

## Common coypu predation on unionid mussels and terrestrial plants in an invaded Japanese river

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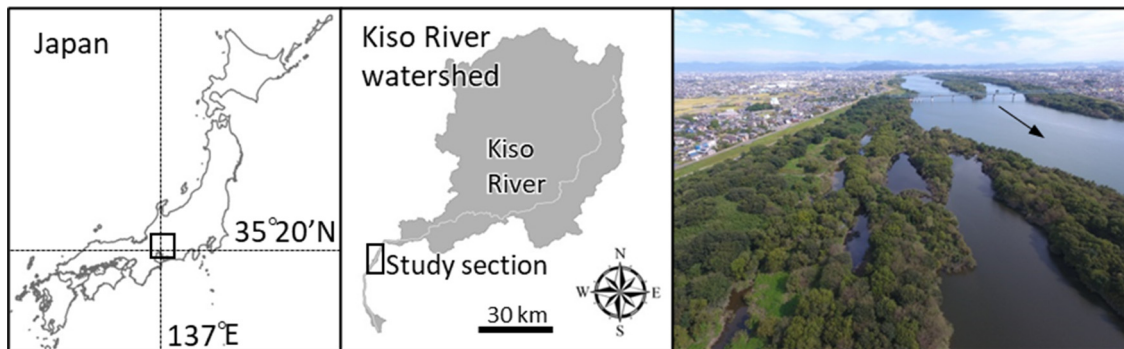
**Abstract** – The coypu (*Myocastor coypus*) is a semi-aquatic herbivorous rodent that has been suspected to prey on freshwater unionid mussels in Japan. Fecal DNA metabarcoding that targeted bivalves and terrestrial plants was performed to examine the diet of exotic coypu in the Kiso River, central Japan. Thirty-two fecal samples from four floodplain waterbodies were collected seasonally. In one of the waterbodies, live and dead mussels were investigated to examine the possible effects of coypu predation on mussel population structures. Common coypu predation on unionid mussels was confirmed in two waterbodies. Midden surveys showed that medium- to large-sized mussels tended to be consumed by coypu; possibly resulting in population structures with few large-sized individuals. Seasonal diet selection of terrestrial plants was also detected: two perennial plants were consumed throughout the year, whereas some perennial plants were consumed seasonally. The number of terrestrial plant species/taxa as primary food was higher in summer than in winter, which was attributed to the high consumption of annual plants in the summer. Future studies need to examine the exhaustive diet of coypu including other animals and plants, as well as its effects on freshwater ecosystems in invaded regions.

**Keywords:** coypu diet / exotic species / fecal DNA metabarcoding / herbivore / omnivore

**Résumé – Prédation du ragondin sur les moules unionidées et les plantes terrestres dans une rivière japonaise envahie.** Le ragondin (*Myocastor coypus*) est un rongeur herbivore semi-aquatique que l'on soupçonne être la proie des moules unionidées d'eau douce au Japon. Un métabarcodage de l'ADN fécal ciblant les bivalves et les plantes terrestres a été effectué pour examiner le régime alimentaire du ragondin exotique de la rivière Kiso, au centre du Japon. Trente-deux échantillons de matières fécales provenant de quatre plans d'eau de plaine d'inondation ont été prélevés de façon saisonnière. Dans l'un de ces plans d'eau, des moules vivantes et mortes ont été étudiées afin d'examiner les effets possibles de la prédation du ragondin sur les structures des populations de moules. La prédation courante du ragondin sur les moules unionidées a été confirmée dans deux plans d'eau. Les études des buttes dépotoir ont montré que les moules de taille moyenne à grande ont tendance à être consommées par le ragondin, ce qui peut entraîner des structures de population comportant peu d'individus de grande taille. La sélection saisonnière du régime alimentaire des plantes terrestres a également été détectée : deux plantes pérennes ont été consommées tout au long de l'année, tandis que certaines plantes pérennes ont été consommées de façon saisonnière. Le nombre d'espèces/taxa végétales terrestres comme aliment principal était plus élevé en été qu'en hiver, ce qui a été attribué à la forte consommation de plantes annuelles en été. Les études futures doivent examiner le régime alimentaire exhaustif du ragondin, y compris d'autres animaux et plantes, ainsi que ses effets sur les écosystèmes d'eau douce dans les régions envahies.

**Mots clés :** régime alimentaire du ragondin / espèces exotiques / métabarcodage de l'ADN fécal / herbivore / omnivore

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**Fig. 1.** Location of the study river and section. The photograph shows a part of the study section. The arrow denotes flow direction.

