Master's Thesis of Science

Susceptibility to Salamander Chytrid Fungus and Self-recognition Behavior of Korean Native Salamanders

한국 고유 도롱뇽들의 도롱뇽항아리곰팡이에 대한 취약성과 자기인식 행동

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Abstract

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Amphibian populations around the world have been devastated by newly discovered chytrid fungal pathogens over the last two decades. *Batrachochytrium dendrobatidis* (Bd) is one of the widely known chytrid fungi which affects anurans, whereas *Batrachochytrium salamandrivorans* (Bsal) is an only recently identified chytrid fungus that affects urodeles. Bsal has been reported to be fatal to a lot of species including those in the Plethodontidae family. Unlike Bd, which is widely distributed and studied in South Korea, the presence of Bsal in Korea and the impact of its infection on native salamanders are still unclear. Thus, it is critical to study whether it is also causing population decline of Korean domestic species. In this research, I

investigated the susceptibility of two Korean native salamanders to Bsal. The Korean crevice salamander (*Karsenia koreana*) is the only Asian species in the Plethodontidae family and to date one species of this family has been reported to be vulnerable to Bsal. The Wonsan salamander (*Hynobius leechii*) increases its susceptibility to Bsal exposure by mostly living in water. In this study, it is found that Bsal was not fatal to both species during the 7 weeks of experiment after its infection. Infected *K. koreana* showed similar changes of their Snout–Vent length (SVL) and total length (TL) before infection and after the end of experiment compared to the controls; however their mass reduced whereas the control group continued to grow. The TL of infected juvenile *H. leechii* decreased slightly more than controls, but both infected and control group experienced similar effects on SVL and body mass. The SVL of the infected adult *H. leechii* decreased slightly more than the controls, but both groups showed similar results for TL and mass. Degrees of survival of *K. koreana* and *H. leechii* (both juvenile and adult) were not significantly different between the infected and control individuals. The results therefore suggest that both species are resistant to Bsal. To prove the process of Bsal infection went properly with live fungus, 2 populations among *Notophthalmus* salamanders (*N. viridescens* and *N. piaropicola*) were infected, species that have been reported as highly vulnerable to Bsal previously, by Bsal in the same way I did for *K. koreana* and *H. leechii*. All individual died within a month with dermal clinical signs with skin sloughs as reported.

Pheromonal markers are used to recognize territories in many species of the Plethodontidae family, which is the most speciose group among all salamander over the world. However, this behavior

is still not investigated in Korean crevice salamanders (*Karsenia Koreana*). I examined whether they can recognize others from nearby to distant populations; (1) salamanders inhabiting the same region in the same mountain, (2) salamanders inhabiting remote regions in the same mountain and (3) total strangers in different mountain. Experimental controls (4) were also examined. Experiment was undertaken with salamander in the halved plastic container with different scent. The first part of the behavioral experiment for recognition was undertaken for 20 minutes and then for another 20 minutes was inspected after rotating the containers 180° right and left side to exclude salamanders' direction preference. For each 20 minutes, initial moving preference for 5 minutes as acclimation period and general preference for staying position for the subsequent 15 minutes were inspected. During the experiment, salamanders moved across given regions by crossing over between the two parts with behaviors including head movement, shaking up and down, and pausing before or after crossing over. The results showed that *K. koreana* demonstrated a small preference to remain in a close neighbor's scent, while there was no preference between self and remote neighbor's part staying same amount of time at both. On the other hand, salamanders highly preferred to remain in a total stranger's scent and they showed the highest preference in an experimental control's scent.

Keyword : Disease susceptibility, salamander chytrid fungus, self-recognition, Korean native salamanders, *Karsenia koreana*, *Hynobius leechii*

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1.1 Introduction

Throughout history, fungal diseases have been threatening both human and animal health (Ainsworth and Austwick, 1973; Nucci and Marr, 2005), and have caused the population decline or extinction of many wild populations (Daszak et al., 2000). Amphibians are known to be critical indicators of biodiversity and ecological balance (Myers et al., 2000; Vitt et al., 1990). However, amphibian populations have been declining due to infection by various pathogens, especially by chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) (Kolby and Daszak, 2016; Martel et al., 2014).

Bd was first detected in sick and dead anurans in the rain forests of both Australia and Central America (Berger et al. 1998). It has also threatened numerous species of frogs in other countries through spread of the pathogen through pet trade businesses with differential host susceptibilities. Bd has also infected the endemic salamander species in central Texas in the United States of America (Gaertner et al. 2009). In Korea, Bd was detected from the samples through PCR and histopathological check of pet frogs which were imported from USA in 2007 (Yang et al. 2009). Jeong et al. then screened the domestic prevalence status for naturally Bd infected amphibians in Korea, including salamanders (Jeong et al. 2010). Through the disease screening process, most of the Korean amphibians were diagnosed as carriers without showing any clinical signs (Bataille et al. 2013). Moreover, recently O' Hanlon et al. suggested that Bd originated in Asia by comparing Bd lineages

isolated in Korea (BdAsia-1) to BdGPL, BdCH, BdBrazil isolated from America, Europe, and Australia mostly (O' Hanlon et al., 2018).

After 10 years of discovering Bd in 2008, the Netherlands experienced a dramatic loss of two salamander species: the fire salamander *Salamandra salamandra* and the Alpine newt *Ichthyosaura alpestris*. Martel et al. (2014) found this was due to a new chytrid fungus species and named it *Batrachochytrium salamandrivorans* (Bsal). In their 4-week experiment, it was found to infect nine salamander species out of 10 and killed 41 infected individuals out of 44 (Martel et al. 2014). Recently, Stegen et al. (2017) emphasized the seriousness of this fungal pathogen which can lead salamanders to extirpation which may in turn influence the ecosystem as a whole.

In Korea, however, most research on endemic salamanders has been done to inspect their ecological characters and general natural history, like the habitat characteristics and the food sources (Yoon et al. 1996). As such, it is still unknown whether they are susceptible to Bsal or not. However, fungus may travel into Korea through various ways, for example, by shipping through multiple national ports to as yet unexpected ways. The animal to prioritize the examination of would be the Korean crevice salamander *Karsenia koreana*, the first and only Asian plethodontid salamander. (Min et al. 2005). In a study in France, Bsal was reported to be lethal to 1 among 3 plethodontid species tested (French cave salamanders, *Hydromantes strinatii*), with the other 2 being resistant (Martel et al. 2014; lethal: infection resulting in lethal disease in all infected animals, resistant: no infection, no disease). Second would be *Hynobius leechii*, which lives in water, where the optimal conditions

for the water-dependent fungus to infect salamanders are provided (Weinstein 2009). Furthermore, the males *Hynobius leechii* shake their body in the water as natural mating behavior when the female approaches, which could make them in higher possibility to meet fungus (Kim et al. 2010). Considering these ecological interactions of the species, if the fungus is imported in any route, it will cause a disastrous decline in the domestic salamander population.

In this study, I performed infection experiments using cultured Bsal on adult Korean crevice salamander (*Karsenia koreana*) and juvenile and adult Wonsan salamanders (*Hynobius leechii*) to determine whether this chytrid fungus leads to chytridiomycosis. Salamanders were checked daily for any clinical signs of disease and survival for 7 weeks after infection and the body sizes and masses were compared between the infected and the control groups before the infection and at the end of study.

1.2. Materials and Methods

Collecting animals and captivity husbandry

A total of 41 individuals of *K. koreana* were collected from 4 mountains; Jangtae mountain, Deajeon-si (36° 11'41.4" N 127° 20'02.8" E), Manin mountain, Daejeon-si (36° 11'58.4" N 127° 26'56.6" E), Songni mountain, Boeun-gun (36° 32'33.5" N 127° 50'01.7" E) and Kaseop mountain, Eumseong-gun (36° 58'17.0" N 127° 41'03.5" E) in 2016 and 2017. Samples were collected by hand by searching under rocks, logs and leaf litter. Each

animal was individually housed in a rectangular plastic container with one dry ground paper towel, one crumbled wet paper towel and one semi-wet paper towel. All paper towels used were unbleached and non-fluorescent. All containers with salamander were kept in an incubator at 15 ℃ (VS-1203P5-L, VISION BIONEX) with a 12/12 LD light schedule. Animals were fed twice a week with 20 ~ 30 of larval crickets with 1 cc sized each time and cleaned containers with UV treated dechlorinated, filtered (5 μm sized filter) water once a week between the two feeding days.

The larval and adult *Hynobius leechii* were both collected from their natural habitats in Korea: Gwanak mountain, Seoul-si (37° 46'84.91" N, 126° 95'51.07" E), Suri mountain, Gunpo-si (37° 34'60.63" N, 126° 89'72.07" E), Buam reservoir, Geumsan-gun (36° 13'79.83" N, 127° 38'11.11" E), and Pyeong-hwa dong, Wansan-gu, Jeonju-si (35° 78'41.70" N, 127° 14'17.69" E) in their active season from mid of January to late November in 2017 and the eggs were also collected from late January to early April of 2017 and 2018. The larvae was kept in the tank with UV filtered water at 15 ~ 20 ℃, divided into three groups of similar sizes to block intraspecific cannibalism as much as possible among them; small, regular and large. Samples were fed with sludge worms (*Tubifex tubifex*) every two days until they were grown up into the juveniles, when they started to show legs and removed their gills. Some larvae were from the collected adults after one male and female were coupled in plastic tank that is filled with filtered water, with the lids closed, in a with 3 ~ 5 ℃. The adults were housed in separate containers with at least 10 cm height and 10 ~ 15 cm^2 once they laid eggs, to prevent any

possible cross contamination of fungus. The inner condition of each container was maintained at the proper humidity inner condition with wet paper. They were fed once a week with 3–5 mm sized cricket larvae, and the towels were changed at the same time.

