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The incidence of abnormalities in the fire-bellied toad, *Bombina orientalis*, in relation to nearby human activity

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Abstract
Declines in amphibian populations are occurring worldwide, and have been attributed to many factors, including anthropogenic environmental changes. One of the ramifications of such declines is abnormalities in many amphibian species. A strong association has been detected between human activities and abnormalities in amphibian populations, but studies on this association are largely focused on lentic species. In this study, it was analyzed whether the degree of local human activity was associated with the rate of abnormalities in *Bombina orientalis* which inhabited lotic environments. We found that the proportions of abnormalities in wild populations of *B. orientalis* increased, when i) the closest human land use was located within 100 m from the frogs' habitat, and ii) the proportion of human land use within a 300-m radius was high. Our findings suggest that human activity has a negative impact on the fitness of nearby amphibian populations, and that wild populations very close to human-induced disturbance are affected.

Key words: amphibian, deformation, human activity, conservation, abnormality

INTRODUCTION

Amphibians are good "bioindicator species" that reflect environmental changes (Hopkins 2007, Lunde and Johnson 2012). Their morphological and behavioural characteristics and inhabiting under/near water with permeable skin during all life-cycle stages make them ideal organisms to assess local environmental health, particularly that of aquatic environments. Accumulating evidence suggests a decline in amphibian populations in many areas worldwide which apparently is attributed to multiple causes (Houlahan et al. 2000, Blaustein and Johnson 2003, Ohmer and Bishop 2011). A number of reasons have been suggested for this decline, including loss of habitat, environmental contaminants, pathogen outbreaks, and diseases (Kiesecker et al. 2001, Blaustein and Johnson 2003).

Herpetological studies have also continuously reported the incidences of abnormally developed body parts in anuran populations (Hebard and Brunson 1963, Lunde and Johnson 2012). Many different abnormalities have been documented, such as digit/limb malformation and missing or extra digits/limbs (Reeves et al. 2013, Lunde and Johnson 2012). Environmental changes, either due to climate change or human activities, such as UV-B radiation, chemical contaminants, or climate-change-induced emergence of pathogens (Skerratt et al. 2007, Lunde and Johnson 2012), are conceivably the main drivers for the emergence of abnormally developed amphibians.

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These abnormalities have been reported primarily in amphibians inhabiting only in lentic habitats, such as ponds or lakes (Johnson et al. 2001, Reeves et al. 2013), whereas abnormalities in lotic-inhabiting amphibians are scarcely reported, either due to rarity or because it may be difficult to survey a sufficient number of frogs to quantify abnormalities in these environments (Lunde and Johnson 2012). *Bombina orientalis* (Bombinatoridae) provides a good opportunity to study the relationship between human land use and abnormalities in amphibians inhabiting a lotic environment, because i) they are a common species found in both lentic and lotic environments such as most creeks and streams, ii) they are vulnerable against human generated pollutants for abnormalities to be developed under the pollutant conditions (Park et al. 2010, Park et al. 2014), and iii) many of the accessible streams and creeks are in close proximity with land used by humans such as agricultural fields, where pesticides and herbicides are often sprayed. Here, we studied the relationship between the degree of human activity and the incidence of abnormally developed frogs on wild populations of *B. orientalis*.

MATERIALS AND METHODS

Study species and field sites

B. orientalis is a common frog species distributed in East Asia and Russia. Hibernating frogs become active beginning in mid-April; they find mates immediately, reproduce until August, and overwinter beginning in October. They are active during the day and night and forage mainly on insects and small arthropods (Ko et al. 2007). We surveyed abnormalities in adult *B. orientalis* at 13 different locations in South Korea (see Appendix for GPS of the study sites) from 18 August to 10 October 2014. These locations were chosen because a previous study (Fong, unpubl. data) confirmed the presence of *B. orientalis* in these areas. Adjacent sites were at least 4.5 km away; thus, constituting separate populations, given the dispersal ability of *B. orientalis*. *B. orientalis* were found exclusively near creeks, either floating on the water surface or sitting on the edge of the creek, or hiding under a rock.

