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Evaluating Movement Patterns and Microhabitat Selection of the Japanese Common Toad (*Bufo japonicus formosus*) Using Fluorescent Powder Tracking

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Bufo japonicus formosus **(Eastern-Japanese common toad) is endemic to Eastern Japan. As with many Japanese amphibians, little is known about its terrestrial life, especially during the non-breeding season. This species persists even in highly disturbed urban areas where many other amphibian species have already been extirpated. An understanding of how such species use habitats within remnant landscapes may help to inform management strategies for the conservation of urban ecosystems. We examined the nightly movement patterns, distance traveled, movement range, and microhabitat selection of non-breeding adult** ***B. j. formosus*** **at an urbanized site, using fluorescent powder tracking. We evaluated the usefulness of this tracking method through this survey. We found that the nightly distances traveled by these toads varied greatly among individuals and nights. No sexual differences in movement pattern, distance traveled, and movement range were detected. However, body size significantly affected distance traveled and movement range. We found that toads tended to use areas covered with grasses and mosses more frequently than expected, and to avoid paved areas. Fluorescent powder tracking was effective for the elucidation of movement patterns and habitat selection of amphibians. Our results provide useful information for the conservation of amphibians, especially for species inhabiting urbanized areas.**

Key words: *Bufo japonicus formosus*, fluorescent powder, tracking, movement patterns, microhabitats, null models

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INTRODUCTION

Information on movement patterns and habitat use of animals is vital to understanding the ecology of species or populations. It can provide important insights for determining the effects of environmental changes on a species, and can support decision-making in the development of optimum management strategies (Valdez et al., 2017). There have been extensive studies on microhabitat use and movement in amphibians. Favorability of habitat features are likely to vary depending on factors such as physiology (Birchfield and Deter, 2005; Graeter et al., 2008), foraging efficiency (Roberts and Liebgold, 2008), and predator avoidance (Milinski and Heller, 1978; Werner et al., 1983; Gilliam and Fraser, 1987; Lima and Dill, 1990). Habitat preferences may also differ between sexes and age classes due to individual behavioral and resource requirements (Bartelt et al., 2004; Bull, 2006; Valdez et al., 2016).

Habitats that promote mate finding and reproductive success will be favored during the breeding season, while those that allow individuals to forage, grow, and survive climatic extremes may be favored during the non-breeding season. Consequently, the habitat requirements of a species often vary seasonally depending on the availability of resources, such as breeding sites or sheltering habitats (Seebacher et al., 2002; Croak et al., 2010; Garnham et al., 2015). While many studies have been conducted on habitat selection, these have generally focused on habitat use during periods of high activity, when individuals are easily detected (MacKenzie et al., 2002; Pellet and Schmidt, 2005). Because most pond-breeding amphibians become more active and easier to detect during the breeding season, when they congregate to breed, many studies are conducted in the breeding season (Lamoureux and Madison, 1999; Hamer and McDonnell, 2008). Since few studies have been done on amphibians during the non-breeding season (Garnham et al., 2015), our knowledge of movement patterns during the non-breeding season has been limited. Although there have been many previous studies of movement pattern and microhabitat use in Europe (e.g., Pellet and Schmidt, 2005), the United States (e.g., Bartelt et al., 2004; Bull, 2006), and Australia (e.g., Croak et al., 2010; Valdez et al., 2016), only a few have been conducted with Asian amphibians, whether in the breeding season or not.

Recent developments in tracking devices, such as bio-logging, in which a logger is directly attached to the animal body, have advanced animal movement studies rapidly (Wilmers et al., 2015). However, the use of such devices has

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been limited to comparatively large-sized animals (Wilmers et al., 2015). The large size and high costs of these devices prevent us from applying them to small animals, and thus there have been few bio-logging studies of small-sized species (Okamiya and Kusano, 2016). One common method for tracking amphibian species is radio-tracking. However, this method has some limitations and shortcomings. For example, the precision of tracking depends on the number of location points acquired. Radio-tags attached directly to animals and the presence of an observer may also disturb the natural movements of the animals studied (Richards et al., 1994; Eggert, 2002).

