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Short communication

Food habits of the urban Japanese weasels *Mustela itatsi* revealed by faecal DNA analysis

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Urban ecosystems all share the overwhelming characteristic that humans "drive the system" (Gehrt et al. 2010). In some cities, regional endemic species including medium to large carnivores occur within highly anthropogenic environments, e.g., Northern raccoon (*Procyon lotor*) and coyote (*Canis latrans*) in California (Gehrt et al. 2010) and stone marten (*Martes foina*) in Luxemburg (Herr et al. 2010). Studies of these species have shown that the carnivores used remnant or newly created green areas, parks, gardens, riverbanks, and agricultural fields in which to rest and forage. Studies monitoring their presence and ecology, and management programs to maintain those habitats have been established and are on-going. Although some mammal species are known to occur in urban areas in Japan, little is known of their ecology there.

The Japanese weasel (*Mustela itatsi*) is reported to have declined throughout Japan (Imaizumi 1986), but is known to survive in both rural and urban areas. In a rural area where they have been studied, Japanese weasels utilize more than two habitat types allowing them to switch between available food items (Kaneko et al. 2009). In contrast, for Japanese weasels in urban areas, riverbanks are among the few remaining important habitats available to them (Fujii et al. 1998), yet no conservation measures have been taken in that habitat.

Past surveys of sympatric carnivores by means of faecal analysis have revealed difficulties in species identification (Birks et al. 2004). For weasels in Japan, experienced researchers have been able to identify droppings based on faecal appearance and diameter (Japanese weasels and Japanese martens *M. melampus melampus*, reviewed by Tsuji et al. 2011), or on species-specific faecal odours (Siberian weasels *M. sibirica* and Japanese martens *M. m. tsuensis*, Tatara and Doi 1994). More recently, DNA analysis has made it possible to accurately identify the faeces of sympatric carnivore species in Japan (Shimatani et al. 2008; Sekiguchi et al. 2010).

In this study, we used DNA analysis to confirm the identification of Japanese weasels from faecal samples collected from weasel habitat along the Tama River in an urban area. We investigated the distribution of Japanese weasels in the riparian environment, and whether male and female Japanese weasels consume different food items. Based on this information, we discuss the management of the Tama River as Japanese weasel habitat.

Material and methods

Study area

The study area was confined to the southwest bank of the middle reaches of the Tama River, between Hamura

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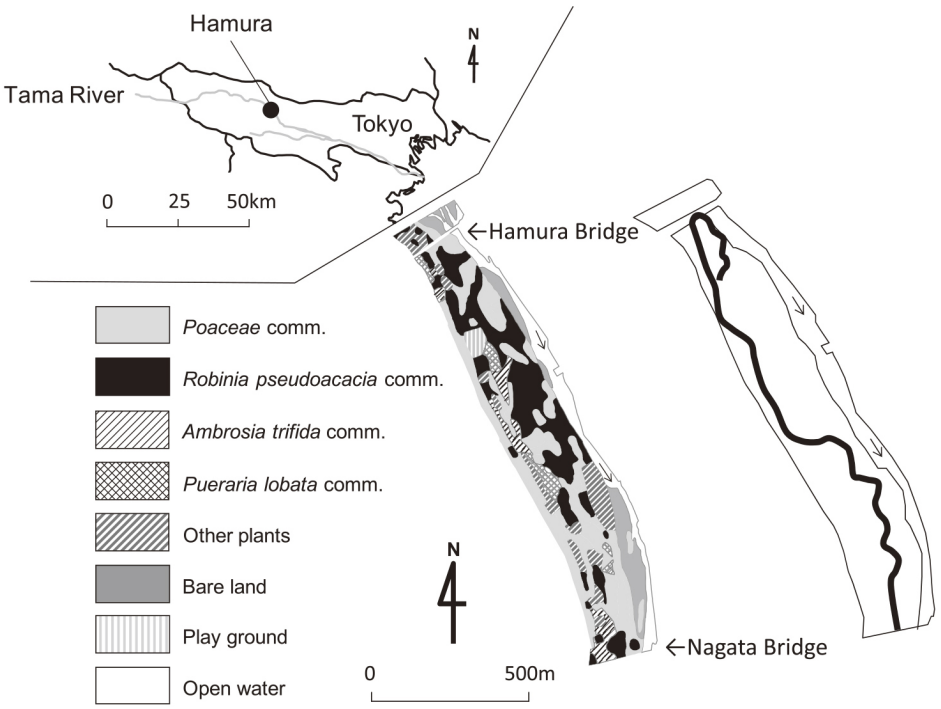

