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RESEARCH

High ranavirus infection rates at low and extreme temperatures in the tadpoles of Japanese treefrogs (*Dryophytes japonicus*) that breed in rice paddies in the summer

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Background: Several species of amphibians in agricultural areas are often infected with ranaviruses; however, the biological or ecological factors that cause this infection are not well understood. In this study, we investigated whether local tadpole density, Gosner developmental stage, and weather conditions affected ranavirus infection in *Dryophytes japonicus* tadpoles in rice paddies over three months.
Results: During the study, eight samplings were undertaken between June 6 and August 21, 2022. No die-off of tadpoles occurred, but 20 of 110 tadpoles (18.8%) were found to be infected with ranavirus. The tadpole density at the sampling site and Gosner stage of the sampled tadpoles were not related to the daily ranavirus infection rate. The mean daily highest temperature during the two weeks prior to the sampling date and the mean daily lowest and highest temperatures during the week prior to the sampling date were negatively related to the daily infection rate.
Conclusions: Our results suggest that low and extreme temperatures caused by flooding and draining of paddy fields or climate change in summer could be a significant risk factor for ranavirus infection in summer-breeding frogs in agricultural areas.

Keywords: Asia, climate change, infectious disease, iridoviridae, rice paddy

Introduction

The mortality of amphibians due to ranavirus infection has been reported in North America and Europe, and recently in South America (Bartlett et al. 2021; Brunner et al. 2021; Duffus et al. 2015). In Asian countries, ranavirus infection and ranavirus-associated mortality have also increased since the first case of ranavirus infection in cultured pig frogs (*Lithobates grylio*) in China (Zhang et al. 1996). Currently, ranavirus infection and mass mortality due to the infection have been confirmed in at least nine Asian countries (Herath et al. 2021). In South Korea, the first ranavirus-associated mass mortality occurred in farmed *Pleophylax chosenicus* in 2009 (Kim et al. 2009). Since then, infection or mortality by ranavirus has been confirmed in six other anuran species (*Rana uenoi*, *Rana huanrenensis*, *Pleophylax nigromaculatus*, *Lithobates catesbeianus*, *Dryophytes japonicus*, and *Kaloula borealis*) (Kim et al. 2022; Kwon et al. 2017; Park et al. 2017; Park et al. 2021; Roh et al. 2022). Both *P. chosenicus* and *K. borealis* are listed as endangered species in South Korea (Lee and Park 2016). In the analyses of the partial major capsid protein (MCP) genes, Frog virus 3 (FV3)-like viruses were responsible for all the infected cases examined in South Korea (Kim et al. 2022). Despite this, the causal biological or ecological factors for the prevalence of ranavirus in agricultural areas have not been well studied and are critical for evaluating the risks of ranavirus infection in amphibians.

Various biological and ecological factors are involved in ranavirus infection and propagation. For example, metamorphosing amphibians, whose Gosner development stage is approximately 44–46, are often more vulnerable to ranavirus infections (Haislip et al. 2011; Kwon et al. 2017). In addition, a high individual density can increase individual contact among tadpoles or frogs, resulting in an increased ranavirus infection rate (Brunner et al. 2015; Millikin et al. 2023; Peace et al. 2019). In addition, ecological factors such as exposure to anthropogenic contaminants and extreme weather conditions could increase the possibility of ranavirus infection in amphibians (Cohen et al. 2020; Daszak et al. 2001; Davis et al. 2020), possibly by suppressing the immune system (Carey et al. 1999; Humphries et al. 2022). Recent climate change has often shifted local weather conditions, such as increasing extreme temperatures and drought periods, resulting in altered seasonal rain patterns (Altizer et al. 2013; Kamruzzaman et al. 2020; Tegegne et al. 2020). These factors may affect the ranavirus infection rate of amphibians in the field.