Based on the Daum Map (Daum Kakao 2015), a web-based map service used to visualize South Korean geography, we mapped out a location within which potential *B. orientalis* habitats, such as creeks and streams, were clearly identified. Two to four researchers searched for frogs extensively for 2–3 days between 0900 to 1900 h at each location. All *B. orientalis* that were found at a site were photographed (Sony A65 camera equipped with SAL1650 lens; Tokyo, Japan) on their ventral and dorsal sides by holding the hindlegs. Therefore, we used the photographs to identify the condition of all visible body parts, except the hindlegs. This loss of information should result in an underestimate of the actual abnormality rates. However, abnormalities of hindlegs would not be different from that of forelegs. We kept the frog in a small container after photography until we finished searching the area to avoid re-capturing the same individual. All individuals were released at their original capture sites. Based on abnormality categories (Reeves et al. 2013), we carefully identified abnormal body parts. In our study, only digit abnormalities (missing or extra digits) were detected.

Quantifying the degree of human land use

We developed two variables to adequately estimate the level of human activity at each site, such as the minimum distance between a focal site and the location of the nearest land used by humans and the proportion of human land use within a 300-m radius. Because our surveyed locations were forested land, the human land use was defined as non-forested area in the map, and by using the map, the human land use was easily distinguished. The 300-m criterion was specified because it is considerably less than the mean migration distances of *B. bombina* and *B. variegata*, which are close relatives of *B. orientalis* (Hartel 2008, Kovar et al. 2009). We used Daum Map to measure the minimum distance from land used by humans to the study site where the frogs were found.

We first specified the area with a 300-m radius at each sampling site. Then we took a screenshot of the 300-m radius area and saved it as a JPEG file. Next, we selected the land area used by humans using the 'polygon selection tool' in ImageJ 1.46r (open source program, National Institutes of Health, Bethesda, MD, USA) and calculated the proportion of land used by humans. We also recorded the type of human activity close to the study site, which varied significantly among locations. Therefore, we did not use this category for statistical analysis but provide it in the Appendix.

Statistical analysis

We used ordinary least square regression to determine the relationship between the rate of abnormalities and two predictor variables, such as the proportion of land used by humans and the minimum distance between a

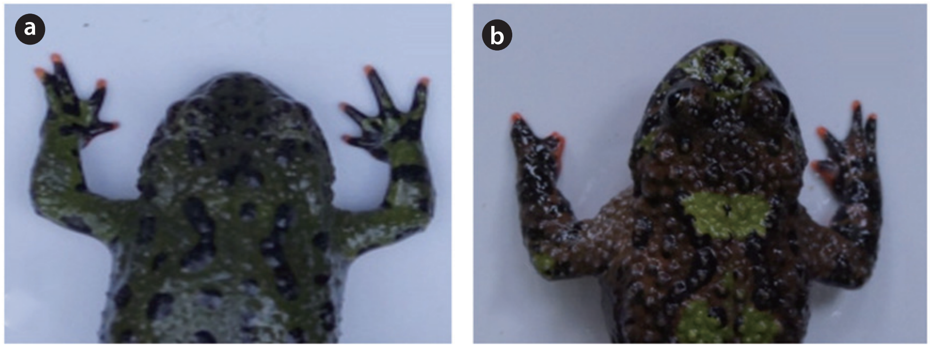

Fig. 1. Example photos of frogs with abnormal digits. Frogs with (a) two digits attached each other in left forelimb, and (b) abnormally shaped left forelimb.

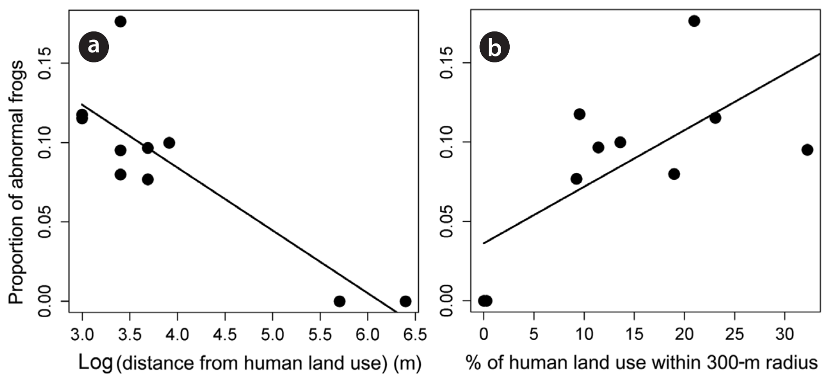

Fig. 2. Relationships between the human activity levels and the proportion of abnormal frogs at each location when the human activity levels were measured as the minimum distance from land used by humans to frog habitat (a), and as a percentage of land used by humans within a 300-m radius from the frog habitat (b).

