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Parasitic development in intestines and oocyst shedding patterns for infection by *Eimeria uekii* and *Eimeria raichoi* in Japanese rock ptarmigans, *Lagopus muta japonica*, protected by cages in the Southern Japanese Alps

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ABSTRACT

The population of Japanese rock ptarmigan (*Lagopus muta japonica*), an endangered species with a habitat above the timberline of the southern Japanese Alps, has declined. As one of the recent conservation strategies for this species, cage protection for broods (hens and chicks) has been introduced in their habitats. Two species of *Eimeria* have frequently been detected in these birds, but little is known about the parasitic circulation in the region, including among birds and in the environment. Here, we conducted histopathology examinations of dead chicks collected under cage protection in 2018, and examined the feces of the hens and chicks of three broods and environmental soils for parasites in 2019 in order to assess the potential sources of infection and pathogenicity. Developmental zoites were found in the epithelial mucosa and/or the submucosa from the duodenum to the colon of all dead chicks. Fecal examination revealed oocysts of *E. uekii* and/or *E. raichoi* in all hens and chicks. Oocysts of *Eimeria* spp. per gram of feces in chicks increased within 2 weeks after hatching and then gradually deceased. Following infection of the chicks, oocysts could accumulate within the cage areas, and oocyst density exceeded more than 1000 oocysts per gram of cage soils. Based on having sporulated morphologies, oocysts could be infective and therefore, be direct or indirect potential sources of infection. However, based on our findings that not all chicks were clinically affected by the infections, other factors such as microbial flora in the chicks established by coprophagy or from the habitat environment, including climate, might be associated with the pathogenicity of *Eimeria* spp., although further studies are needed to assess these correlations.

1. Introduction

Japanese rock ptarmigan, *Lagopus muta japonica*, is one of 23–30 subspecies in *Lagopus muta*, that belong to the order Galliformes, family Tetraonidae (Johnsgard, 1983; del Hoyo et al., 1994). All subspecies of *Lagopus muta* are cold-adapted and live in the arctic tundra or in alpine areas of the northern hemisphere. Japanese rock ptarmigans inhabit only alpine areas of the main island of Japan, which is the southernmost habitat area and isolated from the habitats of other subspecies. Because the number of birds declined from approximately 3000 in the 1980s to about 1700 in the 2000s (Norton and Chard, 1983), the birds were recognized as an endangered species in Japan. Therefore, ecological

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conservation strategies, including national conservation programs, have been implemented (Ministry of the Environment, 2012).

Since protozoan infection of *Eimeria* sp. was reported in 1981 (Kamimura and Kodama, 1981), two species of *Eimeria*, *E. uekii* and *E. raichoi*, have been widely and frequently detected in Japanese rock ptarmigans (Ishihara et al., 2006; Matsubayashi et al., 2018a, 2018b). In general, *Eimeria* spp. are protozoan parasites that are detected in a huge range of vertebrates, such as birds, mammals, and reptiles but they are highly host specific. They parasitize mainly the intestinal mucosa of hosts and are known to cause watery or/and bloody diarrhea as coccidiosis. Thus, the parasites produce a high economic impact, especially in livestock (Shirley, 1995; Shirley et al., 2005) although infection conditions of wild animals remain largely unknown. Notably, younger animals have higher sensitivity to infection and tend to show more severe symptoms, including death (Daugschies and Najdrowski, 2005; Williams et al., 2009). The lifecycle of *Eimeria* spp. is initiated with the ingestion of the mature oocysts through fecal-oral routes and it shows two stages in the host: formation of schizonts in asexual development and of micro- and macro-gametes in sexual development. After the sexual stage, formed oocysts are shed with feces and sporulated (forming four sporocysts each containing two sporozoites) at the appropriate temperature such as 27–28 °C, resulting in acquired infectivity and survival for several months (Waldenstedt et al., 2001; Pyziel and Demiaszkiewicz, 2015). Unlike most *Eimeria* spp., the eimerian parasites of Japanese rock ptarmigan show specific characteristics, e.g., oocysts can mature at lower temperatures at around 10 °C and survive for longer periods (Matsubayashi et al., 2018a). However, detailed cycles between Japanese rock ptarmigans of parents or chicks and the environment, and pathogenicity of parasites, including their impacts on conservation of the birds have not been clarified.

