ORIGINAL ARTICLE

Transmission dynamics of an emerging infectious disease in wildlife through host reproductive cycles

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Emerging infectious diseases are major threats to wildlife populations. To enhance our understanding of the dynamics of these diseases, we investigated how host reproductive behavior and seasonal temperature variation drive transmission of infections among wild hosts, using the model system of cyprinid herpesvirus 3 (CyHV-3) disease in common carp. Our main findings were as follows: (1) a seroprevalence survey showed that CyHV-3 infection occurred mostly in adult hosts, (2) a quantitative assay for CyHV-3 in a host population demonstrated that CyHV-3 was most abundant in the spring when host reproduction occurred and water temperature increased simultaneously and (3) an analysis of the dynamics of CyHV-3 in water revealed that CyHV-3 concentration increased markedly in breeding habitats during host group mating. These results indicate that breeding habitats can become hot spots for transmission of infectious diseases if hosts aggregate for mating and the activation of pathogens occurs during the host breeding season. The ISME Journal (2011) 5, 244–251; doi:10.1038/ismej.2010.123; published online 26 August 2010

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Introduction

Infectious diseases of wild animals are of growing concern in biological conservation. The emergence of these diseases has increased over the past few decades and is now viewed as one of the greatest threats to biodiversity (Daszak *et al.*, 2000; Smith *et al.*, 2009). However, little is known about transmission routes and infection dynamics in the wild, making it difficult to predict their emergence and develop effective countermeasures against outbreaks. To gain a better understanding of the basic properties of emerging diseases in the wild, intensive field studies based on concrete model systems are important. In this study, we focus on the highly virulent cyprinid herpesvirus 3 (CyHV-3), which emerged less than two decades ago and now causes mass mortality in common carp populations worldwide.

CyHV-3 is an emerging infectious agent in common carp (*Cyprinus carpio* L.) and in its ornamental strain koi (*Cyprinus carpio koi*). The first known disease outbreak occurred at a koi farm in the United Kingdom in 1996. CyHV-3 was first isolated in 1998 (Hedrick *et al.*, 2000) and was identified as a novel virus belonging to the family *Herpesviridae* (Aoki *et al.*, 2007). The CyHV-3 disease had spread to fish farms around the world by the early 2000s, causing mass mortality of cultured *C. carpio* (Pokorova *et al.*, 2005). After CyHV-3 was introduced to Japan in 2003, it spilled over to wild populations of common carp (Matsui *et al.*, 2008; Minamoto *et al.*, 2009b), most likely because of the release of infected common carp from aquaculture to rivers and lakes (Iida and Sano, 2005). The largest outbreak in the wild was observed in spring 2004 in Lake Biwa with 70% of the wild carp population (>100 000) dying within a few months (Matsui *et al.*, 2008). Although no additional major outbreak has occurred in Lake Biwa, cases of death due to CyHV-3 are reported every year (Shiga Prefecture, personal communication).

The abundance and dynamics of CyHV-3 in the wild remain largely unknown. However, an increasing number of experimental studies have investigated fluctuations of CyHV-3 in hosts and its possible transmission routes. *In vitro* experiments

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with carp cell lines have demonstrated that CyHV-3 growth depends on host cell temperature; optimal growth occurs at 15–25 °C, whereas temperatures lower than 10 °C or higher than 30 °C result in very low or undetectable virus replication (Gilad *et al.*, 2003). Such temperature dependence also occurs in another viral pathogen of common carp, the spring viremia of the carp virus (Ahne *et al.*, 2002). As fishes are cold blooded, seasonal changes in water temperature are expected to greatly affect CyHV-3 activity in the host. Experiments in aquaria have shown that CyHV-3 is transmitted horizontally from infected to naive hosts through contaminated water (Perelberg *et al.*, 2003; Costes *et al.*, 2009) and that CyHV-3 is discharged from infected hosts through feces (Dishon *et al.*, 2005). A field survey in Lake Biwa revealed that CyHV-3 DNA was detectable in water 5 years after the initial CyHV-3 outbreak (Minamoto *et al.*, 2009a). As the infectivity of free CyHV-3 in water rapidly decreases within a day and disappears within 3 days (Shimizu *et al.*, 2006), successful transmission of CyHV-3 is only possible if naive hosts are exposed to free CyHV-3 relatively quickly after the virus is released from infected hosts.

