안득남석사학위논문

Relationship among call variables and environmental factors, call property differences between Bd infected and non-infected groups, and territoriality in *Hyla japonica*

청개구리의 영역성, 울음소리와 환경적 요인간의 관계, *Batrachochytrium dendrobatidis* (Bd) 감염군과 비감염군의 차이

2014년 8월

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이 논문을 이학석사 학위논문으로 제출함

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Abstract

Relationship among call variables and environmental factors, call property differences between Bd infected and non-infected groups, and territoriality in *Hyla japonica*

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Although there are different types of signals other than vocalization used in amphibian reproductive behavior, acoustic signal is one of the most common and dominant means of anuran amphibian communication and male frog's advertisement call plays an important role in mate choice. Nevertheless, sound production requires a lot of energy and male frog's sound producing behavior and acoustic signaling patterns can be influenced by many different intrinsic or exogenic factors. Meanwhile, chytridiomycosis, which is caused by *Batrachochytrium*

dendrobatidis (Bd) has led to dramatic population decline and extinction in several amphibian species since it was first found in the late 1990s. Although such massive population decline has not been reported in Korean amphibian species, recent studies documented certain level of Bd prevalence in Korea, and treefrog (i.e. *Hyla japonica*), one of the most commonly found anuran amphibian species in Korea was not an exception. Considering all these factors, Bd infection could have affected treefrog sound producing behavior or quality of acoustic signals. In this study, I mainly tried to see whether there is any difference in acoustic signals between Bd infected and Bd non-infected groups of individuals. At the same time, to figure out potential relationships among call properties and environmental factors, and territoriality of treefrog, additional analyses were performed.

In previous studies, female frogs exhibited preference towards certain qualities of call properties, such as call duration and dominant frequency, and these call characteristics were demonstrated as important criterion when it comes to female frog's mate choice. If there is any Bd-induced difference in treefrogs' advertisement calls, and such acoustic changes took place in the quality of major criterional call properties, this could possibly lead to female's frogs mate choice behavior and potentially affect overall sexual selection mechanism in treefrog reproductive behavior.

The result showed that note repetition rate and pulse repetition rate were in positive relationship with atmospheric temperature, and dominant frequency was in negative relationship with snout-vent length. Though this does not indicate any causal effect of environmental factors, we can expect that the speed of note production will move in the same direction as temperature changes. Also negative relationship between snout-vent length and dominant frequency is consistent with what was found from the preceding studies. In general anuran communication, the dominant frequency of sound is determined by the size, tension of the vocal cord and the force of air flow coming from the lung, and larger-size males are likely to have bigger and thicker vocal cord. Considering all these factors, dominant frequency in an inverse relationship with snout-vent length is quite predictable.

When compared call properties of Bd infected and non-infected groups, call duration showed quite notable difference between two groups. However, when t-test was performed to predict the difference between the populations of two groups, only NRR showed significant difference between them. Also when compared linear associations among call properties and environmental factors of two groups, the effect of ND (note duration) on NRR (note repetition rate) and the effect of AT (atmospheric temperature) on ND (note duration) were both greater in non-infected group. The result indicated that Bd infected individuals make notes at a lower speed than non-infected individuals and Bd infected individuals' call properties are less influenced by atmospheric temperature and note duration. This could suggest that some of the call properties might have been affected by Bd infection but still, further studies are required to figure out more precise relationship between Bd infection and quality of calls. Lastly, the coefficient of variation in rice paddy numbers was used to speculate on the territoriality of treefrog and 10 out of 21 recaptured individuals were found in the same rice paddy. Though this cannot be a profound evidence to show the territoriality of treefrog, we can expect that the range of their movement is somewhat limited.

Keyword : *Hyla japonica*, *Batrachochytrium dendrobatidis*, emerging infectious disease, amphibians, bioacoustics, territoriality

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1. Introduction

Anuran bioacoustic signaling has been well studied over many years and serves as a foundation for much of our understanding of animal communication. However, Korean amphibians have been little studied. Therefore, I decided to study the communication system of the common Korean treefrog, *Hyla japonica*. Such studies are especially timely now in light of recent international research suggesting that disease can lead to amphibian population declines.

Mate choice behavior and mating success is often determined by female choice and/or male-male competition. Among factors that could influence mate choice decisions such as call properties, size, color, calls produced by males play an important role in the case of female choosing its mate (Andersson 1994). The same was true in several North American treefrogs as well. In these species, male frogs' long-range advertisement calls serve as important criteria for mate choice and females show little evidence that any sensory cues other than acoustic signals are desirable measures for mate searching (Gerhardt and Huber 2002).

Nevertheless, there are some evidence and cases in which non-acoustic signals are adopted by males as a means of attracting female frogs as well. In case of a diurnal poison-arrow frog and some other anuran species, visual cues are used for reproductive behavior (Hödl and Amézquita 2001, Hartmann, et al. 2005, Summers, et al. 1999) and some aquatic and terrestrial amphibian species were found to use chemical cues to attract and locate potential mates (Pearl, et al. 2000, Belanger and Corkum 2009, Houck 2009, Byrne and Keogh 2007). Especially, the range of visual cues used by anuran amphibian species either in agonistic or mate attracting behavior varies widely, including toe trembling, arm waving, leg stretching, foot flagging, color changing, running and jumping, circling to name a few (Hödl and Amézquita 2001) and some of these frogs often use acoustic signals at the same time (Hödl and Amézquita 2001).

Male frogs' advertisement calls to attract female frogs and successfully achieve mating are costly to produce (Prestwich 1994, Prestwich, et al. 1989, Ryan 1988, Taigen and Wells 1985, Grafe and Thein 2001). Calling males will have to bear at least three threatening risks for the acoustic signaling, which are higher oxygen consumption, exposure to predation, and decline in body mass and condition (Bucher, et al. 1982, Ryan, et al. 1981, Arak 1983, Cherry 1993, Mac Nally 1981, Robertson 1986). Male frogs' oxygen consumption rate is a lot higher than that of resting male frogs (Prestwich, et al. 1989, Bucher, et al. 1982) and conspicuous male frogs' calls can attract predators and parasites like bats and flies feeding on frogs' blood (Jones, et al. 2013, Jones, et al. 2010, Bernal 2006, Page and Ryan 2005, Tuttle and Ryan 1981).

Various types of communication occur in human lives but exchanging messages in an effective and clear way that does not lead to misunderstanding is not easy and signaler and receiver are equally important. The same can be true in treefrogs' advertisement call but a wide range of environmental factors can take effect as well in this case. Among those factors, background noise produced by chorus of conspecific frogs and heterospecific species including insect, abiotic sounds coming from airplane overfly, and motorcycles were demonstrated as influencing factors on male frogs' calling patterns in the previous studies (Wollerman and Wiley 2002, Wollerman 1999, Schwartz 1993, Wong, et al. 2009, Sun and Narins 2005, Lingnau and Bastos 2007). Climatic variables such as water temperature, time of day, moon illumination, rainfall, barometric pressure had statistically significant effects on calling activities of male frogs in the preceding studies (Oseen and Wassersug 2002, Brooke, et al. 2000). Also temperature shift can make changes in female frogs' preference (Gerhardt and Mudry 1980).