## 1 Introduction

Invasive species impact biotic/abiotic components, lead to newly-modulated ecosystems in invaded regions, and often involve biodiversity loss and ecosystem degradation (Strayer *et al.*, 2006; Pyšek and Richardson, 2010). Predation is one of the most prominent behaviors of invasive animals that influence indigenous ecosystems. Predation by invasive animals directly affects the population structure and distribution of native prey species, resulting in changes in community compositions and biological interactions (Strayer *et al.*, 2006; Kurle *et al.*, 2008). Therefore, diet is basic information that will allow us to understand the degree of impact that invasive animals have on biological communities.

Coypu (*Myocastor coypus*), also known as nutria, is a large semi-aquatic rodent that originated from South America (LeBlanc, 1994). This species has been introduced into many other countries outside their native range for fur farming, and feral populations have been established in North America, Europe, and Asia owing to escapes and releases from fur farms (LeBlanc, 1994; Carter and Leonard, 2002; Hong *et al.*, 2015). Currently, the coypu has been selected as part of the “100 of the world’s worst invasive alien species” (Global Invasive Species Database, 2020).

The feeding habit of the coypu is almost entirely herbivorous (LeBlanc, 1994; Carter and Leonard, 2002). Therefore, it has been a concern that indigenous plant communities including endangered and threatened species would be destroyed in regions where coypus were introduced and are now feral (Carter and Leonard, 2002; Prigioni *et al.*, 2005). There have been many studies on aquatic vegetation as the primary food source of coypus (Guichón *et al.*, 2003; Prigioni *et al.*, 2005; Colares *et al.*, 2010), and damage to crops has also been a concern (LeBlanc, 1994; Carter and Leonard, 2002; Sone *et al.*, 2006; Panzacchi *et al.*, 2007; Egusa and Sakata, 2009; Hong *et al.*, 2015).

In Japan, the coypu has been strongly suspected to prey on freshwater unionid mussels, which is an endangered taxon across Japan (Negishi *et al.*, 2008b). Until now, there have only been brief descriptions from other countries, stating that coypu predation on mussels is an occasional and opportunistic behavior (Gosling and Baker, 1991; LeBlanc, 1994). In western Japan, some snapshots of coypu predation on mussels were taken in a pond at Okayama Prefecture in 1999

(Mori, 2002) and in the Yodo River in 2014 (Ishida *et al.*, 2015). Middens, which are a pile of dead mussel shells suspected to be caused by coypu predation, were also found there (Mori, 2002; Ishida *et al.*, 2015) and in the Kiso River, central Japan (Kume *et al.*, 2012). Although these papers reported the characteristics of mussel shells (e.g., shell length and species) from middens as a diet habit of coypu, it was not sufficiently confirmed whether the middens were a result of coypu predation and whether coypu predation was common.

Unionid mussels play various functional roles in stagnant and running water ecosystems (Vaughn and Hakenkamp, 2001; Vaughn, 2018). Therefore, if coypu predation is intensive on mussels, populations of aquatic organisms other than mussels can be also influenced. In addition, coypu predation can further aggravate the deterioration of mussel populations already damaged by habitat degradation. Subsequently, bitterling and *Sarcocheilichthys* fishes can also be threatened because they require unionid mussels for reproduction (Kitamura, 2011; Kitamura and Uchiyama, 2020). The first purpose of this study is to elucidate whether exotic coypu predation on unionid mussels occurs in the Kiso River, Japan. We will examine the possible effects on mussel population structures if coypu predation on the mussels is confirmed. The second purpose is to examine the seasonal changes in the coypu diet in the Kiso River. Terrestrial plants and unionid mussels were the focus of the dietary analysis using fecal DNA metabarcoding.

## 2 Materials and methods

### 2.1 Study site

The study was conducted in a lowland segment of the Kiso River in central Japan (drainage area of 5275 km<sup>2</sup>), located in the temperate zone (Fig. 1). Based on the data from the nearest weather station over the last decade: mean daily air temperature was 15.7°C and summer maximum and winter minimum daily air temperatures were 38.6°C in August 2013 and −7.4°C in February 2012, respectively. Usually, it snows for a few days every year and seldomly accumulates. The riverbed slope of the study segment is approximately 0.02%, and the calculated flow rate from the data over the last decade ranged from approximately 80 m<sup>3</sup> s<sup>−1</sup> of the mean base flow to approximately 4600 m<sup>3</sup> s<sup>−1</sup> of the mean annual maximum (annual maximum during the last decade: 11,054 m<sup>3</sup> s<sup>−1</sup>).



**Fig. 2.** Photographic evidence of coypu predation on mussels (*Nodularia nipponensis*) in a floodplain waterbody (FWB 2) of the Kiso River, central Japan. These photographs were taken at the same position using the same camera trap. Left: coypu preying on a mussel. Right: dead mussel shell eaten by the coypu the previous night.