site and the closest land used by humans. The proportion of land used by humans was normally distributed. However, we log transformed the minimum distance between frog habitat and the closest land used by humans to meet the assumption of ordinary least squares regression. As the two predictor variables of human activity were correlated each other (Pearson's product moment correlation; $t = -3.07$, $df = 8$, $P = 0.02$, $r = -0.73$), we avoided including them in one analysis, but we analysed them separately in two regression models. Then, we adjusted the p-values of each analysis to compensate for the increased type 1 error, following Holm (1979).

Additionally, we employed a nonparametric method for the frog abnormalities to avoid a large datum effect on the proportion data because of the low sample size at each site. All predictor and response variables were binary coded. Abnormality was zero, if no abnormal individual was found, or one, if any abnormal individual was found. Distance was 1 if a study site was < 100 m from human land use, and 0, if it was ≥ 100 m. The proportion was 1 if the proportion of human land use was ≥ 1% of the 300-m radius area, or 0, if it was < 1%. These criteria were chosen because it clearly distinguished the area with human activity and without it. The location with less than 1% of human land use indicates that there were hardly any human activities near the region.

Then, we used Fisher's exact test to identify the association between human land use and the incidence of abnormal frogs.

RESULTS

We found 228 frogs at 13 sites, but only 213 frogs (61 females, 151 males, and 1 unknown) that were collected from 10 sites were used for the analysis after excluding 3 sites with sample sizes < 15. Among the 213 individuals, 19 showed abnormal body parts, of which 18 had the malformed (Fig. 1a) or missing digits (Fig. 1b), and one individual had extra digits.

We found a significant relationship between the human land use and the abnormality rate in adult *B. orientalis*. Specifically, more number of abnormal frogs were found at sites closer to the human land use (Fig. 2a, $F_{1,8} = 22.35$,

P_{adj} = 0.002). The proportion of abnormally developed frogs increased in parallel when the proportion of human land use increased in the 300-m radius area (Fig. 2b, $F_{1,8}$ = 6.878, P_{adj} = 0.031). The analysis of a two × two contingency table also showed significant associations between the incidence of abnormal frogs and the minimum distance from the sampling site to the nearest human land use (Fisher's exact test; n = 10, P_{adj} = 0.044), and between the incidence of abnormal frogs and the proportion of land used by humans (Fisher's exact test; n = 10, P_{adj} = 0.044). We found no sexual differences in the frequency of observed abnormality (Chi-square test; n = 207, χ^2 = 1, P = 1).

DISCUSSION

In accordance with many other studies that have addressed the impact of human activities on frog malformations, our results suggest that the extent of human land use can increase the number of abnormalities in frogs and can negatively impact amphibian conservation in lotic environments. Noticeably, we always found abnormal individuals at sampling study sites where human activity was within a 100-m radius, regardless of the type of human land use (see Appendix for human land use types), but we found no abnormal frogs when human land use was located > 300-m radius from a frog habitat.

The reasons for the observed abnormalities are unknown, and interactions between several factors may have contributed to the observed abnormalities (Blaustein and Johnson 2003). We argue that human-generated chemicals and effluent water from commercial and residential facilities are the main drivers of the abnormalities. UV-B radiation is unlikely the reason for the observed abnormality because i) *B. orientalis* usually inhabits a creek under a thick canopy where most UV light is reflected, and ii) all abnormality patterns were non-symmetrical, unlike the abnormality types revealed by UV-B radiation experiments on amphibians (Ankley et al. 2002, Blaustein and Johnson 2003). We cannot reject the possibility of infectious disease or predation as causes for the *B. orientalis* abnormalities (Blaustein and Johnson 2003). However, no study has reported parasite-induced abnormalities in *B. orientalis*, and secretions from *B. orientalis* have significant effects on microbial (Gibson et al. 1991) and predatory activities in conjunction with their warning colouration. In a nationwide survey, 16.7% of *B. orientalis* were infected with the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), but Bd is not known to cause morbidity or mortality in wild amphibian populations in Korea (Bataille et al. 2013). Thus, we argue that these are not the main factors responsible for the observed high abnormality rate in *B. orientalis*.