What would happen if a caller is infected with Bd then? Would this fungal infection have any effect on male treefrogs' call characteristics? To answer these questions, we need to understand both sound producing mechanisms and impact of Bd infection on amphibian species.

Any type of call produced by male frogs is a result of elaborate collaboration among morphological structures that controls the air flow traveling between lung and buccal cavity through the glottis (Schmidt 1965, Martin and Gans 1972, Emerson and Boyd 1999). Several structures play an important role in sound production of anuran amphibians. These include abdominal muscles, laryngeal muscles, vocal cords, glottis, buccal cavity, and vocal sac. External and internal abdominal muscles push air out of the lungs and the air moves up along the trachea and reaches the larynx, which has laryngeal muscles that control opening and closing of the glottis. The sound is produced when the air stream make the vocal cords vibrate while it is passing through the glottis (Emerson and Boyd 1999, McClelland, et al. 1996).

Ultimately, anuran vocalization is a result of contraction in laryngeal muscles and abdominal muscles (Tobias and Kelley 1987) and behind this morphological coordination, neural and hormonal mechanisms lie. The aforementioned muscles receive input signals from the central nervous system. While neurons that control laryngeal muscle movements are located in the brain stem, abdominal muscle movements are controlled by spinal motor neurons (Emerson and Boyd 1999). Another requisite of anuran amphibian sound production is gonadal steroids (Emerson and Boyd 1999). In the case of the *Xenopus laevis* mate call, male frogs were strongly influenced by androgen regulation and castrated males stopped calling within 3 weeks and resumed calling after the androgen treatment (Tobias and Kelley 1987, Wetzel and Kelley 1983).

Amphibian species in South Korea were first found to be infected with *Batrachochytrium dendrobatidis* (Bd) in 2009 and among all the infected species, *Hyla japonica* belonged to one of the highest prevalence group (Yang, et al. 2009). Though this study demonstrated that some of endemic Korean amphibian species are susceptible to Bd for the first time, the sample size was not large enough to represent overall anuran amphibian population in Korea. According to a recent study, 17.7% (330 out of 1863) of sampled individuals were tested Bd positive and Bd was detected in 52.4% of sampling sites. Among 13 native and one introduced species from which Bd was detected, salamanders (i.e. *Hynobius leechii, Karsenia koreana*) recorded the highest prevalence rate and 53 out of 403 *Hyla japonica* individual samples were Bd positive with prevalence rate of 13.1% (Bataille, et al. 2013).

Bd is the first member of the of Chytridiomycota phylum that is known to be parasitic on vertebrate animals among, and the fungus often is detected on the skin of metamorphosed amphibian and mouthparts of tadpoles where keratinocytes are found (Carey, et al. 2006, Berger, et al. 1998, Rachowicz and Vredenburg 2004). Clinical signs that Bd infected frogs might display include red spots, skin darkening, dorsal side skin shedding, and red coloration on ventral side skin, (강원대학교양서파충류연구실 2009) but typical types of signs observed in Bd infected metamorphosed amphibians are hyperplastic skin, hyperkeratosis (Carey, et al. 2006, Berger, et al. 1998) and tadpoles might exhibit malformation and loss of keratinized mouthparts (Smith, et al. 2007). Although the underlying defence mechanism of Bd needs further research, Bd was proved to be a primary cause of death in experiments with different host species (Garner, et al. 2009, Blaustein, et al. 2005) and there are several hypotheses established to explain the host response to Bd and mortality of Bd infection. Those are lethal toxins produced by Bd, (Blaustein, et al. 2005) impaired osmoregulation resulted from interference with water uptake (Berger, et al. 1998), and decreased plasma electrolyte level (Voyles, et al. 2007).

The outcome of Bd infection, however, is not consistent over populations, regions, species and various factors such as temperature, innate defenses, habitat, and host life history have been demonstrated to affect the magnitude of Bd infection severity (Berger, et al. 2004, Woodhams, et al. 2007, Kriger and Hero 2007, Lips, et al. 2003).

In conclusion, all of the factors I discussed before can possibly affect frogs' sound production and some of it might affect the quality of sound produced by male frogs and make the communication between the two sexes less effective. But still, there has been no study performed to investigate the relationship between Bd infection and sound production of amphibians. Such lack of attention became a main source of motivation for this research.

To verify this hypothesis, I quantified treefrogs' call properties; made a series of comparisons between Bd infected and non-infected groups of individuals' call characteristics; and tried to find corroborating evidence that supports the results of my analyses. Also to further discuss treefrogs' calling behavior and their ecology, field observations and distinctive call patterns were included.

2. Materials and methods

Sampling location and sample size

I recorded a total 59 males' advertisement vocalizations, with 15 males being recorded multiple times, and a total 115 call recordings were made. I recorded the frogs in a rice paddy in Keumgok-dong, Namyangju-si, Kyungki-do, Korea (Latitude: 37.63751963682362, Longitude: 127.22045660018921). The owner of the rice paddy uses organic farming techniques by introducing many freshwater snails instead of pesticide. Thanks to such environmentally friendly farming practice, this 3000 m^2 of artificial wetland is very rich in species and appeared to maintain a healthy ecosystem. On one hand, this could be helpful because such environment could create an ideal breeding site for *Hyja japonica* but on the other hand, this could make a negative impact on overall sampling work by generating unexpected variables.

To record frog calls, I used a Marantz PMD661 recorder and Sennheiser ME80 directional microphone with a Sennheiser windscreen and a small flashlight to locate the frog. The directional microphone with windscreen not only helped us record each targeted frog's call clearly even when there was unnecessary sound, such as faraway trucks, and other amphibian species calling, but also secure enough distance from a frog while minimizing the amount of unwanted noise being recorded due to blowing winds.

Usually, recording started after 8PM and went on until midnight or after from 4th of June 2011 until 15th of Aug 2011, but I tried to visit the site frequently and collect as much data as possible by staying in the field at least 2-3 hours each day unless it was heavily raining. Also I tried to record 10 calls (1 call = start calling until stop calling) per each frog and the duration of each call ranged from just 10-15 seconds to over 15 minutes. The distance between a frog and the microphone was kept at about 20 cm or less and each recorded call was given specific numbers consisting of a combination of numbers given to each rice paddy where an individual was found calling and clipped toe number before being saved to a SD card. The purpose of toe clipping was to do further research on MHC typing and frog calls, but unfortunately, the research is still in process of developing optimal genetic markers and will not be include in this thesis.

Fieldwork – swabbing and tissue sampling

Finally, when sound recording was finished, I caught the frog with a new pair vinyl gloves on each time to prevent contamination, measured the snout-vent length from the ventral side using sterilized inflexible ruler, then swabbed and toe-clipped the frog before releasing it back to where it was first found.

