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Metabarcoding of feces and intestinal contents to determine carnivorous diets in red-crowned cranes in eastern Hokkaido, Japan

Haruka KATAOKA[1)], Nanami KOITA[1)], Natsuko ITO KONDO[2)], Hiroshi C. ITO[2)], Momoko NAKAJIMA[2)], Kunikazu MOMOSE[3)], Hiroko IIMA[4)], Tomoo YOSHINO[4)], Tomoko AMANO[5)], Takio KITAZAWA[1)], Daiji ENDOH[1)], Nobuyoshi NAKAJIMA[2)] and Hiroki TERAOKA[1,3)]*

[1)]School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido, Japan
[2)]Biodiversity Division, National Institute for Environmental Studies, Ibaraki, Japan
[3)]NPO Red-crowned Crane Conservancy, Hokkaido, Japan
[4)]Kushiro Zoo, Hokkaido, Japan
[5)]College of Agriculture, Food and Environment Sciences, Rakuno Gakuen University, Hokkaido, Japan

ABSTRACT. The red-crowned crane *Grus japonensis* in Hokkaido, Japan forms a closed population as a residence that is independent of the mainland population. Based on observations of a limited number of individuals as well as cranes in captivity, red-crowned cranes are omnivores and eat fish, worms, insects and plants in their own territories except in winter, when they are fed with dent corn that is supplied in eastern Hokkaido. DNA metabarcoding based on high throughput sequencing was carried out using universal primer sets for cytochrome oxidase subunit I gene. Feces from 27 chicks collected in June and July in the period from 2016 to 2018 and intestinal contents from 33 adult and subadult cranes that were found dead almost throughout year in 2006–2013 in the field in eastern Hokkaido were used. Although compositions varied considerably in the cranes, both insects and fish were found in adults and subadults to the same extents, while insects were predominant in chicks. Both insects and fish were detected in all seasons for adults and subadults. Horse flies, scarab beetles and weevils accounted for the most of the insects regardless of the life stage. Dace, stickleback, flatfish and sculpin were the major fish species in adults, while chicks ate almost only stickleback. The results provide the first comprehensive data on carnivorous diets in wild red-crowned cranes in eastern Hokkaido as basis for conservation of red-crowned cranes, for which the life style and area continue to change.

KEY WORDS: cytochrome c oxidase subunit I, *Grus japonensis*, high throughput sequencing, Japan, scatology

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The red-crowned crane *Grus japonensis*, which is an endangered species (IUCN Red List, https://www.iucnredlist.org/es/species/22692167/93339099), is a large crane with habitats in the Far East Eurasian continent (mainland population) and in Japan (island population). Most of the red-crowned cranes in the island population are restricted to areas in eastern Hokkaido including Kushiro, Nemuro, Tokachi and Abashiri as non-migratory residences [3, 23, 26]. Red-crowned cranes in eastern Hokkaido feed, build nests and raise offspring in their own territories (area of about 2–7 km^2) from the end of winter until late autumn, while supplied corn is available for them corn delivered in some major feeding stations entrusted by the Ministry of Environment, Japan (MOEJ) and minor private stations in eastern Hokkaido for wintering [23]. They need marshland covered with common reed bush for nesting and cannot live in the forest, although there is a large forest area in eastern Hokkaido [20]. Because their population as well as density of nests dramatically increased from 50 to about 1,600 by 2018 and marshland for nesting has been decreasing due to industrial and agricultural development, the area of land that is suitable for nesting has been decreasing [24].

A large number of red-crowned cranes in eastern Hokkaido have recently changed their habitat from marshlands to farmlands

*Correspondence to: Teraoka, H.: hteraoka@rakuno.ac.jp, School of Veterinary Medicine, Rakuno Gakuen University, Midorimachi 582, Bunkyo-dai, Ebetsu, Hokkaido 069-8501, Japan
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[20]. Farmlands are now indispensable as feeding places for cranes due to their abundance of food for cranes, including insects, other arthropods and worms [30]. Direct observations with binoculars for a limited number of individuals as well as cranes in captivity suggested that red-crowned cranes are omnivores that eat fish, worms, insects and plants including grains [17, 19]. On the other hand, crane pairs with their offspring that feed mainly in human-made environments such as agricultural lands and cowsheds frequently ingest agricultural food including dent corn [30]. Naturally, however, it is difficult to determine prey species as well as eaten plants by direct observation in this situation. One of the other concerns is that there could be a possible bias in judgement of animal species. Larger animals or easily defined animals such as shellfish could be counted more frequently [17]. Direct observation of cranes in a marshland could also be prevented by tall common reed bush except in winter.

Scatological samples including feces and intestinal contents are important samples for nonselective large-scale dietary studies. However, conventional Sanger sequencing of PCR products with universal primer sets may not be adequate because genomic DNA of diet animals are fragmented in scatological samples. Another problem is that a large amount of host animal genomic DNA could interfere with PCR reactions [18]. However, the recent development of ultrasensitive high throughput sequencing (HTS) technology has provided a means for obtaining comprehensive information on the feeding status of wildlife with scatological samples, which had been difficult to study by direct observation in the field, especially for many individuals [2]. While there have been many scatological DNA metabarcoding studies on dietary status, especially that for mammalian carnivores, with marker genes such as mitochondrial cytochrome c oxidase subunit I (COI), studies on bird species have been limited [32, 37]. On the other hand, the number of red-crowned cranes in eastern Hokkaido has reached a peak and begun to decrease in fact (NPO Red-crowned Crane Conservancy (RCC), http://www6.marimo.or.jp/tancho1213/sosutyosa.html). Additionally, The MOEJ [11] decided to gradually reduce the supply of dent corn in major feeding stations with the aim of dispersing the cranes, which may result in drastic changes in their feeding status as well as survival rate. To obtain information for fundamental planning in order to prevent possible problems, the dietary status and food preference of red-crowned cranes should be clarified as precisely as possible.