Relatively high flows were observed in early summer from June to July (the Baiu season), and in early autumn from September to October (the typhoon season); while from November to May, flows were relatively low and stable (Negishi *et al.*, 2012b). Levees were constructed along both sides of the study site, but floodplains and numerous floodplain waterbodies (FWBs) are present in the inter-levee zone. All FWBs become inundated, allowing them to connect to the main channel during floods  $>3200 \text{ m}^3 \text{ s}^{-1}$  (Negishi *et al.*, 2012a).

Until the 1970s, most of the inter-levee floodplain comprised sand bars; however terrestrialization followed by tree establishment has rapidly progressed since the 1980s, resulting in most of the floodplain being covered by trees (Negishi *et al.*, 2008a; Nagayama *et al.*, 2015, 2017). FWBs became small but more abundant from the terrestrialization of the inter-levee floodplains, increasing the number of FWBs isolated from the main channel (Nagayama *et al.*, 2015).

We selected four FWBs for this study, where there were frequent observations of coypus and their trails. Middens were also frequently found in these FWBs, which were suspected to be littered by coypus. Photographs of coypu preying on a mussel were first taken in one of the FWBs on April 11, 2017 (Fig. 2). The muskrat (*Ondatra zibethicus*), a closely related rodent species that commonly preys on mussels (Hanson *et al.*, 1989; Neves and Odom, 1989; Owen *et al.*, 2011), was absent in the study site. In these FWBs, three mussel taxa were present (*Nodularia nipponensis*, *Lanceolaria oxyrhyncha*, and *Cristarini* spp.; Lopes-Lima *et al.*, 2020) and aquatic plants were scarce, but terrestrial plants were abundant.

## 2.2 Collection of coypu feces

Coypu feces were collected from the four FWBs during December 2017 and February, May, and August 2018 to examine their diet using DNA metabarcoding. Two fecal samples that were recently deposited on the ground along the shore were collected at each sampling period from each FWB (32 feces in total). Collected feces were taken to a laboratory in cold storage, frozen on the same day, and stored for subsequent DNA analysis.

## 2.3 Fecal DNA metabarcoding

Each sample of coypu feces was freeze-dried using VD-250R Freeze Dryer (TAITEC, Saitama, Japan) and was crushed

via a Shake Master Neo (BMS, Tokyo, Japan). The DNA was extracted from the crushed feces using the NucleoSpin Plant II Midi (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's protocol. The extracted DNA samples were purified using AMPure XP (BECKMAN COULTER). The final volume of the extracted DNA samples was  $100 \mu\text{L}$ , and the DNA samples were stored at  $-20^\circ\text{C}$  until PCR assay.

Amplicon libraries of *rbcL* and 18S rRNA genes were obtained via PCR amplification using universal primers for terrestrial plants (*rbcL*-F and -R) and bivalves (*gClam*F and R), respectively (Tab. 1). The first PCR was performed using each universal primer pair for terrestrial plants and bivalves. The total volume of the reaction sample was  $10 \mu\text{L}$ :  $1.0 \mu\text{L}$  of 10xEx buffer,  $0.8 \mu\text{L}$  dNTPs,  $0.5 \mu\text{M}$  of each primer,  $2.0 \mu\text{L}$  DNA template,  $0.1 \mu\text{L}$  Ex Taq HS (TaKaRa, Bio Inc.), and DDW. Four replicates of PCR amplification were performed for each sample. The thermal cycle profile for terrestrial plants was  $94^\circ\text{C}$  for 2 min; 30 cycles of  $94^\circ\text{C}$  for 30 s,  $50^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 30 s; and  $72^\circ\text{C}$  for 5 min. For bivalves, it was  $95^\circ\text{C}$  for 3 min; 35 cycles of  $98^\circ\text{C}$  for 20 s,  $65^\circ\text{C}$  for 15 s, and  $72^\circ\text{C}$  for 20 s; and  $72^\circ\text{C}$  for 5 min. For each sample, the first PCR products were mixed for each sample and purified using AMPure XP to obtain a DNA template for the second PCR. The second PCR used 2nd-F and R primers (Tab. 1), including the index to identify samples and adapters for hybridizing on the surface of the Illumina flowcell for the MiSeq sequences. The total reaction volume of the second PCR was  $10 \mu\text{L}$ :  $1.0 \mu\text{L}$  of 10xEx buffer,  $0.8 \mu\text{L}$  dNTPs,  $0.5 \mu\text{M}$  of each primer,  $2.0 \mu\text{L}$  DNA template,  $0.1 \mu\text{L}$  ExTaq HS (TaKaRa, Bio Inc.), and DDW. The thermal cycle profile for the second PCR was  $94^\circ\text{C}$  for 2 min; 10 cycles of  $94^\circ\text{C}$  for 30 s,  $60^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 30 s; and  $72^\circ\text{C}$  for 5 min, for both terrestrial plants and bivalves. The second PCR products were then purified using AMPure XP. The negative control (DDW) was inserted for each step. All the indexed PCR products were pooled in equal volumes. Finally, the pooled library was purified via agarose gel electrophoresis. The purified library was then sequenced ( $2 \times 300\text{bp}$  paired-end) using an MiSeq v3 Reagent kit (Illumina, San Diego, CA, USA).