Fluorescent powder tracking has emerged as one of the most promising tracking methods. This method can provide exact tracks of an individual's movements, and can be useful in discovering how members of population move through environments (Stark and Fox, 2000; Williams et al., 2014; Okamiya and Kusano, 2016). This technique is non-invasive (Lemen and Freeman, 1985; Stapp et al., 1994; Rittenhouse et al., 2006) and inexpensive, and can be performed successfully with little training (Furman et al., 2011). This method has also been successfully applied in small animals, such as small mammals (Frantz, 1972; Lemen and Freeman, 1985; Halfpenny, 1992; Stapp et al., 1994; Windmiller, 1996; Hubbs et al., 2000; McCay, 2000; Corbalan and Debandi, 2009), anurans (Eggert, 2002; Birchfield and Deters, 2005; Graeter and Rothermel, 2007), urodeles (Roberts and Liebgold, 2008; Roe and Grayson, 2008; Orlofske et al., 2009, Williams et al., 2014), tortoises and turtles (Blankenship et al., 1990; Butler and Graham, 1993; Keller, 1993; Tuttle and Carroll, 2005), lizards (Fellers and Drost, 1989; Dodd, 1992; Stark and Fox, 2000), and snakes (Furman et al., 2011). Fluorescent powder has been shown to be safe for small mammals (Lemen and Freeman, 1985), as well as snakes, lizards, frogs, and salamanders (Williams et al., 2014). Moreover, it has been suggested that, for amphibians, being covered with fluorescent pigments is similar to being covered with soil or organic debris (Rittenhouse et al., 2006).

Bufo japonicus formosus (Eastern-Japanese common toad) is endemic to eastern Japan, and common species in Honshu region. The movements of this species during the breeding seasons have been widely studied using various techniques, such as radio-tracking, the bobbin method, and mark–recapture procedures (Yano, 1978; Okuno, 1985a; Hisai et al., 1987; Ishii et al., 1995; Kusano et al., 1995). However, our knowledge of the movement patterns and microhabitat use of this species during the non-breeding season is very limited (Kusano et al., 1995). Although populations have been declining, this species even persists in highly disturbed urban areas where many other amphibian species have already been extirpated (Fukuyama and Kusano, 2013). An understanding how such species use habitats within remnant landscapes may provide some important knowledge for understanding how animals adapt to the urbanized environments, and useful hints for conservation of urban ecosystems.

In the present study, we investigated the movements of *B. j. formosus* with fluorescent powders during the non-breeding season to determine nightly movement patterns, distances traveled, movement ranges, and the factors affecting them. We also analyzed microhabitat use, using a null model approach, and discussed the ecological significance of microhabitat selection of this species.

MATERIAL AND METHODS

Material species and study area

Bufo japonicus formosus is endemic to eastern Japan, from southern Hokkaido to the Kinki and San'in districts of Honshu. While the population in Hokkaido may be introduced, this toad is very common in Honshu region. The toad inhabits a variety of habitats, from sea level to high altitudes in mountains, and breeds explosively during early spring in still waters, such as roadside ditches, lakes, marshes, and small ponds (Maeda and Matsui, 1989). We conducted this study from May to June in 2015 on the campus grounds (area approximately 40 ha) of the Tokyo Metropolitan University in Hachioji-shi, Tokyo (35°37′10″N, 139°22′56″E; datum WGS 84) at an altitude of 110–140 m. This area contains various types of habitats, such as secondary deciduous forests, planted coniferous forests, grass fields, paved areas, and abandoned paddy fields. From late February to March, several hundred *B. j. formosus* individuals aggregate in a human-made pond located near the center of the campus, and subsequently disperse during the non-breeding season to various nearby habitats (Kusano et al., 2015). Detailed descriptions of the study area have been reported by Kusano and Inoue (2008) and Kusano et al. (2006, 2015).

Field tracking

We conducted visual surveys at night between 19:00 and 23:00 within the campus. When *B. j. formosus* individuals were located, they were captured by hand, their sex was recorded, and their snout–vent length (SVL) was measured. We distinguished mature males based on the presence of secondary sexual characteristics, such as thumbpad development. The captured toads were individually marked with passive integrated transponder (PIT) tags, and then fluorescent powder (Nippon Keikou Kagaku Corp., Tokyo, Japan) was applied. We used five different colors: orange (NKP-8304), pink (NKP-8307), green (NKP-8312), yellow (NKP-8315), and white (NKP-8329). These colors were selected to avoid confusion in identification of the individual paths, as suggested by Stark and Fox (2000).