As one of the conservation strategies for Japanese rock ptarmigans, cage protection programs were conducted starting in 2015 in the southern Japanese Alps (Kobayashi et al., 2019). The hens and chicks were kept in cages and were allowed to graze freely outside the cages at several times during the daytime. Cage protection was effective for protecting chicks from predation by predators and from severe climate conditions in the alpine areas. However, the possibility that hens excrete feces containing oocysts of *Eimeria* spp., and therefore, the risk of being exposed to eimerian oocysts in the environments is thought to be increased within the confined areas of the cages. In another report, a few chicks that died after showing weakness during cage protection were histopathologically confirmed to have been infected with *Eimeria* spp. (Matsubayashi et al., 2018a), although detailed analyses could not be done. In the present study, we examined *Eimeria* spp. in the feces of hens and chicks, which were protected by cages, as well as in environmental soils, to assess the potential sources of infection, pathogenicity, and to discuss parasitic circulation for infection in the timber regions.

2. Materials and methods

2.1. Birds and fecal samples

From 29 June to August 4, 2018, 3 broods each with a hen (7 chicks in cage Nos. 1 and 2, and 6 chicks in cage No. 3) were protected in cages (about 1.8 m wide by 3.6 m long) on Mt. Kita (35°40′ N, 138°14′ E), which is within their habitat range in the Southern Japanese Alps in Yamanashi Prefecture, Japan. A total of 5 chicks (Chicks a-e), namely one from cage No. 1, two each from cage Nos. 2 and 3, were found dead during the brooding period. Two of the chicks (Chicks a and d) were killed by accidents and showed no clinical symptoms, and 3 chicks (Chicks b, c, and e) were found dead after showing signs of weakness. The chicks were held at −20 °C in a mountain lodge adjacent to the cages for 2–3 months until the chicks could be fixed in 10% neutral-buffered formalin (Nacalai Tesque, Kyoto, Japan) for a few weeks, necropsied at the laboratory, and have their organs sampled as described below.

Fig. 1. Shelter used for cage protection of Japanese rock ptarmigan broods on Mt. Kita (35°40′N, 138°14′E), Japan in 2019.

During the period from 3 July to August 5, 2019, 3 broods each consisting of 1 hen with chicks (7 chicks in cage No. 4, 6 chicks in cage No. 5, and 5 chicks in cage No. 6) were sheltered on Mt. Kita (Fig. 1). These cages were the same ones used for cage protections in 2018 and kept in the warehouse on Mt. Kita after being fold up while not using. They were not treated with any disinfectant before reuse. The locations for the cage protections were the same as those of the previous year. Broods were protected in the cages were permitted to graze freely outside during the daytime as previously reported (Kobayashi et al., 2019). Fresh rectal feces of hens (within approximately 1 h after being shed) were collected every 1–2 days until 7 days after the start of cage protection. A total of 6 fecal samples for cage No. 4, 3 samples for cage No. 5, and 3 samples for cage No. 6 were collected from hens. Feces of the chicks were sampled from 2- to 32-day-old chicks almost daily except for 3–5 days around the age of 20 days old (due to reasons unrelated to the study). A total of 27 fecal samples for cage No. 4, 25 samples for cage No. 5, and 20 samples for cage No. 6 were collected from chicks. Feces from chicks were collected from the brood and could not be individually identified. During cage protection, birds did not show any clinical symptoms such as diarrhea or weakness, except that two chicks were preyed upon by Japanese stoats (*Mustela erminea nippon*), and the chicks consumed the cecal feces of their mothers as a common coprophagy behavior.