Aggregation and reproductive investment are often considered responsible for increased susceptibility of hosts to infectious diseases in vertebrates (Altizer *et al.*, 2006; Martin *et al.*, 2008). Thus, for animals that aggregate for breeding, transmission of infection is likely to occur during the breeding period. Several observations in the infectious diseases of fish support this assumption. First, in anadromous salmonids, transmission of the infectious salmon anemia virus seems to occur frequently when mature salmon migrate upstream for spawning, and contact between infected mature salmon and susceptible young fish increases (Plarre *et al.*, 2005). Second, infectious hematopoietic necrosis virus increases in sockeye salmon (*Oncorhynchus nerka*) during and after spawning when the host immunity may be weakened (Mulcahy *et al.*, 1984). Sexually matured common carp (>c.350 mm) form groups in breeding habitats for mating when females spawn (Barus *et al.*, 2002). Interestingly, the breeding season coincides with a rise in water temperature to over 15–18 °C in the spring (Barus *et al.*, 2002), which is optimal for the growth of CyHV-3 (Gilad *et al.*, 2003). Furthermore, a survey of the prevalence of CyHV-3 antibodies in the common carp population of Lake Biwa in 2006 indicated that infection occurred primarily in adult fish (Uchii *et al.*, 2009). Together, these findings suggest that the breeding sites are hot spots of CyHV-3 transmission.

In this study, we sought to clarify the transmission dynamics of CyHV-3 in the wild. Our key questions were the following: First, how does the prevalence of CyHV-3 in adult hosts vary over time? Second, how do seasonal temperature changes and host reproductive investment correlate with the abundance of CyHV-3 in wild populations? Third, how does the abundance of free CyHV-3 vary spatially and

temporally in water samples collected from breeding and non-breeding habitats throughout the breeding season? Our results demonstrate that the seasonal temperature shift and host annual reproductive behavior have central roles in the transmission of CyHV-3 among adult common carp.

Materials and methods

Sample collections

We collected 198 wild common carp that were apparently healthy without any disease symptoms from March to November 2008 and from March to August 2009 from the eastern area of the Lake Biwa watershed, including a satellite lake (Ibanaiko), two inflowing rivers (Daidoh and Echi) and the coastal area (Figure 1a). Fish were transported to the

Figure 1 (a) A map of study sites. Common carp were collected from the satellite lake Ibanaiko, the Daidoh and Echi Rivers and from the coastal area of Lake Biwa. Water temperatures were monitored at three points indicated by crosses. (b) Water sampling locations at Ibanaiko. The shaded areas, which were covered with emergent plants such as phragmites, are known breeding sites of common carp. Water samples were collected from two breeding sites (B1, B2) and from two non-breeding sites (N1, N2).

laboratory within 2 h and the standard length was measured to the nearest 1 mm. Fresh blood was extracted from the caudal vessel, left undisturbed for several hours at room temperature and centrifuged (10 min, 1200 g) to collect serum. Serum samples were frozen at -80°C until the assays of anti-CyHV-3 antibodies and testosterone were carried out. The fish were aseptically dissected to collect gills, brain and intestinal contents, which were all stored at -20°C until DNA extraction. Water samples were collected seven times from March to July 2009 from Ibanako. Sampling locations were as depicted in Figure 1b and included two known breeding sites and two non-breeding sites. After collection, water samples were transferred to the laboratory within 2 h and subjected immediately to virus concentration procedures.

Field observations

In 2009, the dates of mating at the two breeding sites in Ibanako were ascertained through direct observation and interviews of local fishermen. Water temperatures were monitored at three points (Figure 1a) every 20 min from March 2008 to August 2009, using temperature loggers (accuracy and resolution were $\pm 0.47^{\circ}\text{C}$ and 0.1°C at 25°C , respectively; UA-001-64, Onset, Pocasset, MA, USA). The maximum temperatures of three points each day were averaged as the representative temperature of that day.

