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Microflora Influence: The Aquatic Environment Changes Grouping Risk and Development Speed of Toad Tadpoles

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In addition to habitat loss and fragmentation, behavioral traits and the deterioration of water environments also contribute to the local extinction of amphibians. Abundant microflora in urban ponds may cause fatal diseases, whereas symbiotic bacteria may protect the host from pathogens; these effects may vary with group size. In this study, I monitored the growth of Japanese common toad (*Bufo japonicus*) larvae in Tokyo using three different group sizes: 1 (solitary), 2 (pair), and 15. Although there was no genetic bias in the major histocompatibility complex (MHC) class II genes or microsatellite loci to the survival of the larvae, the mortality risk of the larvae reared in pond water was higher than that of those reared in tap water. According to the survival analysis, the risk was more significant when the group size was 15. This result would be unwelcomed for the *B. japonicus* tadpoles, which have habits of social aggregation. Furthermore, larval metamorphosis took longer to complete in pond than tap water without any difference in body length or mass. These findings provide fundamental insight into the impact of the aquatic environment and the effect of the group size on animal health and conservation.

Keywords: amphibian larvae, infection disease, population decline, major histocompatibility complex (MHC), urban pond, wild animal health, *Bufo japonicus*, group size

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INTRODUCTION

Wildlife disease management is an important global issue. Two-thirds of mammals, birds, reptiles, and fish, and amphibians face global declines, driven by habitat loss and infectious diseases (WWF, 2020). Local extinctions of wild animals alter ecosystems; this is linked to human activity, animal health, and interactions with microorganisms (Fisher et al., 2012; Rabinowitz and Conti, 2013; Mills et al., 2019; Ode Sang et al., 2022).

In amphibians, fungal pathogens can cause disease-driven extinction; for example, *Batrachochytrium dendrobatidis* has caused or threatened the local extinctions of more than 200 species (Daszak et al., 1999; Skerratt et al., 2007). This has encouraged research on disease management and microorganism diversity in amphibians (Harris et al., 2009; Mcfall-Ngai et al., 2013; Walke et al., 2014; Bataille et al., 2016; Rebollar et al., 2016; Walke and Belden, 2016; Bletz et al., 2017; Jani et al., 2021), where the composition of microbial communities is linked to host resistance to fungal pathogens (Harris et al., 2009; Becker et al., 2015; Rebollar et al., 2016; Bates et al., 2018). Symbiotic bacteria vary by host species, host age, and site

(Kueneman et al., 2014; Walke et al., 2014; Hernández-Gómez et al., 2017; Jiménez and Sommer, 2017). In addition, temperature, humidity, water characteristics, and landscape influence host–microbiota interactions, i.e., the spread of fungal pathogens is related to both biotic and abiotic factors (Beyer et al., 2015; Krynak et al., 2016; Bernardo-Cravo et al., 2020). Environmental well-being is important for wild animal health. The aquatic environment is critical for the growth of aquatic organisms, such as amphibian larvae. Moreover, ponds are crucial for aquatic biodiversity, and urban ponds can maintain the same level of biodiversity as rural ponds (Hill et al., 2017). Finally, managing urban ponds is essential for public health (Lambert and Donihue, 2020) and studying amphibians is necessary to understand pond environments (Menetrey et al., 2005).

The larvae of the Japanese common toad, *Bufo japonicus* Temminck and Schlegel, 1838, exhibit a strong schooling tendency, often aggregating cohesively (Wells, 2010). Aggregation is beneficial in the presence of predators (Watt et al., 1997), whereas it has costs when infectious diseases occur because it prevents social distancing (Sandel et al., 2020; Stockmaier et al., 2021). Generally, grouping increases the risk of transmission of infectious disease through social interactions (Kappeler et al., 2015; Pinter-Wollman et al., 2018; Stockmaier et al., 2021). Furthermore, highly transmissible pathogens can spread more easily, and over longer periods, in gregarious than socially hierarchical species (Sah et al., 2018).