The dye was diluted in paraffin oil (Eggert, 2002; Williams et al., 2014) and applied at the site of capture with a brush to a part of the abdomen and hind limbs of the toads, following the method described in Okamiya and Kusano (2016). Once the powder was applied, we released the toads at their original capture sites. The following night, we revisited the release sites and used a deep violet LED flashlight (Nichia Corp., Tokushima, Japan) to locate the pigments deposited on the ground or vegetation as the toads moved. We followed the pigments to determine individual trajectories, and regarded the points where the direction of their movements changed more than 2.5° as an inflection point. All of the inflection points in individual trajectories were marked with a color spray that was visible in daylight (Calenge et al., 2009). The trajectories were followed from their initial locations until they were lost or the tracked individuals were located. When the tracked individuals were located, fluorescent powder was reapplied. To minimize contact and agitation, nightly surveys were limited to once a night to avoid disturbing the natural behaviors of the toads (Roe and Grayson, 2008).

The following day, we recorded the distance between successive inflection points (DP; the step length) with a tape measure, and the angles at inflection points (AP; the angle between the current step and the previous step) with a compass. We took the initial location of each trajectory using a handheld GPS receiver unit (Garmin International Inc., Olathe, KS). The coordinates of each trajectory was then compiled in the Quantum GIS (QGIS) program to construct a GIS object of the trajectory (http://qgis.org/en/site/forusers/download.html).

Data analysis

We used the software R 3.3.2 (R Development Core Team, 2016) and the package adehabitat (Calenge, 2006) for statistical analyses. To determine the characteristics of the nightly movements of individual toads, we analyzed the distribution of the distances between the successive points (DP) and the angles at the inflection points (AP) of the individual trajectories. A linear mixed effects model was used to test difference in the distribution of the DP between sexes (Zuur et al., 2013), and the randomized version of Watson's two sample test (9999 replications) was used to test for sexual difference in the distribution of the AP (Zar, 1996; Pewsey et al., 2013). In the mixed effects model, Gaussian distribution was used for the probability distribution, and individual ID was used as random effect. The effect of sex was evaluated by a likelihood ratio test based on maximum likelihood estimation, and the p-value was determined using parametric bootstrap approach (999 iterations) due to the small sample size (Faraway, 2006). In the randomized version of Watson's two sample test, we used one trajectory per one individual for analysis.

For nightly movement range, we calculated actual pass length (APL) as an accumulated movement distance by summing DPs of respective trajectories. We also calculated maximum range length (MRL), and nightly movement range (home range size per one night) using the minimum convex polygon (MCP) and kernel density estimation (KDE; 95% probability level; Worton, 1995) methods. Multivariate analysis of variance (MANOVA) was used to test for the effects of sex and size on nightly movement distance and range with APL, MRL, MCP, and KDE as response variables and sex, SVL and their interaction as explanatory variables. For individuals with multiple trajectories recorded, the mean values were used for analysis. All of the response variables were log-transformed to achieve normality prior to analysis.

Habitat selection

To examine microhabitat use and sexual differences in usage, we calculated the frequency of respective habitat types that individual toads passed over during the night. The study area was classified into five habitat types based on the conditions of the soil surface: paved areas, graveled roads, shrubby areas, areas covered with grasses or mosses, and areas covered with fallen leaves. These habitat types were further classified based on the conditions of tree canopies: open or closed, with little overlap observed (<6%). Since all of the geometrical properties of the trajectories could be summarized by studying the variation of the relative angles, we discretized trajectories, such that they are built up by steps with a constant length (Turchin, 1998).

Every trajectory obtained in this study was discretized with a constant step length of 0.5 m, which was determined considering the measurement accuracy of trajectories, the resolution of microhabitat map, and the body size of toads. The coordinates of the points at both ends of each step (0.5 m in length) and the angles (AP) between the successive steps were recalculated.

We then counted the number of the points within each habitat type for each discretized trajectory, and pooled the frequency of habitat types travelled over by toads for all trajectories.