2.2. Soil samples

Soil samples (40–80 g) inside of the cages were collected once or twice each month from July to September 2019 (total of 12 samples). Precisely, samples were collected from cage No. 1 at 12 h after introducing the brood and those from cage Nos. 2 and 3 were collected before introducing the broods in July. Soils were collected during cage protection when birds were outside of the cage in August, and collected at 1.5 months after releasing the broods in September. Additionally, the surface soils were also collected from nine places around the habitat range on Mt. Norikura (36°06′ N, 137°133’ E) from May to June 2019, which is one of their habitats in the Northern Japanese Alps in Gifu and Nagano Prefectures, Japan. Collected feces and soils were stored at 4 °C and examined in the laboratory as described below.

2.3. Histopathological examinations

The heart, liver, kidneys, stomach, duodenum, jejunum, ileum, cecum, and colon were removed from the chicks. The organs were fixed in 10% neutral-buffered formalin (Nacalai Tesque) for several days and processed for routine histological examinations. Tissue sections were stained with hematoxylin and eosin (HE). Stained histological sections were examined under a light microscope (400 ×). Histological scores

were determined based on the number of parasites (-, no parasites; +, a few parasites in intestinal mucosa in some fields; ++, 1–5 parasites in intestinal mucosa per field; +++, > 5 parasites per field).

2.4. Parasitic examinations

For the detection of parasites, the sugar flotation centrifuge method was conducted as described previously (Ekawasti et al., 2020). Briefly, 1 g of fecal sample was diluted in 9 ml of distilled water and centrifuged at 800 × *g* for 5 min after filtration by steel mesh or gauze. The supernatant was discarded, and 10–12 ml of sugar solution with a specific gravity of 1.2 was added to the sediment, followed by centrifugation at 800 × *g* for 5 min. Floated parasites were placed onto a glass slide using an inoculation loop. The entire smear was examined by light microscopy (200 × or 400 ×). Detected oocysts were morphologically identified as *E. uekii* or *E. raichoi* under a microscope (E200, Nikon, Tokyo, Japan) (Matsubayashi et al., 2018a). For estimating the oocysts per gram (OPG) of *Eimeria* spp., the remaining fecal samples were weighed and the oocysts were purified using the fecal samples (approximately 1–10 g) by the sugar flotation centrifuge method. In case of less than 1 g feces, the oocysts were prepared by filtration with steel mesh or gauze after diluted with distilled water. After the purification, 5 μl of 1–3 ml oocyst solution in distilled water was put on slide glass, covered with 9 mm × 9 mm cover glass, and OPG was calculated based on the number of oocysts at the entire smear. The soil samples (40–80 g) were stirred in 300–500 ml of 0.05% Tween 20 (Nacalai Tesque) for 30 min, filtrated through a steel mesh, and parasites were purified and examined by the same methods as described above.

3. Results

Histopathological examinations revealed that most organs showed signs of degradation after death. Although pathological findings were not conclusive, we summarized the extent of parasite presence for each of the examined chicks in Table 1. All of the chicks, including the 2 chicks that were killed by accidents, were infected with *Eimeria* spp. Stages of *Eimeria* spp. were observed in the intestines (from the duodenum to the colon). No parasites were identified in other organs. The stages before mature schizonts (named as trophozoites) (Fig. 2A) and schizonts were found mainly in epithelial cells, and some parasites were also found in the submucosa (Fig. 2B and C). After releasing oocysts, many sexual stages or cavities were observed in the intestinal mucosa (Fig. 2D and 2E), and some hemorrhages were observed in the intestinal mucosa (Fig. 2F). We could not identify the species of *Eimeria* based on developmental forms nor determine the cause of death due to the lack of pathologic findings such as necrosis or ulceration due to the degraded state of the organs.

Table 1
Parasite development scores by histopathological examination of dead chicks during cage protection in 2018.