Measurement of serum anti-CyHV-3 antibodies

Serum anti-CyHV-3 antibodies were quantified using the enzyme-linked immunosorbent assay (ELISA) according to Uchii *et al.* (2009), with some modifications. Pooled sera of 10 wild common carp diagnosed CyHV-3 positive by PCR were used as a positive control and pooled sera of 10 cultured common carp never exposed to CyHV-3 were used as a negative control. Before the analysis, ELISA was optimized using the control sera diluted to 1:2500 or higher, because the extent of cross-reaction with anti-cyprinid herpesvirus 1 antibodies is reduced at those dilutions (Adkison *et al.*, 2005). The optimal ELISA reagent concentrations and serum dilution producing the highest positive-to-negative (P/N) ratio were determined using checkerboard titrations and were subsequently used for further experiments.

One-half each of a 96-well microplate (Costar 3596; Corning, NY, USA) was coated with $50\ \mu\text{l}$ per well of $2\ \mu\text{g ml}^{-1}$ purified CyHV-3 (Adkison *et al.*, 2005) in bicarbonate buffer (pH 9.6) or $50\ \mu\text{l}$ per well of bicarbonate buffer for 1.5 h at 37°C . The plates were blocked with $300\ \mu\text{l}$ per well of blocking buffer for 1 h. After three washes with Tris-buffered saline containing Tween-20, $50\ \mu\text{l}$ per well of the serum samples diluted to 1:2500 was added in duplicate to the antigen-coated and -uncoated wells to correct

nonspecific binding for each serum sample. The positive control was loaded in every plate. After 1 h incubation and three washes, the plates received $50\ \mu\text{l}$ per well of a 1:100 dilution of mouse anti-carp IgM monoclonal antibody (provided by C. Nakayasu, Fisheries Research Agency, National Research Institute of Aquaculture, Japan) and were incubated for 1 h. After three washes, the plates received $50\ \mu\text{l}$ per well of a 1:300 dilution of $0.5\ \text{mg ml}^{-1}$ biotin-labeled goat anti-mouse IgG (H + L) antibodies (KPL, Gaithersburg, MD, USA) and were incubated for 1 h. After three washes, the plates received $50\ \mu\text{l}$ per well of a 1:500 dilution of $0.5\ \text{mg ml}^{-1}$ peroxidase-labeled streptavidin (KPL) and were incubated for 20 min. After four washes, $100\ \mu\text{l}$ per well of 3,3',5,5'-tetramethylbenzidine solution ($50\ \mu\text{g ml}^{-1}$ 3,3',5,5'-tetramethylbenzidine, 0.01% H_2O_2 , 50 mM citrate buffer, pH 4.8) was added to allow color development. After 20 min, color development was stopped by adding $50\ \mu\text{l}$ per well of 1 M H_2SO_4 , and the optical density (OD) at 450 nm was measured using a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA).

An anti-CyHV-3 antibody value for each sample was expressed as a ratio relative to the positive control: $(\text{OD}_{\text{sample } i} - \text{OD}_{\text{NSB of sample } i}) / (\text{OD}_{\text{positive control}} - \text{OD}_{\text{NSB of positive control}})$, where the OD values of antigen-uncoated wells ($\text{OD}_{\text{NSB of sample } i}$) were subtracted from those of antigen-coated wells ($\text{OD}_{\text{sample } i}$) to eliminate nonspecific binding for each sample. The cutoff value was determined as the mean anti-CyHV-3 antibody value + 3 s.d. of 20 cultured common carp never exposed to CyHV-3.

Measurement of serum testosterone

We measured testosterone concentrations in fish serum as an indicator of reproductive investment because blood testosterone levels increase in breeding males and females for many fishes, including common carp (Borg, 1994; Saha *et al.*, 2002). Serum testosterone was measured by competitive ELISA using a Testosterone EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, testosterone in each serum sample and a certain amount of testosterone-acetylcholinesterase conjugate competed for a limited amount of antiserum specific to testosterone in each well of the microplates. The antiserum bound to mouse monoclonal anti-rabbit IgG that was preattached to the wells during 2 h of incubation. After washing the wells to remove any unbound reagents, the substrate for acetylcholinesterase was added to allow color development, and OD at 405 nm was measured using a microplate reader. The magnitude of the OD is inversely proportional to the amount of testosterone in the samples. The serum testosterone concentrations were calculated by creating a standard curve with a series of known concentrations of testosterone for each plate.