A null model approach was used to test for habitat selection by the toads (Gotelli, 2001; Martin et al., 2008). An additional 2999 trajectories were generated for each discretized trajectory (a constant step length of 0.5 m) by randomizing AP (the angles between successive steps) and reconstructing the trajectory while maintaining the initial point of the observed trajectory. Randomly generated trajectories that crossed the places that the toads could not pass (e.g., buildings and walls) were eliminated. Using these randomized trajectories, the expected values for the use of each habitat type was obtained. A randomization test was performed separately for each sex using the expected microhabitat use distribution to examine the differences between the observed and expected values for each habitat type. The significance level for all tests was set at $P < 0.05$ (two-sided).

RESULTS

Field tracking

We obtained 41 trajectories from seven males (mean SVL = 102.1 mm, range = 92–121 mm) and 16 females (mean SVL = 106.1 mm, range = 76–130 mm). Some of the trajectories are shown in Fig. 1. Among the tracked individuals, six (one male and five females) were not marked with PIT tags, but were individually identified by their sex and body size. We successfully recaptured tracked toads in 17 cases: in four of these cases, the toads were resting and in the remaining 13 cases they were active. We successfully followed five individuals (three males and two females) for two or more nights.

Data analysis

Figure 2 shows frequency distributions of the distances between successive steps (DP) and the angles between the current and the previous steps (AP) of 41 trajectories. The

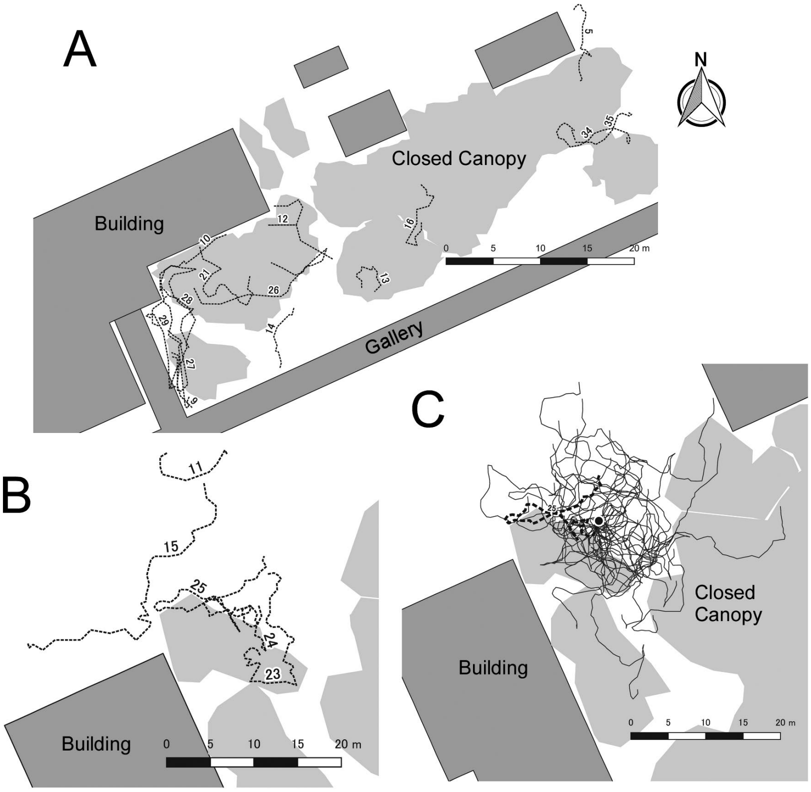

Fig. 1. **(A, B)** Examples of trajectories of *Bufo japonicus formosus* in the surveyed area. The numerical value of each trajectory represents its trajectory ID. **(C)** The observed trajectory of No. 25 (thick broken line) and its randomized trajectories produced by a null model. Among 2999 randomized trajectories, only 30 are shown (thin solid lines). The solid circle shows the common initial point of trajectories.