Ponds that are home to *B. japonicus* larvae contain many microorganisms, some of which are pathogenic. This supports the hypothesis that growing in pond water would be more costly for larvae than growing in water without microorganisms. The risk of mortality due to infection may also be greater for larger than smaller groups. To test this, I conducted a rearing experiment using three different group sizes of *B. japonicus* larvae from an urban pond, Ichiniro-ike (at The University of Tokyo). The number of *B. japonicus* in the Ichiniro pond population has decreased—10 years ago, 120 mating pairs were observed (Hase and Shimada, 2014), compared to fewer than 10 in the last few years (Hase, personal observation). I investigated the influence of water environment and group size on the decline in this local population. In addition, to ascertain whether the genetic background contributed to differences in larval viability, I proceeded with population genetic analyses using the major histocompatibility complex (MHC) class II gene (as an assessment for the immunity against pathogens) and microsatellite loci (as a neutral marker).

METHODS

Rearing Experiment

The rearing experiment was conducted using *B. japonicus* larvae from a field-captured egg string laid by one female in Ichiniro-ike at The University of Tokyo in February, 2021. Only one egg string was available at the time of sampling, which limited replication to the rearing experiment of the present study. Two types of water were used for rearing: dechlorinated tap water and pond water. **Figure 1** shows the rearing experiment. To measure the effect of group size in a limited sample size, I focused on three levels: 1 (solitary, no interaction), 2 (pair, smallest size in which interaction can occur), and 15 (large enough to observe group effects). *Bufo japonicus* larvae were raised in three groups at 18°C with a 12:12 h dark:light cycle. Larval development was monitored for 50 days: survival, hindlimb emergence [developmental stage 38 (Gosner, 1960)], forelimb emergence [stage 42 (Gosner, 1960)], and completion of metamorphosis [i.e., toadlet, stage 46 (Gosner, 1960)] were recorded. Finally, the snout-vent lengths (hereafter, SVL) and body masses of the toadlets were measured. The daily records and developmental data on the reared larvae are listed in **Supplementary Tables 1**, **2**, respectively.

Assessment for Water Characteristics

The chemical composition of the tap and pond water was evaluated using a water quality test (**Supplementary Table 3**). The microflora of pond water was analyzed by 16S-rRNA gene-targeted amplicon sequencing. I have prepared two samples of 2 L of environmental water collected from the Ichiniro-ike (**Supplementary Figure 1**) and filtered through membranes (0.22 μm, MF-Millipore^{TM}). The water samples underwent Next generation sequencing of 16S ribosomal RNA gene PCR amplicons (16S-rRNA gene-targeted amplicon sequencing) at FASMAC Co., Ltd. (Kanagawa, Japan). Bacterial taxonomy (phylum/division) was analyzed using QIIME [ver. 1.9.0, (Caporaso et al., 2010)] based on the representative sequence of operational taxonomic units formed by 97% homology. The result of bacteria taxonomy analysis at the phylum level is shown in **Figure 1B**.

DNA Extract and Genotyping

For dead individuals of the rearing experiment, total DNA content was extracted from the tail-tips (2–3 mm) using DNeasy Blood & Tissue Kits (Qiagen, Inc., Valencia, CA, United States) following the product protocol. For the alive toadlets, toe tips (approximately 1 mm) were left to digest overnight in ethylenediaminetetraacetic acid–sodium dodecyl sulfate (EDTA-SDS) solution (0.3% SDS, 400 mmol/L NaCl, 5 mmol/L EDTA, 20 mmol/L Tris–HCl, pH 8.0) containing 200 μg/mL proteinase K at 55°C.

To evaluate whether the genetic background of immunity links to the survivorship, MHC class II antigen (partial exon) polymorphism of the reared larvae was ascertained. MHC allele sequences were amplified by PCR using locus-specific two primers from previous study (May et al., 2011; Bataille et al., 2015), 2F347 (5′-GTGACCCTCTGCTCTCCATT-3′) and 2R307b (5′-ATAATTCAGTATATACAGGGTCTCACC-3′), and a newly developed primer for *Bufo japonicus*, R_BjMHCII (CCATAGTTG TRTTTACAGWATSTCTCC). PCR was performed according to the product protocol using KAPA2G Fast HotStart Ready Mix (Kapa Biosystems, Inc., Wilmington, MA, United States) to obtain amplified fragments. Sequencing was performed with a newly developed primer, F_BjMHCII (CCTCTGCTCTCCATTACAGATGC), located inside of the fragment. For neutral genetic markers, I selected four microsatellite loci regarding previous studies: *Bbufu23*, *Bbufu39*,