For the eggs, each clutch was kept in a separate plastic tank with filtered water in a refrigerated room at 3–5 ℃. I gradually acclimatized the tanks with the laid eggs to higher temperatures through 8–10 and 14–18 degrees and up to the normal room temperature so that the larvae can hatch. I labeled parent's collected location on their tanks and kept them as I did for collected larvae from natural habitats. Once larvae hatched and survived to 2 weeks, each individual was moved into separate terrestrial tanks as was done for juveniles. Juveniles were fed with 1 cc pin–heads, the smallest cricket twice a week and containers were cleaned once a week with new paper towel after wiping inner part of container with filtered water.

Culturing the fungus

Full liquid hydrolysate–lactose media (TGhL) was made with 800 ml of distilled water, 6.4 g of tryptone, 0.8 g of peptone–E (gelatin hydrolysate), and 1.6 g of lactose in a 1 L of glass bottle. Media was made in the laminar hood after wiping the bench with 70 % of ethanol and with a lit spirit lamp inside so that any aerosol contamination could be blocked from outside. After gently shaking the bottle to mix, the lid was not closed tightly, just half closed, and it was covered with the aluminum foil and was autoclaved. The liquid media stocks were stored at 4 ° C. 10 ml of the liquid media was poured into a cell

culture flask and 1 ml from the liquid fungus stock was added. It was gently shaken and then allowed to grow in proper temperature (for Bsal 15° C) using a thermal chamber (MIR-254, SANYO). After 4 to 5 days, majority of flowing zoosporangia were detected with shaking the container and then stored flasks in 4 ° C for up to 3 months keeping. As a general principle, any contamination in the flask can be detected after putting it for at least 6 hours at growing or room temperature, and if there were any cloudy materials inside it should be thrown away after packaging with bio-hazardous bag. The contaminated liquid was poured in the glass bottle and combined with at least 0.03 % of bleach, sodium hypochlorite, and poured at the sink after overnight (Becker 2017). All the flasks were autoclaved before being disposed with normal experimental waste.

The same composition as full liquid hydrolysate-lactose media (TGhL) was poured into the 1 L glass bottle but also adding 8 g of bacteriological agar to make solid media. After autoclaving, it was poured into petri dishes with 1/3 height full in the laminar hood before the liquid solidified. The agar plates were stored at 4 ° C fridge for keeping. 1 ml of liquid was taken from the culturing flask with pipette and poured it on the agar plate with solid media. The plates were gently shaken so that the poured liquid could cover all the surface of the plate and put the plates for another 4 or 5 days in each growing temperature allowing fungus to grow there also. The culturing flasks were stored again in the same fridge and if the culturing liquid left fewer than 10 ml in the stock container, more liquid media was poured up to 10 ml before placing it in the fridge. 2 ml of the liquid media and UV filtered water combined were poured with 50:50 portion into the

filtering plate and gently shake it so that the zoospores near the zoosporangia can come up and swim in the poured liquid. Plates were put in growing temperature for at least 30 minutes so that zoospores can flow on the liquid surface while zoosporangia can sink down to solid part. Plates were taken back into the biosafety hood and as much liquid as possible was transferred into a 50 ml falcon tube. 2 μl was taken from the falcon tube and added with 18 μl of the combined liquid with liquid media and UV filtered water into the 1.5 ml normal falcon tube so that the zoospore concentration could be diluted 1/10. The liquid in the 1.5 ml tube was gently shaken and mixed a few times with sucking and expelling action with the tip and took 10 μl of it and let it permeate into the Hemocytometer. With 400 magnification of the microscope, the square grid counting the zoospore of transparent and circled shape in 4 squares was checked. The total number was calculated by dividing it by 4 to get the average and multiply by 10^5 to calculate the final concentration. Bsal detection was always undertaken around its growing temperature, 15 ° C. As the optimum concentration is $1 * 10^5$ of zoospores per 1 ml, the progress was repeated until 4 zoospores could be detected from total 4 squares. For every 2~3 months, one uncontaminated stock culture among the last cultures were transferred to duplicated new cell flasks containing TGhL broth to continuously keep them fresh.

Infection

All the animal' snout-vent length (SVL), total length including tail, and mass were measured right before the infection. 1 ml of properly cultured Bsal liquids for experimental group or broth media for

controls was put on the back of each salamander in separate petri dishes. The amount of liquid was enough for the animal's abdomen to continually touch the liquid during the infection for 24 hours in fungus growing temperature. After infection hours, each individual removed from the petri-dish and put back into their original plastic container. After the infection, each animal was checked for any clinical signs or mortality daily and their body size weekly for both the Bsal infected and control groups.

Additionally, I checked whether kept Bsal stock was attenuated or not during sub-culturing passages over 7 times. To prove that Bsal infection process on *K. karsenia* and *H. leechii* went properly, the same Bsal load was used to infect 4 Eastern newts (*Notophthalmus viridescens*) which was reported as fatally susceptible to Bsal already (Martel et al. 2014). In addition, 4 more Peninsula newts (*N. piaropicola*) were tested together to see variation in susceptibility among different population.

Data Analysis

Two-sample t tests were run in Minitab 18 comparing change of SVL, TL and mass before and after infection between Bsal infected and control group of juvenile and adult *H. leechii* and adult *K. koreana*. Survival analysis was performed for all animal groups using the nonparametric Kaplan-Meier procedure with 'survfit' function in R package survival. Log ranks test was used for survival comparison.

1.3. Results

Karsenia koreana

The SVL of Bsal infected and controls of adult *K. koreana* shortened similarly during 7 weeks of experimental period after infection with insignificant difference ($n = 39$, $p = 0.880$) (Fig 1.1). TL of Bsal infected individuals decreased 0. 14 cm more than controls on average but it was not significantly different ($n = 39$, $p = 0.252$) (Fig 1.2). However, salamanders had negative effects on their mass change; Bsal infected individuals lost 0.01 g in average while controls gained more with 0.06 g in average with significant gap ($n = 39$, $p = 0.018$) (Fig 1.3). But PCR tests confirmed that no individuals were left infected at the end of 7−week experiment. Only two individuals among controls died while all of Bsal infected survived and survival rate comparison showed both survived similarly ($n = 41$, $p = 0.9$) (Fig. 1.4). There was not any clinical sign on their skin on dorsal or ventral side with skin lesions until the end of experiment.

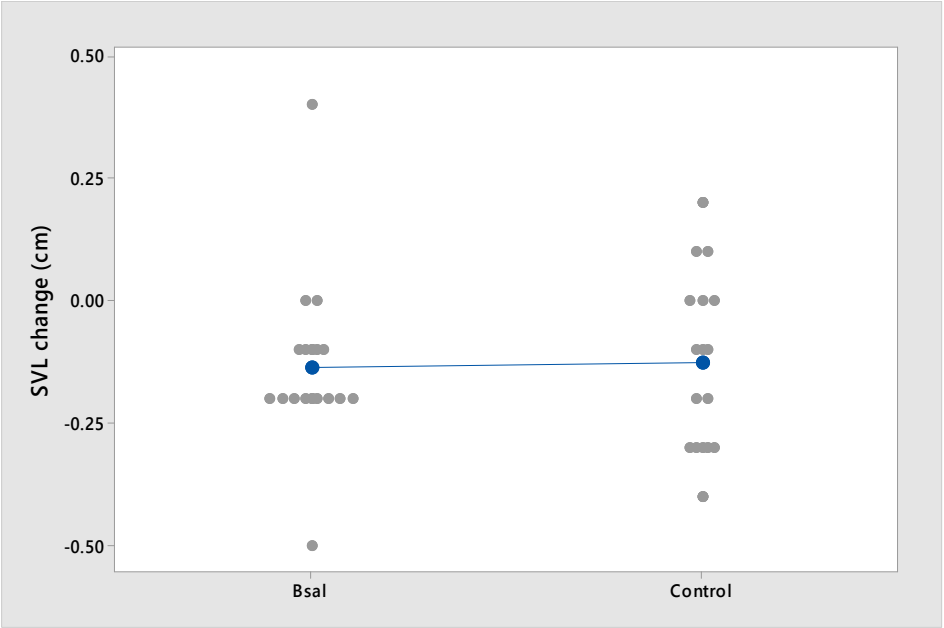


Fig 1.1. SVL change (cm) of Bsal infected (n = 19, left dots) and control group (n = 20, right dots) of adult *K. karsenia*. During 7 weeks experiment period after infection, Bsal infected group' s SVL decreased by a similar amount to controls.

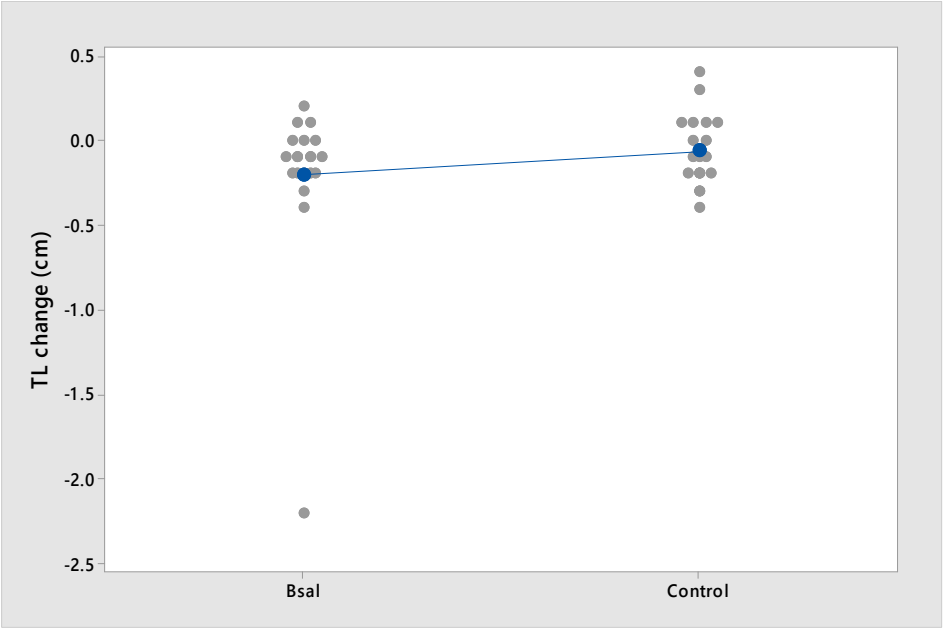


Fig 1.2. TL change (cm) of Bsal infected (n = 19, left dots) and control group (n = 20, right dots) of adult *K. karsenia.* During 7 weeks experiment period after infection, Bsal infected group' s TL was decreased 0. 14 cm more than controls in average but it was not significantly different (p = 0.252).