All data preprocessing and analysis of MiSeq raw reads were performed using the following steps. The FASTX-Toolkit (version 0.0.14; [http://hannonlab.cshl.edu/fastx\\_toolkit/download.html](http://hannonlab.cshl.edu/fastx_toolkit/download.html)) was used to filter out low-quality reads; reads corresponding perfectly to the adapter sequence of the primers were selected, and then primer sequences of the reads were removed using the *fastq\_barcode\_splitter* command. Subsequently, reads with  $<20$  quality values were discarded using sickle tools, and short reads with  $\leq 40$  bp were also discarded with their pairs. The reads remaining after filtering were then assembled using FLASH when read pairs overlapped by  $\geq 10$  bp.

The pipeline program USEARCH (version 11.0.667; <http://www.drive5.com/usearch/download.html>) was used to obtain operational taxonomic units (OTUs), and taxa were assigned using the basic local alignment search tool (BLAST; Edgar, 2010). For each OTU, the top-10-hit taxa with  $\geq 97\%$  sequence similarity were listed, and then a taxon was assigned based on vegetation data obtained from the inter-levee floodplain of the studied river section (River environmental database released by the Ministry of Land, Infrastructure,

**Table 1.** Universal primers for terrestrial plants (rbcL) and bivalves (gClam) in the first and second PCRs.

Primer	Sequence (5' → 3')
1st-rbcL-F	ACACTCTTCCCTACACGACGCTCTCCGATCTAGTCTTGATCGTTACAAAGG
1st-rbcL-R	GTGACTGGAGTTTCAGACGTGTGCTCTTCCGATCTGAAAATCAAGTCCACCRCG
1st-gClamF	ACACTCTTCCCTACACGACGCTCTCCGATCTNNNNNNATGCCTACCATGGTKRTAAC
1st-gClamR	GTGACTGGAGTTTCAGACGTGTGCTCTTCCGATCTNNNNNNCCCGARRCACTCARTCAAG
2ndF	AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTCCCTACACGACGC
2ndR	CAAGCAGAAGACGGCATAACGAGAT-Index1-GTGACTGGAGTTTCAGACGTGTG

**Table 2.** Index numbers of unionid mussel reads (Unionidae spp.) for each feces in each floodplain waterbody (FWB) based on fecal DNA metabarcoding for bivalves. 0: <100 reads, 1: 100 to <1000 reads, 2: 1000 to <10,000 reads, 3: ≥10,000 reads.

FWB	Feces	Dec-2017	Feb-2018	May-2018	Aug-2018
FWB 1	Feces-1	0	0	0	0
	Feces-2	0	0	0	0
FWB 2	Feces-3	0	0	0	0
	Feces-4	0	0	0	0
FWB 3	Feces-5	0	0	0	3
	Feces-6	0	0	2	2
FWB 4	Feces-7	0	3	3	2
	Feces-8	0	0	3	0

Transport and Tourism in Japan: <http://www.nilim.go.jp/lab/fbg/ksnkankyo/>). We were able to assign the family name of the freshwater mussel in this analysis, which was Unionidae including all mussel species observed in the studied river section. DNA sequences recovered from fecal metabarcoding can provide semi-quantitative information on diet composition because of the different digestibility for each prey species (Deagle *et al.*, 2010). Therefore, an index number was given to each taxon based on the number of reads (0; <100 reads, 1; 100 to <1000 reads, 2; 1000 to <10,000 reads, 3; ≥10,000 reads) as a criterion to estimate the abundance of prey species/taxa.

#### 2.4 Survey of coypu middens and live mussels

Dead mussel shells in middens were collected monthly in one of the FWBs (FWB 1 shown in Tab. 2) during 2011 (survey dates: February 11, March 27, April 18, May 21, June 11, July 29). These data were obtained from a series of surveys by Kume *et al.* (2012), where they only used the data from February. Because coypu predation on mussels was detected in this study (see Sect. 3), these middens were considered to be derived from coypu predation. In addition, all middens were found in shallow area or on the ground along the shoreline of the FWB, where live mussels cannot be present (Fig. 3). Almost all dead mussel shells sampled were found in pairs (Fig. 3). These strongly indicated that dead shells found in middens were attributed to coypu predation.

