



Surveillance of *Eimeria* species in wild Japanese rock ptarmigans, *Lagopus muta japonica*, and insight into parasitic seasonal life cycle at timberline regions of the Japanese Alps



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ARTICLE INFO

Keywords:

Conservation
Ecological epidemiology
Eimeria
Japanese Alps
Japanese rock ptarmigan

ABSTRACT

The Japanese rock ptarmigan, *Lagopus muta japonica*, inhabits the alpine zone of mountainous areas at 3000 m above sea level. Since *L. m. japonica* is endangered due to a decline in the overall population, controlling infectious diseases such as those caused by protozoan parasites is a critical factor in the conservation of this species. Although *Eimeria* spp. are considered to have a negative impact on Japanese rock ptarmigan populations, the ecological interactions between the parasites and their hosts have not yet been fully clarified. We therefore conducted seasonal surveys of the prevalence of *Eimeria* spp. in Japanese rock ptarmigan populations. In addition, we recorded the ambient temperature in ptarmigan habitat and characterized the ability of eimerian isolates to acquire infectivity. *Eimeria* spp. were detected in 217 of 520 (41.7%) Japanese rock ptarmigan fecal samples in 2006 and in 177 of 308 (57.5%) fecal samples in 2007. Specifically, we observed two types of oocysts characteristic of *E. uekii* and type B. In adult birds and chicks, infection rates increased towards August (summer) and then decreased as the temperature decreased toward November (winter). Oocyst counts per gram (OPG) of feces peaked in August in adults and chicks, and OPG values were markedly higher in chicks than in adults. Isolated *Eimeria* spp. oocysts sporulated at temperatures as low as 8 °C and remained viable after being stored at 4 °C for 6 months. Our findings suggest that *Eimeria* spp. can complete their annual lifecycle in the cold timberline regions inhabited by the host, the Japanese rock ptarmigan, and that *Eimeria* spp. infection is widespread in the bird populations examined.

1. Introduction

The rock ptarmigan, *Lagopus muta* (Montin, 1781) in the order Galliformes is a cold-adapted species that inhabits the alpine areas of the northern hemisphere. In terms of taxonomy, the species is currently divided into approximately 23–30 subspecies (Johnsgard, 1983; del Hoyo et al., 1994). One of these subspecies, the Japanese rock ptarmigan (*L. m. japonica*), inhabits the timberline regions of the Japanese alpine zone at approximately 3,000 m above sea level. This subspecies is endemic to Japan and is considered to be endangered due to a decline in the overall population (estimated population: ≤2,000 individuals) (Wildlife Division of the Ministry of the Environment, 2012). Given their relative scarcity, the Japanese rock ptarmigan was designated a

special natural monument of Japan in 1955 and is listed as vulnerable in the Japanese Red Data Book (Murata et al., 2007; Wildlife Division of the Ministry of the Environment, 2017).

The conservation of small wild animal populations is difficult, as population numbers can be markedly affected by infectious agents that can cause death or interfere with breeding. Among such infectious agents, parasites can have a potential negative influence on population health as they decrease host physical condition, fecundity, and survival (Anderson and May 1981; Murata et al., 2007). Consequently, controlling host-parasite interactions is an important part of ensuring the survival of threatened animal populations. It has recently been shown that parasite infections, especially by the protozoan parasite *Eimeria* spp. (Phylum: Apicomplexa), are associated with a decrease in overall

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<https://doi.org/10.1016/j.ijppaw.2018.03.004>

Received 27 January 2018; Received in revised form 20 March 2018; Accepted 22 March 2018

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body condition and an increase in mortality in rock ptarmigans (*L. m. islandorum*) in Iceland (Stenkewitz et al., 2016). Although *Eimeria* infection in rock ptarmigans has not been studied extensively, preventing diseases (e.g. coccidiosis in birds) can be one of the most important means by which animal species can be conserved in addition to protecting their environment.

To date, seven *Eimeria* spp. have been identified in the rock ptarmigan (*L. muta*): *E. lagopodi* from Switzerland (Galli-Valerio, 1929), *E. brinkmanni* and *E. fanthami* from Canada (Levine, 1953), *E. uekii* and type B from Japan (Kamimura and Kodama, 1981; Ishihara et al., 2006), and *E. muta* and *E. rjupa* from Iceland (Skirnisson and Th Thorarinsdottir, 2007). Typically, *Eimeria* spp. infections are initiated by oral ingestion of sporulated oocysts, which form two sporozoites within four sporocysts. Each sporozoite then undergoes development in the mucosa of the host intestine before being released in the feces as oocysts. Under ideal environmental conditions (i.e. temperature, humidity and oxygen availability), these noninfectious oocysts undergo a sporulation process resulting in the formation of infectious oocysts. In *Eimeria* spp., the optimal sporulation temperature is generally 27–28 °C (Waldenstedt et al., 2001; Pyziel and Demiaszkiewicz, 2015), with freezing below –15 °C considered to inhibit sporulation or kill the oocysts (Landers, 1953; Lassen and Seppä-Lassila, 2014). The areas of the Japanese alpine zone that are inhabited by the Japanese rock ptarmigan typically experience snowfall from September or October until June in late spring. However, it is not known how the eimerian parasites can survive such harsh environmental conditions and infect their hosts to complete their life cycle. In this study, we examined the seasonal prevalence of *Eimeria* spp. in chicks and adults of the Japanese rock ptarmigan, measured the environmental temperature in the areas in which they were found, and biologically characterized the ability of eimerian isolates to acquire the infectivity.