In our previous study, we found that ranavirus is present in 16.1%–50% of populations of *D. japonicus*, *P. nigromaculatus*, and *L. catesbeianus* in agricultural areas across the Korean peninsula (Roh et al. 2022). Rice paddies, which are a typical habitat in agricultural areas, have very different environments over time because farmers flood and drain them repeatedly to help rice grow in the summer (Borzée et al. 2018). In particular, thousands of dead tadpoles of *D. japonicus* are often found in water-drained rice paddies during the summer (D. Park, personal communication). Therefore, to deal with the risk of infectious diseases, it is important to determine the factors that affect ranavirus infection in frogs in rice paddies. In this study, we investigated whether local tadpole density, Gosner developmental stage, and weather conditions affected the ranavirus infection rate in *D. japonicus* tadpoles in rice paddies over a period of three months. We also wanted to determine if ranavirus infection in the summer directly results in die-offs of the tadpoles of *D. japonicus*.

Materials and Methods

Study site and sampling

D. japonicus tadpoles were collected from a rice paddy (37.57316° N, 126.42033° E, Fig. 1) in Cheongju-si, Chungbuk, Republic of Korea, where a ranavirus infection had previously been confirmed (Roh et al. 2022). We sampled each of the 10 *D. japonicus* tadpoles using a fishing net (12.2 × 10.5 cm) over eight days between June 6 and August 21, 2022. On June 25, June 29, and July 8, we collected tadpoles from two locations more than 10 m apart, which showed different tadpole densities due to lowered water levels in the paddy, and pooled the data for the day. Due to the draining or flooding of the paddy for rice cultivation, our sampling dates are not evenly distributed across the study period.

We determined the tadpole density at the sampling site by dividing the number of tadpoles caught by the number of nettings and the size of each net in square meters (Loman 1997). We conducted, on average, 6.7 nettings (1–10) for the calculation before each sampling. We individually preserved sampled tadpoles in 99% EtOH after euthanasia by submerging them for more than 15 minutes in 0.5% MS222 (Galex et al. 2020) and washing them out using paddy water. In the laboratory, we determined their Gosner developmental stage (Gosner 1960), extracted liver tissues, and preserved the tissues at −80°C.

Additionally, we analyzed the weather data for the average daily lowest, highest (Fig. 2), and mean air temperatures and average daily mean relative humidity during one, two, and four weeks prior to the sampling date. We selected these timeframes to evaluate whether weather conditions during tadpole growth influenced potential ranavirus