In eastern Hokkaido, Japan, the habitats of most red-crowned cranes are in fields near areas where people are living [21]. Thanks to this situation, the dead bodies of about 30 cranes have been collected every year and kept in a freezer in Kushiro Zoo under a protection propagation program for red-crowned cranes formulated by the MOEJ. Fecal samples and blood samples have also been collected when the RCC conducts banding for about 30 chicks in early summer every year [25]. For this study, we were able to obtain intestinal contents from 33 adult and subadult red-crowned cranes that was found dead in the field in the period from 2006 to 2013 and feces from 27 chicks in the same area in 2016–2018. We carried out an HTS-based metabarcoding study on these scatological samples with universal primers for COI in order to clarify carnivorous diets of red-crowned cranes in eastern Hokkaido, Japan.

MATERIALS AND METHODS

Samples and DNA extraction

With permission from MOEJ, feces from 27 chick (estimated 1.5–2 months old) were collected in June and July in the period from 2016 to 2018, when banding was being conducted. Feces were collected over a large clean plastic sheet to avoid contamination of soilborne microorganisms. However, we should admit possible contamination of insects such as small flying insects on feces. Since plastic sheets were washed with a large amount of water and dried indoors after each use to avoid possible contamination of the last feces sample. Feces samples were kept in plastic sample bags for a few hours until freezing at −20°C. Intestinal contents were obtained from 33 adult and subadult cranes where were found naturally dead in 2006–2013 in the field in eastern Hokkaido and had been kept in a freezer in Kushiro Zoo, Kushiro, Hokkaido. Plastic gloves and surgical knives were changed for each. The collected samples in clean plastic bags were stored at −20°C until DNA extraction. The collection sites of chick feces and bodies of adult and subadult cranes in the eastern part of Hokkaido, Japan (major part of the red-crowned crane habitat) are shown in Figs. 1 and 2.

After thawing the frozen samples on ice, total DNA was extracted from about 200-mg samples (wet weight) using the QIAamp DNA Stool Mini Kit according to manufacturer's instructions. Extracted DNA was diluted in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and stored at −20°C until use.

Sequencing

Extracted DNA was used as template for polymerase chain reaction with KOD FX Neo (Toyobo, Tokyo, Japan) to amplify a partial length of cytochrome oxidase subunit I (COI) using the forward primer, mlCOIintF and the reverse primer, HCO2198 (Supplementary Table 1). The primer set was designed to universally recognize animal COI and the size of amplicon excluding primer regions is 313 bp [6, 18]. A blocking primer (WKSCOI, Supplementary Table 1) was newly designed and also used to reduce amplification of COI of the red-crowned crane as a host animal. PCR consisted of an initial activation step at 94°C for 2 min and 16 cycles of denaturation at 98°C for 10 sec, annealing at 62°C for 30 sec, and extension at 72°C for 60 sec, followed by 40 cycles of the same conditions except annealing at 46°C in a thermal cycler (iCycler, Bio-Rad, Hercules, CA, USA). Amplification of PCR products was confirmed with 1% agarose gel electrophoresis. Amplicon libraries without fragmentation were prepared using the Ion Plus Fragment Library Kit according to the instructions of the manufacture (Thermo Fisher, Waltham, MA, USA). Sample-unique barcodes and sequencing adapters A and trP1 for IonPGM (Thermo Fisher) were ligated to products of the 1st PCR with Ion Xpress Barcode Adapters (Thermo Fisher). Then each sample was purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA). The concentrations of samples were quantified using an Ion Library TaqMan Quantification

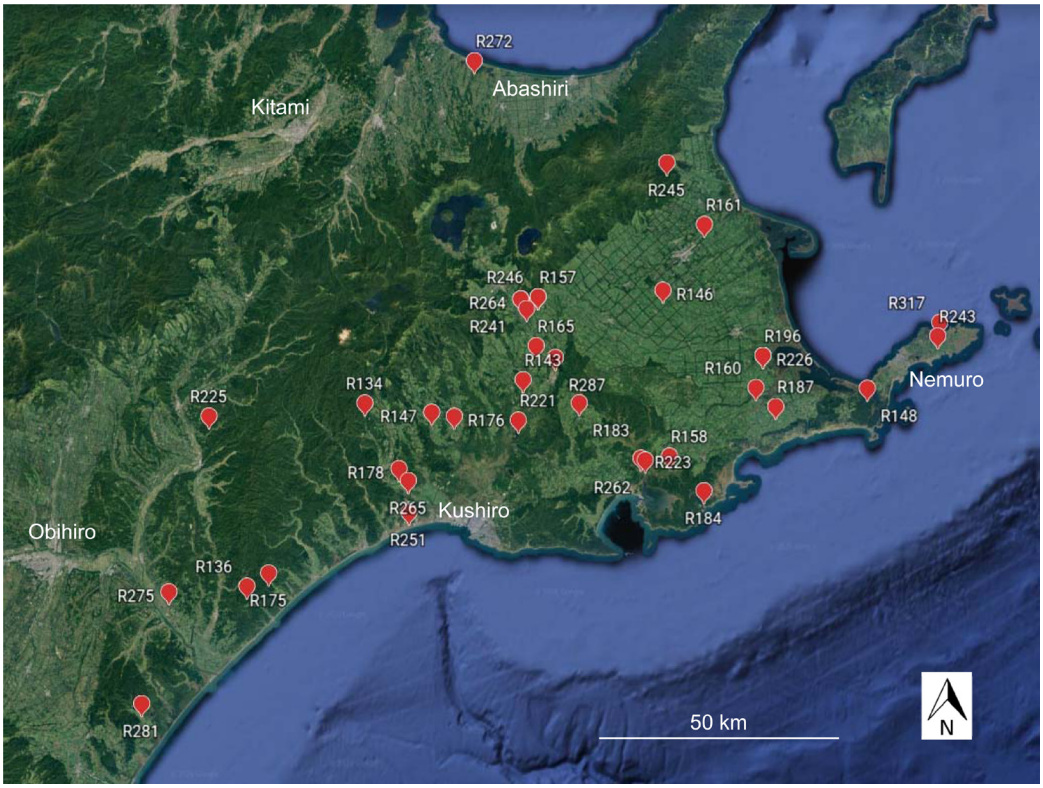

Fig. 1. Sampling sites of bodies of adult and subadult red-crowned cranes in Hokkaido, Japan. Bodies of adult of subadult cranes found dead in the field (n=33) were collected in 2006–2013 and kept in a freezer until intestinal contents were obtained. Collection sites of bodies were plotted on a map that was made using Google Earth.