Fig. 1. Location, vegetation and survey route of Hamura, Tokyo, Japan. Central vegetation map shows dominant plant species (after Ministry of Land, Infrastructure, Transport and Tourism River Bureau Environment Division 2005). Black line on the right map shows the survey route.

Bridge and Nagata Bridge, Tokyo, Japan (35°44'N, 139°17'E, Fig. 1). The area is surrounded by residential areas, but contains scattered ponds and wetlands. *Poaceae* comm. which includes various *Poaceae* species such as common reeds *Phragmites australis*, *P. japonica,* Japanese pampas grass *Miscanthus sinensis* and Amur silvergrass *M. sacchariflorus*, locust tree *Robinia pseudoacacia* comm. and bare land are dominated 38.8%, 30.1% and 10.4% of study area, respectively (River Ecology Research Group 2006, Fig. 1).

Faecal sample collection

A fixed, 1,200 m long survey route was established along the riverbank (Fig. 1). This route was examined two or three times each week and faeces along the route were collected. For each sample the information recorded included: appearance, location, odour, diameter, and freshness. The samples were collected using disposable chopsticks (in order to prevent contamination between samples), preserved in 15 ml tubes with 99.5% ethanol, then transferred to the laboratory where they were stored at –20°C. Seasonal periods were defined as: spring (March to May), summer (June to August), autumn (September to November), and winter (December to February).

DNA analyses of faecal samples

1) PCR Primers for species identification

To establish an effective methodology, we first analysed a subset of the faecal samples using three primer sets specified for the D-loop region (the D-tsd and 195re primer set, Sekiguchi et al. 2010; MT D DIRECT and REVERSE primer set, Matsuki et al. 2006; tana D-F and -R primer set, Matsuki et al. 2008), and with one primer set specified for the cytb region (L14841 and H15149 primer set, Kocher et al. 1989). Because the success rates for amplification using the tanaD-F and -R primer set and the L14841 and H15149 primer set were higher than other primer sets, we used these primer sets mainly thereafter (Table 1). However, some samples could not be amplified using these primer sets, so we devised a new primer set, the D-173d (5'-GCCCCATGCATATAAGCATGTACATA-3') and D-365r (5'-CCTGGCATCTGGTTCTTACTTCA-3') primer set for the Japanese weasel, as the target region on the D-loop is short, making DNA amplification easy.

2) Isolation and amplification of faecal DNA and gel electrophoresis

To determine species and sex, DNA from faecal samples (from 0.5 to 1.0 g) was isolated using the QIAamp® DNA stool mini kit (Qiagen, Hilden, Germany), and

Table 1. Results of the DNA analysis; species and sex identification, results in each primer sets: + clear amplification; – no amplification. Blank: no DNA analysis