For the swabbing and toe-clipping, I used 1.5mL microcentrifuge tubes with 70% EtOH, sterilized swabs, an alcohol-proof pen to write on tubes, scissors, spray bottle with 70% EtOH, and Kimwipe. To prevent contamination, scissors were sterilized each time with 70% EtOH and swabbing was administered all

Figure 1. The study site and rice paddy numbers

around the frog, focusing on the belly, thigh, and webbing. The number of clippedtoes from each hand and foot did not exceed 1 and a total of 2 toes were collected from each individual. Clipped toes were then put into 1.5mL microcentrifuge tubes and 70% EtOH was added for the preservation of tissue. Though labeled tubes were kept in a cooler while staying in the field, these tubes were moved into the freezer and kept at -20℃ as soon as possible.

Bd infection detection

Since this research was designed to make comparisons between males' advertisement call characteristics and Bd infection status, only swabs of individuals whose call recordings were of good quality could be used for Bd infection detection. Therefore, the final number of swabs available decreased from 59 to 42.

For DNA detection, the first part of Bd infection detection, 50 μL of Prep Man Ultra was added to each centrifuge tube with the swab and heated for 10 minutes at 100 °C. After being kept at room temperature for 2 minutes, the tubes were put into a centrifuge, run at 13,000 rpm for 3 minutes, after which the supernatant was placed into second tube was stored at -20°C for nested PCR.

Ensuing nested PCR started with 1:4 dilution of extracted DNA and distilled water. Then 2 μL of diluted DNA was mixed with 13.8 μL of distilled water, 2 μL of buffer, 1.2 μL of dNTP, 0.4 μL of primer 1(bd18SF), 0.4 μL of primer 2(bd28SR), and 0.2 μL of Taq DNA polymerase. After going through each temperature set of a thermocycler, samples were prepared for the second PCR. The following second PCR targeting narrower region was done with master mix of the same materials and ratio, but different primers (bd1a, bd2a) were used this time. Then, tubes were put into a thermocycler and run at programmed cycles of different temperatures. Except for denaturation phase (94℃ for 30 min.), PCR products were exposed to 30 cycles of different temperature ranges each (1st PCR : 94℃ for 30 sec., 50℃ 30 sec., 72℃ 2min., 72℃ 7min.; 2nd PCR : 94℃ for 45 sec., 60℃ 45 sec., 72℃ 1min., 72℃ 7min.) and hold at 10℃. The final PCR products were loaded into agarose gel stained with EtBr and visualized through electrophoresis.

Sound analysis

The software used for the sound analysis is Raven Pro version 1.4 (Laboratory of Ornithology, Cornell University) and I measured 9 call properties. The following are the terms I used and corresponding screenshots of each call characteristics that I measured.

- **PN**: Number of Pulses per each note. Pulse is the most fine-scaled call property and PN was manually counted from waveforms on the screen for 10 notes in the middle of each call.

- **PRR**: The equation used for the calculation of Pulse Repetition Rate is ((PN-1)/Note Duration) (Park, et al. 1996).

- **FII**: Final Inter-pulse Interval for 10 middle notes from each call was measured

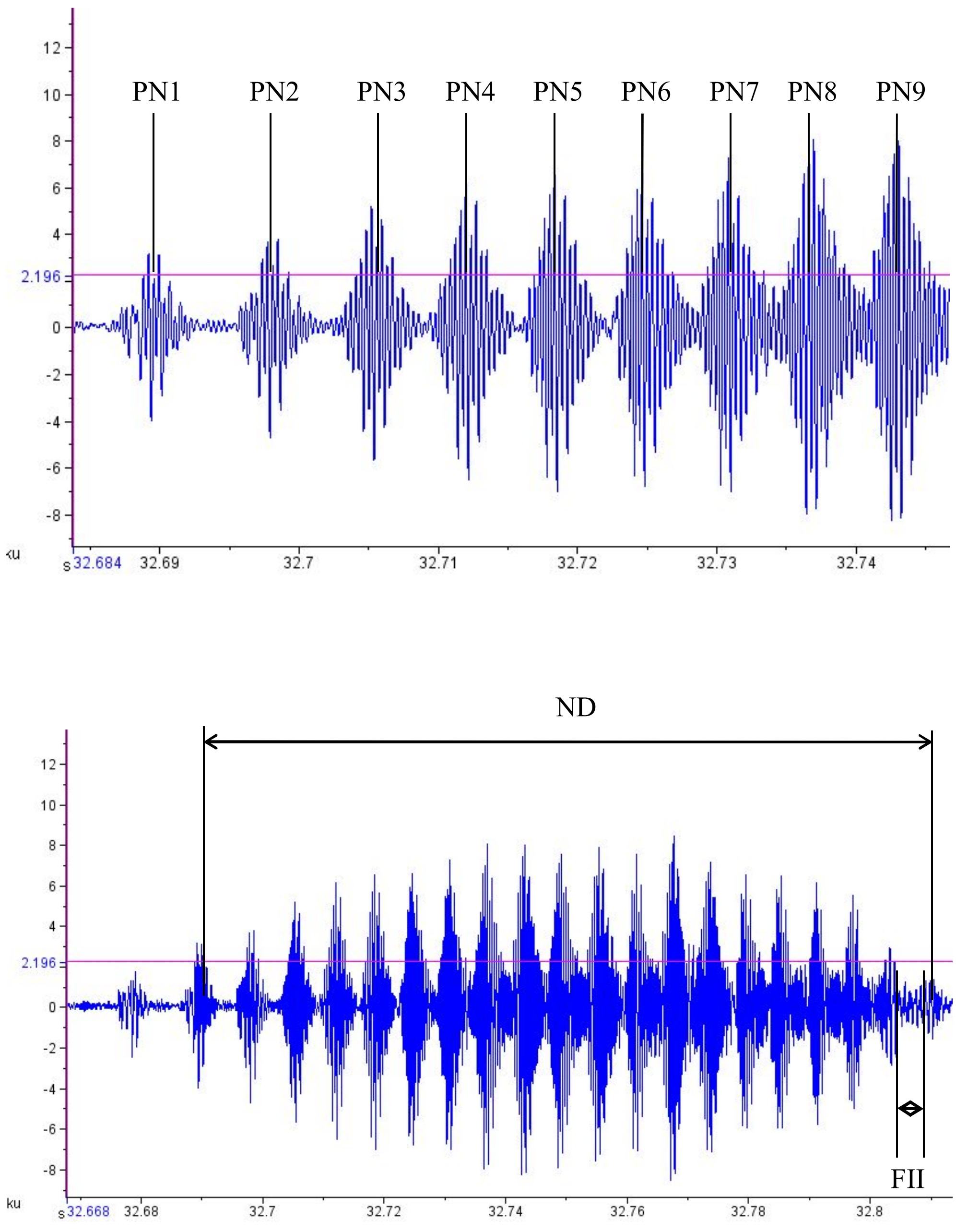
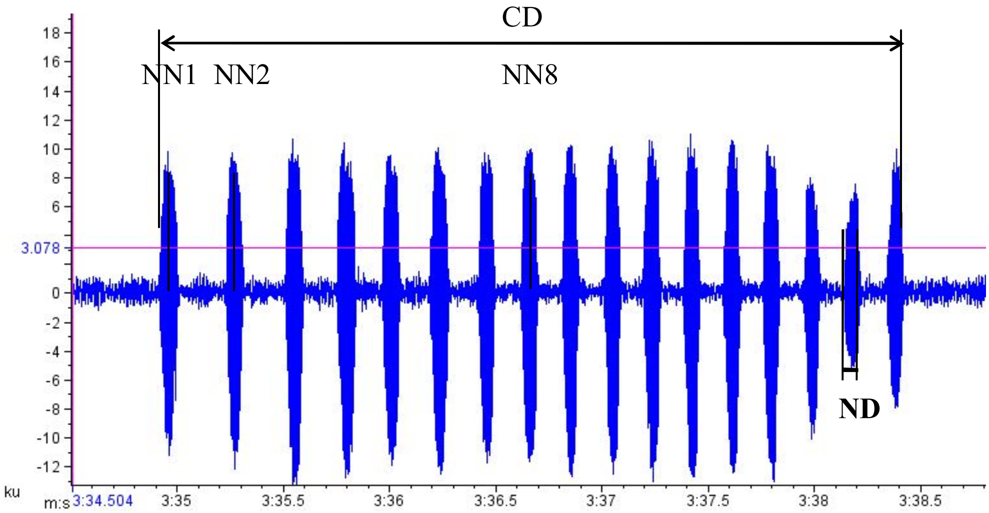