Bbufu62 (Brede et al., 2001), and *Bjap14* (Hase et al., 2013). Following a method described previously (Hase et al., 2013), the loci were amplified with two multiplex reactions using a KAPA2G Fast Multiplex PCR Kits (NIPPON Genetics Co., Ltd.) according to the manufacturer's protocol. The sequencing and fragment size analysis of the PCR products were conducted by 3130xl Genetic Analyzer. In addition, I tried to check for infection with *Batrachochytrium dendrobatidis* of the larvae, referring to the previous study (Goka et al., 2009), but no chytrid presence was detected. All genotype data for 72 individuals are available in **Supplementary Table 4**, and sequences of detected unique MHC alleles are deposited in GenBank (accession number: OM994394–OM994396). Results of the phylogenetic relationship for MHC alleles and genetic clustering for the genotype of microsatellite loci are shown in **Supplementary Figure 2**.

Data Analysis

To evaluate survival in each group, I created Kaplan–Meier survival curves between tap and pond water using log-rank tests. The effect on development was investigated by applying a general linear mixed model (GLMM) with a binomial error distribution. Survival days, days to hindlimb emergence, forelimb emergence, and metamorphosis (toadlet) were used as response variables.

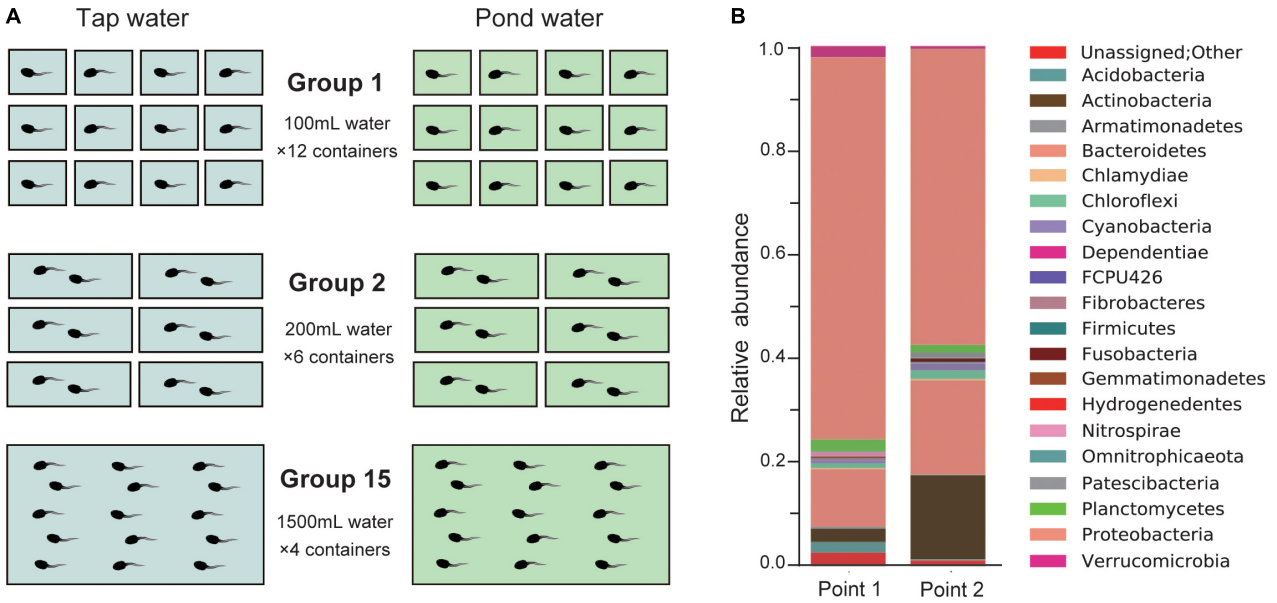

FIGURE 1 | (A) Diagram of the rearing experiment. *Bufo japonicus* larvae reared in three group sizes, 1, 2, and 15 were prepared. The length, width, and height (mm) of the plastic containers were 80 × 80 × 53, 80 × 156 × 53, and 156 × 235 × 83, respectively. Before use, tap water was dechlorinated, and pond water was filtered through a polyethylene net (100 mesh). The amount of water per larva was kept the same at 100 mL, and the water was