Sample ID	Species identification						Sex identification		
	D-loop				cytb	Species	DDX3X	DDX3Y	Sex
	D-tsd and D-195re	MT D DIRECT and REVERSE	tanaD-F and -R	D-173d and D-365r	L14841 and H15149		dbxnt-112d and -373r	dbynt-111d and -296r	
1	+	+			+	Weasel	+	+	Male
2	+	+			+	Weasel	–	+	Male
3	–	+				Weasel	–	+	Male
4		+				Weasel	+	+	Male
5		+				Weasel	+	+	Male
6		+				Weasel	+	+	Male
7		+				Weasel	+	+	Male
8		+				Weasel	+	+	Male
9		+	+			Weasel	+	+	Male
10		–	+		+	Weasel	+	+	Male
11		–	+		+	Weasel	+	+	Male
12			+		+	Weasel	+	+	Male
13	–	–	+		+	Weasel	–	+	Male
14		–	+		+	Weasel	+	+	Male
15		–	+			Weasel	+	+	Male
16		–	+			Weasel	+	+	Male
17			+			Weasel	+	+	Male
18		–	+			Weasel	+	+	Male
19	–	–	+			Weasel	+	+	Male
20	–	–	+			Weasel	+	+	Male
21			+			Weasel	+	+	Male
22		–	+		+	Weasel	+	–	Female
23			+			Weasel	+	–	Female
24			+			Weasel	+	–	Female
25			+			Weasel	+	–	Female
26			–	+		Weasel	+	–	Female
27			–	+		Weasel	+	–	Female
28	–	+			+	Weasel	–	–	Unidentified
29	–	+			+	Weasel	–	–	Unidentified
30			+			Weasel	–	–	Unidentified
31			+			Weasel	–	–	Unidentified
32			+			Weasel	–	–	Unidentified
33			+			Weasel	–	–	Unidentified
34		–	+			Weasel	–	–	Unidentified

purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, England, UK) to increase PCR amplification efficiency.

PCR reactions (in a total volume of 20 µl) were performed with a Biometra T3 Thermocycler under the following conditions: 10 µl 2×Mighty Amp buffer (Takara Bio Inc., Otsu, Japan), 1 µl primer set (containing 10 pmol each primer), 7.8 µl ddH_2O, 0.2 µl *Taq* DNA polymerase (MightyAmp DNA Polymerase, Takara Bio Inc.) and 1 µl DNA. Hot start PCR amplifications included a pause at 94°C, three cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 10 sec, polymerization at 72°C for 30 sec, and 35 cycles of denaturation at 98°C for 15 sec, annealing at 50°C for 10 sec, polymerization at 72°C for 30 sec, and a pause at 4°C.

We checked the PCR product by agarose gel electrophoresis.

3) Sequencing

Cycle sequencing reaction for DNA sequence was performed using BigDye® Terminators v1.1 Cycle sequencing Kit (Applied Biosystems Inc., California, USA) with 3 µl primer (0.8 µM) and 2 µl template DNA.

We used a DNA Sequencer ABI 3130xl Genetic analyzer (Applied Biosystems) for sequencing. Finally, samples were identified as their species by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), a program to search homology of the base sequence.

4) Sex determination

To determine the sex of weasels, we employed the nested PCR method as previously described by Sekiguchi et al. (2010). Hot start PCR amplifications were performed in the same way as in 2) above. In brief, we amplified DDX3X and DDX3Y genes on X chromosome and Y chromosome respectively with the dbxy215d and r primer set. Then the amplified DNA fragment was purified using an illustra GFX PCR DNA and Gel Band Purification kit. The purified DNA fragments were then amplified with either the dbxnt-112d and -373r primer set for DDX3X or the dbynt-111d and -296r primer set for DDX3Y. Amplified DNAs were mixed with 6×gel loading dye and run on 1.4% agarose gel electrophoresis along with 100 bp DNA marker (Nakarai chemicals). Photos were taken with a gel documentation system as described above.