2. Materials and methods

2.1. Study area and birds

The survey in the present study was conducted in the Hida Mountains of the Northern Japanese Alps from April to November in 2006 and 2007; the area extends over Toyama, Gifu, Nagano and Niigata prefectures. We collected a total of 520 fresh Japanese rock ptarmigan fecal samples, including 72 samples from chicks, in 2006, and 308 samples, including 30 chicks, in 2007. Samples were collected from 11 sites: Mt. Tateyama (36°35'N, 137°36'E), Mt. Jonendake (36°19'N, 137°43'E), Mt. Sugorokudake (36°22'N, 137°35'E), Mt. Asahidake (36°49'N, 137°43'E), Mt. Shiroumadake (36°45'N, 137°45'E), Mt. Chougadake (36°17'N, 137°43'E), Mt. Otenshoudake (36°21'N, 137°42'E), Mt. Jigatake (36°35'N, 137°45'E), Mt. Minamidake (36°19'N, 137°39'E), Mt. Norikuradake (36°6'N, 137°33'E), Mt. Yurigadake (36°20'N, 137°38'E), as well as elsewhere in the Northern Alps. Where possible, the age (adult or chick) and sex of the ptarmigan that produced the fecal sample were recorded. Fecal samples were collected by tracking individual birds and collecting any feces that they produced. Additionally, birds could be identified based on unique identification numbers and chicks were identified by observing patterns of their feather color from short distance. In these study areas, it was relatively easy to find and chase a family of the rock ptarmigan during breeding period. Although an effort was made not to collect feces from the same ptarmigans more than twice a month, the possibility exists that some samples were collected from the same birds more than twice in a given month. In addition, the temperatures on the windward and leeward slopes of Mt. Tateyama were measured using data loggers (UA-002-64, Onset Computer Corp., MA, USA).

2.2. Fecal examinations

The fecal samples were placed in a cooler box, transported to our

laboratory, and stored at 4 °C until analysis. The eimerian oocysts were examined by sucrose centrifugal flotation method (Uga et al., 2000). The number of oocysts per gram (OPG) was determined by diluting the feces after filtering through a steel mesh as reported previously (Brackett and Bliznick, 1949). Several positive samples, which contained a large number of oocysts and could be examined within 2–3 weeks after being shedded, were incubated in a 2.5% potassium dichromate ($K_2Cr_2O_7$) solution at 25 °C to allow the oocysts to sporulate. Sporulated oocysts were observed under a differential interference contrast microscope under oil immersion at 1,000 × magnification. Fifty oocysts and their internal structures were then analyzed using a digital color image analysis system (Lumina Vision, Mitani Corporation, Tokyo, Japan). Fecal samples (approximately 50 pooled samples) containing a large number of oocysts (mainly, morphologically *E. uekii*) were filtered by steel mesh and incubated at a range of temperatures (4–45 °C) using 90 cm petri dishes and observed at 24-h intervals over 18 days to determine the timing of sporulation by counting 100 oocysts. In addition, some oocysts were stored at 4 °C for 6 months and then incubated at room temperature to evaluate sporulation.

2.3. Statistical analyses

The statistical tests were performed by the Pearson's Chi-square for comparison of sexes, and the Student's *t*-test for the comparison of the prevalence between adult birds and chicks and seasonal OPG of *E. uekii* and type B. Seasonal comparison of the prevalences, e.g. between spring (April and May) and summer (Jun, July, and August) or autumn (September, October, and November), could not be conducted because of few sample numbers. Statistical significance was set at $p < 0.05$.