Figure 2. Acoustic elements of *Hyla japonica* advertisement call. Wave form of (Upper) 9 pulses (Lower) a single note containing 19 pulses.



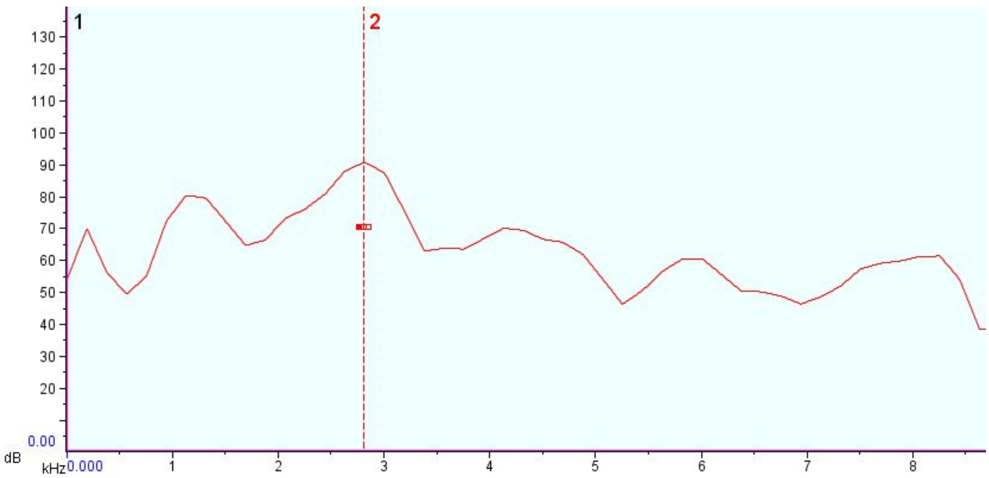


Figure 2-1. Acoustic elements of *Hyla japonica* advertisement call. (Upper) Wave form of a single call containing 17 notes. (Lower) A part of a spectrogram of a note. The peak (dotted line) represents a dominant frequency of 2812.5Hz.

manually.

- **NN**: Number of Note for each call were also manually counted from the waveform on the screen.
- **ND**: Note Duration was measured in seconds and corresponding value for each note was read from spectrum view. Though there is no universally agreed terminology for note, McLister et al. (1995) defined this vocalization element as “the sound units produced by single expiratory events”. In layman’s words, one note is equivalent to one time of “ribbit” or one time of “gaegul” in Korean.
- **NRR**: The equation used for the calculation of Pulse Repetition Rate is ((PN-1)/Note Duration).
- **DF**: Dominant Frequency of each Note measured in Hz were read directly from each spectrogram.
- **R/F**: The ratio between Rise time and Fall time was calculated to quantify the morphological difference among notes of individuals.
- **CD**: Call Duration represents the amount of time consumed by each individual to make each call and Raven automatically shows the value for the selected section of the waveform.

Statistical analysis

All statistical analyses were performed using SPSS (version 21).

Pearson product correlation was used to look for potential relationships between call variables and environmental factors. This was not only to check the direction and strength of such association between two factors, but also to identify and control the environmental factors (temperature, snout-vent length) that might correlate with call properties. Those call properties that are correlated with either temperature or snout-vent lengths were adjusted at the mean temperature or snout-vent length using a regression equation. For example, the equation used for the adjustment of PRR is as follows:

PRR = 68.2 + 5.79 AT

Average temperature : 21.36 °C

Adjusted PRR = Actual PRR + ((68.2 + 5.79*(21.36))-(68.2 + 5.79*(actual temperature)))

To determine whether call properties significantly differed between Bd infected and non-infected individuals, two-sample t-tests were used. Then the regression lines of two groups were compared to further investigate differences between the groups.

Coefficient of variation, which is a variation in relation to the mean were calculated to see the territoriality of *Hyla japonica* and the numbers of rice paddies where frogs were caught were used for the calculation.

3. Results

Bd infection detection

Among 42 individual samples, 9 tested Bd positive. Nested PCR was performed on each sample twice to increase the detectability and samples whose PCR results showed positive bands at least once out of two times of nested PCR were considered infected individuals.

Relationship among call variables and environmental factors

Eighteen significant correlations were found between pairs of a call variable and an environmental factor or two call variables (Table 1). For the visual representation of such relationships, scatterplots of significantly related pairs (at the 0.01 level) are included above (Figure 3, Figure 3-1, Figure 3-2, Figure 3-3).

Although it may seem that many of the variables are correlated with each other, we have to note that non-independent variables must be eliminated to gain an undistorted picture of overall correlations. There were two equations used to calculate the repetition rate of pulses and notes, which are PRR = (PN-1) / ND, NRR = (NN-1) / CD. Therefore, the relations found in pairs of PN (pulses per note) and PRR (pulse repetition rate); ND (note duration) and PRR (pulse repetition rate); NN (notes per call) and NRR (note repetition rate); and CD (call duration) and NRR (note repetition rate) should not be considered meaningful.

