they were transferred to a reproductive aquarium (450 × 170 mm, 200 mm in height) maintained at a water temperature of 29 C° and under a 16L:8D light cycle. River gravel was laid on the bottom of the aquarium in a 10 mm thick layer, and dechlorinated tap water was added over the gravel surface to a depth of 100 mm. Two sticks (20 mm diameter, 150 mm height) were set up as oviposition substrata, and two *K. deyrolli* males were introduced into the reproductive aquarium. After 24 h, the females were returned to their plastic cages, irrespective of whether they had laid an egg mass, and were supplied with prey. This procedure was repeated on alternative days until the females laid an egg mass. The egg masses on a stick were photographed with a cell phone digital camera (W53T, au, Tokyo, Japan) in order to count the number of eggs. We recorded the number of eggs per egg mass, the oviposition interval (the number of days between subsequent two egg masses), the number of eggs produced per day, and the number of prey animals consumed during the oviposition interval. The number of eggs produced per day (*EP*) was calculated as follows:

$$EP = E_i / (D_i - D_{i-1})$$

where E_i denotes the number of eggs in an egg mass on oviposition date D_i . Date that

former egg mass was laid is shown as D_{i-1} . The frog, loach, and dragonfly nymph treatments were replicated 4, 4, and 5 times, respectively. For the number of egg masses, Scheffe's test was performed to assess the differences among the three prey animals when significant effects were detected in the separate one-way ANOVA. For the number of eggs per egg mass, the oviposition interval, the number of eggs produced per day, and the number of prey consumed during the oviposition interval, the data were analyzed with a generalized linear mixed model (GLMM). Prey type (loaches, frogs, or dragonflies) was analyzed as a fixed effect and each individual was fitted as a random effect because these parameters were measured repeatedly in each individual.

III. Comparison of the protein contents of the prey animals

Lethocerinae species including *K. deyrolli* need protein-rich meals because they have three kinds of protease and do not have amylase in their salivary glands (Swart et al. 2006). Thus, we focused on the amount of protein in the three prey types (loach *M. anguillicaudatus*, frog *R. nigromaculata*, and dragonfly nymph *O. albistylum speciosum*). The three prey types were collected from rice fields in June 2009.

Observation of edible parts

The three dietary prey types and the female *K. deyrolli* adult were added to a plastic cup (10 cm diameter × 10 cm height) one by one. After being eaten by *K. deyrolli*, the bodies of the dead prey animals were removed from the plastic cup and then dissected in order to discriminate the eaten and uneaten parts of each prey animal body under a

microscope. Three replicates were performed for each prey type.

Analysis of the protein content of the prey species

The edible parts of the loaches, frogs (muscle tissue), and dragonflies (whole body contents inside the epidermides) (see Results) were dissected away from the inedible parts, freeze-dried after the water on the surface of the dissected tissues had been removed with filter paper, and then weighed on an electronic balance. For the preparation of the total protein extract, each sample (1.0g) was homogenized in a glass homogenizer with 10ml of sample buffer consisting of 30mM Tris (pH 8.5), 7M urea, 2M thiourea, 4% CHAPS, and 5mM magnesium acetate for approximately 3min and then centrifuged at 12,000g for 15min. The supernatant containing the total protein was centrifuged at 100,000g for 60min and assayed. The concentration of the extracted protein was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories). The differences in protein content among three prey types were assessed by Scheffe's test when significant effects were detected in the separate one-way ANOVA.

Determination of the proportion of edible parts of the prey animals

To investigate the proportion of edible parts in each prey type, 10 loach, frog, and dragonfly nymph individuals were collected from the same rice fields at the study site 2009. As the dry weight of each prey type before it was eaten by the *K. deyrolli* could not be weighed, a regression formula concerning the relationship between dry and fresh weight in each prey type was produced. The fresh weights of five prey animals in each

prey type were weighed with an electronic balance, and then the prey animals were freeze-dried and weighed as well. Regression formulas were obtained from the fresh weight of the prey animals (x) and their dry weights (y) for each prey type (Appendix 1).