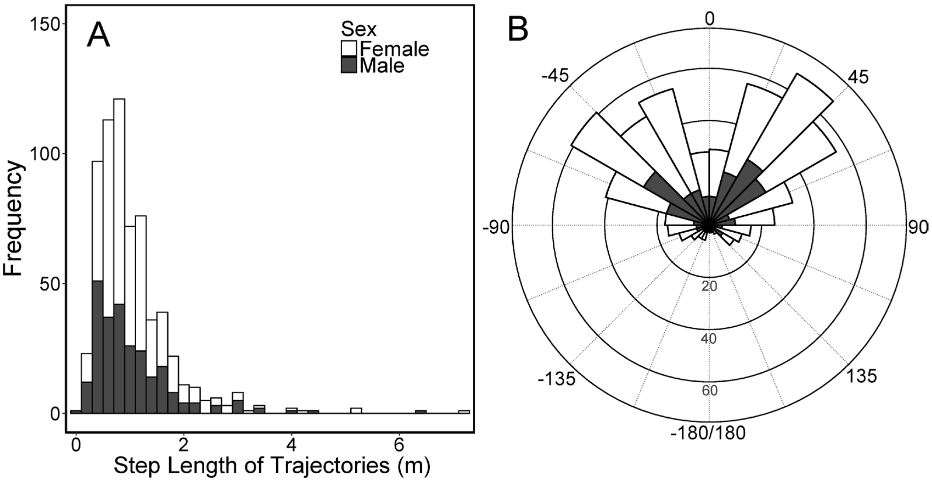

Fig. 2. Frequency distributions of the parameters of movements of the 41 trajectories from 23 individuals of *Bufo japonicus formosus*. **(A)** Step lengths (DP) and **(B)** turning angles (AP). Note that the distribution of turning angles peaks around 45°.

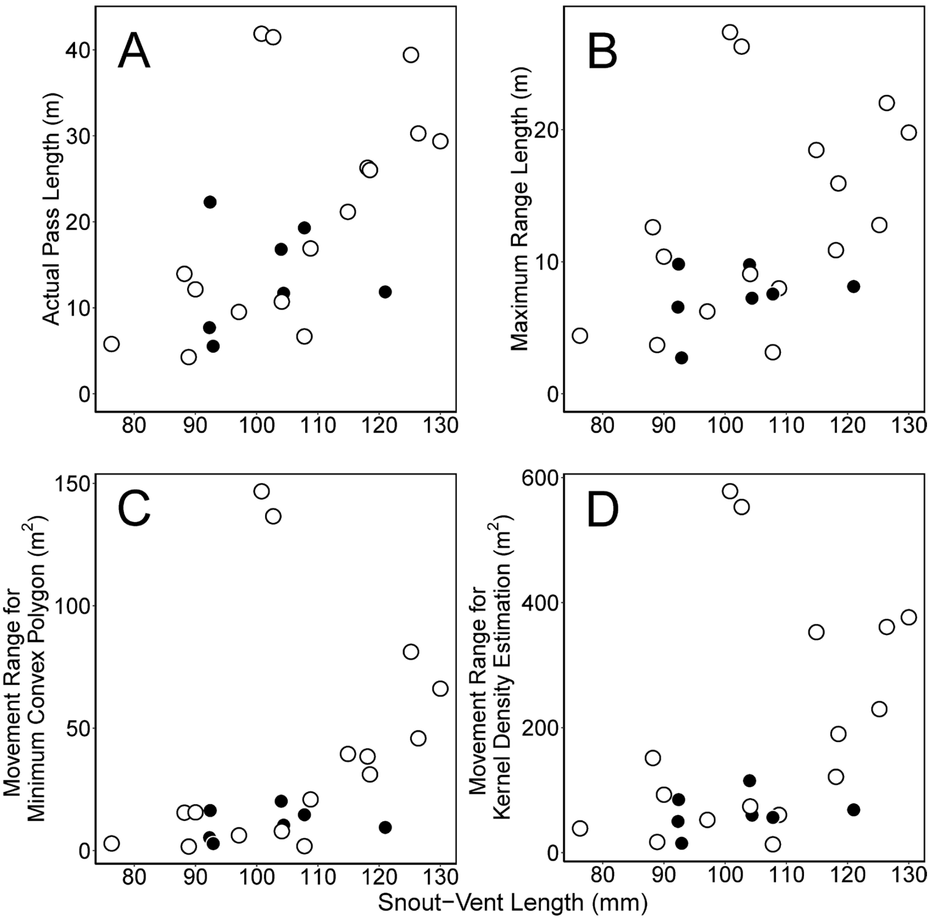

Fig. 3. The relationship between body size (SVL) and movement distance or nightly movement range: actual pass length (APL; **(A)**), maximum range length (MRL; **(B)**), nightly movement range using the minimum convex polygon (MCP; **(C)**) and kernel density estimation (KDE; **(D)**). These estimates were obtained from 23 individuals. Solid and open circles indicate males and females, respectively.