3. Results

We detected *Eimeria* spp. in 217 (41.7%) of 520 Japanese rock ptarmigan samples in 2006, and 177 (57.5%) of 308 samples in 2007 (Table 1). No significant difference was observed in infection prevalence between adults and chicks ($P > 0.05$ in 2006 and 2007). Except for one site (Minamidake), *Eimeria* spp. were found in fecal samples from all of the sampled sites. Regarding to the infection prevalence over time, in adult birds, the rate of infection increased from April to July (early summer), peaking at 69.0% in 2006 and 88.0% in 2007, and then decreased toward November (winter) (Fig. 1). In chicks, infection rates increased after August (summer) (77.5% in 2006 and 90.9% in 2007) and decreased in October (early winter) (9.1% in 2006 and 80.0% in 2007). Chicks generally hatch on June and July, and they are cared by parents for 3–4 months. Infection rates of male and female were 46 of 116 (39.7%) and 49 of 106 (46.2%) in 2006, and 44 of 83 (53.0%) and 26 of 52 (50.0%) in 2007, respectively. Significant differences were seen between the prevalence of male and female only in 2006 ($P < 0.05$).

Microscopic observations revealed two morphologically distinct types of oocysts (Fig. 2): one that was typical of *E. uekii* and one that was similar to the type B oocyst reported previously (Kamimura and Kodama, 1981; Ishihara et al., 2006). The *E. uekii*-type oocysts were ellipsoidal in shape with a smooth colorless wall, no oocyst residuum, and one to three ovoid polar granules (1.6–3.1 μm). The micropyle was indistinct or absent. Sporulated oocysts ($n = 50$; length × width) measured $23.8 \pm 1.7 \mu\text{m}$ ($20.3\text{--}28.9 \mu\text{m}$) × $15.7 \pm 1.3 \mu\text{m}$ ($13.8\text{--}18.8 \mu\text{m}$), and had a shape index (L/W) of 1.5 ± 0.1 (1.3–1.8). Sporocysts ($n = 50$) measured $12.4 \pm 0.8 \mu\text{m}$ ($9.8\text{--}14.4 \mu\text{m}$) × $6.7 \pm 0.5 \mu\text{m}$ ($5.9\text{--}7.8 \mu\text{m}$) and had a shape index of 1.9 ± 0.2 (1.5–2.3). Stieda body and sporocyst residuum were present and two refractile bodies measuring 1.3–4.1 μm in diameter were observed in sporozoites. The type B-like oocysts were subspherical with a smooth colorless wall, no oocyst residuum and micropyle, and one to two ovoid polar granules (1.5–2.8 μm). Sporulated oocysts ($n = 50$; length × width) measured $21.4 \pm 2.4 \mu\text{m}$ ($13.6\text{--}26.0 \mu\text{m}$) × $19.2 \pm 2.2 \mu\text{m}$ ($13.1\text{--}24.6 \mu\text{m}$), and

Table 1
Summary of the prevalence of *Eimeria* spp. in Japanese rock ptarmigan in the present study.

| Mountains | Total No. | Positive No. | 2006* | | 2007* | |
|------------------|-----------|--------------|----------------|--------------|----------------|--------------|
| | | | Adult | Chicks | Adult | Chicks |
| Tateyama | 469 | 219 (46.7) | 121/292 (41.4) | 20/40 (50) | 61/118 (51.7) | 17/19 (89.5) |
| Jiigatake | 340 | 194 (47.1) | 6/12 (50) | 11/20 (55) | 54/71 (76.1) | 1/10 (10) |
| Asahidake | 109 | 64 (58.7) | 9/22 (40.9) | 0/6 (0) | – | – |
| Norikuradake | 58 | 22 (37.9) | 21/54 (38.9) | 1/4 (25) | – | – |
| Sugorokudake | 53 | 22 (41.5) | 21/47 (44.7) | – | – | – |
| Jonendake | 41 | 19 (46.3) | 0/2 (0) | – | – | – |
| Shiroumadake | 37 | 20 (54.1) | 3/4 (75) | 2/2 (100) | 1/5 (20) | 0/1 (0) |
| Yarigadake | 31 | 15 (48.4) | – | – | 15/31 (48.4) | – |
| Otenshoudake | 15 | 10 (66.7) | 2/2 (100) | – | – | – |
| Minamidake | 9 | 0 (0) | 0/9 (0) | – | – | – |
| Chougadake | 6 | 1 (16.7) | 0/4 (0) | – | 1/2 (50) | – |
| Other or unknown | 52 | 27 (51.9) | – | – | 26/51 (51.0) | 1/1 (100) |
| Total | 828 | 394 (47.6) | 183/448 (40.8) | 34/72 (47.2) | 158/278 (56.8) | 19/30 (63.3) |

Parentheses; positive percentage (%), *, positive No./examined No.

No significant difference was observed in infection prevalence between adults and chicks ($P > 0.05$ in 2006 and 2007).