Food habit analysis

Food items taken by Japanese weasels were identified during faecal analysis using the following protocol: a defrosted faecal sample was washed with one liter of water over a sieve with a 1 mm × 1 mm mesh. The residue of undigested items remaining on the mesh were then transferred into a petri dish and identified under a microscope (×30 magnification). Earthworms were recognized based on observations of earthworm chaetae (see Kaneko et al. 2009). Food items found in faeces were then classified into the following categories: 1) rodents, 2) birds, 3) reptiles and amphibians, 4) fish, 5) crustaceans, 6) coleoptera insects, 7) orthoptera insects, 8) insect larvae, 9) other insects, 10) earthworms, 11) other animals, 12) fruits, 13) other plants, 14) anthropogenic foods and 15) others and unknown (hairs, stones or others). We defined "other insects" and "other animals" as remains from unidentifiable insects or animals, such as exoskeletons or bones, but which we could not determine to order or class. "Other plants" were defined as material other than fruit, such as leaves, stems or roots, similarly, "anthropogenic foods" were defined as items likely derived from human garbage or agricultural products.

We used two indices for the food analysis, frequency of occurrence and relative volume. Relative volume was estimated visually as the percentage volume of each category per faecal sample followed Kaneko et al. (2009).

Results and discussion

A total of 184 faecal samples were collected between September 2009 and August 2010. Of those samples, 93 were chosen (n = 93; spring, n = 40; summer, n = 35; autumn, n = 9; winter, n = 9) as in suitable condition for the DNA analysis (freshness, Shimatani et al. 2008; storage in ethanol, Sekiguchi et al. 2010).

Species identification of the faecal samples

Of the 93 faecal samples, 34 were identified as from Japanese weasels (spring, n = 14; summer, n = 7; autumn, n = 8; winter, n = 5) by BLAST search (Table 1). Accordingly, eight out of 34 Japanese weasel samples were analysed with the Mustelidae specific primer set; D-tsd and -195re primer set (Table 1). Though six out of eight faeces could not be identified as Japanese weasels because of no amplification with this primer set, they were identified as Japanese weasels using the other primer sets (Table 1; Sample ID 3, 13, 19, 20, 28 and 29).

In this study, the D-tsd and -195re primer set was made to amplify the Mustelidae D-loop region based on the Japanese weasel tissue samples in Japan's southern island of Kyushu Island (Sekiguchi et al. 2010) to identify faecal samples in eastern island of Honshu Island. The lower success rate of species identification might be caused by two possible reasons; first, some faeces had some inhibitors of PCR, second, sequences of the target region of the D-tsd and -195re primer set might differ between some Kyushu and some Honshu individuals. Indeed, Masuda et al. (2012) found a genetic difference between the Japanese weasel populations of Kyushu and Honshu by mitochondrial DNA analysis.

In rest of 59 faecal samples, 22 samples were identified as Japanese martens, showing that martens distribute sympatrically in the area. Another 37 were other species (n = 23) or unidentified, because both weasels and martens DNA were amplified (n = 14). The quantity of Japanese weasels DNA in faeces of "weasels and martens" was low, so we excluded those from the sex identification and the food habits analysis. The reason of both weasels and martens DNA were PCR-amplified were; 1) one of the two species ate another, and/or 2) both species defecated at the same place. Further ecological studies; identification of hair in faeces, and observations at defecation sites are needed.

Sex identification of the faecal samples

Of the 34 identifiable Japanese weasel samples, 21

were determined to be from males (spring, $n = 6$; summer, $n = 4$; autumn, $n = 7$; winter, $n = 4$), and six from females (spring, $n = 3$; summer, $n = 2$; autumn, $n = 1$; winter, $n = 0$) (Table 1). It was not possible to identify the gender for the remaining seven samples (spring, $n = 5$; summer, $n = 1$; autumn, $n = 0$; winter, $n = 1$), because neither the signal of the X chromosome nor the Y chromosome were amplified by PCR. However, the possibility and accuracy of DNA amplification was maximized by the hot start PCR and the nested PCR, so the possibility of miss detection of DNA is quite small. The success rate of sex identification was 79.4%, confirming that the sex identification method used by Sekiguchi et al. (2010) also works for the faeces of Japanese weasel population in Honshu.