Quantification of CyHV-3 in fish tissues and intestinal contents

DNA was extracted from 100–150 mg of individual gill and brain tissues using a Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was then purified using a QIAquick DNA Purification Kit (Qiagen), which yielded 50 µl of purified DNA. CyHV-3 DNA and a known single-copy gene in common carp (*C. carpio* glucokinase gene) as an internal control were quantified using TaqMan real-time PCR, according to Gilad *et al.* (2004). For each TaqMan assay of CyHV-3 and the glucokinase gene, reaction mixtures of 10 µl of TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of each primer pair, 125 nM of a TaqMan probe and 2 µl of sample DNA in a 20-µl volume were run in triplicate for all samples using the StepOnePlus real-time PCR system (Applied Biosystems).

DNA was extracted from 200 mg of individual intestinal contents using a QIAmp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions, which yielded 200 µl of extracted DNA. Before the extraction, a known amount of lambda phage (1×10^4 virus-like particles per g) was added to each sample to estimate a recovery rate. TaqMan real-time PCR was performed to quantify CyHV-3 (Gilad *et al.*, 2004) and lambda. For lambda, forward primer (5'-TTCTCTGTGGAGGAGTCCATGAC-3'), reverse primer (5'-GCTGACATCACGGTTCA GTTGT-3') and a TaqMan probe (5'-AGATGAACTGA TTGCCGCTCTCCGCT-3') were designed by Applied Biosystems. Each of the TaqMan assays of CyHV-3 and lambda was performed in triplicate for all samples with 4 µl of sample DNA in the reaction mixtures described above. CyHV-3 concentration in intestinal contents was calculated on the basis of the lambda recovery for each sample.

Virus concentration and quantification of CyHV-3 in water

Virus concentration was performed by the cation-coated filter method (Honjo *et al.*, 2010). In total, 1×10^7 virus-like particles per l of lambda phage were added to each water sample as an external standard to calculate the recovery of virus and to estimate the CyHV-3 concentration in the water. Four liters of each water sample containing lambda particles was prefiltered with 3.0-µm and 0.8-µm cellulose acetate filters (C300A142C and C080A142C; Advantec, Tokyo, Japan), and viruses in the prefiltered water were trapped with cation (Al^{3+})-coated 0.45-µm HA electronegative filters (HAWP14250; Millipore, Tokyo, Japan). After a rinse with 0.5 mM H_2SO_4 , the viruses were eluted with 200 ml of 1.0 mM NaOH and precipitated with 8% polyethylene glycol 6000 and 0.4 M NaCl at 4 °C overnight, followed by centrifugation (10 000 g, 1 h). Viral DNA was extracted using proteinase K and sodium dodecyl sulfate, followed

by the phenol–chloroform method (Sambrook and Russell, 2001). The extracted DNA was purified using a DNeasy Blood and Tissue Kit (Qiagen) and was further subjected to QIAquick DNA Purification Kit, yielding 60 µl of DNA solution. For CyHV-3 and lambda quantification, reaction mixtures of 10 µl of TaqMan Gene Expression Master Mix, 900 nM of each primer, 125 nM of a TaqMan probe and 4 µl of sample DNA in a 20-µl reaction volume were run in triplicate for all samples, using the StepOnePlus real-time PCR system.

Results

Seroprevalence of CyHV-3 infection in a host population

The cutoff value separating positive and negative results of anti-CyHV-3 antibodies was set at 0.135 (mean = 0.027, s.d. = 0.036) for the serum samples at 1:2500 dilution. The prevalence rate of anti-CyHV-3 antibodies was 18% (17% (19 of 110) in 2008 and 24% (5 of 21) in 2009) in fish smaller than 350 mm in standard length, whereas it dramatically increased to 61% (71% (15 of 21) in 2008 and 57% (26 of 46) in 2009) in fish larger than 350 mm (Figure 2). These results indicated that CyHV-3 infection was significantly more prevalent in adults than in immature carp.