Organs	Chick				
	a	b	c	d	e
Stomach	–	–	–	–	–
Duodenum	+++	–	+	+	–
Anterior part of jejunum	+++	–	+	+	+++
Posterior part of jejunum	+++	–	+++	++	++
Ileum	+++	+	+++	–	+
Ceca	+++	–	++/+++	+/++	++/+++
Colon	++	–	–	–	+
Heart	–	–	–	–	–
Liver	–	–	–	–	–
Kidneys	–	–	ND	–	–

ND; could not be examined.
-; no parasites, +; a few parasites in intestinal mucosa on some fields, ++; 1–5 parasites in intestinal mucosa per field, +++; > 5 parasites per field.

By fecal examination of hens, oocysts of *E. uekii* were detected in 5 of 6 samples in cage No. 4, 3 of 3 samples in cage No. 5, and 3 of 3 samples in cage No. 6. Those of *E. raichoi* were found in 1 of 6 samples in cage No. 4, 1 of 3 samples in cage No. 5, and 0 of 3 samples in cage No. 6. Among feces collected from chicks, *E. uekii* were positive in 25 samples of 27 samples in cage No. 4, 24 of 25 samples in cage No. 5, and 20 of 20 samples in cage No. 6. *E. raichoi* were found in 19 samples of 27 samples in cage No. 4, 19 of 25 samples in cage No. 5, and 16 of 20 samples in cage No. 6. The shedding patterns of the oocysts are summarized in Figs. 3 and 4. All hens caring for chicks were found to be infected with *Eimeria* spp., and the chicks began to excrete oocysts at 3 days old. OPG values of *E. uekii* in chicks (average of 4.4×10^5, 2.4×10^5, 2.7×10^5 in cage Nos. 4, 5, and 6, respectively) were higher than those of *E. raichoi* (average of 8.1×10^4, 1.7×10^4, 2.2×10^4 in cage Nos. 4, 5, and 6, respectively). OPG values increased within 2 weeks of hatching and then gradually decreased.

We found oocysts in all 12 soil samples collected from cages, and oocysts of *E. uekii* were detected in higher concentration and greater frequency (Table 2). OPG was more than 1000 in August but was relatively low in other months. In the other 9 soil samples of habitats at Mt. Norikura, only 2 samples were positive, and the OPG were 0.2 and 0.06. Oocysts detected from soil of cages had already sporulated and clearly formed sporocysts and sporozoites were observed (Fig. 5a). While oocysts were detected in other areas of Mt. Norikura, the oocysts were not mature (Fig. 5b).

4. Discussion

We examined Japanese rock ptarmigan chicks that died under cage protection, including some that showed emaciation. Histological examination showed that a large number of parasites were found in the intestinal epithelius and in the submucosa of some parts of the intestine, although we could not identify the parasites to the species level of genus *Eimeria* based on morphology. Based on examination of 5 chicks that died while in cage protection, our results suggest that development of zoites could severely damage the mucosa of intestines and disrupt the nutrient absorption along with causing diarrhea. Although little is known about infection in the wild, we speculate that infected chicks might not grow and survive due to inadequate foraging and are more susceptible to predation. Further analyses such as experimental infections are needed to clarify exact clinical symptoms by the infections of *Eimeria* spp.

Coprophagy in chicks of Japanese rock ptarmigans was previously confirmed to occur from 3 to 18 days after hatching (Kobayashi et al., 2019). Consumption of mothers' fresh cecal feces in herbivorous birds is thought to be crucial to the establishment of ceca bacteria as the symbiotic gut bacteria and results in effective degradation of anti-herbivore toxic secondary compounds of the wild plants in the environment (Kohl et al., 2016; Forbey et al., 2018; Grond and Sandercock, 2018). Alpine plants such as Ericaceae or Empetraceae are the main food sources for Japanese rock ptarmigans, and they produce defensive chemicals against herbivores. We confirmed in the present study that coprophagy by the chicks of the hen's cecal feces was immediate or within 1 h after being excreted in the most delayed cases. Generally, oocysts in freshly excreted feces are not infective as reported in poultry *Eimeria* spp. (Norton and Chard, 1983) and thus, coprophagy by chicks might not be the route of transmission although the sporulation time of *Eimeria* spp. infecting rock ptarmigans has not been well documented. Oocysts were first detected in the feces of 3-day-old chicks and increased at around 9–10 days old. The reduction in OPG after about 20 days old might be attributable to an acquired immune response against *Eimeria* spp.