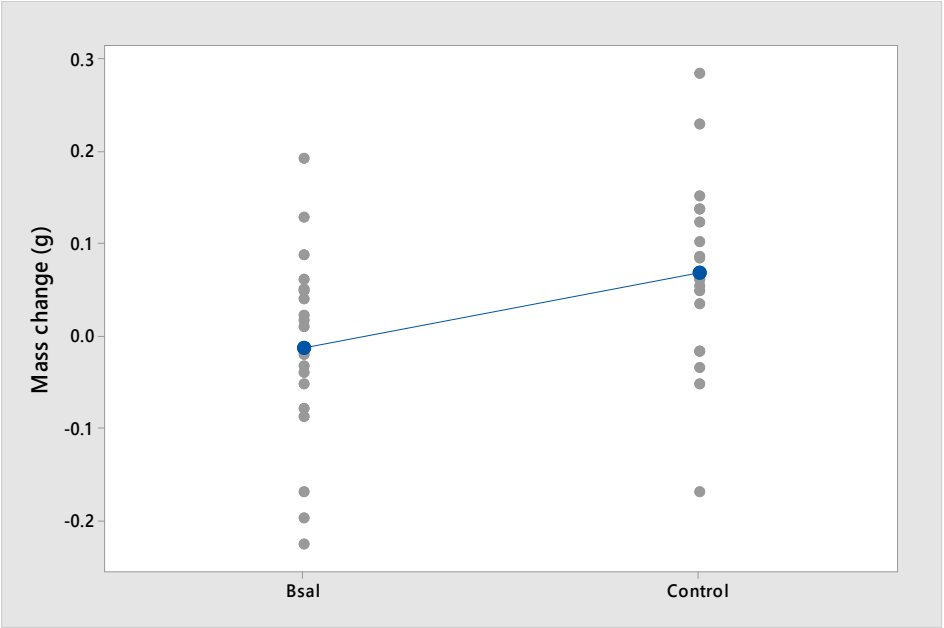


Fig 1.3. Mass change (g) of Bsal infected (n = 19, left dots) and control group (n = 20, right dots) of adult *K. karsenia.* During 7 weeks experiment period after infection, Bsal infected lost its weight while controls got more.

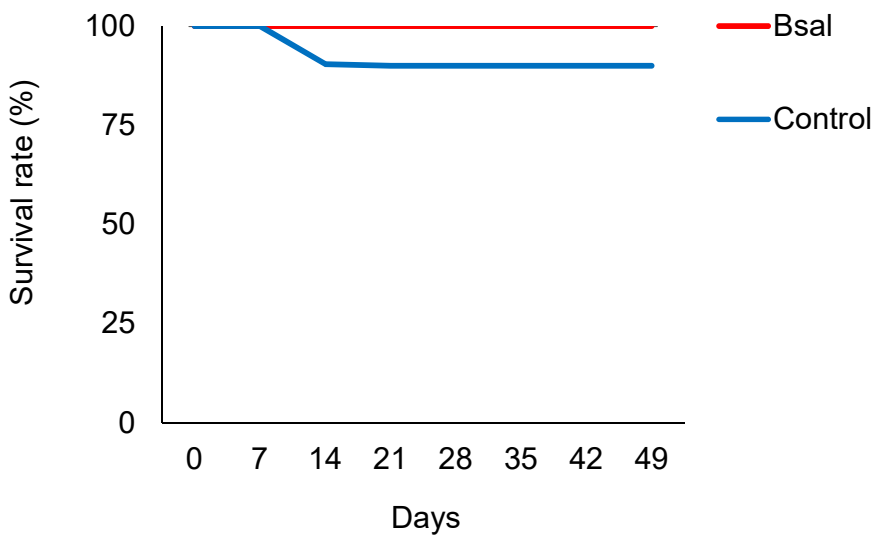


Fig 1.4. Survival rate of adult *K. karsenia.* During 7 weeks experiment period after infection, all of Bsal infected (n = 19, red line) survived until the end of experiment and two died among controls out of 21 (blue line) with 90% of survival rate.

Juvenile *Hynobius leechii*

Bsal infected and controls of juvenile *Hynobius leechii* had similar increase in SVL during their growth over 7 weeks of experimental period after infection with insignificant result ($n = 69$, $p = 0.652$) (Fig 1.5). The TL of Bsal infected decreased 0. 15 cm more than controls on average and it was significantly different ($n = 69$, $p = 0.040$) (Fig 1.6). Both groups lost mass similarly with insignificant difference ($n = 69$, $p = 0.364$) (Fig 1.7). Nine individuals died among Bsal infected and eight did among controls. Survival rate comparison between them showed they died similarly without significant difference ($n = 86$, $p = 0.9$) (Fig. 1.8). There was not any clinical sign on their dorsal or ventral skin (e.g. bared lesions) until the end of experiment.

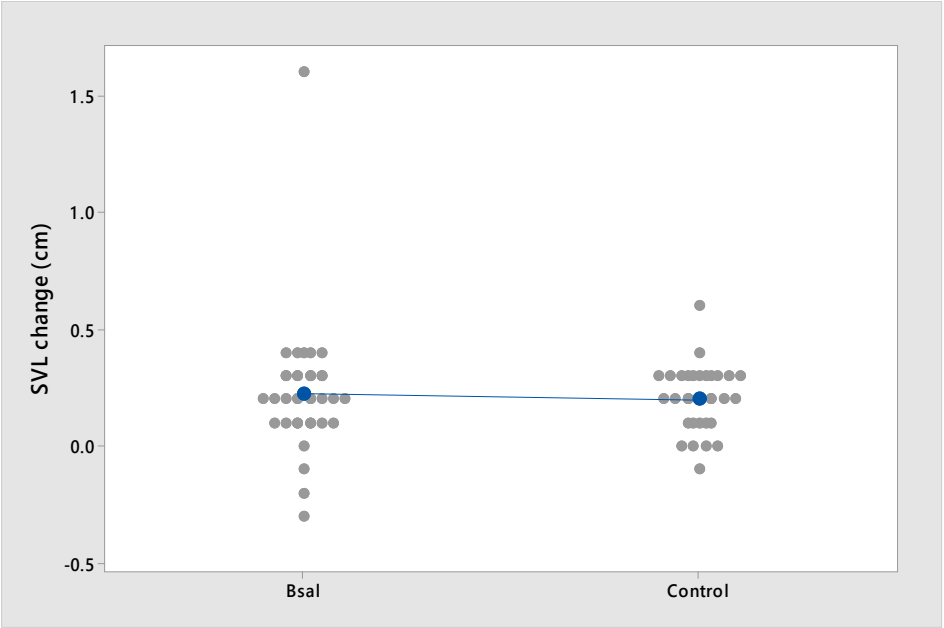


Fig 1.5. SVL change (cm) of Bsal infected (n = 36, left dots) and control group (n = 33, right dots) of juvenile *H. leechii*. During 7 weeks experiment period after infection, Bsal infected group' s SVL increased by a similar amount to controls.

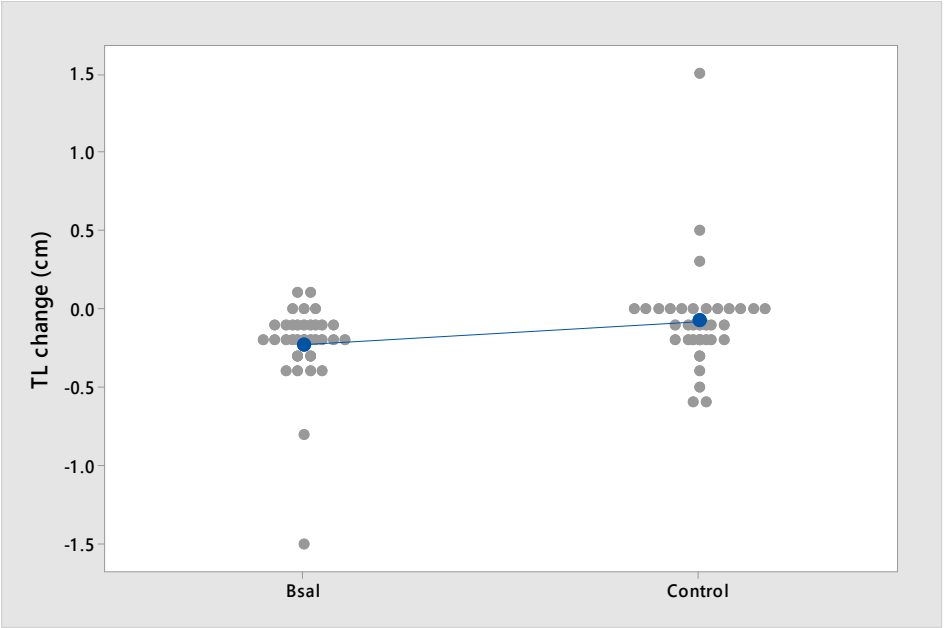


Fig 1.6. TL change (cm) of Bsal infected (n = 36, left dots) and control group (n = 33, right dots) of juvenile *H. leechii*. During 7 weeks experiment period after infection, Bsal infected group' s TL was decreased similarly to control.