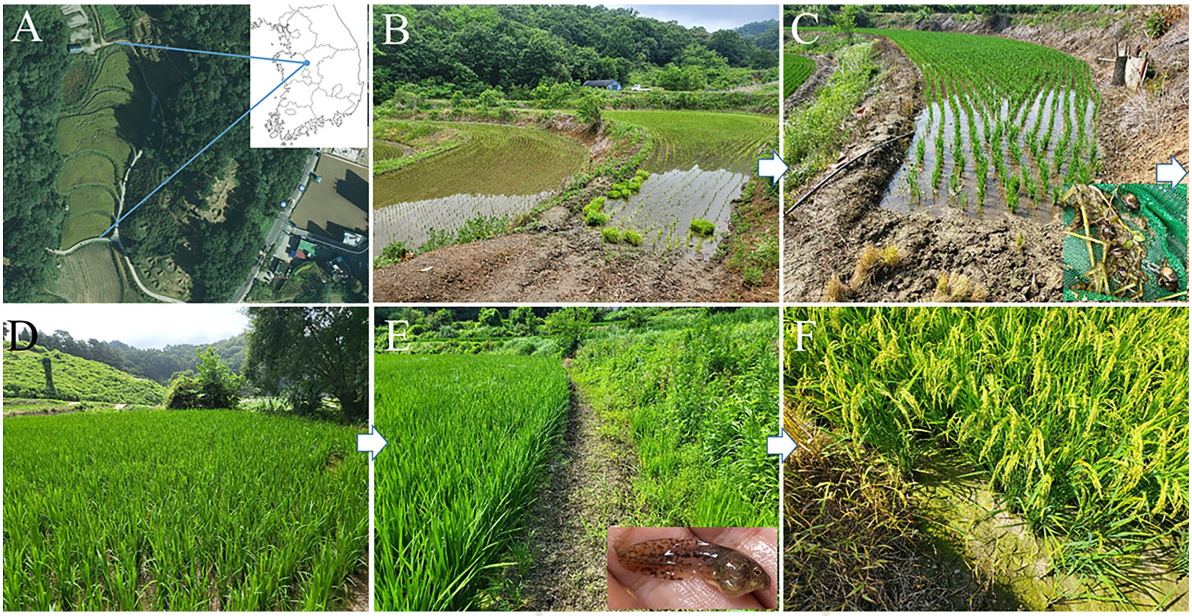

Fig. 1 (**A**) Study site in the rice paddies. The paddies continuously changed during rice cultivation, as photographed on (**B**) June 6, (**C**) June 25, (**D**) July 29, (**E**) August 7, and (**F**) August 21. (**C**, **E**) Insertions are tadpoles collected by a fishing net from the paddy.

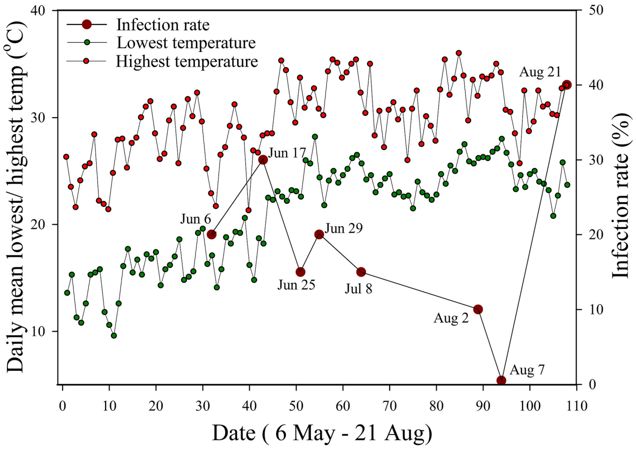

Fig. 2 Ranavirus infection rate of *Dryophytes japonicus* tadpoles over a three-month study period from June 6 and August 21, 2022, which is shown with daily mean lowest and highest air temperature data between May 6 and August 21.

infection and propagation. We downloaded the weather data from the Cheongju Meteorological Center (www.weather.go.kr), which is 7.6 km away from the sampling site.

Quantitative PCR

We extracted genomic DNA from liver tissue with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, quantified it with a Qubit3 Fluorometer (Invitrogen, Waltham, MA, USA) using the Qubit 1X dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA), and then stored it at −80°C until quantitative PCR (qPCR) experiments. For qPCR amplification of the MCP gene, which is conserved among *Ranavirus* species, we used primers RVMCPKim3F and RVMCPKim3R (Kimble et al. 2015). The composition of the amplification reaction solution for qPCR included 10 μL of power SYBR green PCR master mix (Applied Biosystems, Waltham, MA, USA), 0.5 μL of the forward primer, 0.5 μL of the reverse primer, and 4 ng of the extracted DNA. We adjusted the volume to a final value of 20 μL using molecular biology grade water. qPCR was performed in Quant Studio 1 (Applied Biosystems, Waltham, MA, USA) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 62.5°C for 20 seconds.

All samples were run in triplicates, with sterile molecular-grade water as a negative control and a standard set of gBlocks to measure the viral load of each sample in viral copy number equivalents. We constructed a synthetic double-stranded DNA standard as the gBlock copy by synthesizing a 126 bp fragment of the MCP gene (gBlocks Gene Fragments; Integrated DNA Technologies^{TM}, Coralville, IA, USA) and evaluated the standard curve in our system using a 10-fold dilution series from 5×10^9 down to 5×10^1 gene copies of the gBlocks. We used a set of gBlocks (5×10^6, 5×10^4, and 5×10^2 gene copies) as a standard for each sample analysis.

If there was a positive reaction in two or more of the three replicate samples, the melting temperature of the melting curve was the same as the positive control, and the cycle threshold was 35 or less, we considered that the tadpole had been infected by ranavirus. If a positive reaction was confirmed in only one well, the test was rerun and the tadpole was considered to be infected only when the above conditions were satisfied.