Detection of mature oocysts before the introduction of broods (cage Nos. 5 and 6) indicates that oocysts can survive in the field for longer periods of time (perhaps more than half a year). Because oocysts

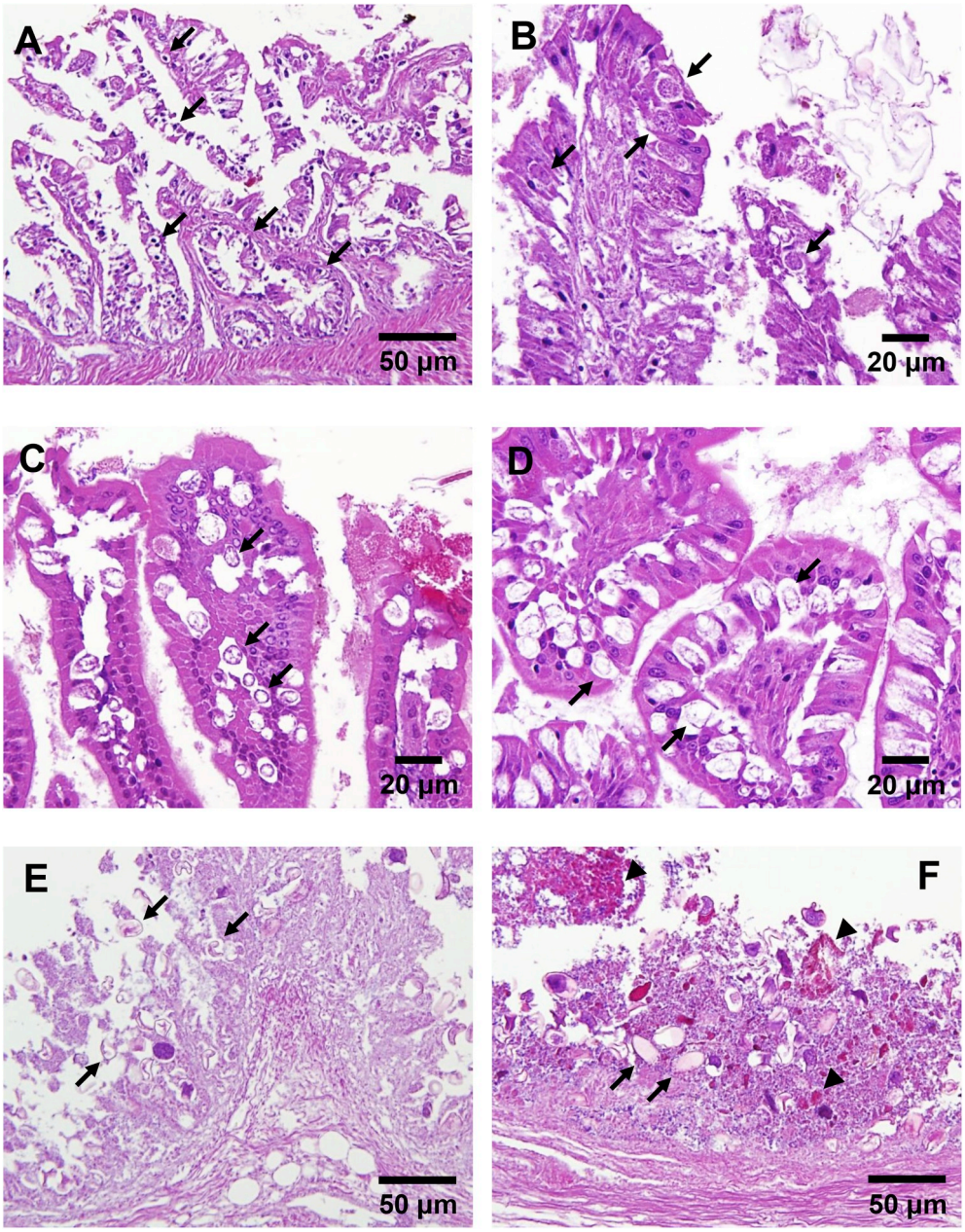

Fig. 2. Histopathological photomicrograph of a section of the intestines of dead chicks during cage protection in 2018. Figs. A and B show developmental trophozoites (arrows) and schizonts (arrows) of *Eimeria* spp. at the epithelial cells of the colon (Chick c) and ileum (Chick a), respectively. Some zoites (arrows) invade into submucosa (ileum of Chick b) (Fig. C). Figs. D, E, and F show the sexual zoites or cavities after releasing oocysts (arrows) of the ileum (Chick a), ceca (Chick d), and ileum (Chick c). Arrowheads in Fig. F indicate hemorrhages in the intestinal mucosa.

(reported as *Toxoplasma gondii*) can be dispersed within the soil column or to other matrices by wind, arthropods, or rain (Dumètre, A., Dardé, 2003), we could detected the oocysts of OPG value with less than 40 on July and August (the first corrections). However, at the second sampling on August, OPG values were appeared to be increased. Although we should examine more soils at many areas in the cages, it is though that oocysts can accumulate in the restricted cage areas due to feces shed from infected chicks as well as hens, and numerous oocysts were consequently found in the soils of cages. The oocysts might be considered to be infective based on their morphologies and, therefore, environments contaminated with oocysts could be sources of infections by direct or indirect routes (contamination of body surfaces). The cage protection can be effective for protecting chicks as described above, and however, the risk of the infection may increase in the limited areas. Although the pathogenicity of *Eimeria* spp. remains unresolved, rapid removal of the feces and changing soils in the cages might be helpful to reduce the infection during the cage protection. In other wild areas, oocysts were rarely encountered in the present study and, furthermore, were morphologically non-infective. The high prevalence of *Eimeria* spp. was previously reported in Japanese rock ptarmigans (adults and chicks) over broad geographic areas (Ishihara et al., 2006; Matsubayashi et al., 2018a, 2018b). One of the potential sources of *Eimeria* spp. in the wild may be environmental, including contaminated soils. However, more detailed analyses of the circulation of parasites in