The fresh weights of five prey animals in each prey type were weighed with an electronic balance, before they were kept in a plastic cup (100 mm diameter $\times$ 100 mm height) with a 24-h fasted female *K. deyrolli*. It was expected that the 24-h fasted female *K. deyrolli* would digest the most edible parts of their prey. Just after being eaten by the *K. deyrolli*, the prey animals were collected and then freeze-dried. To estimate the dry weight of each prey animal before they were eaten by *K. deyrolli*, the fresh weight of each prey animal before they were eaten by the *K. deyrolli* was substituted for F in the regression formula (Appendix 1). The dry weights of the edible parts of each prey type were calculated by subtracting the estimated dry weight after eaten from the estimated dry weight before eaten. Next, the percentage of edible parts was calculated for each prey individual. The percentage of edible parts among the three prey types were compared by Scheffe's test was performed to assess the difference when significant effects were detected in the separate one-way ANOVA. Arcsine-square-root transformations of the exact values of the percentages of edible parts of the prey animals were performed in order to standardize the variance values and improve the normality of the data. Statistical significance was set at 0.05. All statistical tests were conducted using the JMP software (version 8.0).

Results

Occurrence frequency of prey animals

The density of aquatic animals in rice fields varied between the study sites and among the sampling dates (Fig. 1). The two-way MANOVA on the animal species composition revealed significant “site” ($F_{4,61} = 28.7$, $P < 0.001$ for $\text{Log}_{10}(x + 1)$ transformed data), “sampling day” (Roy’s Greatest Root; $F_{8,64} = 7.9$, $P < 0.001$), “site-by-sampling day” interaction effects (Roy’s Greatest Root; $F_{8,64} = 174.4$, $P < 0.001$), and “sub-site” effects (Roy’s Greatest Root; $F_{8,64} = 14.8$, $P < 0.001$). Separate two-way ANOVAs on each animal showed that the density of frogs (site; $F_{1,72} = 14.2$, $P < 0.001$, sampling date; $F_{8,72} = 2.1$, $P = 0.045$, site * sampling date; $F_{8,72} = 1.4$, $P = 0.24$, sub-site; $F_{8,72} = 3.9$, $P < 0.001$) and loaches (site; $F_{1,72} = 104.1$, $P < 0.001$, sampling date; $F_{8,72} = 3.8$, $P = 0.001$, site * sampling date; $F_{8,72} = 3.8$, $P = 0.001$, sub-site; $F_{8,72} = 12.9$, $P < 0.001$) were significantly different between the two sites and among sampling dates. Therefore, there were few loaches in the rice fields at site A, whereas the rice fields at site B contained a relatively high density of loaches. The density of aquatic insects (site; $F_{1,72} = 0.2$, $P = 0.64$, sampling date; $F_{8,72} = 2.3$, $P = 0.032$, site * sampling date; $F_{8,72} = 1.3$, $P = 0.24$, sub-site; $F_{8,72} = 1.9$, $P = 0.08$) and *K. deyrolli* (site; $F_{1,72} = 3.7$, $P = 0.059$, sampling date; $F_{8,72} = 5.5$, $P < 0.001$, site * sampling date; $F_{8,72} = 1.3$, $P = 0.26$, sub-site; $F_{8,72} = 2.3$, $P = 0.030$) was significantly different among the sampling dates, but not between the two sites. The *K. deyrolli* adults were abundant in June at both study sites (Fig. 1). Aquatic insects were present at low proportions at both sites throughout the study period. Hereafter, we term sites A and B as the frog and frog–loach sites, respectively.

Diet of *K. deyrolli*

The diets of the *K. deyrolli* differed between the two study sites ($X^2 = 8.55$, $d.f. = 3$, $P = 0.036$, X^2 test). Although the *K. deyrolli* ate loaches, frogs, and aquatic insects at site B (frog-loach site), they ate frogs and aquatic insects at the site A (frog site) (Fig. 2).

Selectivity of *K. deyrolli*

Although the *K. deyrolli* ate three prey types, the number of consumed animals differed among the prey types ($F_{2, 21} = 4.82$, $P = 0.019$, one-way ANOVA). The number of consumed loaches (mean $\pm$ S.E., 1.6 ± 0.26) was significantly higher than that of either frogs (0.63 ± 0.32) or dragonflies (0.63 ± 0.18 , $P < 0.05$, Scheffe's test), but it did not differ significantly between the frogs and dragonflies ($P = 1.00$).

Effect of prey animal consumption on *K. deyrolli* reproduction

A one-way ANOVA showed that the prey effect on the number of egg masses was not significant ($F_{2, 10} = 0.48$, $P = 0.63$, mean $\pm$ S.E. loach = 3.75 ± 0.25 , frog = 3.50 ± 0.96 , dragonfly = 3.0 ± 0.32). There was no significant difference among the three prey types with respect to the number of eggs per egg mass (prey: $F_{2, 18.5} = 2.26$, $P = 0.13$, individual: $F_{10, 31} = 0.35$, $P = 0.96$, GLMM, loach = 79.4 ± 6.20 , frog = 89.4 ± 4.28 , dragonfly = 72.1 ± 7.56) but there were differences in the oviposition interval among the prey types (prey: $F_{2, 12.2} = 5.22$, $P = 0.02$, individual: $F_{10, 31} = 0.35$, $P = 0.96$). The oviposition interval of the adults given loaches (5.8 ± 0.48) in their diet was the shortest of the three prey types, followed by those fed frogs (6.4 ± 0.46), and those given

425 dragonflies (7.8 ± 0.60).