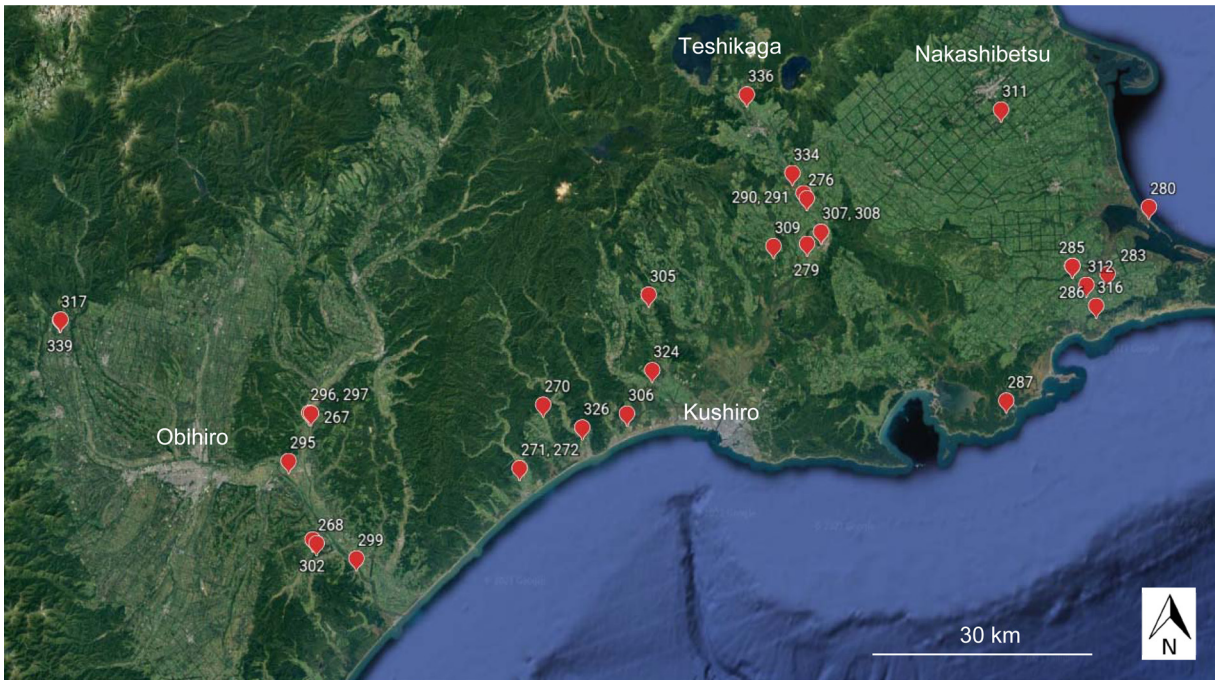

Fig. 2. Sampling sites of feces of red-crowned crane chicks in Hokkaido, Japan. Feces of red-crowned crane chicks (n=27) were collected in June and July in the period from 2016 to 2018 and kept in a freezer until analysis. Collection sites were plotted on a map that was made using Google Earth.

Kit (Thermo Fisher) and LightCycler 480 (Roche, Basel, Switzerland). Each sample was mixed and appropriately diluted and prepared for sequencing with an Ion PGM Template OT2 400 kit (Thermo Fisher) and IonPGM Hi-Q Sequencing Kit (Thermo Fisher) following the manufacture's protocols. Sequencing was performed on an IonPGM System (Thermo Fisher) with Ion 318 v2 Chip (Thermo Fisher).

Processing of sequencing output and identification of query sequences

The sequence data were demultiplexed on the Torrent Suite (Thermo Fisher), and the program package Claident v0.2 [34] was used for subsequent analyses. Following trimming of the primer regions, the sequences were trimmed from the end until there were three or more consecutive bases with a Phred-like quality value of 27 or higher. The remaining sequences were filtered by the length (min=280 bp, max=313 bp) and if the percentage of bases with a quality value less than 20 was greater than 0.1, they were also removed. Although it is general to leave a margin before and after the sequence length in metabarcoding, we set the analysis length to 280–313 bp to reduce the computational load, because no obvious effects were obtained in the preliminary analysis using longer sequences. After noisy and chimeric sequences had been removed with default parameters, sequences were clustered into OTUs at 97% identity. Clustered sequences with less than 0.3% of total read counts were discarded to eliminate false positives.

The obtained OTUs were subjected to a blast search using the dataset animals_COX1_species (updated on Apr. 7, 2018) provided in Claident. The dataset animals_COX1_species is a reference dataset of animal COI sequences with species names registered in GenBank. For the taxonomic assignment of OTUs, the lowest common taxonomic name was used for the top ten taxonomic names that matched 97% or more. If an adequate species was not found in red-crowned crane habitats in Hokkaido, the corresponding genus was selected. Identified species were compared with habitat information on animal species. Sequence data that were obtained in this study are available in the DDBJ Sequenced Read Archive under the accession number DRA012450.

Specific PCR

In order to detect some frog and crayfish species, conventional nested-PCR reactions were carried out with primer sets specific for each animal designed in this study (Supplementary Table 2). Because we did not find clear positive bands in agarose electrophoresis by the first PCR, GoTaq Green Master Mix (Promega, Fitchburg, WI, USA) was used according to the manufacture's instructions for the first and nested PCR reactions with a step down procedure (3 cycles of denaturation at 95°C for 45 sec, annealing at 65°C for 45 sec, extension at 72°C for 45 sec; 3 cycles of denaturation at 95°C for 45 sec, annealing at 59°C for 45 sec, and extension at 72°C for 45 sec; 30 cycles of denaturation at 95°C for 45 sec, annealing at 53°C for 45 sec, and extension at 72°C for 45 sec). Some of the bands were extracted and used for conventional Sanger sequencing for confirmation of the specificity with an ABI PRISM 310 Genetic Analyzer (Thermo Fisher) after reactions with a Big Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher).