turning angles (APs) of 30–45° were frequent on both sides (left and right), and hard turns with APs of more than 90° were rare (Fig. 2B). We did not detect significant sexual differences in DPs (likelihood ratio test; $\chi^2 = 2.688$, $P = 0.121$) or APs (Watson's two sample test; $U^2 = 0.035$, $P = 0.879$). Movement distances (APL) and nightly movement ranges (MRL, MCP, and KDE) varied greatly among the 23 individuals (Fig. 3): APL, MRL, MCP and KDE ranged from 1.7–41.9 m (mean = 16.8, SD = 11.4), 2.7–27.4 m (mean = 11.4, SD = 7.1), 1.7–146.8 m^2 (mean = 32.1, SD = 40.3), and 13.6–578.5 m^2 (mean = 161.7, SD = 168.1), respectively. The results of MANOVA revealed that SVL had a significant effect on movement distance and nightly movement ranges, but sex and it's interaction with SVL did not. (sex: $F_{4,16} = 0.770$, $P = 0.560$; SVL: $F_{4,16} = 3.047$, $P = 0.048$; sex*SVL: $F_{4,16} = 0.354$, $P = 0.838$).

Habitat selection

All 41 trajectories were discretized, and we obtained a total of 1294 points at both ends of the respective steps. The results showed that 174 points fell within open paved areas, 54 within open graveled roads, 47 within open shrubby areas, 447 within open areas covered with grasses and mosses, 521 within areas covered with grasses and mosses under closed canopy, 106 within areas covered with fallen leaves under closed canopy, and eight within other areas under closed canopy. The total number of points was 1357. Different habitat types overlapped slightly, and the sum of the number of points fallen within each habitat type was slightly larger than the actual total number of points within the trajectories (1294).

A total of 2999 simulations produced the frequency distribution of microhabitat use under the null model (Fig. 1C), and we compared the observed frequency of microhabitat use and the expected frequency separately for each sex. Males tended to use areas covered with grasses and mosses more frequently than expected irrespective of the conditions of canopy cover (closed, $P = 0.0107$; open, $P < 0.0001$), whereas they avoided paved areas ($P = 0.0013$) when they foraged during the night (Fig.

