

The role of golf courses in maintaining genetic connectivity between common frog (*Rana temporaria*) populations in an urban setting

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Abstract We studied population size, genetic diversity and differentiation of common frog (*Rana temporaria*) populations at urban golf courses and reference natural ponds in the greater Helsinki region, southern Finland. A total of 248 tadpoles from 12 locations (six golf courses, six reference sites) were genotyped with 13 polymorphic microsatellite markers. The most urban populations, situated in northern Helsinki, were the largest breeding sites having >120 (golf courses) and >200 (reference sites) spawn clumps at the time of sampling. On average, there was no difference in the number of spawns between the anthropogenic ponds at golf courses and the natural water bodies. Genetic variation within populations was substantial ($H_O = 0.68$) while genetic differentiation between populations was low ($F_{ST} = 0.016$; average distance = 17.6 km). The golf course populations did not differ from natural populations in terms of genetic variability or differentiation. Hence, our results suggest that golf courses contribute positively to urban amphibian populations by

providing suitable water bodies for reproduction and green corridors for dispersal, thus preventing isolation and loss of genetic variability within populations.

Keywords Urban · Microsatellites · F_{ST} · Common frog · Population structure · Golf course

Introduction

It is estimated that by 2030 almost five billion people worldwide will live in urban environments (Cohen 2004; United Nations Population Division and World Urbanization prospects 2011). Urbanization not only causes habitat loss and fragmentation for flora and fauna (McKinney 2006), but also increases the levels of air, soil and wetland pollution (Hamer and McDonnell 2008). This makes urbanization, together with global warming, one of the biggest threats to biodiversity and ecosystem functioning (McKinney 2002).

One in three (32.5 %) of close to 6000 amphibian species are threatened, i.e. listed in the 2004 IUCN Red List categories of Vulnerable, Endangered or Critically endangered (Stuart et al. 2004) and many (7.4 %) are on the brink of extinction, listed as Critically Endangered (CR—the IUCN Category of highest threat). In comparison, 3.8 % of mammals and 1.8 % of birds, respectively, are listed as critically endangered (Stuart et al. 2004). Even though the typical causes of biodiversity loss, such as habitat loss and overexploiting, do not completely explain the rapid and enigmatic decline in amphibian species and populations (Stuart et al. 2004), the role of urbanization in the process should receive more attention. Despite worldwide declines reported over past decades (Hamer and McDonnell 2008), there is still insufficient information on

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the ecology of amphibians in urban and suburban areas, particularly in the tropics and sub-tropics.

The greatest threat to species survival in urban environments is the loss and degradation of suitable habitats (Mitchell and Jung Brown 2008). The viability of frog populations is dependent not only on the size, number, and connectivity of terrestrial habitats interspersed throughout the environment, but also on the availability of aquatic breeding habitats (e.g. Beebee 1979; Vizyova 1986; Van Buskirk 2005). In order to avoid predation by fish, pond-breeding frogs (such as the common frog, *Rana temporaria*) often use temporal wetlands or isolated ponds for the larval stages of their life cycle (Gamradt and Kats 1996; Scott et al. 2008). However, in urban areas these habitats are in serious decline (Semlitsch and Bodie 1998; Johansson et al. 2005; Piha et al. 2007; Baldwin and deMaynadier 2009). Furthermore, roads, buildings and other unsuitable terrain act as barriers to dispersal, thus reducing genetic variation within isolated populations (Vos and Chardon 1998; Cushman 2006; Andrews et al. 2008). As a habitat generalist, the common frog is one of the very few species that can tolerate moderate levels of urbanization and, thus, maintain viable populations in cities (Hitchings and Beebee 1997; Saarikivi 2008). For example, these frogs can colonize created ponds if suitable source populations are in the vicinity (Rannap et al. 2009). As such, the species is often found in natural or semi-natural urban areas, including parks, private gardens, cemeteries, vacant lots and golf courses (Colding et al. 2006; Gledhill et al. 2008).