Table 1. Pearson product correlation between call variables and environmental factors (N=42)

Abbreviations: AT (atmospheric temperature), SVL (snout-vent length), PN (pulses per note), PRR (pulse repetition rate), FII (final inter-pulse interval), ND (note duration), NRR (note repetition rate), DF (dominant frequency), NN (notes per call), RF (rise/fall time ratio), CD (call duration)

	AT	SVL	PN	PRR	FII	ND	NRR	DF	NN	RF	CD
AT											
SVL											
PN	-.278	-.055									
PRR	**.626****	-.038	**.311***								
FII	-.274	-.124	.032	**-.349***							
ND	**-.767****	-.099	**.581****	**-.555****	**.382***						
NRR	**.659****	.132	**-.553****	**.351***	**-.373***	**-.787****					
DF	.282	**-.406****	.187	.298	-.018	-.052	.052				
NN	.005	-.047	.060	-.119	.270	.186	-.029	-.042			
RF	.049	-.121	**.332***	.288	-.136	.044	-.118	**.392***	-.114		
CD	-.109	-.071	.146	-.181	**.323***	**.314***	-.191	-.055	**.986****	-.098	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

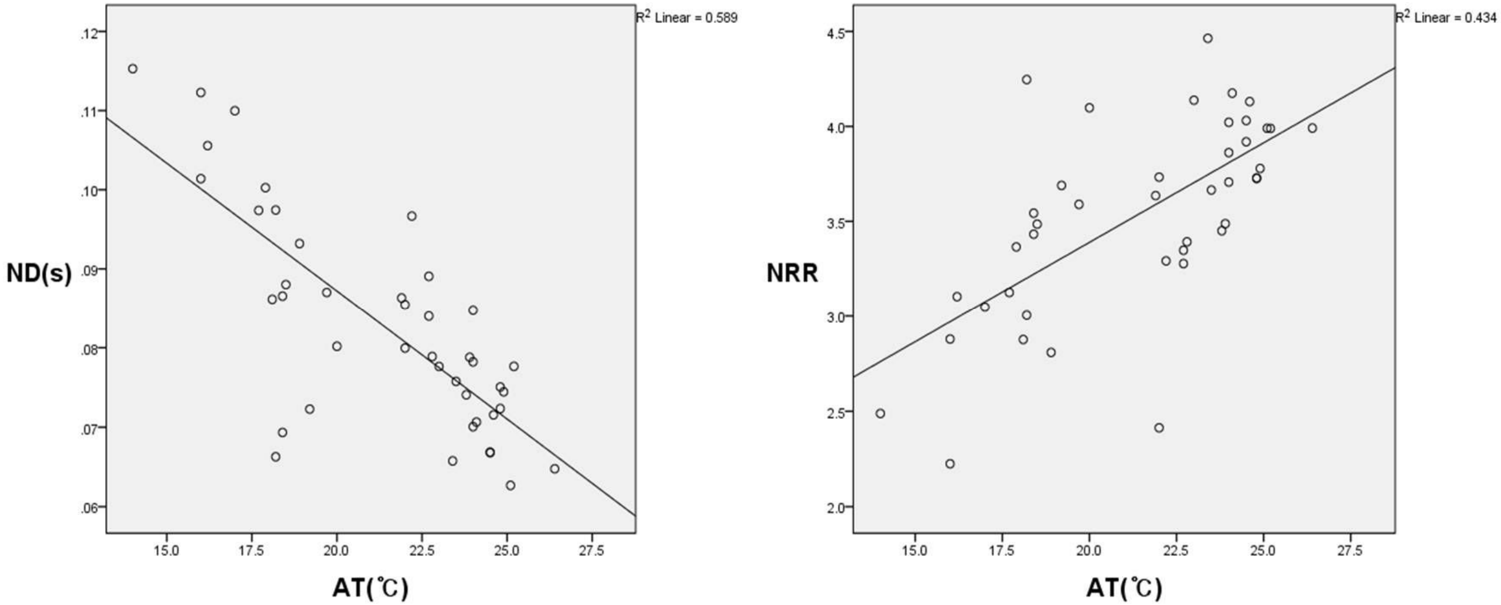


Figure 3. Relationships between AT (atmospheric temperature) and ND (note duration), AT (atmospheric temperature) and NRR (note repetition rate)

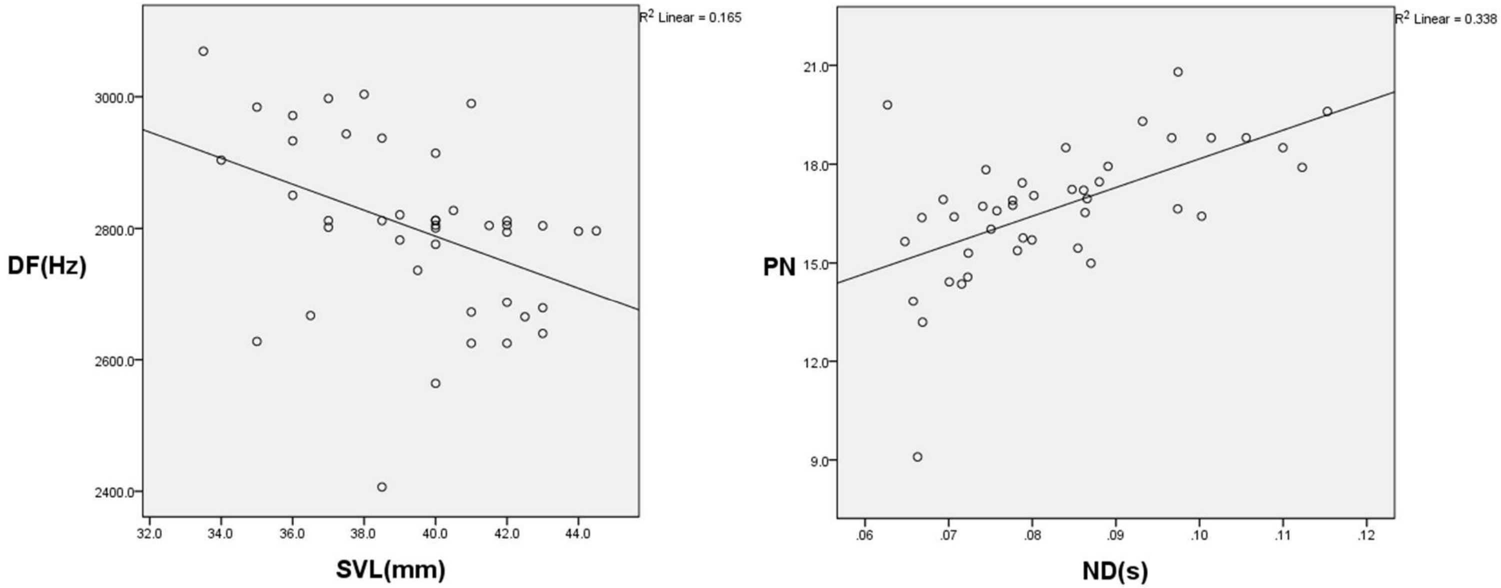


Figure 3-1. Relationships between SVL (snout-vent length) and DF (dominant frequency), ND (note duration) and PN (number of pulses per note)