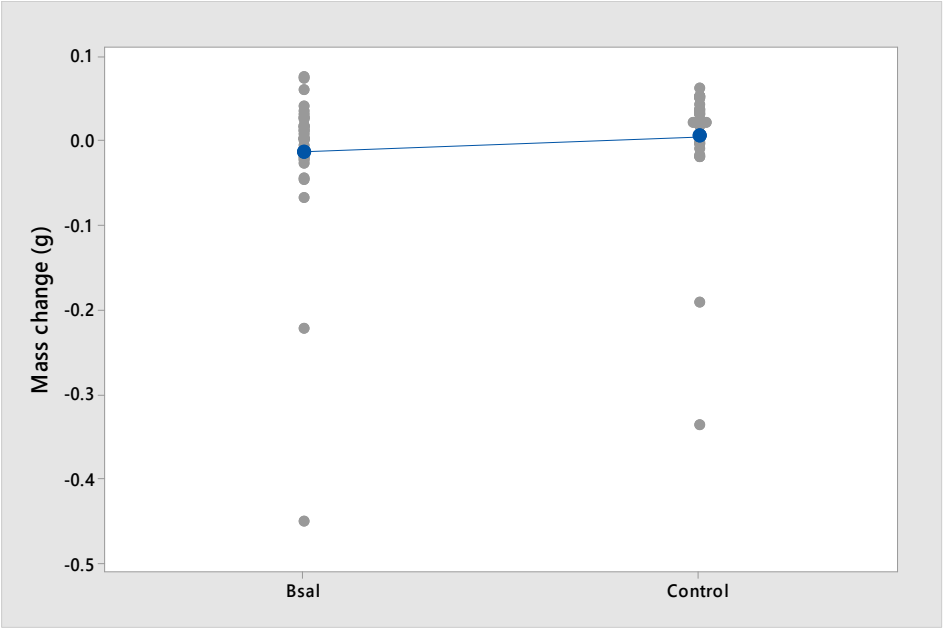


Fig 1.7. Mass change (g) of Bsal infected (n = 36, left dots) and control group (n = 33, right dots) of juvenile *H. leechii*. During 7 weeks experiment period after infection, Bsal infected lost mass similarly to controls.

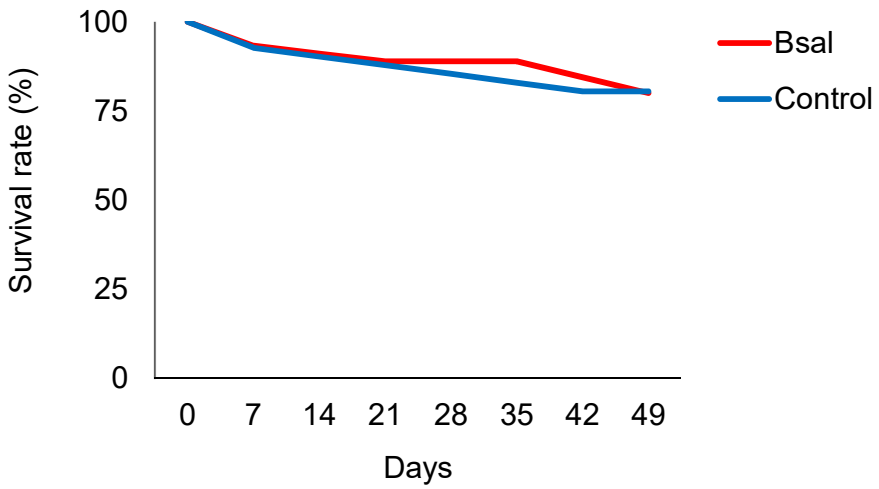


Fig 1.8. Survival rate of juvenile *H. leechii* over the 7 week experiment period after infection. Bsal infected (n = 36, red line) survived similarly to controls (n = 33, blue line).

Adults of *Hynobius leechii*

The SVL of Bsal infected adult *Hynobius leechii* decreased by 0.16 cm more than controls during 7 weeks of experimental period after infection with significant difference between both groups ($n = 43$, $p = 0.044$) (Fig 1.9). However, the TL of Bsal infected and controls decreased similarly with insignificant difference ($n = 43$, $p = 0.369$) (Fig 1.10). Also, both s lost mass similarly ($n = 43$, $p = 0.291$) (Fig 1.11). 16 individuals died among Bsal infected while 13 did among controls and survival rate comparison between them showed they died with the almost same rate ($n = 72$, $p = 0.9$) (Fig 1.12). There were no clinical signs on their dorsal or ventral skin (e.g. bared lesions) until the end of experiment.

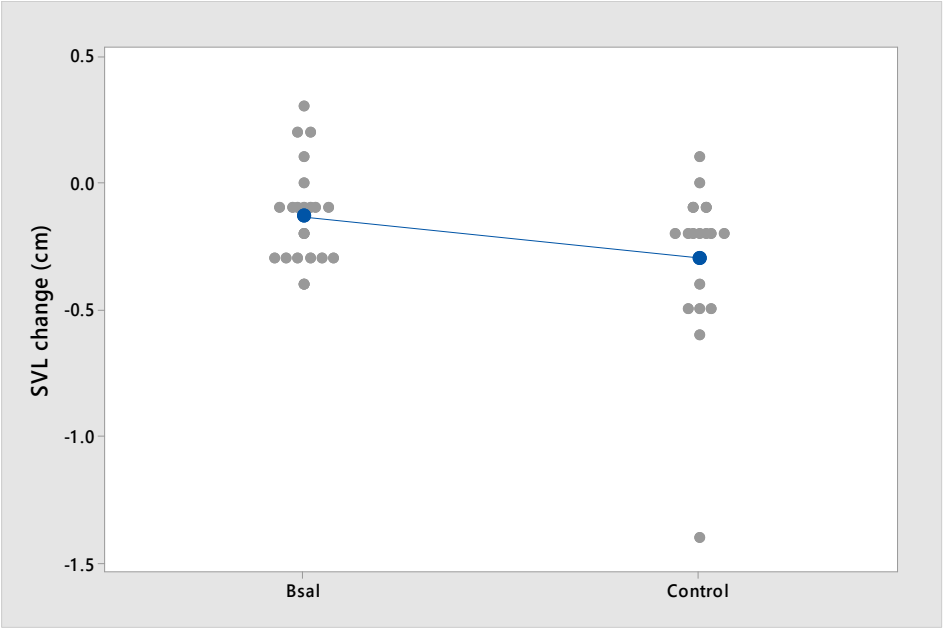
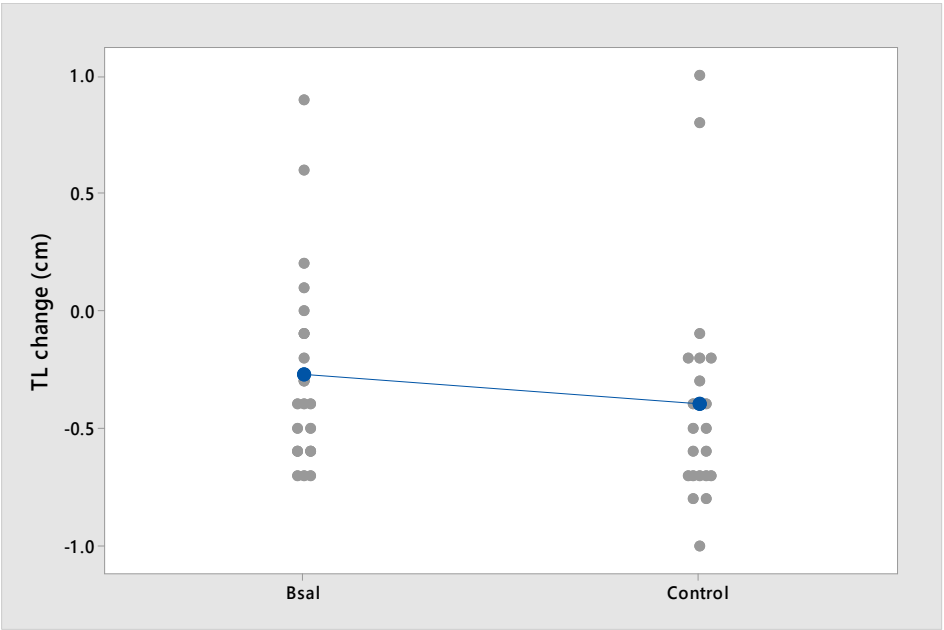


Fig 1.9. SVL change (cm) of Bsal infected (n = 22, left dots) and control group (n = 21, right dots) of adult *H. leechii*. During 7 weeks experiment period after infection, Bsal infected group' s SVL was decreased more than controls.



Fi 1.10. TL change (cm) of Bsal infected (n = 22, left dots) and control group (n = 21, right dots) of adult *H. leechii.* During 7 weeks experiment period after infection, Bsal infected group' s SVL was decreased more than controls.