RESULTS

Adults and subadults

The total number of raw data used for analysis in this study was 6,817,479 reads with a mean of 113,625, a maximum of 718,991, and a minimum of 10,326 reads. Clustering with 97% agreement resulted in 1,377 OTUs, of which 137 OTUs (613454 reads) could be identified by organism names under the phylum. Thirty-three samples of intestinal contents from adult and subadult cranes were obtained throughout the year except August and September in this study. The identified animals were arbitrarily classified by corresponding common names (mostly order or infraorder, Table 1), although there was marked individual variation (Supplementary Table 3). There were 20 or more animal categories that contained one or more species. Eight fish categories were identified [1]. The animal categories that were most frequently detected were stickleback and dace (n=19). Horse fly placed the third as the most insect (n=16). Other fish species including flatfish and sculpin followed horse fly as fourth and fifth places (n=9 for both). Far Eastern smooth flounder (*Liopsetta pinnifasciata*), which mainly lives in marine water and sometimes in brackish water as well as eelpout (*Zoarces elongatus*), was the major flatfish identified [1]. Except two beetles, scarab beetle (n=7) and weevil (n=5) [35, 36], goby (n=5) and char (n=4) followed flat fish. Collectively, sticklebacks, dace, flatfish and sculpin were the major fish species consumed by cranes.

Lamprey *Petromyzontidae* sp., which belongs to the class an Agnatha, was detected in a sample from a crane (R225) that was found in an inland area far from the sea [1] (Supplementary Table 3). Whelk shellfish *Fusitriton oregonensis* (mainly marine shellfish) (R187) and pond snail *Radix auricularia japonica* (freshwater shellfish) (R158) were each detected in one sample [7, 29].

The identified animals were summarized into wider categories such as insects and fish based on their detection rates (Fig. 3A). Insects and fish were the two predominant categories, both having a detection rate with almost 70%. Earthworms, snakes and spiders were not detected in intestinal contents from adults and subadults.

Total reads (%) indicate the percentage of read counts of each sequence of total reads of all sequences that were detected in each scatological sample (Fig. 3A). In total reads (%) indicated by open bars, insects and fish were the two major carnivorous diets again. Total reads of insects seemed to be higher or comparable to those of fish in adults and subadults (Fig. 3A).

Aquatic insects that live in a water environment throughout their lives were never found. Frogs and mammals such as rodents were also not detected in any of the samples by HTS analysis in this study.

Table 1. Animal categories identified in intestinal contents of adult and subadult cranes

Common name	Number of samples identified	Sum of % total reads	Average ± SEM in all samples	Average ± SEM in identified samples	Median in identified samples	Species included in animal category
Stickleback	19	201.16	5.81 ± 2.13	10.06 ± 3.23	5.81	*Pungitius* sp. (18) a, *P. tymensis* (4) a
Dace	19	224.98	6.82 ± 1.89	11.84 ± 2.10	5.39	*Tribolodon* sp. (19) g, *T. hakonensis* (2) g
Horse fly	16	437.68	13.26 ± 4.70	27.35 ± 8.54	11.21	*Eristalis* sp. (1) i, *Haematopota pluvialis* (8) i, *Platycheirus* sp. (3) f, *Tabanus* sp. (6) j
Flatfish	9	41.81	1.27 ± 0.47	8.13 ± 1.12	6.43	*Liopsetta pinnifasciata* (9) g, *Pseudopleuronectes yokohamae* (2) g, *Platichthys stellatus* (1) g
Sculpin	9	15.51	0.47 ± 0.17	1.72 ± 0.41	1.59	*Cottus amblystomopsis* (8) g, *Myoxocephalus stelleri* (2) g, *Myoxocephalus brandti* (6) g
Scarab beetle	7	159.69	4.84 ± 3.08	22.81 ± 13.77	4.50	*Popillia japonica* (7) c
Weevil	5	294.25	8.92 ± 4.56	58.85 ± 20.26	50.02	*Sipalinus gigas* (5) c
Goby	5	4.54	0.14 ± 0.07	0.91 ± 0.29	0.80	*Gymnogobius* sp. (5) g
Char	4	3.75	0.11 ± 0.06	0.94 ± 0.13	0.95	*Salvelinus* sp. (4) g
Froghopper	3	114.46	3.47 ± 2.48	38.15 ± 24.17	32.94	*Lepyronia coleoptrata* (3) f
Fly	3	28.68	0.87 ± 0.52	9.56 ± 2.40	8.20	*Graphomya maculata* (1) f, *Hybomitra* sp. (1) f, *Lucilia* sp. (1) f
Lamprey	2	94.21	2.85	47.11	-	*Petromyzontidae* sp. (2) g
Ladybird	2	14.92	0.45	7.46	-	*Coccinella septempunctata* (1) c, *Harmonia axyridis* (1) c
Chironomid	2	9.00	0.27	4.50	-	*Psectrocladius* sp. (1) d, *Polypedilum masudai* (1) d
Eelpout	2	5.72	0.17	2.86	-	*Zoarces elongatus* (2) g
Grass hopper	2	3.45	0.10	1.73	-	*Oxya* sp. (1) e, *Tetrigidae* sp. (1) b
Crucian carp	1	15.08	0.46	-	-	*Carassius* sp. (1) g
Leaf beetle	1	3.66	0.11	-	-	*Basilepta fulvipes* (1) c
Whelk shellfish	1	1.63	0.05	-	-	*Fusitriton oregonensis* (1) k
Pond snail	1	1.08	0.03	-	-	*Radix auricularia* (1) h

Animal categories identified in intestinal contents from 33 adult and subadult red-crowned cranes are presented. "Number of samples" indicates number of intestinal contents in which each animal category was identified. As some samples contain two or more species in each animal category, each animal category may contain more than the sum of species. Parenthesis in "Species included in animal category" indicates number of individuals. Species were determined on the basis of the following references: a [1], b [10], c Hokkaido. 2016. Beetles in Hokkaido: a list of species and subspecies. https://www.pref.hokkaido.lg.jp/fs/2/2/8/4/9/7/2/_/syuasyumokuroku_konchu.kouchu.pdf, accessed to the previous version at July 24, 2021.; d [11], e [13], f [16], g [28], h [31], i [35], j [36], k [40].