Breeding status of wild common carp

Concentrations of serum testosterone were high during April 2008 and during April and May in 2009 (Figure 3). Group mating of common carp was

Figure 2 Seroprevalence of CyHV-3 infection in the common carp population of Lake Biwa in 2008 and 2009. Bars indicate numbers of anti-CyHV-3 antibody-positive/negative individuals, and the solid line depicts the proportion (%) of antibody-positive fish for each size class.

Figure 3 Seasonal fluctuation in serum testosterone in the common carp population of Lake Biwa in 2008 (○) and 2009 (□).

Figure 4 (a) Seasonal abundance of CyHV-3 in gill and brain tissues of common carp: +, CyHV-3 concentrations in gill tissues of fish captured in 2008; ×, those in brain tissues in 2008; ○, those in gill tissues in 2009; □, those in brain tissues in 2009. Water temperature changes in 2008 and 2009 are indicated by dashed and solid lines, respectively. (b) CyHV-3 concentrations in intestinal contents of common carp captured in 2009. Solid line indicates water temperature.

observed at the two selected breeding sites on 7–8 April and 31 April–1 May 2009.

Seasonal abundance of CyHV-3 in host tissues and intestinal contents

In gill or brain tissue, the CyHV-3 genome was detected in 12% (16 out of 131) of fish < 350 mm in

Figure 5 Variation in water CyHV-3 concentrations at breeding and non-breeding sites before, during and after mating of common carp in 2009. Mating occurred on the days indicated by arrows. CyHV-3 concentrations of water samples from N1 on 2 April and from N2 on 9 June, 2009 were not determined because of low virus recovery (<0.5%).

standard length, in 51% (34 out of 67) of fish > 350 mm, in 16% (21 out of 133) of seronegative fish and in 45% (29 out of 65) of seropositive fish. The highest levels of CyHV-3 in gill and brain tissue (that is, 3×10^4 – 5×10^5 copies per 10^6 host cells) were observed from April to June when water temperatures ranged from 15 to 25 °C (Figure 4a). The tissue CyHV-3 quantities were less than 1×10^4 per 10^6 host cells during other periods. In the intestinal contents, the CyHV-3 genome was detected in 19% (4 out of 21) of fish < 350 mm, in 24% (11 out of 45) of fish > 350 mm, in 3% (1 out of 36) of seronegative fish and in 50% (15 out of 30) of seropositive fish. The highest levels of CyHV-3 concentrations in the intestinal contents (that is, 2×10^4 – 5×10^4 copies per g) were detected in March and April. Intermediate CyHV-3 concentrations in the intestinal contents (that is, of the order of 10^3 copies per g) were consistently observed during the sampling period (Figure 4b).

CyHV-3 dynamics in water around mating

The average recovery rate of viruses from water samples was 4.7% (range: 0.1–23.4%). We did not quantify CyHV-3 concentrations for samples with recovery rates less than 0.5%. Water CyHV-3 concentrations at the two breeding sites changed significantly during the breeding season. Concentrations ranged from 0 to 670 copies per l before mating (11 March and 2 April), peaked at 3817 and 1820 copies per l at the two sites during mating (1 May) and decreased again to low levels of 0–744 copies per l 2 weeks after mating (14 May, 9 June and 7 July; Figure 5). CyHV-3 concentrations at the breeding

sites were low between the first and second mating (19 April). CyHV-3 concentrations at the two non-breeding sites exhibited small peaks of 937 copies per l and 271 copies per l during the second mating (1 May); these values were much lower than those at the breeding sites (Figure 5).

Discussion

Mechanisms involved in the dynamics of emerging infectious disease in wildlife are still poorly understood. Only a few studies have investigated the factors that determine disease outbreaks and transmission routes in nature. For example, annual recruitment of juveniles has been shown to stimulate transmission of *Mycoplasma gallisepticum* in house finches (Altizer *et al.*, 2004), and the prevalence of chytridiomycosis infection in frogs increased during the cool and dry season (Woodhams and Alford, 2005). Using common carp and CyHV-3 as a model system, this study provides novel empirical evidence for a key role of the host reproductive cycle and seasonal temperature shift in infection dynamics and the transmission of emerging pathogens.