Data analyses

For analyses, we log-transformed the tadpole density and viral concentration of the infected samples, which were determined using the quantity of gBlock copies. Considering that most of the data did not pass the normality test ($p < 0.05$) and the sample sizes were as small as eight, we applied nonparametric statistics for the analyses. We tested relationships between the ranavirus infection rate and viral concentration of infected tadpoles and the tadpole density at the sampling site, the Gosner developmental stage of the investigated tadpoles, and 12 weather conditions using the Spearman correlation test ($\alpha = 0.05$) in SPSS v. 24.0 (IBM Corp., Armonk, NY, USA). The results are presented as means ± standard error unless otherwise stated.

Results

The mean tadpole density at the sampling site was 219.2 ± 83.8 ind./m^2 (range: 78–780 ind./m^2; n = 11) (Table 1). The mean Gosner developmental stage of the collected tadpoles was 33.6 ± 1.3 (range: 24–42; n = 110). Among the 110 tadpoles examined, we found a total of 20 tadpoles (18.2% ± 3.3%; range: 0.0%–40.0%; n = 8) (Table 1), which were infected with ranavirus over seven samplings, except a sample on 7 August. No mass mortality of *D. japonicus* tadpoles was observed during the study. Infected tadpoles had a daily mean Gosner developmental stage of 31.6 ± 1.2 (range: 25–40; n = 20). The daily mean viral concentration in infected tadpoles was 549.3 ± 152.1 copies/μL (range: 8.5–2,631.1 copies/μL; n = 20). The highest individual viral concentration, 2,631.1 copies/μL, was detected on August 21.

Neither the daily infection rate nor the viral concentration in the infected tadpoles had a significant relationship with tadpole density at the site or their Gosner developmental stage ($p > 0.05$; Fig. 3A, B). Among the 12 weather conditions, however, the mean daily highest temperature ($r = -0.711$; $p = 0.048$; n = 8) (Fig. 3C) during the two weeks prior to the sampling date and the mean daily lowest ($r = -0.735$; $p = 0.038$; n = 8) (Fig. 3D) and highest ($r = -0.711$; $p = 0.048$; n = 8) temperatures during the week prior to the sampling date were negatively and significantly related to the daily infection rate. The remaining relationships were not statistically significant ($p > 0.05$).

Table 1 Daily values (mean ± standard error) of the factors that potentially affect the ranavirus infection rate of *Dryophytes japonicus* in rice paddies