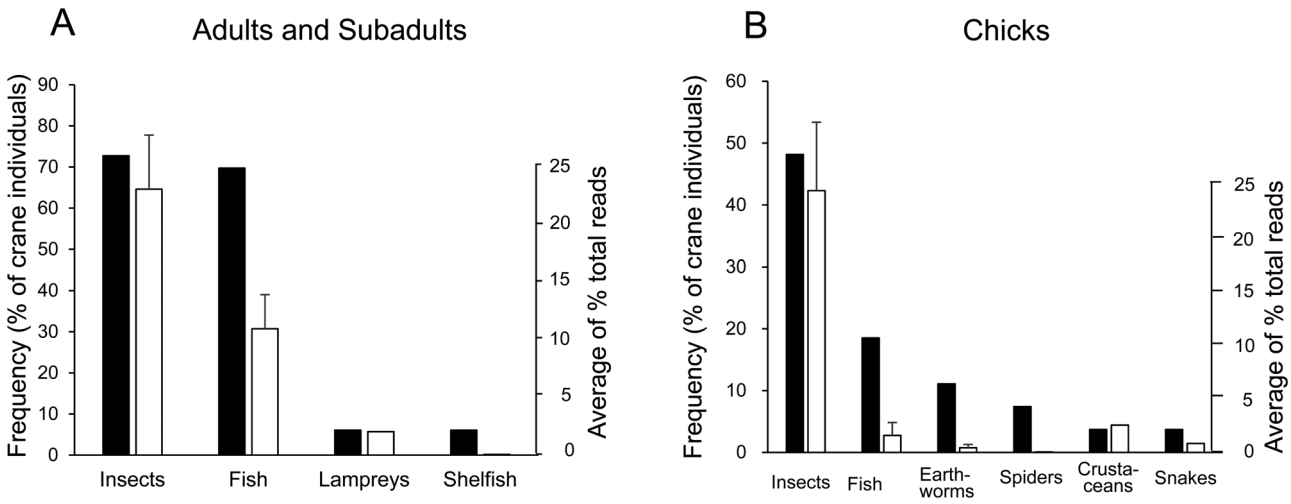

Fig. 3. Frequent orders of animals in the diet of red-crowned cranes in eastern Hokkaido. Frequencies of animal categories identified in intestinal contents of adults or subadults of red-crowned cranes (**A**) and in chick feces (**B**) are presented as percentages (black bars). A white bar indicates the average of percent reads with SEM of each sample for intestinal contents of 33 adults and subadults (A) and for feces of 27 chicks (B).

Monthly feeding status of adults and subadults

The percentages of adult and subadult cranes that ate insects and fish, the two major categories, each month are shown in Fig. 4A and 4C. We were not able to obtain samples in August and September. Although no fish were detected in February, both fish and insects were detected throughout each year. While most cranes would have received corn in the feeding stations (December–February), dace (n=5), stickleback (n=4) and others (eelpout, flatfish and sculpin, n=1 for each) were detected as major fish species in these months. The detection rates in winter were similar to those for the entire year. In addition, five horse flies, two weevils, one grasshopper (*Tetrix japonica*), and four species of beetles (leaf beetle, scarab beetle, froghopper and ladybird) were found in samples collected in December−February.

Averages of % total reads of both insects (Fig. 4B) and fish (Fig. 4D) by month showed considerably large standard errors of means (sem); however, they showed roughly the same % total reads on average. Percent total reads of insects tended to be higher than those of fish.

In individual data (Supplementary Table 3), many fish species were found in some cranes, while no fish was detected in other cranes. Compared to fish feeding status, insects were similarly found in each crane sample.

Chicks

Since banding of red-crowned cranes was carried out in early summer (June and July), chick feces obtained were restricted to this period. Identified animals were arbitrarily classified by common names (mostly order or infraorder, Table 2). There were 17 animal categories that contained one or more species. The animal category that was most frequently detected (n=9 of 27) was horse fly. Horse fly species included *Haematopota pluvialis* (n=7), *Tabanus* sp. (n=5), *Platycheirus* spp. (n=2), and *Eristalis* sp. (n=1) [35, 36].

The second most frequently detected animal categories was stickleback (*Pungitius* sp.) (n=6). Stickleback was almost the only fish that was detected in chick feces in this study, except for loach in No. 295 (Supplementary Table 4). Most of the chicks with feces containing fish were localized inland more than 10 km from the seashore (Nos. 267, 295, 307, 308 and 324), except for No.