Two investigators waded along the shore of the waterbodies and collected dead mussel shells when they found coypu middens (consisting of more than 4 paired shells). The collected dead shells were totaled for each taxonomic identity and shell length was measured. When we found one shell (valve), we searched a partner shell to avoid double counting



**Fig. 3.** Photographs of middens deposited in nearshore area. An arrow on the left photograph indicates a midden. The right photograph shows a large midden found in our midden survey.

and measuring. Dead shells were removed from the study site during every survey date. Therefore, the collected dead shells were newly deposited shells following the previous survey date. The number of dead shells collected on February 11 was considered as a cumulative collection over a certain period before the beginning of the study.

Data of live mussels obtained in the FWB 1 on May 25, 2007 (Negishi *et al.*, 2012a) and May 28, 2018 (this study) were used to examine the possible effects of coypu predation on the population structures of live mussels. The method for the collection in 2018 followed that of the 2007 survey by Negishi *et al.* (2012a). The actual time spent for searching mussels in 2007 and 2018 was equal (115 min). Mussels were searched for bare-hands by personnel with dry suits in a belt transect (2–3 m wide) laid out along the longest axis of the waterbody. The survey progressed from one end to the other of a belt transect. When mussels were found, supporting personnel recorded taxonomic identities, measured shell length, and then released the mussels at the point of collection.

**Table 3.** Mean index numbers (N = 8) from reads of each taxon, based on the fecal DNA metabarcoding for terrestrial plants. Taxa with a total index number >0.5 from the four survey periods are shown as primary foods.

Family	Species/taxon	Dec-2017	Feb-2018	May-2018	Aug-2018
Amaranthaceae	<i>Achyranthes</i> sp.				1.50
Apiaceae	<i>Oenanthe javanica</i>			0.63	
Asteraceae	<i>Ambrosia artemisiifolia</i>				0.63
Asteraceae	<i>Astereae</i> sp.	1.25	0.88	1.50	
Cannabaceae	<i>Humulus japonicus</i>				0.75
Commelinaceae	<i>Murdannia keisak</i>				0.75
Cyperaceae	<i>Carex</i> spp.	2.25	2.25	1.13	0.50
Fabaceae	<i>Glycine soja</i>			1.13	1.13
Fabaceae	<i>Pueraria montana</i>			0.63	
Poaceae	<i>Andropogon virginicus</i>				0.63
Poaceae	<i>Elymus</i> spp.	1.50	2.00		
Poaceae	<i>Poa</i> sp.		1.88		
Polygonaceae	<i>Polygonum lapathifolium</i>			0.63	1.38
Polygonaceae	<i>Persicaria thunbergii</i>				2.00
Rosacea	<i>Potentilla indica</i>	0.63	0.75		
Rosacea	<i>Rosa multiflora</i>	2.25	1.63	0.88	0.63
Rosacea	<i>Potentilla</i> sp.	0.75			

The Kolmogorov-Smirnov test was performed to examine the difference between shell length distributions of live mussels between the 2007 and 2018.

### 3 Results

#### 3.1 Diet composition

Unionid mussels (Unionidae spp.) and 17 terrestrial plant taxa were detected as primary food by PCR amplification of coypu feces (Tabs. 2 and 3). Unionid mussels were detected from 7 feces: 1 in FWB 4 in February (the coldest winter), 3 in FWB 3 and 4 in May (spring), and 3 in FWB 3 and 4 in August (the warmest summer) with relatively higher index numbers (read numbers) (Tab. 2). Although mussels were not detected from the fecal samples of FWB 2 (Tab. 2), photographs of a coypu preying on mussel were taken in April, 2017 (Fig. 2). Mussels were also not detected in FWB 1, where the midden survey (2011) was conducted (see below).

The highest taxon number of terrestrial plants detected from feces was 10 in August, followed by 7 taxa in May and 6 taxa in December and February (Tab. 3). Voluble *Rosa multiflora* and perennial *Carex* spp. were detected in all sampling periods, with relatively higher index numbers in December and February (cold seasons) (Tab. 3). Some perennial plants such as *Elymus* spp., *Poa* sp., and *Potentilla indica* were only detected in the cold seasons, whereas some other perennial plants such as *Achyranthes* sp., *Andropogon virginicus*, *Oenanthe javanica*, and *Pueraria montana* were only detected in the warm seasons (May and August) (Tab. 3). Most of the other plants were annual plants that were only detected during warm seasons (Tab. 3).