Urban development often represents an intense form of fragmentation and affects many species negatively (McKinney 2002). Populations can show characteristics being morphologically, physiologically or behaviorally distinct from their rural counterparts (Kotze et al. 2011). A number of studies have also shown genetic effects of fragmentation at a fine scale within cities, where a combination of restricted gene flow, founder effects and small effective population size shape the genetic structure of urban populations (see Kotze et al. 2011). For example, studying the genetic structure of the cockroach (*Blattella germanica*), Cloarec et al. (1999) found strong genetic substructuring within two cities in France. Reduced gene flow and loss of genetic variability in urban areas have been shown in many other groups of arthropods as well, such as ground beetles (Keller and Lurgiader 2003; Keller et al. 2004), crickets (Vandergast et al. 2009) and spiders (Schäfer et al. 2001). In reptiles, three species of lizards (and one species of birds) displayed 2.5–3.6 times higher F_{ST} -values (average 0.040–0.095) in urban areas as compared to areas of continuous habitat (average 0.013–0.020; Delaney et al. 2010). Using allozyme electrophoresis, Hitchings and Beebee (1997) showed similar results for the common frog in the UK. Barriers associated with urban habitats had promoted genetic population differentiation, and despite the lack of any absolute barrier to

movement between ponds, substantial genetic differentiations were found between sites separated by (on average) 2.3 km only (Hitchings and Beebee 1997).

The increasing popularity of golf has resulted in the rapid establishment of golf courses near urban settlements (Colding and Folke 2009). Being relatively large urban green areas, golf courses satisfy the needs for many species, for example birds (Gillihan 2000) and carabid beetles (Saarikivi et al. 2010). The small water bodies found on many golf courses provide challenging obstacles for players, simultaneously offering suitable habitat for many aquatic species (Paton and Egan 2002; Colding and Folke 2009). Shallow ponds in the Northern hemisphere can be especially favorable for frogs, as wintertime freezing eliminates fish and other predators (Brönmark and Hansson 2005). However, while the diversity of terrestrial species on golf courses has been studied quite extensively, the contribution of ponds to the occurrence of aquatic species has been less studied (Colding et al. 2009; Hodgkison et al. 2007). Amphibians have a tendency to establish populations on urban golf courses (Colding et al. 2006; Saarikivi 2008), and thus, golf courses most likely form important green spaces in maintaining urban amphibian diversity. Yet, only two comparative studies concerning amphibian biodiversity on golf courses versus natural habitats have been conducted (Colding et al. 2006; Hodgkison et al. 2007); neither of which focussed on the possible role of golf courses in maintaining genetic diversity and connectivity of urban populations. While the global decline of amphibians has received significant attention (e.g. Alford and Richards 1999; Blaustein and Wake 1990; Houlahan et al. 2000), knowledge on amphibian activity and habitat use in anthropogenic-altered environments is, with the exception of studies focusing on roads (e.g. Fahrig et al. 1995; Hels and Buchwald 2001), forestry (e.g. deMaynadier and Hunter 1995; Gibbs 1998) and agricultural effects (e.g. Hamer et al. 2004; Piha et al. 2007), essentially lacking (Mifsud and Mifsud 2008).

In this study, the aim was to assess the role of golf courses in maintaining genetic diversity and connectivity within and between amphibian populations in urban surroundings. For this purpose we compared the population sizes, genetic variability and differentiation of the common frog (*R. temporaria*) populations in five urban golf courses and nearby natural habitats within the greater Helsinki region in southern Finland with the aid of microsatellite markers.

Materials and methods

Study species

Rana temporaria is a widespread anuran with a range extending from northern Spain up to the coast of the Arctic

Sea (Gasc et al. 1997). In many areas of northern Europe it is the most numerous amphibian species and occurs commonly also in urban landscapes (Johansson et al. 2005; Saarikivi 2008). *R. temporaria* is the most abundant amphibian species in the Helsinki area, and is often encountered at ponds and ditches in golf courses (Saarikivi 2008). It is a generalist species using both permanent and temporary aquatic habitats for breeding. In southern Finland, it breeds from mid April to early May. Each female lays a single egg clutch and the larvae hatch within 2 weeks. The larval period lasts ca. 50–80 days, after which the metamorphs start a terrestrial life. Since the frogs require both terrestrial and aquatic habitats during their life cycle, migrations between spatially separated habitats occur naturally. However, being ground-dwelling species, frogs have generally a low dispersal capacity, which is further reduced by the fragmentation and limited connectivity of urban habitats, that may lead to high genetic differentiation even between adjacent populations (Reh and Seitz 1990; Hitchings and Beebee 1997; Vos et al. 2001; Cushman 2006; Johansson et al. 2005, 2006, 2007).