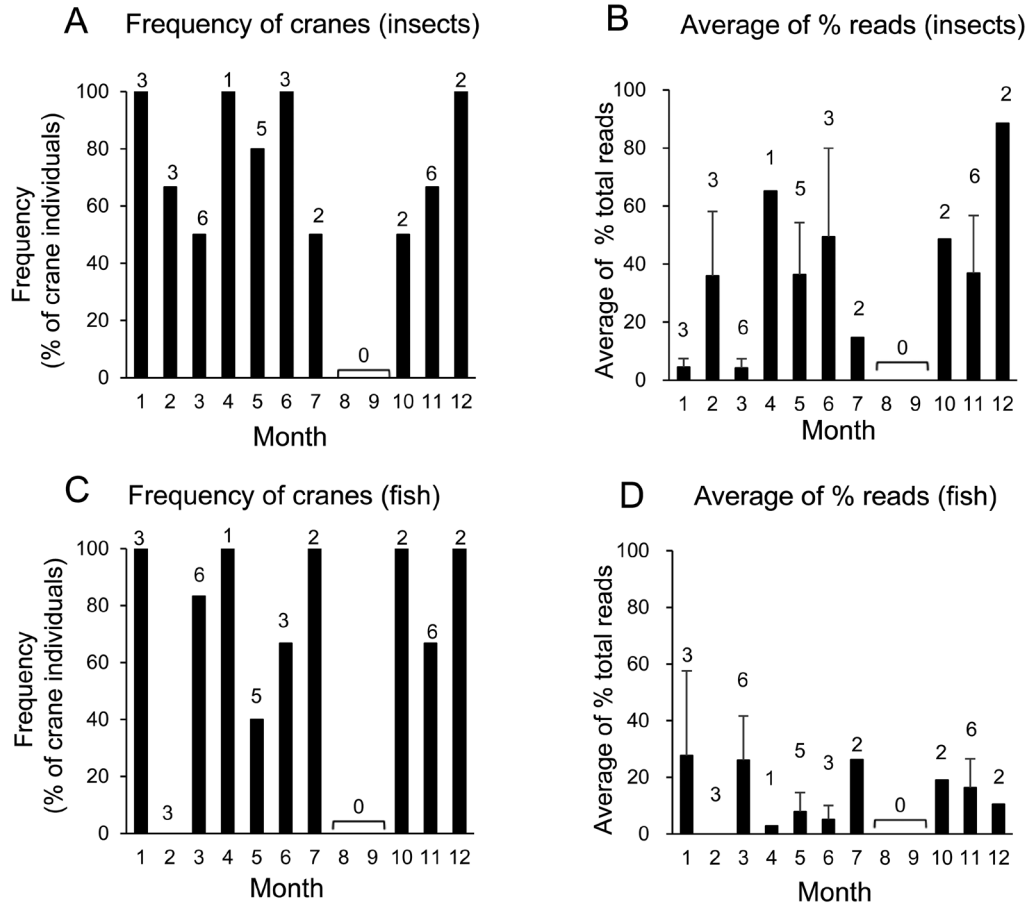

Fig. 4. Comparison of monthly status of feeding of insects and fish in adult and subadult cranes. Percentages of adult and subadult cranes that fed on insects (**A**) and fish (**C**) each month are presented. Averages of % reads of insects (**B**) and fish (**D**) that were identified each month are also presented. Vertical bars and numerical values above black bars indicate SEM of means and case number of cranes, respectively. Samples were not obtained in August and September in this study.

Table 2. Animal categories identified in chick feces

Common name	Number of samples identified	Sum of % total reads	Average ± SEM in all samples	Average ± SEM in identified samples	Median in identified samples	Species included in animal category
Horse fly	9	423.14	15.67 ± 5.52	47.02 ± 10.70	47.12	*Haematopota pluvialis* (7) k, *Tabanus* sp. (5) k, *Platycheirus* sp. (2) g, *Eristalis* sp. (1) c
Stickleback	6	48.40	1.79 ± 1.39	8.07 ± 6.31	2.31	*Pungitius* sp. (6) a
Butterfly	5	11.52	0.43 ± 0.22	2.30 ± 0.79	2.33	*Inachis io* (4) f, *Papilio* sp. (1) f
Moth	4	153.20	5.67 ± 3.62	38.30 ± 20.66	0.00	*Ptycholomoides aeriferana* (2) f,*Catocala dissimilis* (1) f, *Malacosoma neustria* (1) f, *Plemyria rubiginata* (1) f
Weevil	4	75.64	2.80 ± 1.61	18.91 ± 7.76	19.40	*Sipalinus gigas* (3) b, *Hypera nigrirostris* (1) b
Scarab beetle	4	30.49	1.13 ± 0.70	7.62 ± 3.86	5.89	*Popillia japonica* (4) b
Froghopper	3	17.52	0.97 ± 0.60	8.72 ± 3.03	10.06	*Lepyronia coleoptrata* (3) g
Fly	3	14.21	0.53 ± 0.41	4.74 ± 3.65	4.74	*Psychoda* sp. (1) e, *Scathophaga stercoraria* (1) g, *Sepsis monostigma* (1) g
Earthworm	3	13.39	0.50 ± 0.36	2.68 ± 1.59	1.17	*Amynthas yunoshimensis* (1) d, *Eisenia* sp. (1) d,*Metaphire agrestis* (1) d, *Metaphire hilgendorfi* (1) d
Locust	2	5.96	0.22	2.98	-	*Tetrigidae* sp. (1) j, *Tetrix japonica* (1) e
Spider	2	1.27	0.05	0.63	-	*Phalangium opilio* (1) l, *Tetragnatha* sp. (1) m
Crayfish	1	79.58	-	-	-	*Pacifastacus leniusculus* (1) n
Snake	1	26.17	-	-	-	*Elaphe quadrivirgata* (1) i
Mosquito	1	18.61	-	-	-	*Prionocera* sp. (1) h
Leaf beetle	1	2.20	-	-	-	*Galerucella grisescens* (1) b
Loach	1	1.37	-	-	-	*Lefua* sp. (1) i
Ladybird	1	0.31	-	-	-	*Harmonia axyridis* (1) b

Animal categories identified in 27 red-crowned crane chick feces are presented. "Number of samples" indicates number of feces in which each animal category was identified. Fly also includes drain fly. As some feces contain two or more species in each animal category, each animal category may contain a large number of species. Parenthesis in "Species included in animal category" indicates number of individuals. Species were determined on the basis of the following references: a [1], b Hokkaido. 2016. Beettles in Hokkaido: a list of species and subspecies. https://www.pref.hokkaido.lg.jp/fs/2/2/8/4/9/7/2/_/syuasyumokuroku_konchu.kouchu.pdf, accessed at July 24, 2021.; c [11], d [14], e [10], f [12], g [16], h [27], i [28], j [35], k [36], l [39], m [38], n [41].

306. For all of the other chicks, there were small rivers or ponds less than 1 km from the sites of capture for banding (Fig. 2).