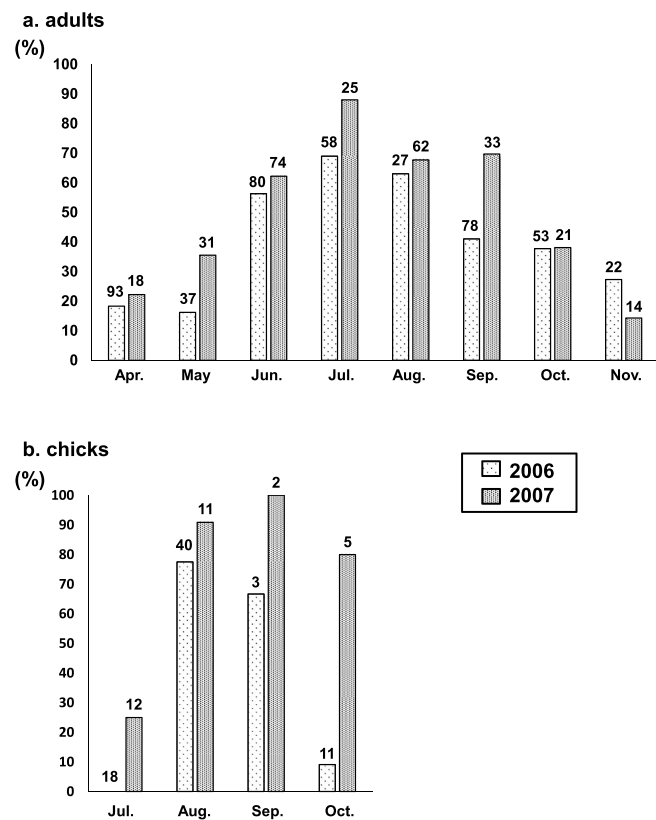


Fig. 1. Seasonal prevalence of *Eimeria* spp. infection in Japanese rock ptarmigan from April to November in 2006 and 2007. (a) and (b) show the prevalence of infection in adult birds and chicks, respectively. Numbers above bars indicate the total number of fecal samples analyzed.

had a shape index (L/W) of 1.1 ± 0.1 (1.0–1.4). Sporocysts ($n = 50$) measured $12.1 \pm 1.1 \mu\text{m}$ ($9.7\text{--}13.8 \mu\text{m}$) \times $7.5 \pm 0.7 \mu\text{m}$ ($5.9\text{--}9.2 \mu\text{m}$) and had a shape index of 1.6 ± 0.2 (1.2–2.1). Stieda body and sporocyst residuum were present. Two refractile bodies measuring 1.5–4.1 μm in diameter were observed in sporozoites. After comparisons against previous studies (Kamimura and Kodama, 1981; Ishihara et al., 2006), we identified the oocysts isolated in this study as *E. uekii* and type B oocysts.

Detection rates for the two *Eimeria* spp. in adults and chicks are shown in Fig. 3. Mixed infection by *E. uekii* and type B oocysts was

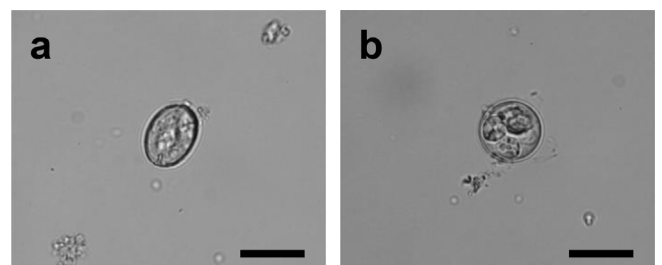


Fig. 2. Photomicrograph of eimerian oocysts detected in the feces of Japanese rock ptarmigans; (a) *E. uekii* and (b) type B. The scale bar indicates 20 μm .

frequently observed, with type B oocysts being most abundant in August. The infection rate by type B oocysts in chicks was high; 17 of 23 (73.9%) positive birds were observed in August 2006 and 8 of 11 (72.7%) positive birds were observed in August 2007. We calculated the OPG for some fecal samples and summarized the findings in Fig. 4. In adults, the OPG values for *E. uekii* oocysts increased in August and September (Fig. 4a), while values for type B oocysts peaked in August (Fig. 4b), although the number total of type B oocysts examined was limited. Average OPG values could be determined for some positive chicks in August; OPG values for *E. uekii* oocysts were 106,532 (400–742,000 (minimum–maximum); $n = 29$) in 2006 and 161,800 (800–608,000; $n = 9$) in 2007. Similarly, average OPG values for type B oocysts were 43,979 (2000–148,000; $n = 17$) in 2006 and 4338 (800–12,000; $n = 8$) in 2007. The OPG values obtained for chicks in 2006 were significantly higher than those of adults ($P < 0.05$).