changed every 2 days in all containers simultaneously. When the larvae began to swim [stage 25 (Gosner, 1960)], I started feeding them with food pellets (PLECO; Kyorin, Inc., Ltd., Hyogo, Japan). After the forelimbs grew out, the amount of water was reduced by half, and the container was slanted to make space for the toadlets to land. **(B)** Relative abundances of bacterial taxonomy at phylum level of Ichiniro pond water. Points 1 and 2 correspond to the rearing water's sampling location at Ichiniro-ike (**Supplementary Figure 1**).

TABLE 1 | Summary of the rearing experiment.

Water	Group size	Survival		Days to development of			SVL (mm ± SD)	Mass (mg ± SD)
		Rate	Days	Hindlimb	Forelimb	Toadlet		
Tap	1	0.58	39.1 ± 14.8	28.3 ± 1.6	35.2 ± 2.2	42.0 ± 1.2	11.1 ± 0.8	157.1 ± 71.7
	2	0.75	44.8 ± 10.0	27.7 ± 0.6	34.3 ± 1.0	41.4 ± 1.2	11.7 ± 1.3	178.7 ± 90.8
	15	0.87	41.9 ± 8.4	29.6 ± 2.1	36.1 ± 1.7	44.1 ± 1.4	12.5 ± 1.2	262.1 ± 51.8
	Total	0.81	45.8 ± 10.1***	29.1 ± 2.0*	35.6 ± 1.8***	43.4 ± 1.7***	12.2 ± 1.3	198.2 ± 60.6
Pond	1	0.58	35.1 ± 18.0	30.0 ± 0.0	36.4 ± 0.8	43.6 ± 1.1	11.7 ± 0.6	197.6 ± 68.2
	2	0.67	38.3 ± 17.6	30.6 ± 3.3	37.8 ± 4.4	43.9 ± 1.7	12.2 ± 0.4	194.3 ± 70.7
	15	0.53	26.5 ± 14.5	30.8 ± 1.2	38.2 ± 1.0	46.4 ± 1.0	13.0 ± 1.0	238.6 ± 44.5
	Total	0.56	37.6 ± 15.4***	30.6 ± 1.7*	37.8 ± 2.1***	45.6 ± 1.7***	12.4 ± 0.9	199.2 ± 72.8

Water, type of rearing water; group size, classification of rearing group; survival rate, alive days (mean ± SD, ends in 50 days). Days to development (mean ± SD): hindlimb, hindlimb emergence (stage 38, Gosner, 1960); forelimb, forelimb emergence (stage 42, Gosner, 1960); toadlet, metamorphosis completion (stage 46, Gosner, 1960). SVL, mean snout-vent length as a toadlet. Mass, mean body mass as a toadlet.$p < 0.05$; ***$p < 0.001$ (GLMM).*