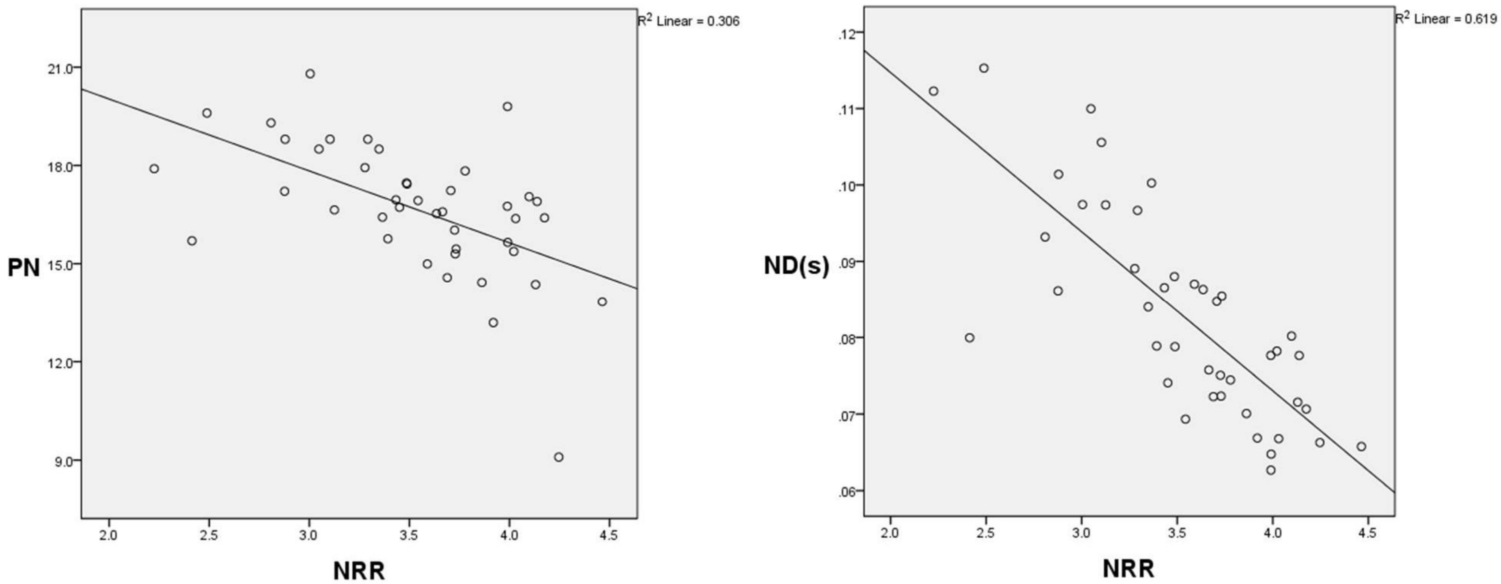


Figure 3-2. Relationships between NRR (note repetition rate) and PN (number of pulses per note) and NRR (note

repetition rate) and ND (note duration)

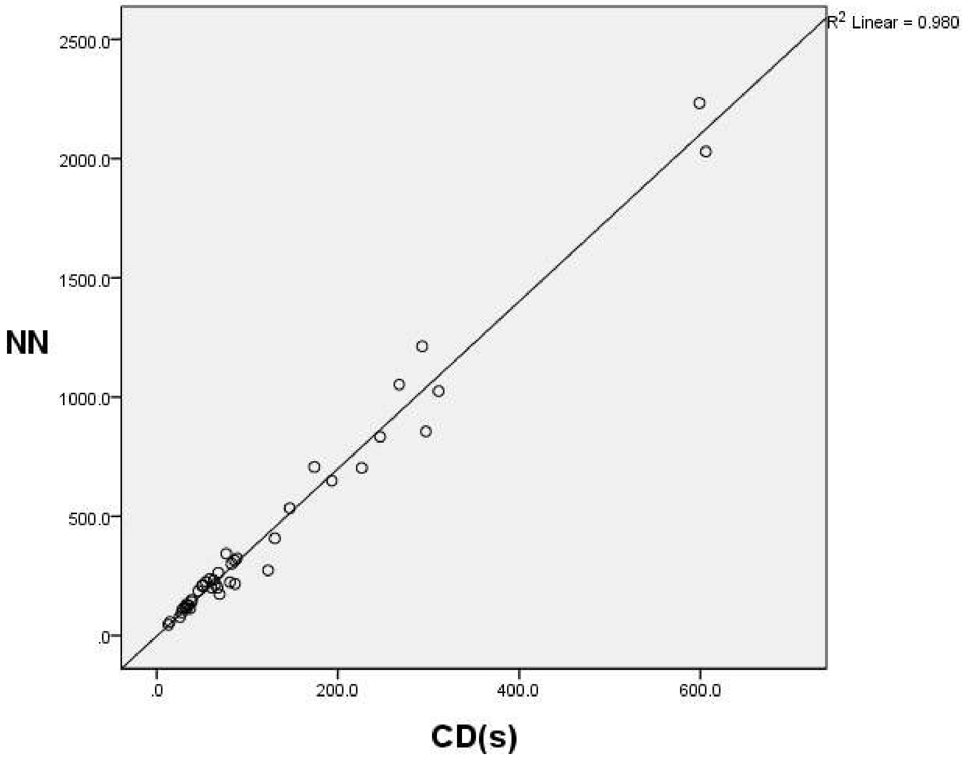


Figure 3-3. Relationships between CD (call duration) and NN (number of notes per call)

While AT (atmospheric temperature) was positively related with NRR (note repetition rate), a negative relationship was found between AT and ND (note duration). Also SVL (snout-vent length) was in negative relationship with DF (dominant frequency). Notes and calls with longer durations tended to have larger number of pulses and notes, and ND (note duration) and NRR (note repetition rate) were negatively related.

Call property and Bd infection

Looking at group statistics, it was quite notable that CD (call duration) of Bd infected and Bd non-infected groups exhibited difference (Table 2). When independent samples T-test was performed to see whether there is a population mean difference between Bd infected and Bd non-infected groups, Only NRR of Bd infected group (3.256±0.409) was significantly lower than Bd non-infected group (3.580±0.359) ($P = 0.025$, Table 2-1)

Linear association between ND (note duration) and NRR (note repetition rate); AT (atmospheric temperature) and ND (note duration) in regard to BD infection status were different (Figure 4). Though NRR decreased as ND increases, the effect coming from the ND was greater in non-infected group. Likewise, though ND decreased as AT increased in both groups, the influence of AT was bigger in non-infected group.

Hyla japonica territoriality

Twenty-one individuals were captured more than twice and the number of rice paddies where they were caught and coefficient of variation in rice paddy numbers within individual are shown in Table 3. Although this might not prove *Hyla japonica*'s territoriality, the same individuals were very often caught in the same rice paddy (10 out of 21) and it seemed some individuals were returning to their previous location even after they had left in the middle of recording (Personal observation).

Table 2. Group statistics of Bd infected and Bd non-infected group.