We also examined the sporulation characteristics of the isolated *Eimeria* spp. (mainly *E. uekii*) and how they were affected by temperature. More than 80% of oocysts sporulated after 24-h incubation at temperatures of between 20 and 30 $^{\circ}\text{C}$, except at 23 $^{\circ}\text{C}$. After 48-h incubation, oocysts sporulated at slightly lower temperatures (17 and 18 $^{\circ}\text{C}$) and higher (31 $^{\circ}\text{C}$) temperatures (Fig. 5). However, oocysts did not sporulate at temperatures above 33 $^{\circ}\text{C}$ and the morphology of most oocysts was observed to change, especially at temperatures above 38 $^{\circ}\text{C}$. Statistically, positive correlation was observed between temperatures (4–30 $^{\circ}\text{C}$) and sporulation, $y = 4.5081x - 21.051$, $R^2 = 0.7227$, correlation coefficient: 0.85. At incubation temperatures between 8 and 16 $^{\circ}\text{C}$, oocyst sporulation occurred from 72 to 384 h. After storage at 0 $^{\circ}\text{C}$ for 6 months, 82.0% of oocysts sporulated successfully after incubation at room temperature.

We measured the average, maximum and minimum environmental temperatures on the windward and leeward slopes of Mt. Tateyama (Fig. 6). Temperatures peaked in August, and temperatures on the leeward slopes of the mountain sites were lower than those on

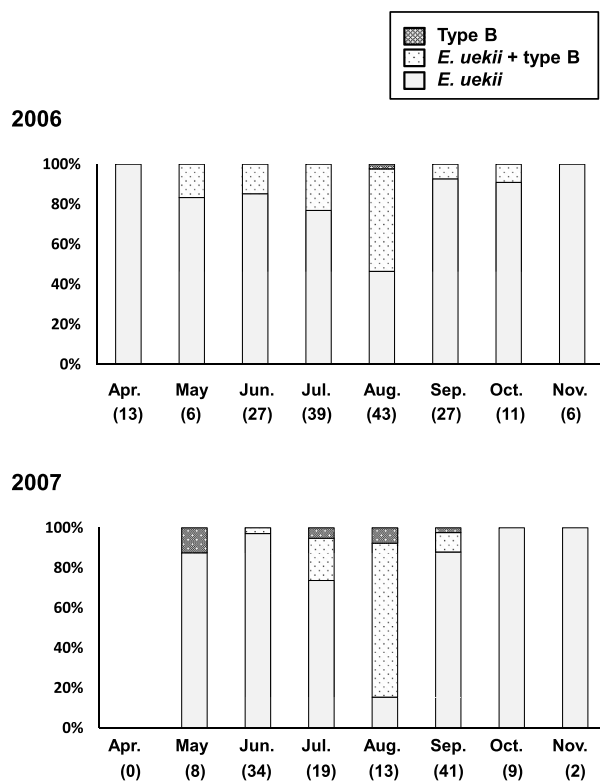


Fig. 3. Seasonal detection rate for *E. uekii*, type B, and mixed *Eimeria* spp. oocyst infection in both adults and chicks in 2006 and 2007. Numbers in parentheses below months indicate the total number of fecal samples analyzed. Data for the number of chicks (23 in 2006 and 11 in 2007) were only available for August.

windward slopes between May and October.

4. Discussion

To date, two reports have been published on *Eimeria* spp. infection in the Japanese rock ptarmigan (Kamimura and Kodama, 1981; Ishihara et al., 2006). The prevalence of parasites described as *E. uekii* was 61–85% in adult birds and 84% in chicks on Mt. Tateyama from 1971 to 1974, increasing toward September and decreasing in October and November (Kamimura and Kodama, 1981). In the other report, the prevalence of *E. uekii* and type B oocysts at 13 sites including Mt. Tateyama was 60.9% and 29.7%, respectively, from 2003 to 2005 (Ishihara et al., 2006). The findings of these studies suggested that *Eimeria* spp. are widely distributed in Japanese rock ptarmigan populations. We examined 828 birds at more than 11 sites and determined the prevalence to be 47.6%, with infections increasing toward summer and peaking in July. Among examined sites, only one (Minamidake) was found to be negative. There is less possibility that birds stay at the limited area because birds including chicks after hatching easily moved to other places. The reason remains unknown but the number of oocysts shed in feces of the examined birds could not be the peak. Oocysts of *E. uekii* were detected more frequently than those of type B, which corroborates the findings of previous studies.