The seroprevalence survey of CyHV-3 indicated that infection occurred significantly more frequently in adult fish than in immature carp. This pattern was previously described for the common carp population in Lake Biwa in 2006 (Uchii *et al.*, 2009). In this study, we found an identical trend in 2008 and 2009 (Figure 2), suggesting that newly matured hosts were subject to CyHV-3 infection in every year sampled. The seroprevalence profile also indicates that the majority of adult carp were immunized by natural infection in 2006, 2008 and 2009. Immunity at the population level, called herd immunity, would provide indirect protection to the unimmunized portion of the population by reducing contact between contagious and susceptible individuals (Fine, 1993). Such herd protection, as well as natural selection of more resistant carp strains after the CyHV-3 outbreak, may have contributed to the fact that no severe outbreaks of CyHV-3 disease have occurred in Lake Biwa since the initial outbreak in 2004.

CyHV-3 displays optimal growth at 15–25 °C *in vitro*, whereas growth is very low at <10 °C and undetectable at >30 °C (Gilad *et al.*, 2003). As expected from the temperature dependence, CyHV-3 increased in host tissues in the spring after the water temperature exceeded 15 °C (Figure 4a). However, an increase in CyHV-3 was primarily observed in spring, although water temperatures were also optimal in the fall. This observation may be explained by a trade-off between the immune and reproductive systems; that is, investment in reproduction results in weaker immune defenses and enhanced host susceptibility to infectious diseases (Altizer *et al.*, 2006; Martin *et al.*, 2008).

Suppression of immune functions by sex hormones has been reported in common carp (Watanuki *et al.*, 2002). The high testosterone level in the common carp population in the spring (Figure 3) thus indicates high investment in reproduction and reduced immune defense during this period. Thus, spring would be the most favorable season for CyHV-3 to multiply in carp tissue and spread throughout the host population.

Dishon *et al.* (2005) demonstrated experimentally that infectious CyHV-3 is excreted in the feces by infected hosts. We also detected the CyHV-3 genome in the intestinal contents of common carp. CyHV-3 in the intestinal contents could be acquired from ingested food; however, CyHV-3 was more likely to be secreted into the intestinal tracts, because it was detected primarily in seropositive hosts (15 out of 16). Recently, latency and reactivation of CyHV-3 have been demonstrated *in vitro* (Dishon *et al.*, 2007). Thus, hosts with acute or recurrent CyHV-3 disease may function as a source of infection by releasing CyHV-3 into water through feces.

We found considerably different dynamics of CyHV-3 concentrations in water between breeding and non-breeding sites. CyHV-3 markedly increased in breeding sites during mating, whereas it increased only slightly in adjacent non-breeding sites (see Figures 1b and 5). Moreover, mating occurred during the period of highest abundance of CyHV-3 in tissues of captured fish (Figures 4a and b), suggesting that breeding sites accumulate CyHV-3 released from infected hosts when hosts aggregate for group mating. In contrast, non-breeding sites would not accumulate CyHV-3 because of the lower host density. Our results suggest that CyHV-3 concentrations in water change rather rapidly; concentrations at breeding sites were low on 19 April and 14 May (that is, 11 and 13 days after mating, respectively; Figure 5). Such rapid decreases could be attributed to the short survival of CyHV-3, that is, <3 days in water at temperatures >15 °C (Shimizu *et al.*, 2006). To summarize, these observations indicate that CyHV-3 concentrations in water change in response to the breeding behavior of common carp on a small spatio-temporal scale.

In conclusion, the seasonal temperature shift and host reproductive investment both operate in favor of CyHV-3 growth during the breeding season. Host aggregation for mating in the breeding habitats most likely caused local increases of CyHV-3 concentrations, with adult hosts participating in mating experiencing a greater risk of infection. Our findings highlight the importance of reproductive cycles for transmission dynamics of infectious diseases and further demonstrate how breeding habitats can, on a small spatio-temporal scale, develop into hot spots of infection transmission during mating. As wild animals often aggregate for mating in spatially limited breeding habitats, we expect many wildlife species to face the same or a similar risk of infection. One implication of our study is that conservation

strategies against wildlife infectious diseases must pay special attention to breeding habitats. Furthermore, promising directions for future studies include examination of how the loss, fragmentation and degradation of breeding habitats affect the spread of infectious diseases and the vulnerability of wildlife populations to these diseases.

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