Sampling

Five golf courses within the Helsinki-area (viz. Helsinki [H], Espoo [E], Vantaa [V] and Sipoo [S]) were selected for sampling (Fig. 1). These particular golf courses were chosen on the basis of presence of potentially suitable small water bodies as breeding habitat for frogs (Table 1). Also, the current land use practices in the surroundings of these golf courses were similar to those in typical

residential areas and recreational coniferous forest. From each golf course one population was sampled, except from Talma golf, which due to its substantially larger size (130 ha; Table 1) was considered to host two populations of *R. temporaria*. Detailed information on the size and age of each golf course, as well as number of suitable breeding ponds are reported in Table 1. Within 1–2 km from the golf courses six natural habitats with long-established breeding sites were sampled as reference sites, leading to a total number of 12 sampled populations (Fig. 1).

Sampling was conducted during 10 days in late April 2009. At each population, the total number of spawn clumps was counted and two eggs were taken from each of the 20–50 clumps (for a total of 40–100 eggs/population). Only relatively large and complete looking clutches were sampled to minimize the risk of sampling two clutches laid by the same female. Similarly, in order to avoid the mixing of full sibs, the eggs were stored and raised in two containers for each population (totally 24 containers). Only one of the two individuals sampled per clutch were used in genetic analyses, the other served as a back up. When reaching >1 cm in length, the tadpoles were anesthetized with MS-222 and stored in 70 % ethanol at +4 °C. Permission for sampling was granted from the local environmental authority (Suomen Ympäristökeskus, decision nr. LUO 213).

Molecular methods

From each population 20–25 tadpoles were randomly selected (from a single container) for further analyses.

Fig. 1 Map of the study area showing the five golf courses (filled circle) and their nearby reference sites (filled square) that have long-established breeding populations. Urban areas are indicated in grey, main roads as black lines and the location of the Helsinki city centre is marked by a star. The small inset map has a black box to show the location of the larger fine-scale map. Abbreviations are in Table 1

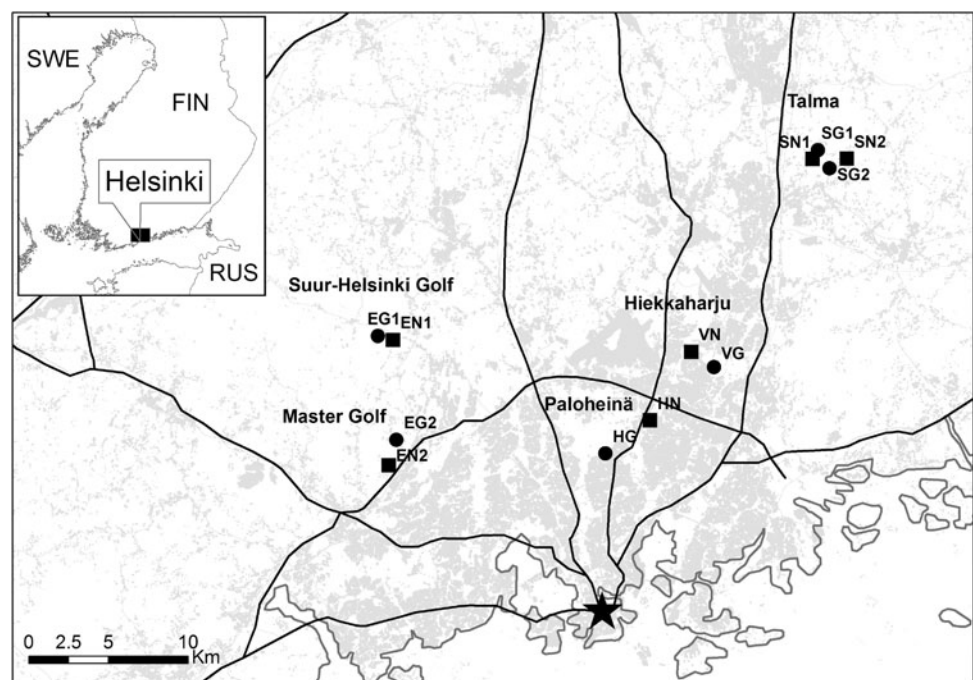


Table 1 Basic information about golf course sites and numbers of spawn clumps found in them and nearby reference sites