Japanese rock ptarmigans are restricted to the alpine zone at around 3,000 m above sea level. They are typically found on the windward slopes of mountains after winter in March to May where their evergreen food plants are more accessible as the snow thaws relatively quickly in these areas (Nakamura, 2007). Males then compete for females and the pairs establish a breeding territory on the leeward slopes where they feed on sprouts and arthropods. Between June and September, the birds

lay and brood their eggs, and the precocial young remain with their parents for 3–4 months after hatching. It is considered that the transmission of *Eimeria* parasites occurs when adult birds come into close contact with each other during breeding, with infections among birds likely occurring most often in August and September when temperatures are optimal for sporulation. The increase in the density of the birds as the temperature increases may facilitate completion of the *Eimeria* lifecycle.

Like in adult birds, the prevalence of *Eimeria* spp. in chicks also peaked in August and September. However, OPG values among chicks were markedly higher than among adult birds. During breeding, female birds primarily take care of chicks. Although no clear tendency in *Eimeria* prevalence was observed between sexes, the parents are the most likely source of infection to chicks. However, since adults are expected to have acquired immunity against *Eimeria* spp., it is considered that the number of oocysts shed by mature birds would not have been large. In addition, coprophagy is commonly found in vertebrates, and the intestinal microbial flora acquired in this way can provide significant benefits to nutrition and growth performance due to the beneficial effect that the flora have on the gut (Soave and Brand, 1991; Barrow, 1992). Although coprophagy is uncommon in birds (Hurd et al., 1991), we observed that some Japanese rock ptarmigan chicks consumed their parents' feces (data not shown). Such behavior would facilitate infection by *Eimeria* spp. Furthermore, gut microflora in chickens has been shown to mitigate the symptoms associated with *Eimeria* spp. infection (Dalloul et al., 2003; Lee et al., 2007; Giannenas et al., 2012), and however, further studies are necessary to evaluate that changes in the microbiome transmitted from parents to chicks might reduce the number of oocysts that are shed by the adults.

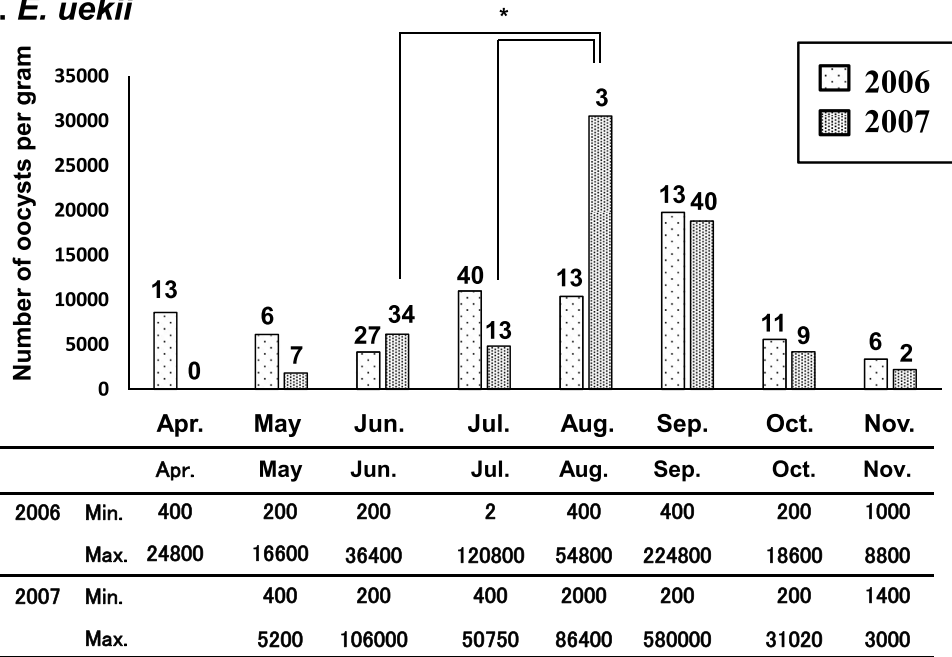
After being shed from the host, oocysts obtain infectivity by sporulation under favorable conditions. In bovine and ovine *Eimeria* spp., freezing at -10 to -18 °C has been shown to kill the oocysts (Landers, 1953; Lassen and Seppä-Lassila, 2014). The surface temperature of the soil when there is snow cover will not drop far below 0 °C; however, soil temperatures can fall below -15 °C in the absence of snow (Sharatt et al., 1992). Based on our finding that oocysts remained viable after storage at 0 °C for 6 months, it is possible that the oocysts could have survived in the soil over winter and that they then sporulated after June when the environmental temperatures in the study area exceeded 0 °C. Although several studies have examined the effect of low temperature on eimerian oocyst sporulation in other animals, incubating eimerian oocysts at 8 °C for 8 weeks did not make these oocysts sporulate (Edgar, 1954). However, in our study, eimerian oocysts from Japanese rock ptarmigans were capable of sporulating at 8 °C if they were incubated for more than 48-h. It is therefore considered possible that *Eimeria* spp. from cold-adapted birds may be relatively tolerant to, and capable of sporulating at low temperatures.