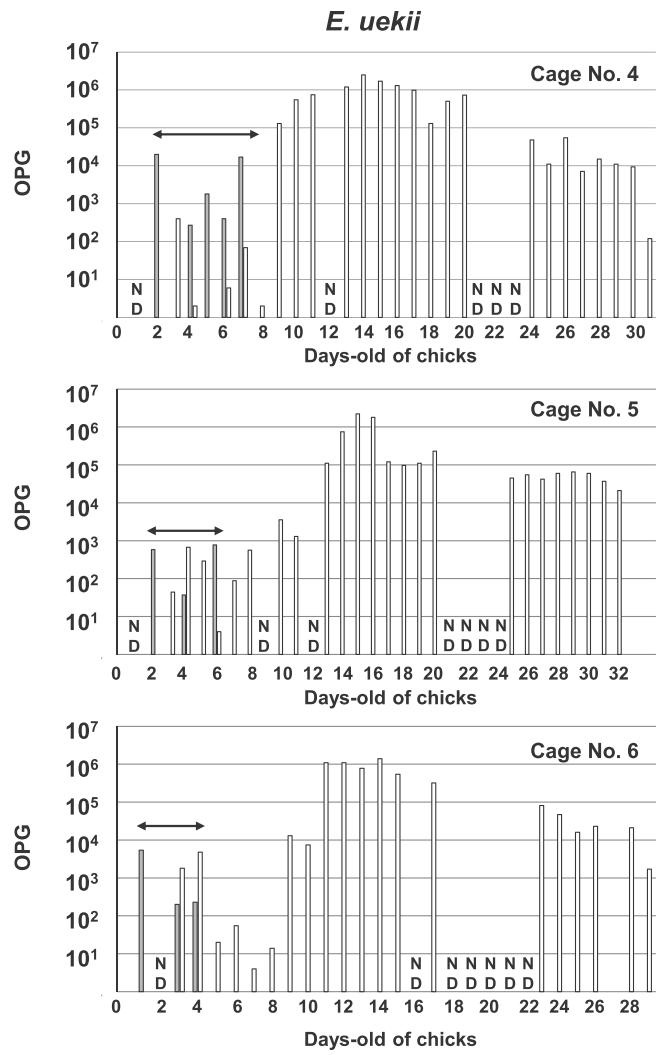

Fig. 3. Number of oocysts per gram (OPG) as seasonal detection rate for *E. uekii* of hens (solid bars) and chicks (open bars) of cage Nos. 4–6 in 2019. Double arrows indicate the periods when feces of hens were examined. ND indicates that we could not collect feces and did not determine the OPG.

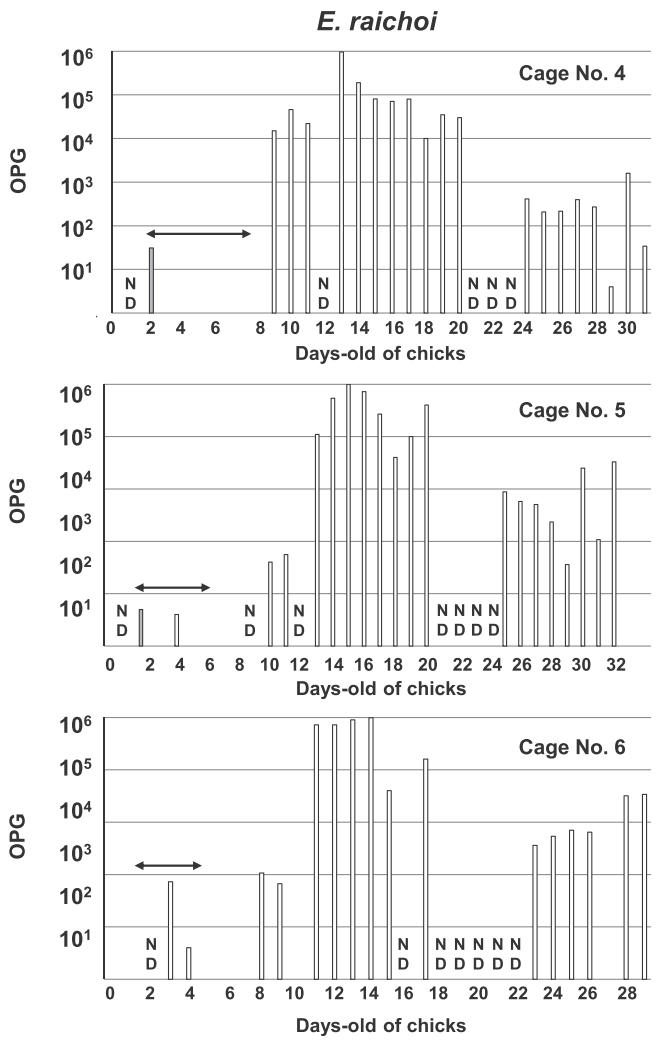

Fig. 4. Number of oocysts per gram (OPG) as seasonal detection rate for *E. raichoi* of hens (solid bars) and chicks (open bars) in cage Nos. 4–6 in 2019. Double arrows show the periods during which feces of hens were examined. ND indicates that we could not collect feces and did not determine the OPG.

the field must be conducted.