In this study, we identified fewer female faeces than male faeces. Some previous studies have shown the same tendency for lower acquisition rates of female Japanese weasels (Fujii 1998; Kaneko et al. 2013), but because of our small sample size further analysis is necessary before we can discuss sex ratio or the ecological difference between males and females. However, this study suggests for the first time that it is possible to provide information about the sex of Japanese weasels in their natural habitat by using faecal DNA analysis.

Food habits

A male faeces in summer did not contain any food items, so we excluded this faeces from following analysis (Table 2). Fruits ($n = 11$), coleoptera insects ($n = 10$), and other insects ($n = 9$) were the yearly major food categories of Japanese weasels in frequency of occurrence ($n = 33$). Other plants seem to have been eaten accidentally (Table 2), as seen also by Fujii et al. (1998) and Kaneko et al. (2009). Japanese weasels switched their food seasonally, the seasonal top food categories were: coleoptera insects (6 out of 14) in spring, fruits (4 out of 6) in summer, orthoptera insects and crustaceans (5 out of 8) in autumn, and fish and fruits (4 out of 5) in winter (Table 2). Fish and other insects were found in weasel faeces in all seasons, whereas insect larvae and anthropogenic foods were not found in the faeces. In terms of relative volume, fish (13%), orthoptera insects (10%), and coleoptera insects (7%) were the yearly major food categories of Japanese weasels. The seasonal top food categories were: coleoptera insects in spring (10%) and summer (16%), rodents also in summer (16%), orthoptera insects (37%) in autumn and fish (52%) in winter.

In the 20 faecal samples of male Japanese weasels, the most frequent four food items were other insects ($n = 7$), fish, coleoptera insects, and fruits ($n = 6$, respectively). In terms of relative volume, fish (13%), orthoptera insects (10%), and reptiles and amphibians, crustaceans and other insects (6%, respectively) were the major food categories (Table 2). In the six faecal samples of female Japanese weasels, the most frequent three food items were coleoptera insects, other insects, and fruits ($n = 2$, respectively). In terms of relative volume, orthoptera insects and fruits (10%, respectively) and crustaceans (5%) were the major food categories.

In Asian countries, weasels consume more arthropods than Europe (Tatara and Doi 1994; Fujii et al. 1998; Kaneko et al. 2009), perhaps resulting from a difference in overall arthropod availability between Europe and Asia. In this study, we found that male and female Japanese weasels mainly ate arthropods and fruits, whereas in a study in rural area Kaneko et al. (2013) showed a different trend, with male Japanese weasels specializing on mammals and crustaceans, and females as generalists switching between orthoptera and coleoptera insects, earthworms and fruits, along with mammals and crustaceans. The difference of dietary diversity of available food items between rural and urban habitat may affect food choice especially in males. However, there were insufficient data in sample size to discuss sexual differences in the diet properly. Our first trial using species identification of faeces using molecular techniques is able to access population level approach in this topic as future direction. However, it still contains several issues for the lab analysis protocol. First, in our limited work time in the DNA lab, we did not examine consistency of the experiment. Second, we did not analyse individual identity of the faecal samples this time. Kaneko et al. (2013) used stomach samples for food analysis as it is the most consistent samples in species and sex identification, but the survey ended in only 32 samples even after 12 years effort to correct the samples, especially in 4 samples of females. Using molecular technique for Japanese weasels in this study is going to be breakthrough in the area. Nevertheless, the consistency of the experiment and advance in lab techniques might remain as future topics to encourage studies of the Japanese weasel with ecological fieldwork.