Although it has been reported that *E. uekii* may cause lesions in the host (Kamimura and Kodama, 1981), the virulence of *Eimeria* spp. in the Japanese rock ptarmigan has not been examined in detail. Since collecting diarrhea samples or finding weak or dead birds in the field is rare, clarifying the pathogenicity of the parasites is difficult. In addition, the number of ptarmigans in Japan is decreasing and several populations have been extirpated. Within the context of ptarmigan conservation, further studies are therefore necessary in order to evaluate the ecology, biology and pathogenicity of *Eimeria* spp.

Acknowledgements

The authors gratefully acknowledge Dr. Hiroshi Nakamura (Professor Emeritus of Shinshu University), Dr. Takaaki Sakanakura (The Institute for Alpine Environment and Biota), Mr. Tsutomu Matsuda (Toyama Raicho Kenkyukai: Toyama Rock Ptarmigan Research Association), and Ms. Yoko Ichikawa, Ms. Asumi Tamada, Ms. Rina Nagai, Ms. Mio Hagihara, and Ms. Mizuki Takahashi (Former College of Bioresource Sciences, Nihon University) for assistance with conducting

a. *E. uekii*



b. type B

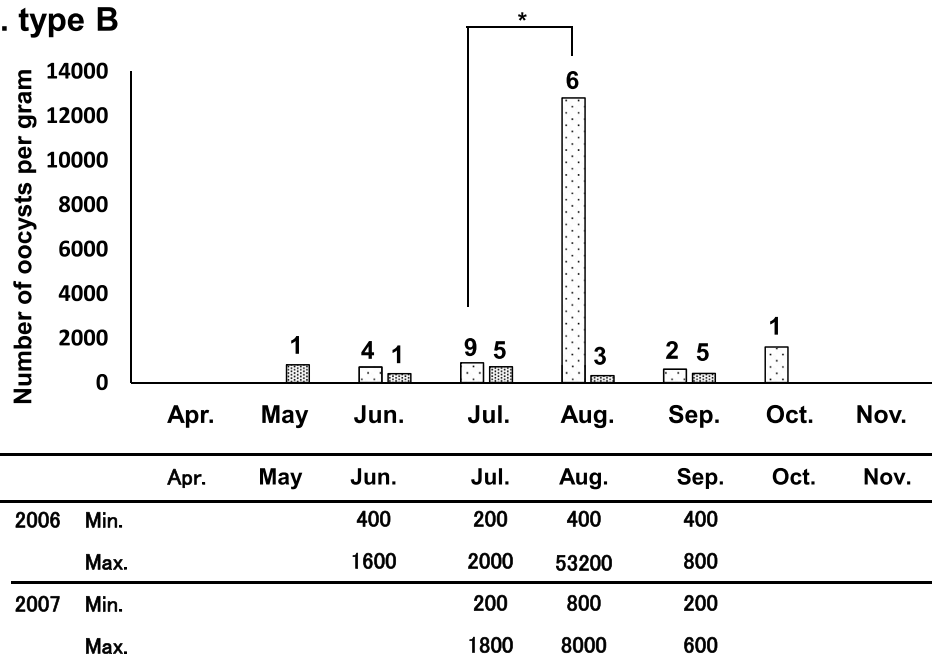


Fig. 4. Average seasonal OPG values for (a) *E. uekii* and (b) type B in adult birds in 2006 and 2007. Numbers above bars indicate the total number of fecal samples analyzed. Tables below graphs show the maximum and minimum OPG values. Significant differences of OPG indicate as (*) between the months ($P < 0.05$).

surveys in the present study. A part of this study was supported by grants from the Life Science Center of Nihon University, JSPS KAKENHI (Grant No. JP16510179) and the Environment Research and Technology Development Fund (No. 4-1604).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijppaw.2018.03.004>.

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 sacrificed for the purpose of this study and the study did not have any human participants.