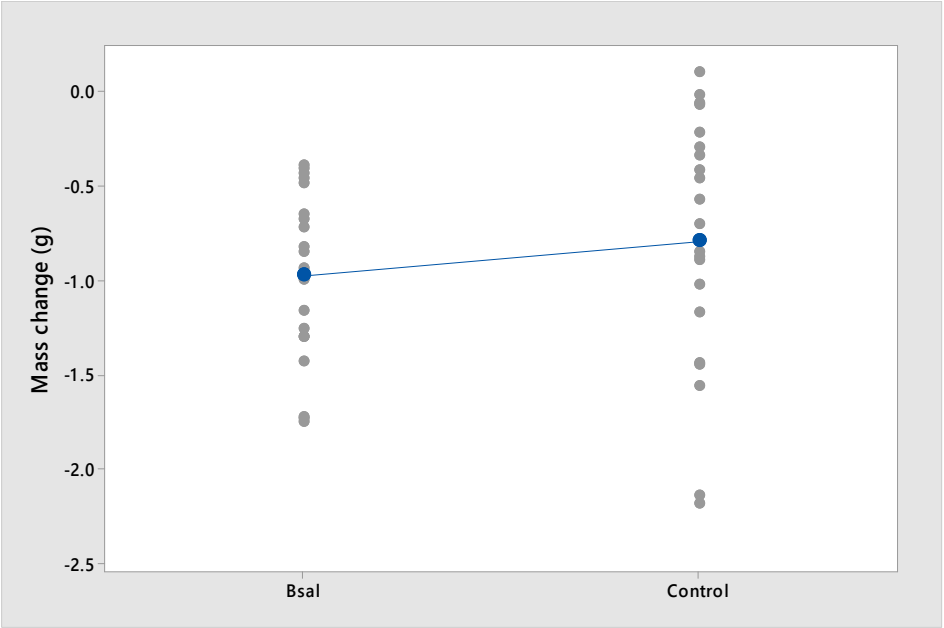


Fig 1.11. Mass change (g) of Bsal infected (n = 22, left dots) and control group (n = 21, right dots) of adult *H. leechii*. During 7 weeks experiment period after infection, Bsal infected and controls lost their weight similarly.

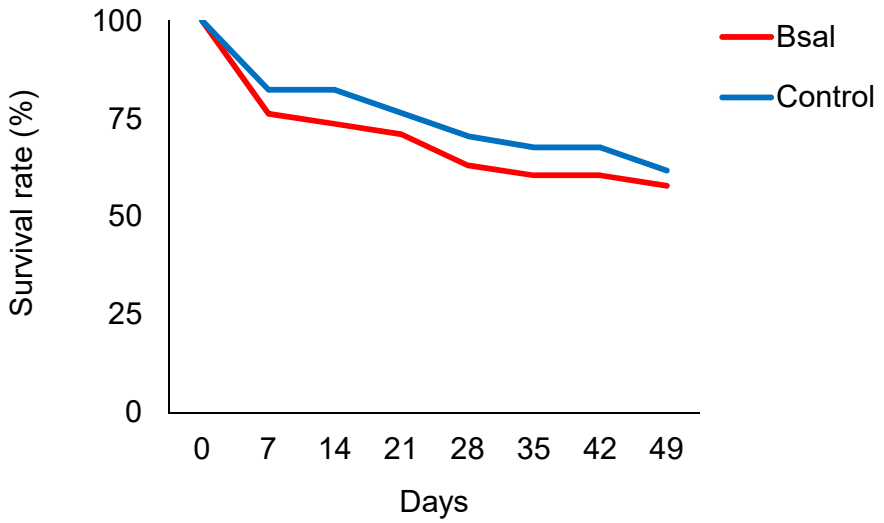


Fig 1.12 Survival rate of adult *H. leechii* during 7 weeks experiment period after infection. Bsal infected (n = 22, red line) survived similarly to controls (n = 21, blue line).

Notophthalmus salamanders

After 4 eastern newts (*Notophthalmus viridescens*) and 4 peninsula newts (*N. piaropicola*) were infected by Bsal on the same date, all of individual showed scrubbing on their dermal area, especially on dorsal side within a week (Fig 1. 13(a), (b)). Both populations showed similar patterns of mortality until all died and there was not significant difference between two population groups (log rank test, $X^2 = 0.4$, 1 df, $p = 0.5$) (Fig 1.14).

Fig 1. 13 (a, left) Skin slough which was accumulated around the tail of Bsal infected *Notophthalmus* salamanders. Red arrows.
(b, right) Pieces of skin sloughs on Bsal infected *Notophthalmus*' s body.

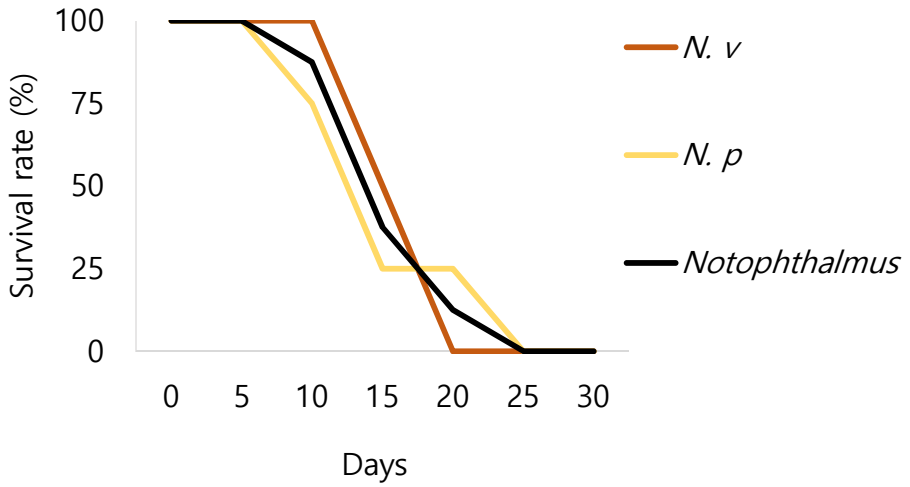


Fig 1.14. Survival rate of 4 eastern newts (*Notophthalmus viridescens*, brown line) and 4 peninsula newts (*N. piaropicola*, yellow line) during 30 days after Bsal infection. Black line refers survival rate of total 8 individuals (0 % at the end) and all died from 10 up to 24 days.

1.4. Discussion

This is the first study to test whether adult Korean crevice salamanders (*Karsenia koreana*) and juvenile and adult Wonsan salamanders (*Hynobius leechii*) are susceptible to Bsal. In this study, I tested disease susceptibility to salamander chytrid fungus, Bsal, on individuals by looking for clinical signs – especially on dermal part – and checking daily survival rates for each group. Changes of snout–vent length (SVL), total length (TL) and mass comparing before the infection and after 7 weeks were also inspected between infected and controls. Throughout the experiment, I found both species have resistance to Bsal with the fungal pathogen having a minimal impact on body size and mass with less than 0.2 cm changes in SVL or TL and 0.02 g in mass. There was only one index of significant change between Bsal infected and uninfected controls whilst other indexes experienced insignificant. There were no clinical signs on the dorsal skin or lesions on the ventral side until the end of experiment of 7 weeks on all individual among three treatment groups. Besides, the rapid mortality of Eastern newts (*N. viridescens*) supported that Bsal fungus stock were kept in active passages and infection process went properly in laboratory condition.

Bsal susceptibility of Plethodontidae salamander has been in question since Martel et al. reported one species (*Hydromantis stirnatii*) among three tested is highly susceptible to fungus, but my result is consistent with the finding that the other two species that were resistant to it (*Plethodon glutinosus* and *Gyrinophilus porphyriticus*) (Martel et al. 2014). Also the results showed as Bsal

is not fatal to both juvenile and adult *H. leechii*, it makes sense considering that none of the three species among family Hynobiidae were fatally susceptible to Bsal with one tolerant (*Salamandrealla keyserlingii*) and two resistant species (*Hynobius retardatus* and *Pachyhynobius shangchengensis*) (Martel et al. 2014; tolerant: infection in the absence of disease). Rapid mortality of *Notophthalmus* within 24 days (at maximum) showed Bsal culturing and infection stages were done properly as previous reference reported that Eastern newts (*Notophthalmus viridescens*) are a fatally susceptible species to Bsal (Martel et al. 2014). Insignificant difference in Bsal susceptibility between Eastern and Peninsula newts, *N. viridescens* and *N. piaropicola*, implies that there is no variation between the two salamander populations; North America and Florida panhandle, but tests among other 2 populations of *Notophthalmus* should be verified; *N. v. dorsalis* and *N. v.* louisianensis.

Given that Bd purportedly originated in the Korean peninsula as the genetic ancestor for the whole biodiversity of Bd around the (O' Hanlon et al. 2018), it is possible that Korean anurans have been gained resistance towards Bd after long-term coevolution (unpublished data, Waldman lab). Korean urodeles might also have developed immune system to Bd as they are resistant to it now as carrier; *Hynobius leechii*, *H. quelpaertensis* and *K. koreana* (Bataille et al. 2013). If a species has no experience of exposure to fungal pathogens, there may not have been proper selective pressure to evolve an immune system that can respond appropriately towards the pathogen. Since Bsal is the closest fungus with Bd in genetic tree, it

is somewhat likely that Korean salamander species could have evolved resistance to Bsal at the same time (Martel et al. 2013). But still susceptibility screening has to be done for all uncovered species as the two pathogens can derive different immune reaction since they are genetically different and there is a 10 year gap between their discoveries (Martel et al. 2013).