#### 3.2 Size structures of live and dead mussels

Eight-to-fourteen middens were found in each of the midden survey dates in 2011, and a total of 918 dead mussel

shells were found: 513 *L. oxyrhyncha*, 384 *N. nipponensis*, and 21 *Cristariini* spp. From live mussel surveys, 134 (26 *L. oxyrhyncha*, 96 *N. nipponensis*, and 12 *Cristariini* spp.) and 87 (54 *L. oxyrhyncha*, 28 *N. nipponensis*, and 5 *Cristariini* spp.) mussels were captured in 2007 and in 2018, respectively.

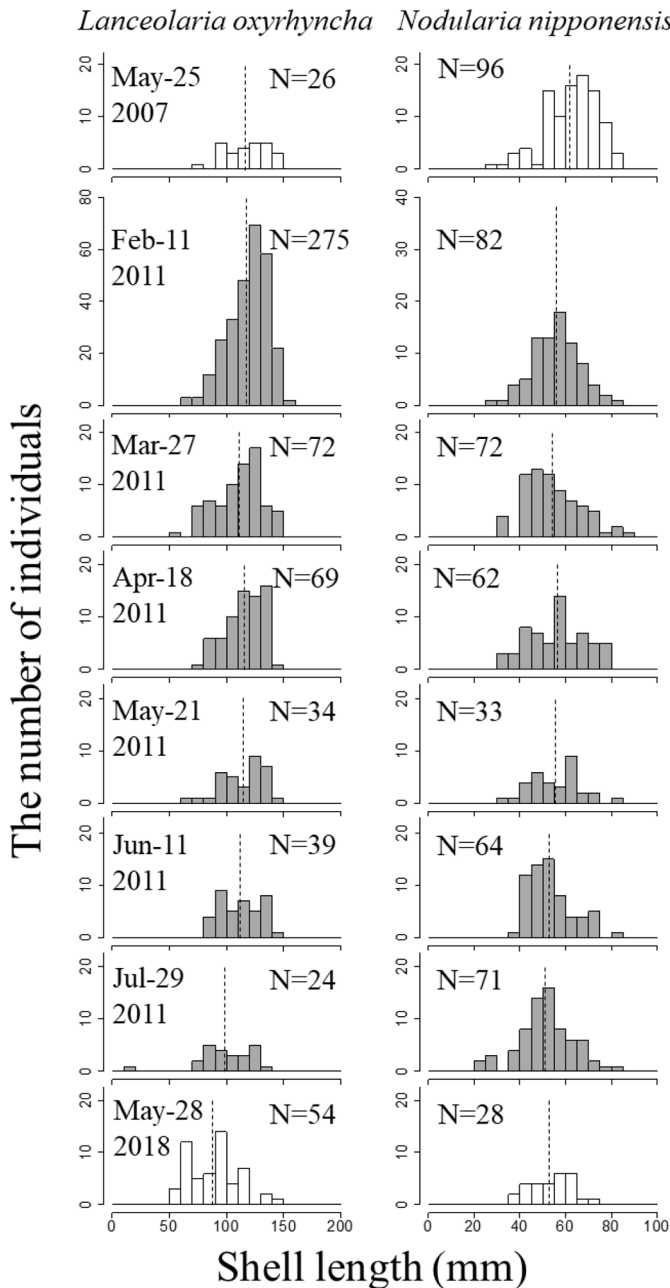
Size structures of the two dominant live mussel taxa were statistically different between 2007 and 2018 (*L. oxyrhyncha*:  $p < 0.001$ , *N. nipponensis*:  $p = 0.001$ ) (Fig. 4). Mean shell lengths of *L. oxyrhyncha* and *N. nipponensis* decreased from 116.7 mm in 2007 to 88.8 mm in 2018, and from 61.9 mm in 2007 to 53.6 mm in 2018, respectively. The proportion of <100 mm *L. oxyrhyncha* increased from 0.27 in 2007 to 0.74 in 2018. Additionally, the proportion of <60 mm *N. nipponensis* also increased from 0.36 in 2007 to 0.71 in 2018 (Fig. 4).

Mean shell length of dead *L. oxyrhyncha* was 110.6–118.7 mm from February to June 2011, and finally decreased to 99.0 mm in July 2011 (Fig. 4). The proportion of ≥100 mm shells of dead *L. oxyrhyncha* decreased over time (0.84 in February, 0.72 in March, 0.81 in April, 0.74 in May, 0.67 in June, and 0.50 in July). The number of shells of dead *L. oxyrhyncha* also decreased over time during the midden survey year. Mean shell length of dead *N. nipponensis* was similar during the midden survey year (51.3–56.1 mm). Overall, the shell length of *N. nipponensis* revealed a hump-shaped distribution with a peak of medium ranges. There was no evidence for the high proportion of large shells (≥60 mm) of dead *N. nipponensis* (0.22–0.35), and for the decrease in the number of shells of dead mussels during the midden survey year. However, large shells of dead mussels were constantly found throughout the midden survey year.