Alternatively, anthropogenic chemicals may be responsible for abnormalities in *B. orientalis* in South Korea. Although we do not know the quality of water in our study sites, many creeks are in close proximity with potential pollution sources which may contain human generated chemicals, such as pyrethroid insecticides (tourist attractions, pensions, and camping sites), agricultural chemicals (agricultural fields, orchards), or effluent (dwelling houses), which can affect frog development (Berrill et al. 1993, Mann et al. 2009, Park et al. 2014). In the study by Park et al. (2014), *B. orientalis* tadpole abnormality rates increased in municipal effluent water that was not fully treated, with a mean abnormality rate of 20%, compared to those raised in unpolluted upstream water (mean abnormality rate, 3%). They also showed that rate of abnormalities was positively correlated with total water nitrogen, which has a detrimental effect on amphibian development (Rouse et al. 1999). Therefore, we argue that the differences in human induced chemicals should be one of the main factors that resulted in the observed variation in abnormality rates in this study.

We note here that the abnormality rates in our study should be considerably lower than true abnormality rates in nature. First, because our survey started in August, which is after most *B. orientalis* have grown fully, it is likely that the malformed tadpoles or froglets (also including embryos that failed to develop) had already died off. The survival rates of malformed tadpoles or froglets are much lower than those of normally developed ones (Park et al. 2014). This can be also deduced by the fact that we only found individuals with digit abnormalities, which may be less crucial for survival than other types of abnormalities, such as eye/limb/skeletal abnormalities. Second, we used photographs to detect abnormalities, which could underestimate abnormality rates because we may have missed hindleg abnormalities and minor skeletal abnormalities. However, it is practically difficult to estimate true abnormality rates in nature (Lunde and Johnson 2012) because the entire developmental period should be monitored to obtain the true rates. Because most abnormality studies, including ours, used a similar sampling scheme it is possible that the overall level of abnormalities was significantly higher than that reported previously.

To the best our knowledge, this is the first attempt to address amphibian abnormalities in a lotic environment as related to human activity levels. These kinds of studies are important because they reflect ecological health, which will consequently affect human health. In line with the previous studies (Kiesecker et al. 2001, Taylor et al. 2005, Hopkins 2007, Mann et al. 2009), our results further support the idea that human activities negatively impact amphibian conservation. Our finding that the sites with frog abnormalities were in close proximity to land used by humans suggests that ecological health can be retained/improved by maintaining human land use away from the area of interest (Taylor et al. 2005, Reeves et al. 2008). It is desirable to incorporate a more detailed field survey and chemical analysis to firmly conclude the negative impact of human land use on amphibian abnormalities and the effects of how different types of land use. These studies will offer a future direction for amphibian conservation and to improve ecological health.

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Appendix. GPS coordinates of each sampling location and the types of nearest human land use. We categorized the human activity regions as agricultural land use (including orchards and paddy fields) and urban use (including residential area, tourism area)

Location (survey date)	GPS coordinates	Nearest human activity (distance from collection sites)	no. observed abnormal individuals (sample size)
Gapyeong (2014. 9. 9)	37°54′01.2″N, 127°35′52.0″E	Urban use (50 m; commercial accommodation)	3 (30)
Gangreung (2014. 9. 4-5)	37°38′64.5″N, 128°55′04.2″E	Urban use (600 m; commercial accommodation)	0 (16)
Goesan (2014. 8. 19-20)	36°41′65.4″N, 127°50′76.4″E	Agriculture use (300 m; farming field)	0 (17)
Sooncheon (2014. 8. 21-22)	34°99′19.7″N, 127°34′49.9″E	Urban use (30 m; tourist attraction)	2 (25)
Youngam (2014. 8. 23)	34°46′47.8″N, 126°43′20.2″E	Urban use (20 m; camping site)	2 (17)
Wanju (2014. 9. 25)	35°41′69.6″N, 127°05′63.7″E	Agriculture use (20 m; paddy field)	3 (26)
Andong (2014. 9. 17-18)	36°23′23.9″N, 128°55′16.1″E	Agriculture use (30 m; orchard)	4 (30)
Woolsan (2014. 10. 1-2)	35°27′20.0″N, 129°06′24.0″E	Urban use (40 m; temple)	3 (31)
Jeju (2014. 8. 26-28)	33°18′49.0″N, 126°33′54.1″E	Urban use (30 m; dwelling house)	2 (21)