Group Statistics

BD		N	Mean	Std. Deviation	Std. Error Mean
SVL	Infected	9	40.167	2.2079	.7360
	Non-infected	33	39.242	2.9131	.5071
PN	Infected	9	17.2194	1.14371	.38124
	Non-infected	33	16.5123	2.22911	.38804
PRR	Infected	9	193.1069	15.22892	5.07631
	Non-infected	33	191.3789	25.44215	4.42891
FII	Infected	9	.0023	.00066	.00022
	Non-infected	33	.0024	.00085	.00015
NN	Infected	9	590.0560	653.48041	217.82680
	Non-infected	33	381.0870	430.90797	75.01145
ND	Infected	9	.0832	.00690	.00230
	Non-infected	33	.0827	.01513	.00263
NRR	Infected	9	3.2554	.40905	.13635
	Non-infected	33	3.5804	.35944	.06257
DF	Infected	9	2833.2199	94.58473	31.52824
	Non-infected	33	2789.9876	129.98045	22.62669
RF	Infected	9	1.9019	.65316	.21772
	Non-infected	33	1.9640	.88286	.15369
CD	Infected	9	172.5779	191.42592	63.80864
	Non-infected	33	108.1610	118.15528	20.56819

Table 2-1. Result of independent samples T Test

Independent Samples Test

	T-test for Equality of Means					
	t	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval	
					Lower	Upper
SVL	0.882	0.383	0.924	1.048	-1.193	3.042
PN	0.914	0.366	0.707	0.774	-0.857	2.271
PRR	0.193	0.848	1.728	8.933	-16.325	19.781
FII	-0.243	0.809	0.000	0.000	-0.001	0.001
NN	1.149	0.257	208.969	181.891	-158.646	576.584
ND	0.944	0.351	0.003	0.003	-0.004	0.010
NRR	-2.336	0.025	-0.325	0.139	-0.606	-0.044
DF_N	0.929	0.358	43.232	46.523	-50.794	137.258
RF	-0.196	0.845	-0.062	0.317	-0.702	0.578
CD	1.260	0.215	64.417	51.145	-38.950	167.784

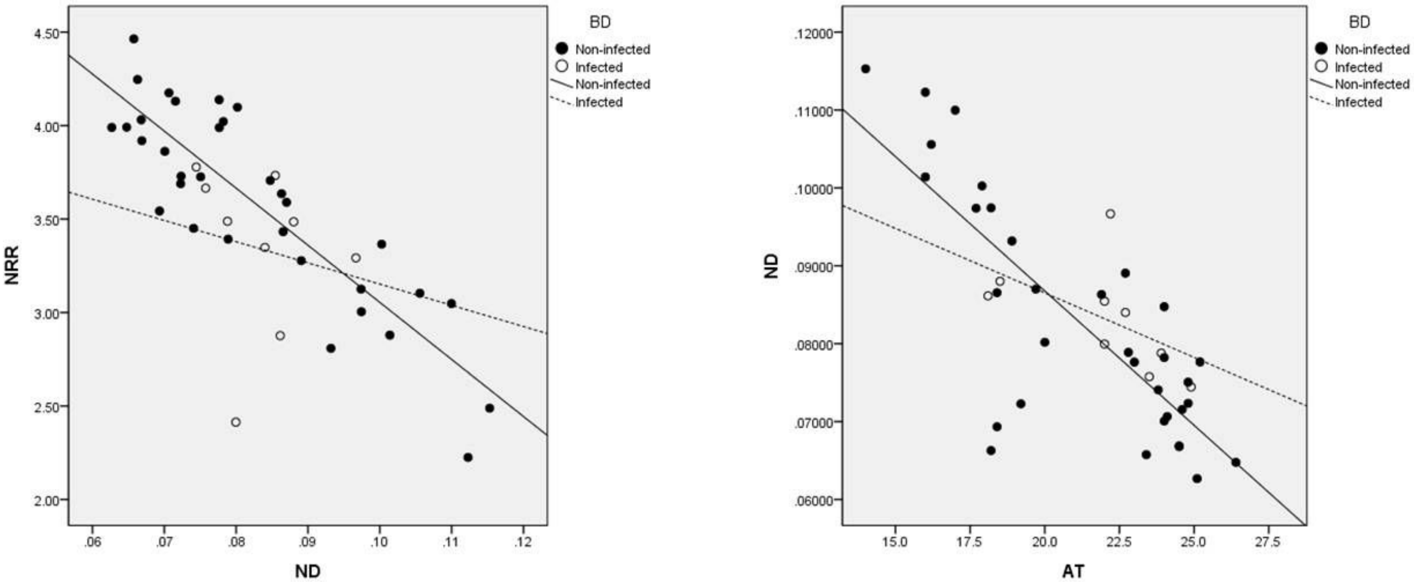


Figure 4. The effect of ND (note duration) and AT (atmospheric temperature)on NRR (note repetition rate) and ND (note duration) in Bd infected and Bd non-infected groups.

Table 3. Rice paddy number of recaptured individuals

Toe No.	Rice paddy No. 1st capture	Rice paddy No. 2nd recapture	Rice paddy No. 3rd recapture	Rice paddy No. 4th recapture	CV (coefficient of variation, %)
1-5	1	3	1		69.3
1-9	1	1	2	2	38.5
1-10	2	3	3	3	18.2
1-13	1	1			0.0
1-17	5	1			94.3
1-18	1	1			0.0
2-5	1	3			70.7
2-7	4	4	4		0.0
2-17	2	4			47.1
3-5	4	3			20.2
3-6	3	3	3		0.0
3-9	5	4			15.7
3-10	3	2	3	3	18.2
3-11	3	3			0.0
3-13	3	3			0.0
3-14	3	2	2		24.7
3-15	2	2			0.0
3-17	2	3			28.3
4-9	3	3	3		0.0
4-18	3	3			0.0
5-9	3	3			0.0
				CV Mean	21.2

4. Discussion

Relationship between environmental factors and call properties

The result from the Pearson product correlation demonstrated that some *Hyla japonica* call properties were correlated with atmospheric temperature. Considering that treefrogs are poikilotherms, some of the acoustic components would be influenced by atmospheric temperature (Gerhardt and Huber 2002). Therefore, measuring the cloacal temperature would have been more accurate in terms of examining the relationship among acoustic units and temperature but still, several relations were observed between atmospheric temperature and call variable measurements.

While PRR (pulse repetition rate) and NRR (note repetition rate) tended to increase with rising temperature, ND (note duration) was negatively related with atmospheric temperature. This could mean that the direction of NRR and ND variation would be different when there is a temperature shift. Given that NRR is production speed of a note in a broad sense and ND is a temporal size of note, we can intuitively conclude that this is correct. Additionally, though it was not very strong, a negative relationship between SVL (snout-vent length) and DF (dominant frequency) was observed and this is consistent with what previous studies have found (Zweifel 1968, Zimmerman 1983, Wagner Jr 1989).

Gerhardt (2002) once suggested that tension on the vocal cords, signaler's position in the air stream, pressure of the driving force of air coming from the lungs are thought to be primary determinants of dominant frequency (Gerhardt and Huber 2002). The point we have to note here is that the actual relationship might exist between the dimensional structure of sound producing organs and DF, not between SVL and DF. This might provide a partial explanation for the relatively low correlation efficient found between SVL and DF. Indeed, some anuran amphibians' calls display dominant frequencies lower than what can be expected from their body size (Gerhardt and Huber 2002).

BD infection and call property

NRR, which means the number of note repetitions in a single unit time can, represents the speed of note production. Thus independent samples T-test result can draw a conclusion that Bd infected population from which the samples were collected would make notes at a lower speed than Bd non-infected population in average. Earlier, I discussed that calling male frogs' oxygen consumption is a lot higher than that of resting males. Taking into account the fact that male frogs are heavily dependent on aerobic metabolism (Ryan 1988), this could mean that Bd infected individuals' oxygen consumption and metabolism efficiency or rate is lower than that of non-infected individuals. Hypothetically, this suggests that Bd itself, or host response to Bd infection might have inhibited such energy extracting pathways and led to slower production of notes.