As a related further study, as I made samples of dermal tissues with medical cotton swabs on each individuals at least bi-weekly I will also do real-time PCR to detect changes in infection intensity of Bsal to verify my conclusions. This will be more specific investigation on whether the two endemic salamander species are totally resistant to this fungus or tolerant as infection intensity goes down as time goes by after infection. In addition, susceptibility to Bsal on another domestic salamander species should be further investigated as well. Korean clawed salamander (*Onychodactylus koreanus*) is belonged to family Hynobiidae as *H. leechii* does and breeds in or near the stream with temperature around 10 ℃, which is similar to the breeding range of *H. leechii*, 7-10 ℃ (Park 2005, Park 2010). Considering around 50 individuals of *O. koreanus* breed at the same time and the water temperature also can be optimal growth temperature for Bsal, 10-15 ℃, the Bsal resistance of *O. koreanus* should also be tested under the hypothesis that living conditions can make salamanders more susceptible to infection in their natural habitat (Park 2005, Martel et al. 2013). Simultaneous infection of Bd and Bsal can also be performed on domestic anuran and urodele species since nothing is reported so far with any amphibian species and there is applicable PCR method to process with DNA samples

from skin tissues (Blooi et al. 2013).

Furthermore, legal practice should be set up and started to protect domestic native salamanders whose immune system may not be sufficiently evolved to defend from infection by Bsal. Many other countries including the United States of America, Australia and Thailand have already officially listed chytridiomycosis as a communicable disease for amphibians and have been tracking them domestically by records and the USA has already blocked the importation of foreign salamanders by law. Recently, Canada joined this list and accepted a policy banning the importation of amphibians from June in 2017 (Kim et al. 2017). They also emphasized there should be quantitative assessment for the danger of chytridiomycosis and properly set the legal policy to manage the importation of live amphibians (Kim et al. 2017). Indeed, when live amphibians come into Korea with various importation reasons including ornamental, academic or consumption purposes, normally any proper and actual check has not been performed from international airport, only with paper sheet with importation permission. Considering that the Wonsan salamander, *H. leechii*, is the own species whose collection from the wild is legally prohibited and also listed to the IUCN Red List as Least Concern, management and preservation of their domestic population is vital especially checking for susceptibility to Bsal.

Chapter 2. Self-recognition Behavior of a Korean Native Salamander: *Karsenia koreana*

2.1. Introduction

Olfactory recognition has been observed in several vertebrate groups; Brennan et al. (1990) found that mice can use chemosignals and olfactory pheromones, which allows their females to recognize which male she has previously mated. Within anurans, Lee and Waldman (2002) tested whether the frog, *Leiopelma hamiltoni,* can recognize its own fecal chemosignal by exposing each subject to either a smear of its own feces or another' s feces. After *L. hamiltoni* was reported to communicate by means of intraspecific chemosignals Waldman and Bishop (2004) studied whether *L. hamiltoni* can discriminate different conspecific odors by investigating whether they show any preference for substrates marked with their own chemosignal or those of others. The results showed that frogs can memorize odors and that they preferred their own odor to a blank and that they universally avoided unfamiliar conspecifics.

The Plethodontidae family is the most speciose group of salamanders globally, having 471 reported species within 28 genera and some species in this family was studied for self-recognition. Two species in the Plethodontidae family, *Desmognathus monticola* and *Desmognathus fuscus* inhabit the same habitats and have been observed to view each other as potential interspecific competitors (Organ 1961, Keen 1982). Keen et al. (1984) found that they show aggressive attitude towards intruders – primarily by biting – after individuals establish their home sites. The red backed salamander, *Plethodon cinereus*, also shows territoriality and use pheromonal markers which allows them to differentiate between familiar

(neighbors) and unfamiliar individuals (Jaeger 1986). Salamanders recognize their territory and communicate intraspecifically by marking them with dermal olfaction glands (Tristram, 1977). Plethodontid salamanders use their special structure, the vomeronasal organ, to detect olfactory chemicals; this is related to a unique character of the group – nose–tapping (Dawley and Bass 1988). Given this olfactory behavior, it seems likely that plethodontid salamanders can memorize and recognize odors. The Korean crevice salamander, *Karsenia koreana*, is the salamander of the Plethodontidae that is endemic to Asia, specifically in montane regions of South Korea (Min et al. 2005). At present, no studies on their home recognition or any behavior have been conducted. As the only Asian plethodontid salamanders, the study of their olfactory behavior should be prioritized, e.g. whether they communicate with conspecifics using chemosignals or pheromones.

In this study, I tested *K. koreana* for their ability to distinguish conspecifics. I presented each subject to substrates with either its own or a conspecific' s odor to see whether they show differential preference for odor following a similar principle to Waldman and Bishop (2004). I also observed for any unique or repeated behaviors e.g. nose–tapping or head movement during the inspection period. Furthermore, by repeating the experiment multiple times, I aimed to establish whether they can memorize and recognize their own odor after repeated exposures to conspecific odors.

2.2. Materials and Methods

Collecting animals and captivity husbandry

Karsenia koreana individuals were collected from 4 different sites in South Korea; Jangtae mountain (36° 11'41.4" N 127° 20'02.8" E, n=24) in Deajeon-si (where the first individual was found by Min et al. 2005), Manin mountain (36° 11'58.4" N 127° 26'56.6" E, n=6) in the same city, Kaseop mountain (36° 58'17.0" N 127° 41'03.5" E, n=2) in Eumseong-gun, and Songni mountain (36° 32'33.5" N 127° 50'01.7" E, n=1) in Boeun-gun (Fig 2.1). Animals were collected by hand by searching under rocks, logs and leaf litter. They were maintained in an environmental chamber (VS-1203P5-L, VISION BIONEX) at 15 ° C with 12/12 LD cycle for light control. Unbleached brown paper towels were wetted using UV treated dechlorinated, filtered (5 μm sized filter) water, and placed in the plastic containers used to house the salamanders. The containers were a minimum of 10 cm in height and 10-15 cm^2 of ground area, and were cleaned weekly. Animals were fed 20-30 1 cc pin-head crickets (the smallest cricket size) weekly by directly dropping them into the container so that animals can see prey easily.

Fig 2.1. *Karsenia koreana* collection sites. Grey circles represent known habitat and red pins the actual collection sites.

Experimental setting

A transparent rectangular plastic container was placed on the desk with its base facing upward, so that the inside was clearly visible in footage recorded by a video camera (Sony DCR-SR82) fixed to a tripod. The container was divided into 2 halves with a marker line. There were a total of 4 different experimental settings. All settings used the scent of the experimental animal in one half and 1 of 4 different 'non-self' odors in the other half: (1) close neighbor salamanders inhabiting the same sampling site (6 * 12 meters) in the same mountain, (2) remote neighbor salamanders inhabiting different sampling sites in a remote region of the same mountain, (3) total stranger salamanders from different mountains and (4) experimental control (blank scent with humid towel). The experimental animals scent was marked in the plastic container (48 * 35 * 40 centimeters) with on the 'A' half of the container whilst the other half ('B') was marked with the 'non-self' odor. The odor was marked using paper towels from the living container of the salamander subject and towels were taken at least 3 days after cleaning the container. Scents were set by rubbing paper towel on all inner surfaces (excluding lid) on each half the container and the towels were also laid flat on the base. All experimenters wore poly-gloves to avoid contamination with human scent or between the towels from different salamander containers.

Once the experimental setting was ready, the experimental individual (the same as was used to mark the 'self' or 'A' half) was taken and put on the center of the experimental container. The individual was gently placed so that the head was pointing parallel to

the mid-line between the A and B parts. Experiment was done between 6:00 p.m. and 1:00 a.m. after sunset and in complete darkness with an ambient temperature of between 10 and 15 ° C (the same temperature of their incubator). The entire experiment was recorded by video camera using the night vision setting. The total duration of the experiment was 40 minutes. After 20 minutes the recording was paused, the experimental individual temporarily removed and the container rotated 180° so that any individual preference on direction can be ignored. After rotation the individual was replaced and recording resume for a further 20 minutes. In the interval between every experiment, all inner surfaces were cleaned with 70% of ethanol and totally dried so that previous odors or pheromones were removed.

The first 5 minutes of recording was ignored as an acclimation period. After that point, the direction an individual started to go forward at first, how much time they spent in each half totally, and any other specific behaviors e.g. nose-tapping (Dawley and Bass 1988) was indicated by recorded screen. Settings (1), (2), (3) and (4) were done with 21, 21, 16 and 30 individuals of *K. koreana* respectively. Each individual was tested a minimum of 3 days after cleaning the container.

Data Analysis

For the description of salamander odor preference behaviors, all videos were checked using Windows Media Player 12 for the remaining 15 minutes after the acclimation period of first 5 minutes both before and after rotating the container. All behaviors performed

by each individual (e.g. head shaking, nodding, hesitation behaviors) were observed and were grouped into patterns of similar behaviors. The time spent in each half by the salamander was recorded. Pair t-tests were run using Minitab 18, comparing the total staying time spent in each half in the 30 minute experimental period (i.e. excluding the acclimation period).

2.3. Results

Initial and general behavior

After the initial 5 minutes (acclimation period) salamanders began to demonstrate possible olfactory behaviors including shaking their heads up and down or left to right. Regarding directional preference, most salamanders immediately headed directly in the direction their head was pointed after their placement in the experimental apparatus. After the acclimation period, the selection pattern varied between individuals from no movement to continually crossing the borderline or wall inside. If a salamander continually crossed the borderline during the 15 minutes, they switch the location by order like A, B, A, and B again. Generally, salamanders investigate both sides within the first 5 minutes of acclimation and then after that they seem to show a strong preference for one side or the other to remain.

Staying preference

For experiment setting (1) with salamanders from the same site, salamanders stayed less in the self-part with an average gap of 400

seconds but this time the difference was not statistically significant (n = 21, p = 0.082) (Fig 2.2). In setting (2) with remote neighbor salamanders from differing sites on the same mountain, on the other hand, salamanders showed similar preference for both halves with only 6 seconds of gap (n = 16, p = 0.968) (Fig 2.3). But in setting (3) between stranger salamanders from different mountains, salamanders showed similar gap with significant difference in staying time as settings (1) and (2) with an average 426 second gap (n = 21, p = 0.033) (Fig 2.4). In experimental control (4), salamanders stayed in B (blank) more than A (self-part) with an average gap of 431 seconds which is a significantly different preference (n = 30, p = 0.008) (Fig 2.5).