Date	Tadpole density (ind./m^2)	Gosner stage	SVL (mm)	Ranavirus infection rate (%)	Viral copies (copy/μL)	During 1 week prior				During 2 weeks prior			
						Mean temperature (°C)	Lowest temperature (°C)	Highest temperature (°C)	Mean humidity (%)	Mean temperature (°C)	Lowest temperature (°C)	Highest temperature (°C)	Mean humidity (%)
June 6	78	27.9 ± 0.2	7.8 ± 0.1	20	1,013.4 ± 731.6	22.3 ± 0.8	17.0 ± 0.7	28.3 ± 1.2	53.7 ± 5.5	22.2 ± 0.5	16.7 ± 0.4	28.6 ± 0.8	51.5 ± 3.2
June 17	78	28.0 ± 0.3	7.7 ± 0.1	30	334.4 ± 279.4	22.5 ± 0.8	18.2 ± 0.7	27.6 ± 1.0	61.8 ± 3.3	22.0 ± 0.7	17.7 ± 0.5	27.1 ± 0.9	61.9 ± 2.5
June 25	86	36.3 ± 1.0	12.7 ± 0.3	20	802.3 ± 104.7	26.5 ± 0.5	22.7 ± 0.1	31.7 ± 0.9	69.4 ± 2.3	24.5 ± 0.7	20.6 ± 0.7	29.7 ± 1.0	65.9 ± 2.4
	780	36.2 ± 1.3	10.9 ± 0.8	10	415.6								
June 29	78	34.8 ± 1.5	9.9 ± 0.6	20	200.4 ± 174.9	27.5 ± 0.6	24.4 ± 0.7	31.9 ± 0.6	71.3 ± 3.4	26.0 ± 0.7	22.5 ± 0.9	30.8 ± 0.7	69.8 ± 2.1
	780	35.7 ± 1.7	10.9 ± 0.6	20	352.7 ± 232.5								
July 8	78	26.6 ± 1.5	5.8 ± 0.7	20	57.7 ± 3.1	29.4 ± 0.3	25.2 ± 0.3	34.4 ± 0.4	69.1 ± 1.2	28.5 ± 0.4	24.9 ± 0.4	33.0 ± 0.5	70.6 ± 1.8
	100	31.0 ± 2.0	7.5 ± 1.2	10	91.9								
August 2	98	37.7 ± 1.2	13.3 ± 0.4	10	1,191.8	29.3 ± 0.4	25.8 ± 0.4	33.3 ± 0.7	67.5 ± 2.7	27.7 ± 0.6	24.5 ± 0.5	31.8 ± 0.7	72.0 ± 2.3
August 7	143	39.2 ± 0.7	15.7 ± 0.7	0	0	29.4 ± 0.3	26.5 ± 0.3	33.2 ± 0.6	74.8 ± 0.7	27.3 ± 0.4	25.9 ± 0.4	33.1 ± 0.6	70.9 ± 1.9
August 21	112	36.6 ± 0.8	13.2 ± 0.5	40	857.7 ± 615.4	27.3 ± 0.4	23.6 ± 0.5	31.3 ± 0.5	76.8 ± 2.0	25.9 ± 1.0	24.4 ± 0.5	30.8 ± 0.6	78.2 ± 1.8
Total	219.2 ± 83.8	33.6 ± 1.3	10.5 ± 0.9	18.2 ± 3.3	549.3 ± 152.1	26.8 ± 1.0	22.9 ± 1.2	31.5 ± 0.8	68.0 ± 2.6	25.9 ± 1.0	22.1 ± 1.2	30.6 ± 0.7	67.6 ± 2.8

For clarity purposes, we did not include weather conditions during four weeks prior to the sampling date.
SVL, snout-vent length.

Discussion

In this study, we examined the effects of tadpole density, Gosner developmental stage, and weather conditions on the rate of ranavirus infection in *D. japonicus* in rice paddies, where mortality rates are high for unknown reasons. We found that 18.2% of *D. japonicus* tadpoles were infected with ranavirus, and the infection rate was similar to the 16.1% reported in our previous study (Roh et al. 2022). During the three-month study, no mass mortality of the tadpoles occurred. Among the examined factors, only the lowest and highest air temperatures during one and two weeks prior to the sampling date were negatively and significantly correlated with the ranavirus infection rate. Our results suggest that low and extreme temperatures during summer may increase the risk of infectious diseases in *D. japonicus*.

The effect of tadpole density on ranavirus infection in *D. japonicus* tadpoles remains unclear. A high rate of ranavirus infection is often associated with a high density of tadpoles in an area. This is because tadpoles have more contact with each other and stress levels may be high (Brunner et al. 2015; Peace et al. 2019). For example, ranavirus infection in tiger salamanders (*Ambystoma tigrinum*) increases when drought causes more of them to aggregate (Brunner et al. 2007). However, in this study, we did not observe such a relationship. The dynamics of irrigation in rice paddies may be responsible for these different outcomes. In paddy fields, where rice is cultivated, water flooding and draining are repeated to facilitate successful growth of rice (Borzée et al. 2018). This system dynamically changes the topographical features of paddy floor, subsequently changing the water levels in shallow patches where *D. japonicus* tadpoles mostly inhabit. Tadpoles continuously move across various patches; therefore, tadpole density might vary considerably and continuously over time. In this study, although we calculated the tadpole density at the sampling site, our measurements may not reflect the specific tadpole density to which the tadpoles were actually exposed during initial contacts with ranavirus. To appropriately test how different tadpole densities affect the ranavirus infection rate, spatial dynamic models of tadpole movement in rice paddies need to be developed.