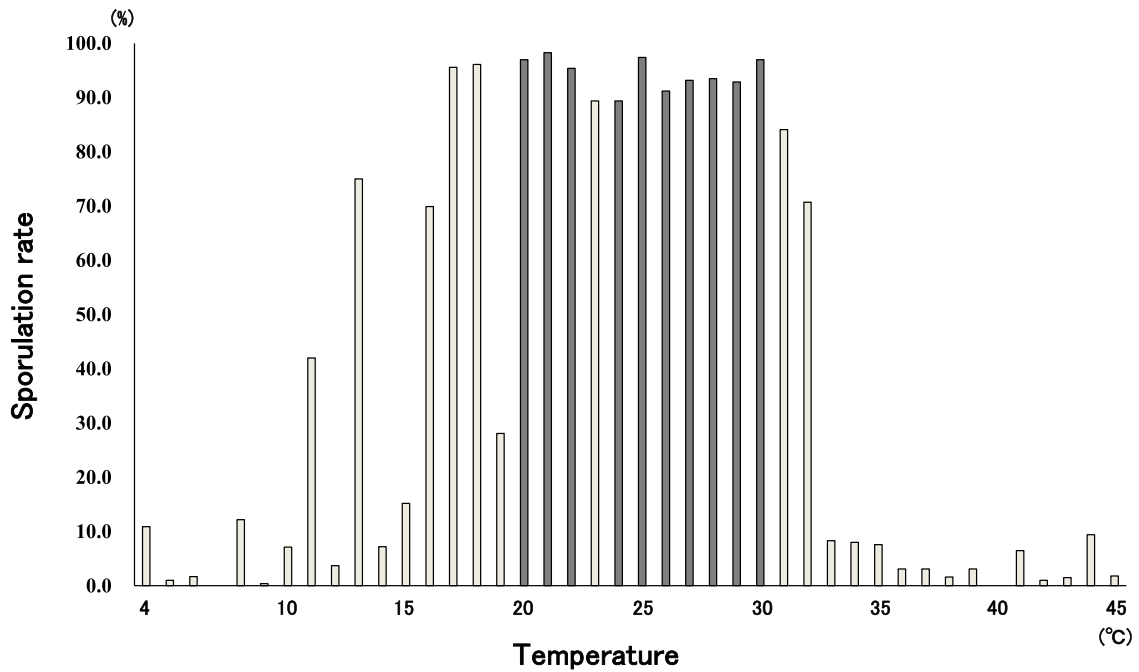
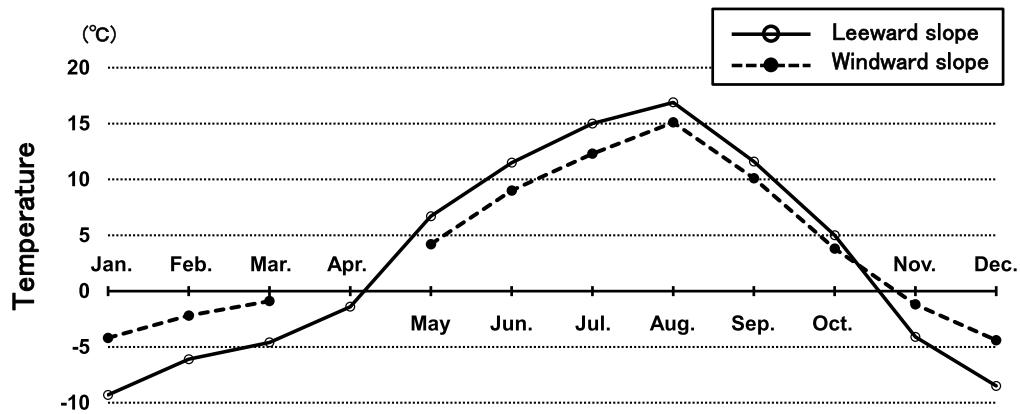


Fig. 5. Sporulation rate for *Eimeria* spp. (mainly *E. uekii*) after incubation at different temperatures for 48 h. Dark bars indicate sporulation rates of > 85% after incubation for 24 h.



| Areas | | Jan. | Feb. | Mar. | Apr. | May | Jun. | Jul. | Aug. | Sep. | Oct. | Nov. | Dec. |
|----------------|------|-------|-------|-------|------|------|------|------|------|------|------|-------|-------|
| Leeward slope | Max. | -1.0 | 0.0 | 0.0 | 11.0 | 29.5 | 39.0 | 52.5 | 42.4 | 36.7 | 26.9 | 0 | -1.5 |
| | Min. | -22.5 | -14.5 | -10.5 | 13.0 | -8.0 | -0.5 | 4.5 | 3.3 | -1.8 | -1.6 | -12.5 | -17.5 |
| Windward slope | Max. | -1.5 | -0.5 | -0.5 | ND | 18.5 | 25.5 | 25.0 | 29.0 | 24.5 | 17.0 | 12.5 | -1.5 |
| | Min. | -10.0 | -3.0 | -1.5 | ND | -2.5 | -0.5 | 4.0 | 4.5 | -1.0 | -1.0 | -2.5 | -8.0 |

Fig. 6. Average monthly environmental temperatures on the windward and leeward slopes of Mt. Tateyama from 2006 to 2007. The temperatures on the windward slopes were not measured in April. The table below the graph shows monthly maximum and minimum temperatures.

Conflicts of interest

The authors declare that they have no conflict of interest.

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