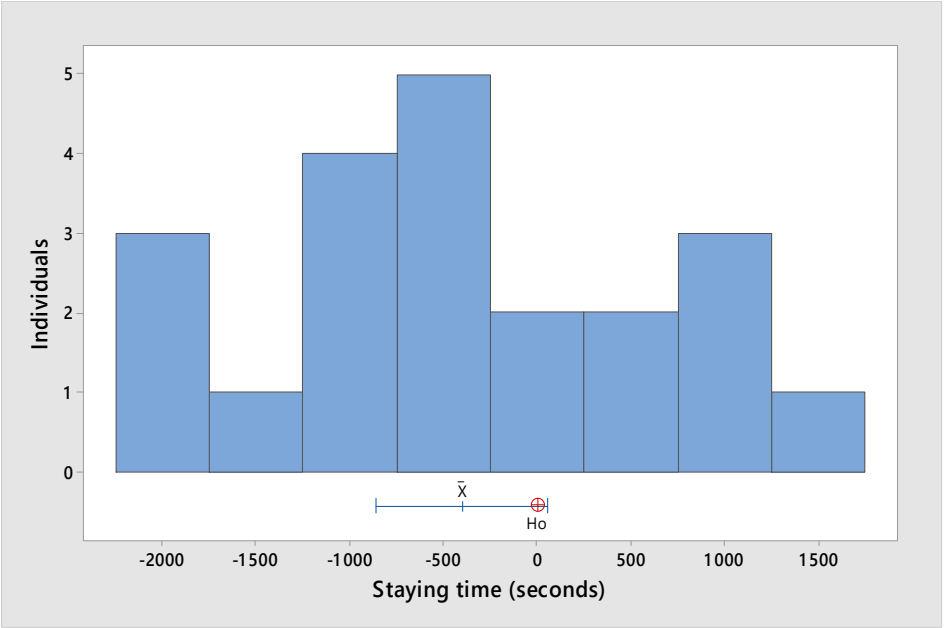


Fig 2.2. Setting (1) comparing staying time with scent preference between self and close neighbor. Salamanders stayed more in neighbor (B) than self-scent with 400 seconds the average gap, which is close to significance (n = 21, p = 0.082). X axis, staying time (seconds) refers A-B; A, self, right side of X axis and B, neighbor, left. X bar ($\bar{X}$), mean of staying time (A-B).

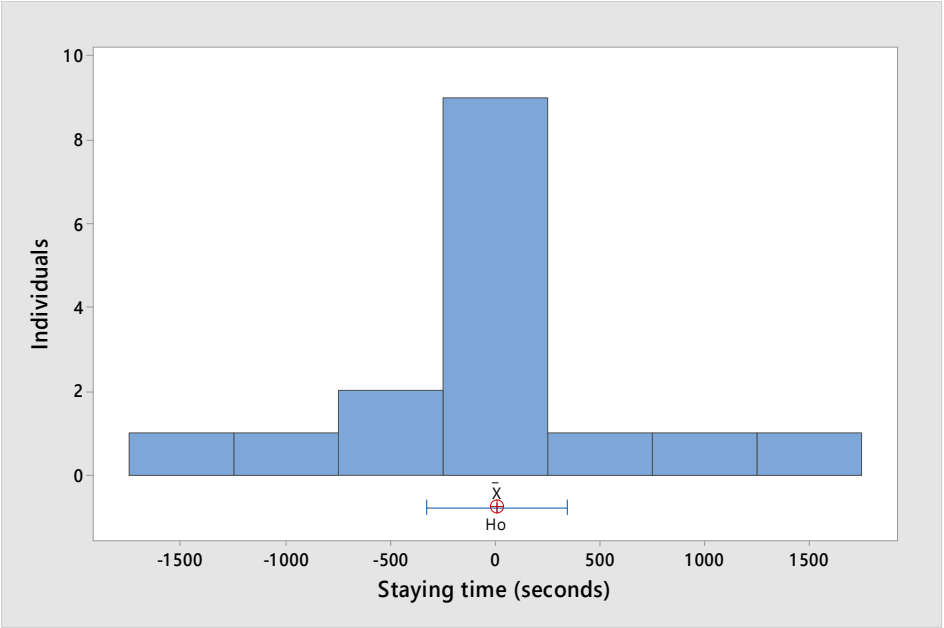


Fig 2.3. Setting (2) comparing staying time with scent preference between self and remote neighbor (stranger). Salamanders stayed very similarly long at both part with only 6 seconds of gap (n = 16, p = 0.968). X axis, staying time (seconds) refers A−B; A, self, right side of X axis and B, stranger, left. X bar ($\bar{X}$), mean of staying time (A−B).

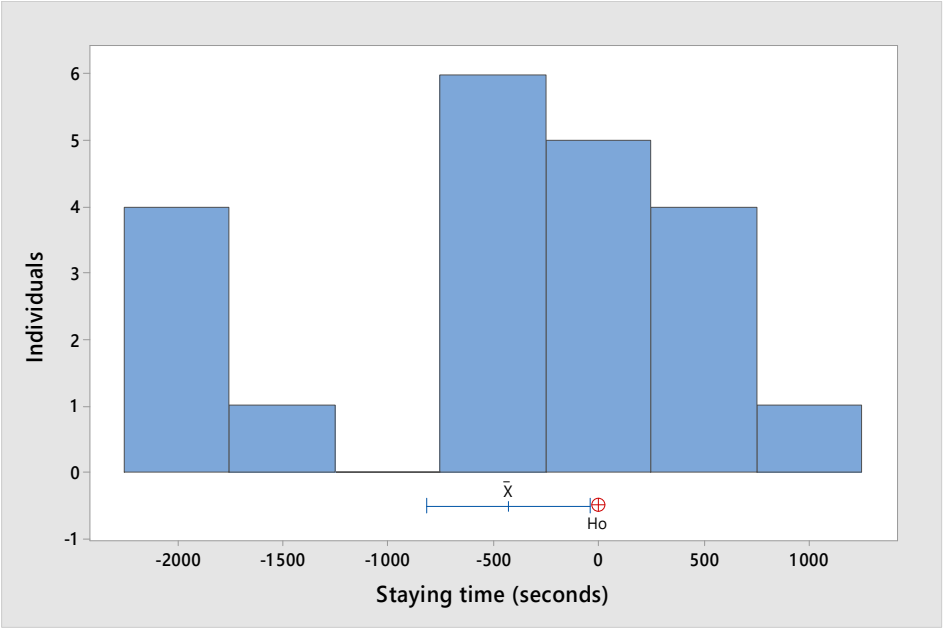


Fig 2.4. Setting (3) comparing staying time with scent preference between self and genetic total stranger. Salamanders Stayed more in B than itself (A) (n = 21, p = 0.033) with 426 seconds gap. X axis, staying time (seconds) refers A−B; A, self, right side of X axis and B, total stranger, left. X bar ($\bar{X}$), mean of staying time (A−B).

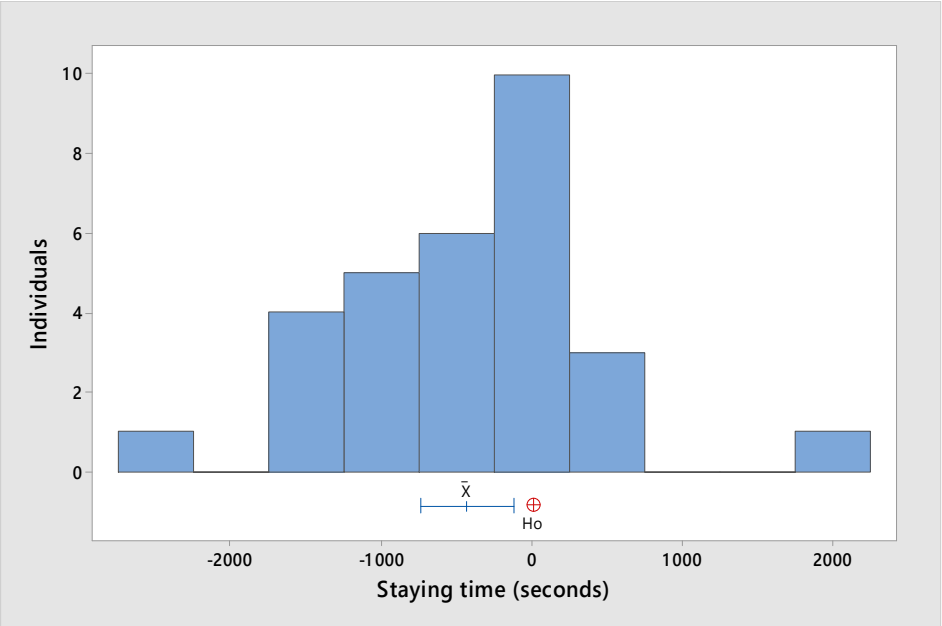


Fig 2.5. Setting (4) comparing the staying time with scent preference between self and blank as control. Salamanders stayed more time in Blank part than itself (n = 30, p = 0.008) with 431 seconds the average gap. X axis, staying time (seconds) refers A−B; A, self, right side of X axis and B, blank, left. X bar, mean of staying time (A−B).

Borderline reaction description: hesitation

Observed behaviors in all 4 settings can be grouped into 3 major patterns: first, hesitation on the borderline for a few seconds at least. Second, they walk towards the borderline but hesitate and sometimes raise their heads up. Third, they cross the borderline but stop right after their head is crossed at least 1/3 of the way up to the whole head. While stopping on the borderline, some salamanders shook their heads up and down, but no clear nose-tapping behavior was observed. After hesitating at the borderline, there were two divisions in behavior: some continued slowly into the other half but others returned to the original half. Total number of crossing time on the borderline was 5.3, 4.5, 4.5, and 5 times in average at four different setting. The hesitation was shown in various timing from the first (second 0) and also after the acclimation is done up to 15 minutes, each end of recording.