Also, it possible to conjecture that individuals who make lower-speed notes were infected by Bd in premetamorphic stage. Parris and Cornelius(2004) demonstrated that Bd infected tadpoles body mass at the time of metamorphosis was smaller and larval-period length was longer than non-infected tadpoles. They suggested that such developmental difference between two groups might have resulted from poorer life-history performance induced by Bd infection. If this can be applied to *Hyla japonica*, those who experienced Bd infection in earlier stage of development might have also gone through underdevelopment of organs involved in sound production.

Given that anuran amphibian advertisement calls are under the effect of diverse biotic and abiotic factors, we can assume that the health status of frogs would affect the mate attracting signals. Nevertheless, what I have found out from this research project can be further developed through additional studies. Most importantly, the advertisement call is for mate attraction and its value can be realized by female choice. With the female frog's preference taken into account, the result would be more interesting and meaningful.

Hyla japonica territoriality

Territoriality of male frogs has been demonstrated in former studies already (Martins and Haddad 1988, Fellers 1979, Toledo and Haddad 2005, Zina and Haddad 2005) and such defensive behaviors are known to be common in amphibian species with prolonged breeding season (Vilaça, et al. 2011). Though when and how male frog's territorial defense mechanism is activated would be different, there are several direct and indirect benefits a male frog is expected to obtain from keeping a specific area for himself. Obviously, a male frog will be able to have less number of competitors around him and increase the success rate of mating. Also a male frog will be able to occupy more advantageous spot for signaling and make his acoustic message travel further, and in result, attract more female frogs to his territory.

Many insects and anuran species are good examples of acoustic communication and such mate-attracting functions of their sound signals have been proved long ago. Yet, considering that high-frequency signals are more likely to be attenuated and degraded and small-sized signalers generally produce relatively high-frequency sounds (Wiley and Richards 1978, Bennet-Clark 1998), insects and anurans need special tactics to overcome such constraints. In case of insect species, using plant leaves as baffles (Forrest 1991) and singing at an elevated location (Arak and Eiriksson 1992) were reported before.

Though the result from the correlation analysis presented a negative relationship between DF (dominant frequency) and SVL (snout-vent length), which could mean that there is an intrinsic limits on the quality of sound *Hyla japonica* produce, and still, there is a possibility of the frogs using surrounding vegetation or other environmental features as means of improving sound transmission efficiency. Particularly, male frogs who are older and more familiar with the conditions of breeding sites by participating in mate choice competitions taking place in the same sites more frequently might have preferred singing spots. If this is true, more convincing explanation will be available for the result.

However, the sizes of rice paddies were different and the numbers do not represent the exact location of individuals at the time of recapture. Therefore, additional field observation and sampling is needed to examine the size of territory or territoriality of *Hyla japonica* precisely.

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초 록

청개구리의 영역성, 울음소리와 환경적 요인간의 관계, *Batrachochytrium dendrobatidis* (Bd) 감염군과 비감염군의 차이

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양서류의 생식 행동에 있어 무미양서류는 음향 신호를 이용한 의사소통이 가장 지배적이며, 수컷 개구리의 교미 음성은 짝짓기에 있어서 중요한 역할을 하는 것으로 알려져 있다. 소리생성에는 많은 에너지가 요구되며 소리생성에 수반된 행동과 발생된 음향 신호 패턴은 다양한 내.외적 요인에 의해 영향을 받을 수 있다. 한편, 항아리곰팡이 (*Batrachochytrium*

dendrobatidis, Bd)에 의해 발병하는 항아리곰팡이병 (chytridiomycosis)는 1990년대 후반에 최초로 보고된 이 후 여러 양서종의 극적인 개체수 감소와 멸종을 일으켰다. 최근 국내 양서종에서도 Bd에 감염이 확인되었으나 대규모의 개체수 감소가 보고된 바는 없다. 국내에서 가장 흔히 발견되는 무미양서종인 청개구리 (*Hyla japonica*) 역시 감염이 확인되었다. 본 연구에서는 Bd의 감염이 청개구리 소리생성행동과 음향신호의 품질에 미치는 영향을 확인하고자 했다. 또한 소리특성, 환경요인, 청개구리의 세력권 간의 잠재적인 관계를 파악하기 위해 추가적인 분석을 수행하였다.

이전 연구들을 통해 암컷은 수컷의 소리지속시간과 지배적 주파수 파장 등과 같은 특정 소리 특성에 선호도를 보였으며, 이러한 특성은 암컷의 배우자 선택에 중요한 요소임이 밝혀졌다. 이를 바탕으로 Bd 감염이 청개구리의 교미음성에 영향을 주었다면 이는 암컷 개구리의 배우자 선택에 영향을 주어 청개구리 생식행동에 있어서 성선택 방법에 영향을 줄 수 있다. 실험 결과는 소리 반복률과 파동 반복률은 대기 온도에 비례하며, 지배적 주파수는 입-항문 길이와 반비례 하였다. 이 결과는 환경 요인에 따른 영향을 말해주진 않지만, 소리 생산 속도는 온도 변화와 같은 방향으로 움직일 것으로 예상할 수 있다. 또한 입-항문 길이와 지배적 주파수의 반비례하는 경향은 이전 연구 결과와 일치하였다. 일반적으로

무미양서종의 소리에서 지배적 주파수는 성대의 크기와 탄력, 폐에서 나오는 공기의 힘에 의해 결정되며 이 때 크고 탄력적인 성대는 수컷의 크기에 의존적일 가능성이 크. 이를 고려할 때, 지배적 주파수와 입-항문길이 간의 반비례 관계는 예측 가능하다. Bd감염에 따른 소리 특성의 변화를 보면 소리 지속시간은 감염 집단과 비감염 집단 사이에 상당한 차이를 보였다. 그러나 t-test 결과는 오직 NRR (소리반복률)만이 상당한 차이가 있음을 보여주었다. 또한, 두 그룹의 소리 특성과 환경 요인 간의 선형관계을 비교했을 때, NRR에 대한 ND (소리 지속시간)의 영향과 ND (소리 지속시간)에 대한 AT (대기 온도)의 영향은 모두 감염되지 않은 그룹에서 더 컸다. Bd 감염 개체들이 감염되지 않은 개체들 보다 더 낮은 속도로 소리를 내며 그들의 소리 특성은 대기온도와 소리 지속시간에 영향을 덜 받고 있었다. 이는 소리 특성의 일부가 Bd 감염에 의해 영향을 받았음을 말해줄 수도 있으나, Bd 감염과 소리 품질의 정확한 관계를 이해하기 위해서는 더 많은 연구가 필요하다. 마지막으로, 논 갯수의 변동 계수를 이용하여 청개구리의 세력권을 추정하였다. 재포획된 21마리 개체 중에 10마리는 같은 논에서 발견 되었다. 이것이 청개구리의 세력권 표시에 관해 결정적 증거가 될 순 없겠지만, 이들의 운동 범위가 다소 제한되어 있음을 예상할 수 있다.