Shimizu et al. (2009) identified three essential human-driven components of urban river conservation: controlling river flow, maintaining river basins, and preserving ecological environments. Japanese weasels along the Tama River consumed a wide range of food items varying seasonally from 2009 to 2010. In the urban area of the

Table 2. Frequency of occurrence and relative volume of male and female Japanese weasels revealed by the faecal analysis in Hamura between September 2009 and August 2010

a. Frequency of occurrence

	n	Rodents	Birds	Reptiles and amphibians	Fish	Crustaceans	Coleoptera insects	Orthoptera insects	Larvae	Other insects	Earthworms	Other animals	Fruits	Other plants	Anthropogenic foods	Others and unknown
Japanese weasel																
Spring	14	1	0	0	1	1	6	1	0	2	0	2	3	11	0	11
Summer	6	1	0	0	1	1	3	0	0	2	2	0	4	4	0	3
Autumn	8	0	0	3	1	5	1	5	0	4	0	1	0	3	0	3
Winter	5	0	1	0	4	0	0	0	0	1	0	1	4	4	0	2
Total	33	2	1	3	7	7	10	6	0	9	2	4	11	22	0	19
Male Japanese weasel																
Spring	6	1	0	0	1	0	2	1	0	2	0	1	1	5	0	6
Summer	3	1	0	0	0	1	3	0	0	1	1	0	2	2	0	1
Autumn	7	0	0	3	1	4	1	4	0	3	0	1	0	3	0	2
Winter	4	0	0	0	4	0	0	0	0	1	0	0	3	4	0	1
Total	20	2	0	3	6	5	6	5	0	7	1	2	6	14	0	10
Female Japanese weasel																
Spring	3	0	0	0	0	0	2	0	0	0	0	1	1	1	0	2
Summer	2	0	0	0	0	0	0	0	0	1	0	0	1	2	0	2
Autumn	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1
Winter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	6	0	0	0	0	1	2	1	0	2	0	1	2	3	0	5

b. Relative volume (%)

	n	Rodents	Birds	Reptiles and amphibians	Fish	Crustaceans	Coleoptera insects	Orthoptera insects	Larvae	Other insects	Earthworms	Other animals	Fruits	Other plants	Anthropogenic foods	Others and unknown
Japanese weasel																
Spring	14	4	0	0	3	3	10	4	0	2	0	5	4	44	0	22
Summer	6	16	0	0	15	9	16	0	0	5	0	0	7	20	0	12
Autumn	8	0	0	24	3	12	1	37	0	14	0	5	0	3	0	2
Winter	5	0	10	0	52	0	0	0	0	3	0	2	15	16	0	2
Total	33	4	2	6	13	6	7	10	0	6	0	3	5	25	0	12
Male Japanese weasel																
Spring	6	8	0	0	7	0	13	8	0	5	0	8	1	27	0	23
Summer	3	32	0	0	0	18	32	0	0	4	0	0	5	6	0	4
Autumn	7	0	0	27	4	9	1	34	0	16	0	6	0	3	0	1
Winter	4	0	0	0	65	0	0	0	0	4	0	0	9	20	0	3
Total	20	4	2	6	13	6	7	10	0	6	0	3	5	25	0	12
Female Japanese weasel																
Spring	3	0	0	0	0	0	5	0	0	0	0	5	13	30	0	47
Summer	2	0	0	0	0	0	0	0	0	10	0	0	10	50	0	30
Autumn	1	0	0	0	0	30	0	60	0	5	0	0	0	0	0	5
Winter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	6	0	0	0	0	5	3	10	0	4	0	3	10	32	0	34

Tama River, the home range area of Japanese weasels is restricted to the riverbanks (Fujii 1998). Furthermore, anthropogenic foods were not consumed by Japanese weasels in this study, which is consistent with previous studies (Fujii et al. 1998; Kaneko et al. 2009). Therefore, the environment of riverbank which keeps a seasonally varying prey should be conserved against introduced locust trees which predominate 30.1% of the study area (Fig. 1), and are still rapidly increasing (River Ecology Research Group 2000, 2006). Naturally, disturbances (e.g., floods) prevent ecological succession of vegetation in riverbank, but in urban area, the river banks were not disturbed because of local flood management works (e.g., dredging river basin, water course control). Consequently, we should enforce the constructive disturbance (e.g., deforestation) to keep the urban river remaining as natural habitat for Japanese weasels.

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