### 4 Discussion

#### 4.1 Coypu predation on mussels

The coypu has been well known as an herbivorous rodent in its native range (Colares *et al.*, 2010) and some invaded



**Fig. 4.** Size structures of live mussels (open bar) in 2007 and 2018, and dead mussel shells (dark gray bar) during the midden survey of 2011. Dashed lines denote mean shell length of the species in each survey date.

regions (Abbas, 1991; Prigioni *et al.*, 2005). However, the results of fecal DNA metabarcoding revealed that invasive coypu in the Kiso River preyed on freshwater unionid mussels from the coldest winter to the hottest summer. Until now, the predation of mussels by coypu has been considered as an occasional and opportunistic behavior (Gosling and Baker, 1991; LeBlanc, 1994), and thus, there has not been any report on this predation event with scientific verification. In Japan, there have been some previous reports of coypu preying on mussels in central and western Honshu (Mori, 2002;

Kume *et al.*, 2012; Ishida *et al.*, 2015). However, these reports only investigated dead mussel shells in middens, presumed to be littered by coypu. This study is the first report that verifies common, non-occasional predation of native unionid mussels by coypu in an invaded site.

Diet shifting, where herbivores begin to prey on animals in invaded sites, has also been observed in other rodent species such as the black rat (*Rattus rattus*) (Kawakami *et al.*, 2010; Shiels *et al.*, 2014). This rat preyed on seabirds, insects, frogs, snakes, and plants in invaded islands (Yabe *et al.*, 2009; Shiels *et al.*, 2014). Such a diet shift of rodent species from herbivory to omnivory may be induced by the changes in the environment they inhabit. Coypu predation on mussels may not be that surprising when considering the common muskrat's predation on freshwater mussels and plants (Haag, 2012). In the Czech Republic, coypu predation is considered as one of the main factors of recent decline of mussel populations (Beran, 2019). We should recognize the predation risks of coypu on freshwater mussels to fully understand the damage to ecosystems in invaded regions, including not only Japan but also North America and Europe.

Coypu preying on mussels might be triggered by the lack of food items during winter and might subsequently continue through other seasons and beyond generations. The lower taxon number of terrestrial plants in winter (6 taxa) identified as primary food by fecal DNA metabarcoding, is likely attributed to the absence of annual plant species in winter. Some reports indicated that plant roots and rhizomes are the primary food diet of coypu during winter (Abbas, 1991; Gosling and Baker, 1991). Compared to the amount of effort necessary to dig into the ground, mussels are likely to be easily available prey for coypu. Moreover, it was reported that muskrat predation on mussels increased during winter when plant material was scarce (reviewed in Zahner-Meike and Hanson, 2001). These imply that coypu likely learned to prey on mussels in winter owing to necessity.

In this study, however, winter predation on mussels by coypu was detected from only one fecal sample in February, but with a high index number. In general, food items detected from feces reflect the diet from within a few days (Deagle *et al.*, 2010). Therefore, our results of fecal DNA metabarcoding might underestimate the degree of coypu predation on mussels during winter. Whenever we visited the study FWBs, coypu trails and middens were always observed. Further studies with more sampling effort will be necessary to elucidate coypu predation on mussels throughout the year.

Short-term but intensive predation on newly established mussel populations by the muskrat was observed in a lake in western New York State, USA (Diggins and Stewart, 2000). Similarly, the intensive predation on a mussel population by invaded coypu was also reported in a pond in Okayama Prefecture, western Japan (Mori, 2002). These studies imply that intensive coypu predation on mussels began suddenly when both populations encountered one another. A large number of mussels were consumed in 2011 in the FWB 1 site (see also Kume *et al.*, 2012). However, the DNA metabarcoding did not detect mussels from coypu feces in 2018, although coypu middens were still observed in the FWB 1. This may indicate that intensive predation on mussels by coypu discontinued in the FWB 1; likely owing to the decline in large mussel availability caused by previous intensive

predation by coypu. When the availability of mussels becomes high, intensive predation may restart in the FWB 1.