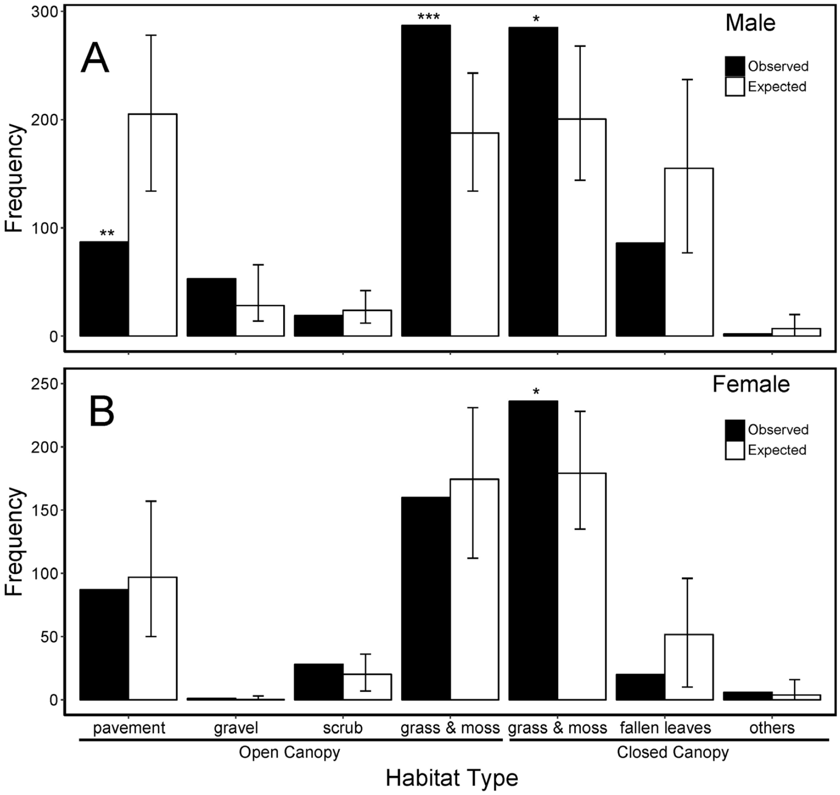

Fig. 4. Microhabitat selection by *Bufo japonicus formosus* The frequencies of microhabitat use for five habitat types in two canopy types are shown in males **(A)** and females **(B)**. Solid and open bars indicate observed and expected frequencies by the null model, respectively. Error bars indicate 95% confidence intervals. A randomization test was performed (No. replications = 2999), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (two-sided test).

4A). Females tended to use closed canopy areas covered with grasses and mosses frequently than expected (*P* = 0.016) when they foraged during the night (Fig. 4B). Slight sexual differences of microhabitat use were observed in paved area and open canopy areas covered with grasses and mosses (Fig. 4).

DISCUSSION

Movement pattern

In the present study, the field survey was conducted during the period (from May to June) when *B. japonicus* was reported to be the most active in the non-breeding season (Okuno, 1984), and the movement and microhabitat use patterns of *B. j. formosus* during the season were clarified in detail.

The distribution of the distance between the inflection points (DP) showed that most of the DPs were shorter than 2 m. On the other hand, long-distance straight movement exceeding 4 m was observed at low frequency. Such movement patterns are commonly observed in a variety of animal species (e.g., Ramos-Fernandez et al., 2004; Simon, 2007; Sims et al., 2008). This pattern, known as the Lévy walk, is considered to be an adaptive searching behavior for uncertain resources (Edwards et al., 2007). This result suggested that the observed movements are similar to Lévy walk pattern, which has not yet been reported in amphibians to the best of our knowledge. Generally, toads may not know where the prey is abundant. In such situations, Lévy-like movement patterns may contribute to enhance foraging efficiency.

This study also clarified the linear tendency of the nightly movements of toads. The analysis of the distribution of the turning angle (AP) of trajectories indicated the turning angles of 30–45° were frequent on both sides (left and right), and hard turns with APs of more than 90° were rare. This suggests a slightly meandering (straight) pattern of movements of toads. Many radio-tracking studies have already reported that amphibian species tend to move in a linear manner during their migration (e.g., Semlitsch, 1981; Madison, 1997). However, radio-tracking studies tend to produce linear paths, because the intervals between relocations cannot be set short enough to prevent disturbance of the behaviors of the animals tracked. On the other hand, by using fluorescent powder tracking, researchers can obtain more detailed and precise trajectories, although information on time is not recorded. These results clearly show the linear movement pattern of amphibians.

Movement distance and movement range

We found that the distance of the nightly movement of *B. j. formosus* had a wide range of variation among individuals. In addition to the present study, earlier fluorescent powder tracking studies on amphibian and reptile species have shown that nightly movement distances varied greatly within and among species: e.g., 1–70 m in salamanders (Roe and Grayson, 2008) and 3–203 m in snakes (Furman et al., 2011). The longest distance of the nightly movement that was observed in the present study was 41.9 m. Since the individual was not recaptured, the individual presumably traveled a longer distance. In a previous research on toads, an individual of the southern toad (*Anaxyrus terrestris*) was reported to move several hundreds of meters within a single night (Graeter et al., 2008). We also found that many individuals were traveling relatively short distances (less than 20 m). The average of the APL was only 16.8 m. Okuno (1985a, b) conducted a long-term mark-recapture study of *B. j. japonicus* in Kanazawa, Ishikawa Prefecture, and reported that the movement distance during a single night was approximately 20 m in concurrence with the present study.

Some individuals returned to the original sites after nightly activities. High site fidelity to summer home ranges has been reported in this species (Chiba, 1978; Okuno, 1985b). These results suggest that toads have their own stable home ranges. Other radio-tracking studies on *B. j. formosus* also reported similar results (Shirane, 1982; Kusano et al., 1995).