The next most frequently detected animals were butterflies (n=5) and moths (n=4) (Table 2) [12] followed by beetle insects including weevils, scarab beetles (n=4 for both), and froghoppers (n=3). Earthworms (n=3), spiders (n=2) and snakes were also detected in chick feces, although they were never found in adults and subadults [1, 28, 35, 36]. Four species of earthworms, *Amynthas yunoshimensis*, *Eisenia* sp., *Metaphire agrestis*, *M. hilgendorfi*, were detected [14]. Foreign crayfish (*Pacifastacus leniusculus*) was also detected in a chick feces near two swamps and a small river in Shiranuka (No. 306, Supplementary Table 4, Fig. 2) [41].

The identified animals were summarized into wider categories (Fig. 3B). Filled bars indicate detection rates of animal categories (Fig. 3B). Insects were predominant, being detected in about 50% of the chicks. Fish were the second-most frequently detected animals, but their detection rate was less than 20%, and the next most frequently detected animals were earthworms and spiders. The percentage of chicks that did not eat prey was much higher than the percentages of adults and subadults (370.% for chicks and 3.0% for adults and subadults) (Supplementary Table 3 and 4).

Sum of total reads % (open bars, Fig. 3B) clearly indicate that insects are the major category for chicks.

Detection of frogs and crayfish by PCR with specific primer sets

As mentioned above, frogs were never detected by the HTS-based metabarcoding study, although frogs were frequently reported as one of the representative carnivorous diets for red-crowned cranes [17]. Thus, we tried nested-PCR with specific primer sets for COI of two major frog species in eastern Hokkaido: Ezo frog (*Rana pirica*) and Japanese tree frog (*Dryophytes japonica*) [28]. As shown in Table 3, only Ezo brown frogs were detected in two chicks (285, 311), three subadults (R264, R269, R281) and five adults (R143, R158, R175, R176, R246).

For comparison, we also tried nested-PCR with primer sets specific for signal crayfish (*P. leniusculus*), which are prevalent in eastern Hokkaido as alien crayfish as well as a native crayfish, Japanese crayfish (*Cambaroides japonicus*) [41]. As a result, only a signal crayfish was detected in a chick captured between Koitoi River and Koitoi Swamp (306), the results being in good agreement with the results of the NGS barcoding study (Supplementary Tables 3 and 4).

Table 3. Detection of frogs by nested-PCR with specific primer sets

Sample No.	Stage	Collection date	Collection site
285	Chick	28 June, 2006	Hamanaka
311	Chick	30 May, 2007	Bekkai
R143	Adults	28 June, 2006	Shibecha
R158	Adults	30 May, 2007	Akkeshi
R175	Adults	16 November, 2007	Urahoro
R176	Adults	20 November, 2007	Tsurui
R246	Adults	18 May, 2010	Shibecha
R264	Subadults	16 December, 2010	Teshikaga
R269	Subadults	28 May, 2011	Teshikaga
R281	Subadults	2 November, 2011	Taiki

Ezo brown frog Rana pirica was detected in some samples in chick feces (Chick) and adult or subadult intestinal contents (Adults, Subadults) by nested-PCR with specific primer sets (Supplementary Table 2). Collection dates and sites (municipality) of chick feces and adult or subadult bodies are also indicated. Japanese tree frog Dryophytes japonica was not detected by nested-PCR with specific primer sets.

DISCUSSION

In this study, we obtained the first quantitative information on carnivorous diets of red-crowned cranes in eastern Hokkaido, a main area for the island population, by analysis of DNA extracts of intestinal contents and feces. Although we used nested-PCR products before NGS analyses, which might distort the original amount of target DNA, we used only average values of % reads for comparison among different samples. Adult crane pairs have their own territory for raising their offspring and feeding. They usually feed inside their territory within an area of about 2–7 km^2 [23]. Although the size of the home range of chicks is still unclear, it cannot be more than the territory for adult pairs because chicks can only walk or run on foot. Also, there is no information on the foraging range of cranes that receive corn supplied for them in winter. Additionally, it is well known that birds excrete very frequently in order to keep their weight light for flight. It was reported that daily rates of excretion by subadult (60 days after hatching) and adult (120 days after hatching) domestic fowls (*Gallus gallus*) were 35.5 ± 3.1 and 33.7 ± 6.1, respectively [15]. Retention time in the digestive system of domestic fowls is quite short, the average time being 5 to 6 hr [33]. Although we do not have any literal information on the excretion rate in red-crowned cranes, it must be very high compared to that of most mammalian species. Since we collected digested contents in the small intestines of adults and subadults, accumulated diets should reflect diets in a shorter period than those in chick feces. It is known that some digested contents stay in the cecum and need a longer time before excretion (fecal feces) in birds [9]. This might increase the retention time of some of the intestinal contents. Collectively, however, it is thought that diets found in their intestinal contents and feces mostly reflect their diets in quite close to sampling sites.

Frogs were not detected in any of the samples in our metabarcoding study despite the fact that frogs including Ezo frogs and Japanese tree frogs are considered a major part of the diet for red-crowned cranes in eastern Hokkaido [17, 19]. An additional conventional nested-PCR study with primer sets specific for these frogs showed that many cranes ingested Ezo frogs. On the other hand, signal crayfish were detected in the same two chick feces in both the metabarcoding and nested-PCR studies. Ando *et al.* [2] reported some conditions that are required for a dietary study by fecal DNA metabarcoding analysis. They recommended the use of animal category-specific markers and plural universal primers for each animal category for a metabarcoding study with feces. In this study, we used only a set of universal primers targeting the COI region of a wide range of animals [18]. In addition, universal primers that target the COI region of a wide range of animals have been developed with higher amplification efficiency, and they should be selected on the basis of their suitability for the target taxon [4, 5]. One of the possible reasons for discrepancy in results of the metabarcoding and nested-PCR studies was low match rate for universal primers used to the recognition sequence of Ezo frogs. Although the corresponding sequence of Ezo frogs is unavailable at present, those of three relative species, *Rana kunyuensis, R. dybowskii* and *R. uenoi* are different from universal primer for one, three and three base pairs of 26 base pairs (Supplementary Fig. 1). Among the animals detected in many samples, however, one species of flatfish, Far Eastern smooth flounder (*P. pinnifasciatus*), and a signal crayfish (*P. leniusculus*) showed differences in four base pairs and three base pairs, respectively, while the forward universal primer was highly degenerated. As for the blocking primer used to prevent interference by the enormous amount of red-crowned crane genome fragments in samples, corresponding sequences of those three Rana frogs showed the exact match (Supplementary Fig. 2). However, some species detected in many samples including Japanese dace (*T. hakonensis*), horse fly (*H. pluvialis*) and Japanese beetle (*P. japonica*) also showed an exact match. There was no clear trend of vertebrates showing a higher matching rate against the reverse universal primer and blocking than that shown by invertebrates, or vice versa. Thus, the inconsistent results of the metabarcoding and nested-PCR studies cannot be accounted for by only a mismatch of primer sequences. The negative results (not detected) in the metabarcoding study do not always confirm no inclusion of the animal species in the sample, while positive results (detected) strongly indicate the presence of the animal species in the scatological sample. Naturally, it can be said that negative results indicate the possibility that the scatological sample does not contain the animal species. Further metabarcoding study with plural primer sets specific for each animal category should be carried out in order to increase the accuracy of experiments in following studies.