#### 4.2 Impact of coypu predation on unionid mussels

The results of midden surveys showed that large-sized *L. oxyrhyncha* and medium-sized *N. nipponensis* tended to be consumed by coypus, likely resulting in an alteration of mussel population structures. Such trends are well known as a feeding strategy of muskrats, which promises high energy benefits, and thereby heavily alters mussel population structures (Hanson *et al.*, 1989; Neves and Odom, 1989; Diggins and Stewart, 2000; Owen *et al.*, 2011). We also observed in our study that the size structures of both mussel species significantly changed from 2007 to 2018, with reductions in mean shell lengths and the proportions of large-sized individuals.

If size-selective predation by coypu occurs commonly, this might cause a deterioration in population size of mussels through the decrease in mature adult mussels. Mature mussel density is an important factor for fecundity rate (Downing *et al.*, 1993; McLain and Ross, 2005). Decrease in their abundance could affect reproductive success, followed by diminishing mussel population size. Possibly, coypu predation does not necessarily cause extirpation of mussel populations because predation pressure gradually decreases when mussel density becomes low, as observed in *L. oxyrhyncha* in our study. However, as long as coypu predation continues, the recovery of the mussel population could be inhibited.

Coypu predation on unionid mussels could indirectly influence bitterling fish populations. In the study site (Kiso River) for example, the deepbody bitterling (also known as the Itasenpara bitterling; *Acheilognathus longipinnis*), which is a national monument and national endangered species of wild fauna in Japan, is present. *A. longipinnis* deposits eggs inside the branchial cavity of host unionid mussels in autumn, and hatched larvae spend time there until the next spring when they emerge from the host mussels (Uehara, 2007; Nishio *et al.*, 2015; Kitamura and Uchiyama, 2020). Unionid mussels, *N. nipponensis* and *L. oxyrhyncha*, are available host mussels for *A. longipinnis* (Uehara, 2007; Kitamura *et al.*, 2009). In the Kiso River, the *A. longipinnis* population is already threatened by the altered river environment (Sagawa *et al.*, 2011; Nagayama *et al.*, 2017), and coypu preying on these mussels could further aggravate the status of *A. longipinnis* populations, and other bitterling populations in Japanese rivers.

#### 4.3 Seasonally flexible diet of coypu

The high seasonal variation in the coypu diet consisting of plant species has been reported in both native and invaded ranges (Abbas, 1991; Prigioni *et al.*, 2005; Colares *et al.*, 2010). These seasonally flexible diets were also detected from our fecal DNA metabarcoding analysis for terrestrial plants, with a greater richness in plant predation during warm periods. The coypu in our site preferred *Carex* spp. and *Rosa multiflora*, which were consumed throughout the year. Some perennial plants were only consumed in warm seasons, likely because their flowers and leaves might be preferred. Because perennial *Elymus* spp. and *Potentilla indica* were consumed only in winter, they might be involuntary food during periods when

annual plants are absent. Annual plants that were consumed as primary foods might be seasonally favorable foods.

Coypu primarily consumed aquatic plants in both native and invaded sites (Guichón *et al.*, 2003; Prigioni *et al.*, 2005; Colares *et al.*, 2010). In this study, aquatic plants were not targeted in the fecal DNA metabarcoding analysis. However, our results generally represent the diet of coypu in this study site. Coypus consume terrestrial plants when aquatic plants are scarce (Guichón *et al.*, 2003; Prigioni *et al.*, 2005). For example, terrestrial plants were sufficiently consumed by coypus inhabiting agricultural lands, where aquatic plants were scarcer (Abbas, 1991). The FWBs in our study were present in terrestrialized floodplains (see Sect. 2.1). Trees surrounded the FWBs, the aquatic-terrestrial transition zone with wet conditions was very narrow, and overall water transparency was low. Therefore, aquatic plants were scarce and could not be used as primary food by coypu in our study site.

## 5 Conclusion

The present study demonstrated that feral coypus prey on freshwater unionid mussels in Japan. In our study site, the exotic coypu can inhibit the increase in mussel populations. It is possible that several small mussel populations have been seriously threatened by feral coypus at other local sites in Japan. Unionid mussels play various functional roles in freshwater ecosystems (Vaughn and Hakenkamp, 2001; Vaughn, 2018). Therefore, the impacts on freshwater ecosystems by exotic coypu may be higher and broader than expected in Japan and possibly also in other countries where coypus are invasive. The common feeding on various terrestrial plants observed in this study further amplifies this concern. The black rat that invaded oceanic islands shifted their diet from herbivory to omnivory, with many animal species as a part of their common diet (Yabe *et al.*, 2009; Shiels *et al.*, 2014). Our results imply that the exotic coypu may also shift its diet and consume animals other than mussels, such as crustaceans and amphibians. Future studies should exhaustively examine the diet of exotic coypu to better understand its impacts on communities and ecosystems.

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