Golf course	Abbr	Year established	Course area(ha)	Number of ponds/ ponds with spawn	Number of spawn clumps	
					Course	Ref.sites
Hiekkaharju	VG	1999	42	1/1	58	51 (VN)
Talma	SG1	1989	60	10/2	19	27 (SN1)
Talma	SG2	1989	70	11/3	73	55 (SN2)
Luukki	EG1	1990	32	5/3	22	23 (EN1)
Master-golf	EG2	1987	80	5/4	74	29 (EN2)
Paloheinä	HG	1996	30	6/6	135	220 (HN)
Mean	SE				65.5 (16.1)	67.5 (28.7)

DNA was extracted from nose tissue using Glass fiber plate isolation (Ivanova et al. 2006; populations VG, VN, SG1, SN1, SG2, SN2, EG1, EN1, EG2 and EN2; Table 1) or the Chelex-100 protocol according to Walsh et al. (1991) (HG and HN; Table 1). After DNA extraction the samples were stored in -20°C .

For genotyping, a total of 48 microsatellite loci were tested on ten individuals, and the following 16 loci were chosen for further analyses: BFG005, BFG009, BFG031, BFG046, BFG053, BFG057, BFG082, BFG097, BFG115, BFG130, BFG131, BFG146, BFG157, BFG203, BFG232, BFG237 (Matsuba and Merilä 2009). For all primers the PCR was performed in a total volume of 10 μl containing 10 ng of genomic DNA, 1 \times Multiplex PCR Master Mix (Qiagen), 1 \times Q-solution (Qiagen) and 0.5 μM of each primer (three to six primer pairs per multiplex). One of each primer pair was labelled with a fluorescent dye (FAM, TET or HEX). The PCR conditions for all loci were as follows: Initial denaturation at 95°C for 15 min, followed by 35 cycles of 30 s at 95°C , 1 min 30 s at 58°C and 1 min at 72°C , and final extension step of 5 min at 72°C . Gel separation of the pooled loci was conducted with a MegaBACE 1000 automated capillary DNA-sequencer. Individual allele scoring was done with Fragmented Profiler 1.2 software (Amersham Biosciences). One person (AG) did all the scorings and an independent reader verified the genotypes.

Data analysis

Prior to data analysis, all loci were tested for the presence of null-alleles with Microchecker 2.2.3 (van Oosterhout et al. 2006) and linkage disequilibrium between each pair of loci with FSTAT 2.9.3.2 (Goudet 1995, 2001). The within population genetic diversities were quantified in terms of allele number, expected (H_E) and observed heterozygosity (H_O) with microsatellite toolkit (Park 2001) and allelic richness (AR) in FSTAT. Probabilities for Hardy–Weinberg equilibrium were tested for each locus and population using the probability test implemented in GenePop 4.0.1.0 (Raymond and Rousset 1995). The relatively recently established golf course populations were tested for signs of recent shifts in

population size with the Wilcoxon test with TPM as implemented in Bottleneck 1.2.02 (Cornuet and Luikart 1997). A comparison of genetic diversities between golf courses versus natural habitats was conducted with the group test in FSTAT. The substructuring of all populations was quantified by Weir and Cockerham's (1984) standardized F_{ST} in FSTAT, with a permutation test using Bonferroni correction to adjust the statistical significances of the F_{ST} estimates. Applying GenePop 4.0.1.0 (Raymond and Rousset 1995) an analysis of isolation by distance based on the F_{ST} estimates was performed using the Mantel's test (Mantel 1967). Substructuring of the populations was further analysed with a Bayesian approach using the group level mixture analysis in BAPS 5.2 (Corander and Marttinen 2006).

Results

Occurrence of *Rana temporaria* at the golf courses

All golf courses offered suitable breeding sites to *R. temporaria*. The number of ponds on each course varied from one to 11, hosting on average 66 spawn clumps per golf course (Table 1). The most urban golf course, situated in northern Helsinki (Paloheinä), was the largest golf course breeding site and had 135 spawn clumps at the time of sampling (Table 1). Its reference site, a pond in an old park, had 220 spawn clumps. In the other reference sites (ditches and lakes), situated 1–2 km from the edge of the golf courses, on average 49 spawn clumps were counted (Table 1). There was no difference in the number of spawn clumps between the golf courses and reference (natural) sites (Wilcoxon test, $V = 11$, $P = 1$; Table 1).