The results of the present study clearly showed that insects and fish were the two major carnivorous diets for red-crowned cranes in eastern Hokkaido, Japan. The area of marshlands in eastern Hokkaido has been decreasing, and most red-crowned cranes now feed in farmlands instead of marshlands [30]. Thus, it is thought that the results of the present study mainly reflect the feeding habitats of cranes in farmland. Among insects, horse flies were predominant. Some beetles including scarab beetles, weevils and froghoppers were also detected. Although this might be due to the thick cuticular layer of beetles, butterflies and moths, which have a relatively thin cuticular layer, were also frequently detected. Insects that live in an aquatic environment for their whole life cycle such as Dytiscidae or Gerridae species were not detected in this study. Similar to the case for insects, some species of fish were found in almost 70% of adult and subadult cranes. Dace, stickleback, flatfish and sculpin were the major fish species detected. As

a remarkable trend in individual data (Supplementary Table 3), many fish species were found in some cranes, while no fish were found in the other cranes. One of the possible reason is that small fish that were eaten by larger piscivorous fish were also detected by metabarcoding. However, we were not able to obtain information on the size or life stage of fish detected, and fish species as prey of other large fish might have been included in some samples. Many shellfish species have been reported as representative animals in the diet of cranes in fresh water (such as Viviparidae and Lymnaeidae), estuary and marine water (such as Ruditapes) and also on land (such as Succineidae) [17, 30]; however, only a small number of shellfish was detected in this study. Shellfish with a noticeable shell can be easily confirmed by direct observation with binoculars as one of the possible causes of this discrepancy. Mammalians such as rodents [17] were never detected in this study. Earthworms, which are considered to account for a major part of the carnivorous diet, were not found in adult and subadult crane samples. Rugworm species, another Annelida such as *Arenicolidae* sp. [17, 19], were also not detected in this study, although many cranes were captured near the seashore or an estaury area. The efficacy of universal primers used for an NGS study in these invertebrates should be determined [4].

One of the surprising results in our study was that red-crowned cranes in eastern Hokkaido ate fish and insects, two major parts of the carnivorous dies for cranes with a similar frequency all year around, although it should be noted that samples in summer were very limited. We speculated that fish that cannot swim so quickly might be major preys in winter as well as earthworms in the piled compost. Almost the same fish species were detected in the period from December to February (five daces, four sticklebacks and three other fishes) at ratios similar to those all year around. Insects detected in the winter included five horse flies (including a *Platycheirus*), two weevils, a grasshopper and four different beetle insects. Scarab beetle seemed to be fewer in winter. Ingested horse flies could be larvae because they spend the winter as larvae [8]. Scarab beetles (*P. japonica*) and weevils (*S. gigas*) spend winter as larvae or adult because they need two years for maturation in Hokkaido (National Agriculture and Food Research Organization, http://www.naro.affrc.go.jp/org/fruit/apdb/coleop/P_japo1.htm). Although cranes in winter are depended on corn supplied for them as their major food, it is thought that they may consume almost the same amounts and types of fish and insects in their carnivorous diet in winter as those in other seasons.

We have provided the first comprehensive data on carnivorous diets of wild red-crowned crane chicks in Eastern Hokkaido. Insects were predominant carnivorous diet of red-crowned crane chicks, which is very different to adult and subadult cranes, which seem to consume similar amounts of insects and fish. In chicks, the most frequently detected insects were horse flies, followed by butterflies, moths, weevils and scarab beetles to similar extents. Chicks also ingested earthworms and spiders, which were never found in older cranes. On the other hand, stickleback was the almost only fish species detected in some chicks. Although we did not have any scatological samples from adults and subadults in August and September, it can be concluded that insects and other invertebrates are also major components of the carnivorous diet of chicks, unlike the diets of adults and subadults, in June and July. Since they need water to survive, there must have been rivers or marshes near sites where they were captured in this study. It was mentioned that chicks sometimes receive fish from their parents [19]. Thus, it was speculated that chicks still do not have good fishing skills yet. Fishing might be more difficult than catching insects and other animals. It is also conceivable that sticklebacks live in shallow ponds or rivers unlike other fish living in a deep estuary part of a river or in the sea.

In conclusion, the present study has provided the first comprehensive data on carnivorous diets of red-crowned cranes and their chicks. Since we were not able to collect feces and intestinal contents in the back regions of a large wetland such as Kushiro Wetland, some cranes in those areas may have different carnivorous diets, although most cranes now live in peripheral areas of a large wetland [22]. The information we obtained on the dietary status of most red-crowned cranes in eastern Hokkaido is not completely consistent with conventional descriptions highlighting aquatic and terrestrial animals such as fish, crabs and earthworms as their carnivorous diets [19, 24]. Our data should be basic data for making an effective conservation plan for red-crowned cranes in the island population in eastern Hokkaido, for which the life style and area are continuing to change.

CONFLICT OF INTEREST. We have nothing to declare for this study.

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