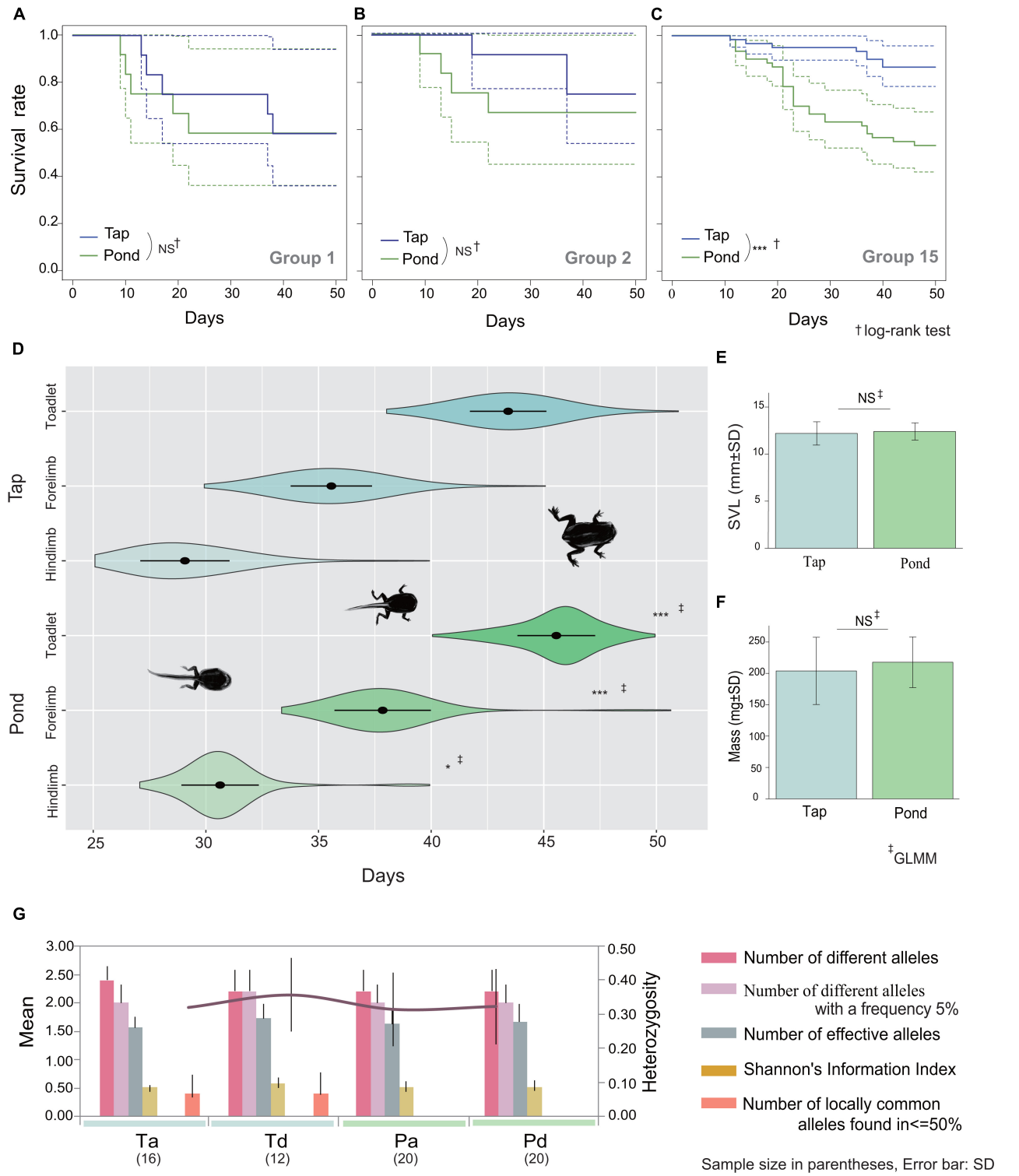

FIGURE 2 | Kaplan-Meier Curves of rearing tadpoles over 50 days for group sizes 1 **(A)**, 2 **(B)**, and 15 **(C)**. Dashed lines represent 95% confidence intervals. **(D)** Violin plot with mean and standard deviation for the days taken to develop between tap ($N = 72$) and pond ($N = 47$) waters, from left to right, hindlimbs emergence, forelimbs emergence, and completion of metamorphosis (toadlets). SVL **(E)** and mass **(F)** of toadlets. **(G)** Allelic patterns by category: Ta, tap water and alive; Td, tap water and dead; Pa, pond water and alive; Pd, pond water and dead. NS, not significant; *$p < 0.05$; ***$p < 0.001$. Detailed information on allele frequency is presented in **Supplementary Table 5**.