Ranavirus infection rates of *D. japonicus* tadpoles were not directly correlated with the Gosner developmental stage. The ranavirus infection rate in amphibians is often high at Gosner stages 44–46, when metamorphosis is close, often leading to die-off events (Greer et al. 2005; Kwon et al. 2017). Nevertheless, ranavirus infection generally occurs throughout all developmental stages (Haislip et al. 2011; Roh et al. 2022), and no relationship is often detected (Gray et al. 2007). In this study, the Gosner developmental stage of the infected tadpoles varied widely between 25 and 40 stages. In addition, tadpoles at different stages, possibly

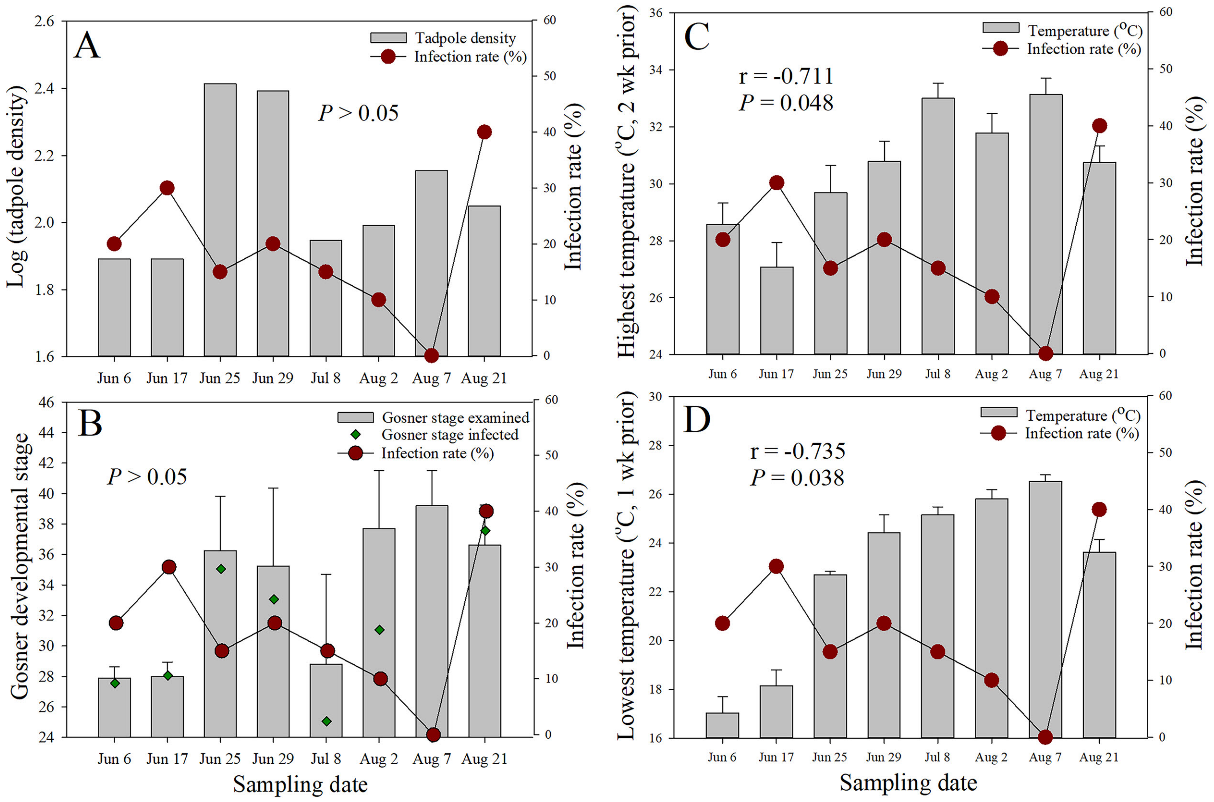

Fig. 3 (**A**) Relationships between the daily ranavirus infection rate of *Dryophytes japonicus* tadpoles and the tadpole density at the sampling site, (**B**) the Gosner developmental stage of collected tadpoles, and weather conditions of the daily mean highest (**C**) and lowest (**D**) temperature during two weeks and one week prior to the sampling date, respectively.

from different clutches, are often found at the same time or site. Therefore, our results, which showed no trend between Gosner stage and ranavirus infection rate, are not surprising considering such field situations and previous studies. In contrast, our results showed that *D. japonicus* tadpoles could be at different developmental stages at specific times and sites. Ranavirus transmission among different tadpole stages should be evaluated in future studies. Newly hatched or lower-stage tadpoles, in which metamorphosing tadpoles are present, may be more vulnerable to ranavirus infection.