Kusano et al. (1995) estimated that the home range of *B. j. formosus* was approximately 220 m^2, based on radio-tracking data for several months. The mean nightly movement range (MCP) estimated in the present study was much smaller than the value described above, and the difference was the result of the shorter tracking period (only one night).

We found that movement distance and nightly movement range increased with SVL. However, we did not detect any significant effect of sex on movement distance or nightly movement range, although four different measures were applied: APL, MRL, MCP, and KDE. This may have resulted from incomplete tracks (Roe and Grayson, 2008).

Habitat selection

A significant preference for grasses and mosses vegetation with canopy cover was observed in both sexes. However, preference for the open canopy area was observed only in males. Toads have a number of adaptations for terrestrial life, including an ability to store water in their lymph sacs and bladder (Boutilier et al., 1992) and the ability to withstand relatively high amounts of dehydration (Shoemaker et al., 1992). However, because they have little physiological control over evaporative water loss, toads need to periodically rehydrate and select protected sites that conserve body water (Carey, 1978; Tracy et al., 1993). Because grasses and mosses can keep the soil surface moderately moist, these habitat types are suitable for preventing the toads' bodies from drying (Seebacher and Alford, 2002). Grasses and mosses appear to contain sufficient moisture, regardless of the conditions of the canopy cover in the study site. The observed sexual difference may be due to sampling bias. However, Valdez et al. (2016) reported that only females actively select for terrestrial vegetation in the golden bell frog (*Litoria aurea*). In addition to protection from desiccation, terrestrial vegetation may provide opportunities for foraging (Pyke and White, 1996; Pyke and White, 2001) and shelters from predators (Valdez et al., 2016). The preference for terrestrial vegetation is a general trend of many amphibian species (e.g., Pyke and White, 2001; Valdez et al., 2016).

The significant avoidance of paved areas was observed in only males. Smaller bodies have a lower capacity to hold water and thus an increased risk of dehydration, which may limit them in moving through drier areas (Bartelt et al., 2004). Paved areas are generally dry, open spaces and lack hiding places, such as underground burrows and cavities, which are important microhabitats for toads (Bartelt et al., 2004). High humidity at such shelter sites reduces the loss of body water from toads (Tracy, 1976). Avoidance of open paved areas may also be due to higher potential predation risks. In the study site, the potential predators of toads were the raccoon dog (*Nyctereutes procyonoides*) and the Asian tiger snake (*Rhabdophis tigrinus*), which actively preys on toads to sequester defensive steroids (Hutchinson et al., 2007; Kojima and Mori, 2014). Although open spaces such as paved areas certainly lack secure refuges against predators, we do not have any evidence of higher predation risk in the open paved areas of the study site at present.

Conclusion

In the present study, the movement patterns and microhabitat use of *B. j. formosus* were examined using fluorescent powder tracking. This is the first report in which this method was used in a Japanese amphibian species. Information included in the present study will be helpful in our understanding of the functional aspects of habitat ecology and implementing these features in future conservation management strategies.

This study demonstrates that *B. j. formosus* forages frequently, even in highly human-disturbed areas without canopy cover beside buildings. A total of seven species of amphibians reproduce at the study site (Kusano and Inoue, 2008; Kusano et al., 2006, 2015); all of the other species rarely emerge from the forested areas (see Okochi, 2001). Almost all amphibian species have disappeared from highly disturbed urban areas of Tokyo, but *B. j. formosus* has persisted even in these areas (Fukuyama and Kusano, 2013). These results have also been found in other amphibian species that continue to persist in disturbed habitats (Price et al., 2011; Smallbone et al., 2011; Scheffers and Paszkowski, 2012; Valdez et al., 2015). This may be because of the ability to utilize a wide range of habitats, from disturbed open habitats to natural forested habitats (Warren and Büttner, 2008; Canessa et al., 2013; Fukuyama and Kusano, 2013). However, we found that they avoid excessively disturbed areas such as paved areas despite adaptation for urbanized environments. The results of this study may provide some important knowledge for understanding how animals adapt to the urbanized environments, and useful hints for conservation of urban ecosystems.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

HO conceived and designed the study. HO and TK performed the statistical analysis. TK prepared the figures. HO wrote the manuscript.

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