Group size was used as a random variable, while water type was a fixed explanatory variable. SVL and body mass were evaluated using a GLMM with Gaussian distribution and were used as response variables. The statistical analysis was performed using R 4.1.2 (R Core Team, 2021). The population genetic analyses between dead and alive individuals were carried out based on the MHC class II exon and four microsatellite loci by the program GenALEx v. 6 (Peakall and Smouse, 2006).

RESULTS

Table 1 shows the results of the rearing experiment. Water quality was significantly related to differences in larval survival: *B. japonicus* larvae were alive better when raised in tap water than in pond water ($z = 19.37$, $p < 0.001$). However, Kaplan-Meier estimator, the group size effect was evident only for group 15: the results of the log-rank test for group sizes 1, 2, and 15 were $p = 0.8$, $p = 0.8$, and $p < 0.001$, respectively (**Figures 2A–C**). The development rate was significantly lower in pond water (days to hindlimb, $z = -2.38$, $p = 0.017$; forelimb, $z = -3.83$, $p < 0.001$; toadlet, $z = -5.07$, $p < 0.001$; **Figure 2D**). Neither the SVL nor the body mass of the toadlets differed according to water type (SVL, df = 107.17, $t = -1.20$, $p = 0.23$; mass, df = 103.42, $t = -1.903$, $p = 0.06$; **Figures 2E,F**).

There was no association between genotype and the results of the rearing experiment (**Figure 2G**). The unique MHC alleles were not explicitly relevant to larval survival and the microsatellite genotype did not explain the differences according to viability and water type (**Figure 2G**, **Supplementary Figure 2**, and **Supplementary Table 5**).

DISCUSSION

Bufo japonicus larvae reared in pond water were more likely to die and developed more slowly than their siblings in tap water (**Table 1**). Large groups of tadpoles in pond water were at greater risk of death (**Figures 2A–C**). Changes in larval growth depended only on the water conditions (**Figure 2D**); no distinctive genotypes were detected in the individuals that died (**Figure 2G**). Two factors likely contributed to these results.

First, the pond water did not inherently favor the development of the larvae. Ichiniro pond water contains abundant microflora, particularly Proteobacteria and Bacteroidetes (**Figure 1B**). Some deaths may be attributable to the same disease because deaths in the group 15 often occurred in succession, suggesting them to be infection-related (**Figure 2C** and **Supplementary Table 1**). Twenty-one bacterial taxa were detected (**Figure 1B**); even if bacteria are linked to the cause of death, the bacteria responsible are likely to vary among groups, containers, and individuals. A wide variety of causes of death would also make it even more difficult to assess the association with genetic background, especially in the immune system associated locus, i.e., MHC genes. In addition, microflora can alter the chemical composition of the water (Garcia-Ochoa and Gomez, 2009; Garcia-Ochoa et al., 2010). The water quality testing showed that the biological oxygen demand of pond water was three times higher than that of tap water (**Supplementary Table 3**), which indicates the possibility of microbial modification. Deterioration of the quality of the pond water would delay the development of larvae.

Second, a local population decline could debilitate larvae. The number of *B. japonicus* in Ichiniro pond is decreasing rapidly, and at the time of sampling for this study, only one egg string was found. Therefore, the genetic diversity of toads at this site has decreased. In a previous rearing experiment using tap water conducted at the same site, the larvae had a higher survival rate [99% (Hase et al., 2022)] than in the present study (81%; **Table 1**). Even in the absence of pathogenic microorganisms, larval viability has decreased. Genetic deterioration is likely occurring in *B. japonicus* in this area due to the rapid population decline (Willi et al., 2006).

More research is needed to reveal the link between environmental microflora and animal health. Under optimal conditions, animals grown with symbiotic bacteria are protected from pathogens (Sommer and Bäckhed, 2013). Even in animal taxa that are not highly hierarchical, disease management efforts should consider behavioral traits such as grouping.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: OM994394–OM994396).

ETHICS STATEMENT

All tadpoles were treated carefully to minimise physical stress, in compliance with the ethical guidelines of the institute. After genotyping, all toadlets were released into their field near the home pond.

AUTHOR CONTRIBUTIONS

KH designed the research, conducted the experiments, ran the analysis, and prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.917067/full#supplementary-material

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