Temperature affects the rate of ranavirus infection in *D. japonicus*. The infection and transmission of ranaviruses are temperature-dependent, although different species have different optimal temperatures for propagation (Bayley et al. 2013; Brand et al. 2016; Hall et al. 2018). In the laboratory, FV3, which has also been found in South Korea, propagated well at 24°C–25°C air temperatures in epithelioma papulosum cyprini (EPC) or in inoculated anuran tadpoles (Ariel et al. 2009; Brand et al. 2016). In fields in Tennessee, USA, the prevalence of FV3 in green frog (*Rana clamitans*) and American bullfrog (*L. catesbeianus*) tadpoles was greater in October and February than in June, during which the mean water temperatures were 14.2°C, 5.1°C, and 21.2°C, respectively (Gray et al. 2007). In our study, the infection rate was high when the lowest or highest air temperature to which tadpoles were exposed was low, suggesting that in ambient environments, low extreme temperatures could be a more important determinant of ranavirus infection than mean or high extreme temperatures. Interestingly, the highest infection rate of 40% was detected on August 21, when the daily mean air temperature was as high as 27.3°C. This result could be explained by the sudden decrease (from 29.4°C to 27.3°C) in the lowest temperature on the days of the summer, which from June to August in South Korea. Low and extreme temperatures generated during high-temperature periods can be stressful to tadpoles. The ranavirus-associated mass mortality of *P. chosenicus* tadpoles, which was the first die-off case in South Korea, also occurred during the rainy period (July 2009) in summer when extreme low temperatures occurred (Kim et al. 2009). These results suggest that low and extreme temperatures in summer due to the rainy season or climate change could increase ranavirus infection in summer-breeding frogs such as *P. chosenicus*, *D. japonicus*, and *K. borealis*, of which breeding season extends to June, July, or August (Lee and Park 2016).

Even though 40% of *D. japonicus* tadpoles were infected with ranaviruses in August, there was no mass mortality. Ranavirus infection rates and mortality are often affected by the phylogeny, life history, and ecology of the species (Hoverman et al. 2011). In a susceptibility test of 19 amphibian species to ranavirus, FV3 caused more infections and deaths in ranid frogs than in other types of amphibians (Hoverman et al. 2011). In our previous study, we also found that *D. japonicus* was infected at 16.0% of 43 sites, but there were no cases of die-off (Roh et al. 2022). In South Korea, no overall mortality of *D. japonicus* due to ranavirus infection has been reported. Considering these results, the Japanese treefrog *D. japonicus* may be less likely to be affected by ranavirus infections. Nevertheless, to fully exclude a possible link between the summer mass mortality of *D. japonicus* tadpoles and ranavirus infection, we need to test more possibilities using dead tadpoles in the field.

Conclusions

In this study, we found no die-offs in *D. japonicus* despite a detected ranavirus infection rate of up to 40%. The tadpole density and their Gosner stage largely varied significantly over time and in different locations in the field. This makes it difficult to determine the exact cause of the increased rate of ranavirus infection in *D. japonicus*. Nevertheless, we found that low and extreme temperatures were correlated with higher ranavirus infection rates in *D. japonicus*. Considering recent climate change shifts in rain patterns in South Korea from the rainy season to the monsoon-type rainy season, the sudden drop in extreme temperatures in summer poses a great risk for ranavirus infection in summer-breeding frogs. Special attention should be paid to the possible link between the prevalence of infectious diseases in amphibians and climate change during summer.

Abbreviations

FV3: Frog virus 3

MCP: Major capsid protein

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Authors' contributions

NHR did data curation, investigation, and writing-original draft. JK did data curation, formal analysis, and writing-review and editing. JP did data analysis, funding acquisition, and writing-review and editing. DP did conceptualization, supervision, writing-original draft, and writing-review and editing.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Kangwon National University (KW-200618-3).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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