In contrast to the chick deaths observed in protective cages in 2018, we did not find any chicks in protective cages in 2019 showing clinical signs by infection or mortality. Previously, commensal microflora populations in the digestive tract were reported to modulate numerous physiological processes, including immune development and nutrition and metabolism, and pathogen exclusion (Macpherson and Harris, 2004; Bäckhed et al., 2005). Recently, it has been demonstrated that intestinal coccidial infections of *Eimeria* spp. in mice and chickens perturb the microbiota and that microbiome dysbiosis can be correlated with lesion severity of infections (Macdonald et al., 2017; Huang et al., 2018). Although the reasons remain unknown, successful establishment of the microbiome in chicks by coprophagy may reduce the pathogenicity of *Eimeria* spp. The number of oocysts present in the environment before acquiring immunity or habitat conditions such as temperature or weather might also be associated with the pathogenicity of *Eimeria* spp. Further studies are required to assess these correlations and contributions for the survival of Japanese rock ptarmigans in the timber regions.

Table 2
Number of oocysts per gram (OPG) detected from the soil of the cages in 2019.

Month (days after hatching)	Cage No.					
	4		5		6	
	E. ue	E. ra	E. ue	E. ra	E. ue	E. ra
July (0–3)	36.4	0	14.0	0	2.1	0
July (24–27)	ND	ND	ND	ND	0.2	0
August (30–32)	2.5	0	ND	ND	0.05	0
(31–33)	1393.6	0	1098.4	0	1600.0	0
September (84–87)	21.0	0	28.7	0	586.4	7.7

E. ue; *E. uekii*, E. ra; *E. raichoi*.
*Soils of cage No. 4 in July were collected 12 h after inducing the brood and those of Nos. 5 and 6 were before inducing the broods.
ND; could not be examined.

Ethics statement

All experiments were carried out without using live animals, and the collection of feces was conducted in a non-invasive manner. Thus, ethical approval for animal experimentation was not necessary. All of the examinations in this study were permitted by the Ministry of the Environment, Government of Japan. No animals were sacrified for the purpose of this study and the study did have any human participants.

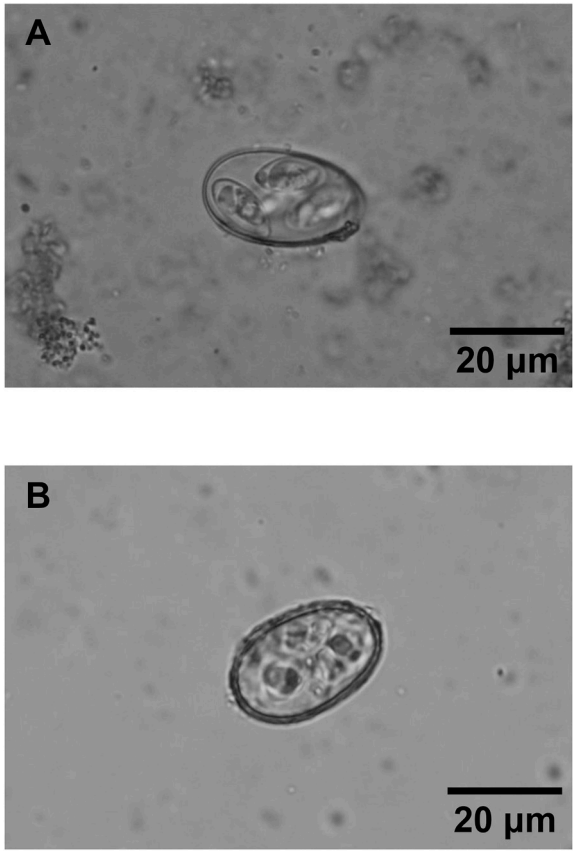

Fig. 5. *Eimeria* oocysts (*E. uekii*) isolated from soil inside the cage (cage No. 6) (A) on Mt. Kita (35°40′N, 138°14′E), one of their habitats in the southern Japanese Alps and on Mt. Norikura as other habitats on northern Japanese Alps (B). In Fig. A, the sporocysts and sporozoites are clearly formed.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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