At the setting (1) with close neighbors captured in the same site as non-self, only 33 % of individuals (n = 7) recorded hesitated at the borderline. 5 individuals among them showed hesitation with pausing going forward both before and after the turning over the container. At the setting (2) with remote neighbors captured in the different site in the same mountain as non-self had 7 individuals among 16 recorded showed borderline hesitation (43 %) and 2 individuals among them showed twice of hesitation during total recording while 1 of them showed it three times. At the setting (3) with total stranger captured in the totally distant mountain at least 15 km had 9 individuals among 21 recorded showing borderline hesitation (42 %) and 3 individuals among them showed two times of

pausing. The last setting (setting 4) as experimental control had self-scent for the 'A' half and blank, similarly damp brown paper for the 'B' half. 16 individuals among 30 recorded showed borderline hesitation (53 %) and 4 individuals among them showed pausing behavior more than two times while 12 individuals hesitated once or twice during the total recording period.

2.4. Discussion

This is the first report that the Korean plethodontid salamander, *K. koreana*, also used their olfactory sensory system to recognize and to distinguish between conspecifics from different territories.

As an initial reaction to the experimental setup, salamanders generally investigated both sides within each the first 5 minutes of acclimation and after that, they display a strong preference for one side to stay longer. This can imply that *K. koreana* use their olfactory sensory system to distinguish conspecifics as other Plethdontidae salamanders have been shown to do with pheromonal markers (Jaeger 1986). By using paper towel from each individual's container as a scent-marked substrate, the results of this study suggest that *K. koreana* also shows intraspecific chemosignal memorizing and recognition (Jaeger 1977). Here, individuals showed a much greater preference to stay on the part with an unfamiliar scent. Moreover, their behavior (shaking their heads up and down or left to right) while they were investigating each side suggests olfactory involvement in exploration activity. Since the family Plethodontidae is reported to use the vomeronasal organ to detect chemicals

(Dawley and Bass, 1988), *K. koreana*'s head moving behavior suggests that they use the vomeronasal organ even though there was no specific nose-tapping behavior.

Regarding the total time spent in each side, the salamanders showed an absolute preference for the blank/ control half in in setting (4) and in setting (3) the scent of the salamander from a different mountain with more than 400 seconds gap comparing to time spent in the 'self' half. Since they continually crossed between halves whilst moving/ shaking their heads, it is possible that they were memorizing or processing the unfamiliar scent. In setting (1) they also stayed more than 400 seconds longer in the part with the salamander from the same site' s scent which was approaching statistical insignificance ($p = 0.082$). This result is somehow different from the previous finding on other plethodontid salamander that show aggressive behavior towards intruders considering that if they were protecting their home site, they would also be inside part of their habitat (Keen et al. 1984, Staub 1993). However, when salamanders were presented the scent of a salamander captured from a different site in the same mountain in setting (2), they showed an almost equal preference for staying at both side ($p = 0.968$).

The behavior patterns of salamanders in the vicinity of the borderline showed that they were using their whole body or at least always heads including shaking or raising their head. Even though salamanders rarely tapped their head on the ground as 'true' nose-tapping, the head movements that they did display can also suggest that they are using olfactory sensory system towards the new part since they do ultimately show a preference for one side.

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국문 초록

한국 고유 도롱뇽들의 도롱뇽항아리곰팡이에 대한 취약성과 자기인식 행동

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지난 20여 년간, 새롭게 발견된 항아리곰팡이 병원체들은 전세계의 양서류 집단을 황폐화 시켜왔다. 먼저 발견된 항아리곰팡이 (*Batrachochytrium dendrobatidis*, Bd)는 주로 개구리 등의 무미목을 감염시키는 병원체로 알려졌다. 반면, 그보다 더 최근에 발견된 도롱뇽항아리곰팡이 (*Batrachochytrium salamandrivorans*, Bsal)는 도롱뇽 등의 유미목을 감염시키며, 무폐도롱뇽목 (Plethodontidae)의 몇 종을 포함한 전세계 많은 종의 도롱뇽에게는 치명적인 영향을 미치는 것으로 보고되었다. 그간 항아리곰팡이에 대한 국내 연구가 널리 이뤄져 왔고 국내 자연환경에도 널리 퍼져있는 것으로 보고된 것과 달리, 도롱뇽항아리곰팡이의 국내 존재 여부와 국내 고유 도롱뇽에의 감염 시

영향 등은 여전히 알려져 있지 않다. 이에 도롱뇽항아리곰팡이가 국내 종들의 개체군 감소에도 영향을 미치는지에 대한 연구가 촉구되는 실정이다. 본 연구에서는 해당 곰팡이에 대한 국내 도롱뇽 두 종의 취약성을 확인하였다. 이끼도롱뇽 (*Karsenia koreana*)은 아시아 유일의 무폐도롱뇽목에 속하는 종이며, 무폐도롱뇽목의 한 종은 이미 도롱뇽항아리곰팡이에 매우 취약한 것이 발견되었다. 도롱뇽 (*Hynobius leechii*)은 도롱뇽항아리곰팡이와 같이 수생생활을 주로 하는 종으로서 해당 곰팡이에 대한 노출의 위험을 안고 있다. 본 연구를 통해 두 종의 도롱뇽이 도롱뇽항아리곰팡이에 감염된 후 7주 간의 실험기간 동안 취약성을 보이지 않음을 확인하였다. 감염 전과 실험 종류 후의 비교에서, 감염된 이끼도롱뇽은 대조군과 주둥이-총 배설강 길이 (Snout-Vent length, SVL)와 전체 길이 (Total length, TL)의 비슷한 변화를 보였다. 그러나 대조군은 실험기간 동안 무게 (body mass)가 는 반면, 실험군의 무게는 전체적으로 감소하였다. 동일한 실험기간 동안, 도롱뇽 준성체 중 감염군의 전체 길이가 대조군보다 더 줄어들었으나 주둥이-총 배설강 길이와 무게에서는 비슷한 양상을 보였다. 도롱뇽 성체의 경우, 실험기간 동안 감염군의 주둥이-총 배설강 길이가 대조군보다 더 줄어들었으나 전체 길이와 무게에는 비슷한 영향을 받았다. 이끼도롱뇽과 도롱뇽 준성체, 성체 모두 감염군과 대조군 사이의 사망률 비교에서 유의한 결과가 나오지 않았다. 이 결과를 통해 두 종의 도롱뇽 모두 도롱뇽항아리곰팡이에 저항성을 가지고 있음을 주장할 수 있다. 살아있는 도롱뇽항아리곰팡이의 도롱뇽 감염 과정이 적절히 이뤄졌는지 확인하기 위해, 이전 연구에서 도롱뇽항아리곰팡이에 굉장히 취약한 것으로 보고된 *Notophthalmus* 속의 두 도롱뇽 집단 (*N. viridescens*와 *N. piaropicola*)을 국내 도롱뇽에 한 것과 동일한 방법으로 감염시켰다. 이후 모든 개체가 각질 등의 진피층의 임상 징후를 보이며 한 달 내 사망하였다.

무폐도롱뇽목 (Plethodontidae)은 전세계에서 가장 많은 종을 가지고 있는 큰 집단이며, 이 중 많은 종들이 서식지를 인식하는 데 페로몬 신호를 사용하곤 한다. 그러나 국내의 이끼도롱뇽 (*Karsenia koreana*)에서는 이러한 행위가 아직 발견되지 않았다. 본 연구에서는 이끼도롱뇽이 가까운 이웃이나 먼 개체군을 인식하는지를 연구하였다. (1) 실험 개체와 같은 지역 내에 서식하는 개체 (가까운 이웃), (2) 같은 산 내 다른 지역에 서식하는 개체 (먼 이웃), (3) 아예 다른 산에서 서식하는 개체 (완전한 이방인)에 대한 반응을 각 실험하였고, (4) 실험적 대조군 또한 진행하였다. 실험은 플라스틱 통 내부에 중앙선을 표시한 뒤 각 반쪽에 실험 개체와 다른 개체의 냄새를 묻혀 실험 개체의 반응을 보는 것으로 진행하였다. 통 내 좌우에 대한 이동 선호도를 배제하기 위해, 처음 20분 간 실험 개체의 행동 관찰 후 통의 좌우를 돌려 다시 20분 간 개체를 두고 반응을 확인하였다. 각 20분에서 적응기간인 처음 5분 간의 이동 선호도와 나머지 15분 간 전반적인 위치 선호도가 모두 관찰되었다. 이끼도롱뇽은 머리를 위, 아래 등으로 흔들거나 중앙선 전후에서 멈추는 행동 등을 보이며 실험 통 내의 두 부분을 돌아다녔다. 실험 결과, 이끼도롱뇽이 특정 냄새가 없는 실험적 대조군이나 다른 산 내 서식하는 개체의 냄새가 있는 부분에 머무는 것을 가장 선호하는 것을 알 수 있었다. 동일 지역에 서식하는 개체의 냄새가 있는 곳에도 본인의 냄새가 묻은 곳에서보다 더 오래 머물렀지만, 유의한 차이는 없었다. 같은 산 속 떨어져있는 지역의 개체 냄새와 본인의 것 사이에서는 어떠한 선호도도 보이지 않았다.

주요어 : 질병 취약성, 도롱뇽항아리곰팡이, 자기인식, 한국 고유 도롱뇽, 이끼도롱뇽, 도롱뇽

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