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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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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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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. Jiigatake (36°35′N, 137°45′E), Mt. Minamidake (36° 19′N, 137°39′E), Mt. Norikuradake (36°6′N, 137°33′E), Mt. Yarigadake (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 ± 1.7 μm (20.3–28.9 μm) × 15.7 ± 1.3 μm (13.8–18.8 μm), and had a shape index (L/W) of 1.5 ± 0.1 (1.3–1.8). Sporocysts (n = 50) measured 12.4 ± 0.8 μm (9.8–14.4 μm) × 6.7 ± 0.5 μm (5.9–7.8 μm) 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 ± 2.4 μm (13.6–26.0 μm) × 19.2 ± 2.2 μm (13.1–24.6 μm), and