426 There were differences in the number of eggs produced per day among the
427 three prey types (prey: $F_{2,14.2} = 9.58$, $P = 0.002$, individual: $F_{10,31} = 0.65$, $P = 0.76$). The
428 number was significantly higher in the adults given loaches (15.5 ± 1.24) or frogs (13.8
429 ± 1.17) than in those given dragonflies (9.1 ± 1.30 , $P < 0.05$, Scheffe's test), but it did
430 not differ significantly between the loach and frog groups ($P = 0.62$).

431 The number of prey consumed during the oviposition interval differed among
432 the three types (prey: $F_{2,10.7} = 11.06$, $P = 0.003$, individual: $F_{10,32} = 3.68$, $P = 0.002$).
433 The number of consumed loaches (7.7 ± 0.58) and frogs (9.3 ± 0.63) were almost the
434 same ($P = 0.79$, Scheffe's test) but that of dragonflies was higher (22.9 ± 2.57 , $P <$
435 0.001).

436 437 Observation of edible parts

438
439 Three prey types eaten by the *K. deyrolli* were dissected and observed. The muscles of
440 the loaches and frogs were digested, whilst their skin, bones, and guts remained intact.
441 In contrast, most parts of the dragonfly nymphs were digested, excluding their
442 epidermides.

443 444 Analysis of protein content of the edible parts of prey

445
446 There were differences in the protein contents of the edible parts of the three prey types
447 ($F_{2,21} = 19.15$, $P < 0.001$). The amount of protein (mg / sample 1mg) in the loaches
448 (0.75 ± 0.058) and frogs (0.72 ± 0.078) was significantly higher than that in the

dragonflies (0.30 ± 0.017 , $P < 0.001$, Scheffe's test), but it was not significantly difference between the loaches and frogs ($P = 0.94$).

Comparison of the proportion of edible parts of the prey animals

The percentage of edible parts differed significantly among the three prey types ($F_{2,12} = 9.62$, $P = 0.003$, one-way ANOVA, for arcsine-square-root transformed data). The percentage was significantly higher in the loaches than in the frogs ($P = 0.003$, Scheffe's test). Sixty-nine (± 3.1) percent of the loach's body was eaten by the *K. deyrolli* whereas only half ($49 \pm 4.5\%$) of the frog's body and $59 \pm 1.2\%$ of the dragonfly's body were eaten (Fig. 3).

Discussion

In the present study, we carried out a field census and laboratory experiments to determine the dietary preference of *K. deyrolli* adults among three potential prey types, the effects of their consumption on reproductive performance, and their protein content. When the three prey types were supplied in laboratory conditions, the *K. deyrolli* ate more loaches than frogs or dragonflies. As Lethocerinae such as *K. deyrolli* are vertebrate specialists (Smith 1997), *K. deyrolli* may prefer loaches and frogs to dragonflies. However, *K. deyrolli* ate more loaches than frogs. This can be explained by differences in the frequency at which they encountered loaches and frogs: the frogs were usually found on the land territory in the aquarium, whereas the loaches were present in the water with the *K. deyrolli*. Or, the result may simply reflect catchability

under the laboratory conditions rather than preference under natural conditions.
However, the field survey complements the result of the laboratory experiment: *K. deyrolli* adults ate loaches in the rice fields (Fig. 2) when they were available (Fig. 1).

Some researchers reported that frogs are common prey of *K. deyrolli* in rice fields (Hirai and Hidaka 2002; Ohba and Nakasuji 2006; Hirai 2007). The differences between our results and theirs have been caused by differences in handling times among the prey types. The time required for prey consumption was markedly different between the insects and frogs. For example, it took longer than 5 h for a *K. deyrolli* to complete a frog meal, but only 3 h to eat a dragonfly nymph (S. Ohba, unpublished data). As researchers are more likely to discover long-handling time prey such as frogs, researchers who investigated the diet of *K. deyrolli* by direct observation (Hirai and Hidaka 2002; Ohba and Nakasuji 2006; Hirai 2007) might have overestimated the frequency of frogs in the diet of *K. deyrolli*. This over- and under-estimation by direct observation among the prey types can be solved by a stable isotope approach (Fry 2006).

There was no significant differences in the number of egg mass and the number of eggs per egg mass among the three prey types, but the oviposition interval of the loach-supplied *K. deyrolli* was the shortest followed by those of the *K. deyrolli* given the frog and dragonfly treatments. The *K. deyrolli* females tested in this laboratory study laid egg masses containing more than 70 eggs, which was almost same number as that found in natural populations in 2010 (66.5 ± 3.67 eggs/ egg mass, mean $\pm$ S.E., $n=23$, S. Ohba unpublished data). This suggests that *K. deyrolli* females continue to eat prey animals until the number of mature eggs in her ovary reaches approximately 70. The number of eggs produced per day by the *K. deyrolli* supplied loaches or frogs were

twice as high as that in those given dragonflies. In addition, in order to lay an egg mass, *K. deyrolli* needs to consume about 3 times more dragonflies than loaches or frogs. These results suggest that *K. deyrolli* females shorten their oviposition interval when they eat loaches or frogs. Therefore, loaches and frogs are important prey animals for *K. deyrolli* because they allow them to lay more egg masses within their limited breeding season of about 2 months (late May to July).

The protein content of the edible parts was higher in the loaches and frogs than in the dragonflies, but it did not differ between the loaches and frogs. This agrees with the results for the oviposition interval and the number of eggs produced per day. However, the percentage of edible parts was highest in the loaches (Fig. 3), suggesting that loaches are the best food for *K. deyrolli*. Thus, *K. deyrolli* that have captured a loach may gain more benefit than *K. deyrolli* that have captured a frog. This is the key finding of the present study and could be springboard towards more detailed behavioural ecological studies of *K. deyrolli* and improved conservation measures.

From the viewpoint of the frequency of encountering the various prey animals, the oviposition interval, and the percentage of edible parts in the prey's bodies, loaches are the best of the three prey types with regards to *K. deyrolli* reproduction. Therefore, it is possible that the dietary components of *K. deyrolli* adults have recently shifted from loaches to frogs because the population density of loaches has declined due to farm land consolidation and the establishment of vertical gaps of more than 10 cm between the water levels of rice fields and drainage ditches (Suzuki et al. 2001). There were vertical gaps of more than 10 cm between the water levels of the rice fields and the drainage ditch at site A (frog site), which contained few loaches (Fig. 1). As the *K. deyrolli* adults inhabiting the rice fields at site A could not encounter loaches, they ate frogs instead

(Fig. 2).

Although loaches are appropriate prey for reproduction in *K. deyrolli* adults (this study), anuran larvae (tadpoles of *R. nigromaculata*, *R. schlegelii*, and *H. japonica*) are indispensable for rapid growth in younger *K. deyrolli* instar nymphs (Ohba et al, 2008). Therefore, loaches and frogs are utilized by *K. deyrolli* adults and nymphs respectively. These results strongly suggest that environments that contain an abundance of both frogs and loaches are indispensable for maintaining *K. deyrolli* populations. To halt the recent decline in fish populations in rice ecosystems, several conservation measures have been implemented in Japan such as installation of “fishways” (Suzuki et al. 2004). “Fishways” connect rice fields and ditches with large gaps can be useful for successful migration of loach and other fish (Suzuki et al. 2004) because the loss of this connectivity (i.e. large gaps) between fields and ditches has a serious impact on abundance and diversity of fish (Katano et al. 2003; Katayama et al. 2011). The installation of fishways seems to be the most effective conservation technique for not only fish but for higher order predators including *K. deyrolli*.

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Figure legends

Fig. 1. Seasonal changes in the frequencies of *K. deyrolli* adults and the three prey categories at the two study sites. Two-way ANOVAs on each animal showed that the density of frogs and loaches were significantly different between the two sites and among sampling dates (see Results).

Fig. 2. Three prey groups included in the diet of *K. deyrolli* adults at the two study sites. The sample sizes were 19 at site A and 25 at site B. The diets of the *K. deyrolli* differed between the two study sites.

Fig. 3. The percentage of edible parts among the three prey types. Different letters denote significant differences ($P < 0.05$, Scheffe's test).

Appendix

Appendix 1. Dry and fresh weights of each prey animal.

Prey animals	Regression formula ^a	R^2
Loach	$D = -0.012 + 0.214F$	0.988*
Frog	$D = -0.021 + 0.207F$	0.993*
Dragonfly	$D = -0.038 + 0.277F$	0.895*

^a D and F refer to dry weight and fresh weight, respectively.

* $P < 0.001$

Fig. 1. Ohba et al.

Fig. 2. Ohba et al.

Fig. 3. Ohba et al.