Genetic variation

Eight of the 16 loci displayed signs of having null alleles at least in some of the populations. Of these, three loci (BFG005, BFG082, BFG146) had a high probability of null-alleles in the majority of the populations, and hence, they were removed from the data. Five loci (BFG046, BFG057,

Table 2 Genetic variability of the common frog in golf course and reference populations

Population	N	H_E	H_O	F_{IS}	AR
Golf course populations					
VG	24	0.72	0.69	0.05	6.60
SG1	19	0.73	0.73	0.00	6.58
SG2	21	0.69	0.65	0.06	6.53
EG1	22	0.72	0.67	0.06	6.62
EG2	22	0.73	0.66	0.12*	7.00
HG	16	0.72	0.68	0.06	6.69
All	124	0.72	0.68	0.06	6.67
Reference populations					
VN	25	0.75	0.71	0.05	6.75
SN1	19	0.67	0.68	-0.01	6.35
SN2	22	0.71	0.70	0.01	6.18
EN1	22	0.72	0.70	0.03	6.78
EN2	19	0.69	0.62	0.10*	6.35
HN	17	0.70	0.70	0.00	6.42
All	124	0.71	0.69	0.03	6.47

H_E average expected heterozygosity, H_O average observed heterozygosity, F_{IS} fixation index, AR allelic richness

* $P < 0.01$

BFG097, BFG157, BFG232) had possible null alleles present in one or maximum three populations and thus, were not considered to significantly affect the results and were included in all further analyses. None of the loci were in significant linkage disequilibrium with each other. In the 13 microsatellite loci used, the total number of alleles per locus varied from 4 to 38, and the mean number of alleles per population (\pm SD) from 7 (\pm 3.2) to 9.08 (\pm 4.4). Allelic richness, rarefied (Goudet 1995, 2001) to ten individuals, was on average 6.57 (\pm 0.22) per population. The observed and expected heterozygosities in golf course populations were high, being on average 0.68 (\pm 0.03) and 0.72 (\pm 0.01), respectively (Table 2). A significant departure ($P \leq 0.001$) from Hardy–Weinberg equilibrium was detected in two populations (EG2 and EN2). This could be due to possible null alleles present at locus BFG057 and BFG232, respectively, as suggested by the results from Microchecker 2.2.3 (van Oosterhout et al. 2006). For all measures of within population genetic variation, the patterns were similar between golf courses and natural habitats (H_E , $P = 0.61$; H_O , $P = 0.41$; F_{IS} , $P = 0.16$; AR, $P = 0.17$) and there were no signs of bottlenecks in the relatively recently established golf course populations.

Population structuring

Population structuring was weak. The pairwise exact tests of population subdivision revealed F_{ST} values no higher than 0.036 (average $F_{ST} = 0.016$; Table 3). None of the

pairwise F_{ST} values among populations were significant. The Bayesian clustering method supported these results: the optimal partition included only one cluster. No isolation-by-distance was apparent in the data ($R^2 = 0.03$, $P > 0.07$).

Discussion

Common frogs used the artificial and relatively recently established golf course ponds for breeding as often as the nearby natural sites; similar numbers of egg clutches were observed in both types of habitats. Genetic differentiation between the golf course populations and their corresponding natural sites was very low, implying frequent gene flow between the different habitats. Somewhat surprisingly, similar results were encountered across the entire study area (\sim 1000 km²), and the genetic differentiations between all population pairs was very weak.

The within population genetic diversities, including allelic richness, observed and expected heterozygosity and F_{IS} , were also similar in all populations, reinforcing the view that gene flow is frequent between the localities. In fact, low genetic differentiation across the study area suggests a sufficient supply of natural and/or semi-natural water bodies and green corridors to enable common frogs to form a single large population, or metapopulation (Hanski 1999), within the greater Helsinki region.

Of the many studies focussing on genetic population structuring in amphibians, only a handful have been conducted in urban or suburban regions. Both agriculture (Johansson et al. 2005) and roads (Hels and Buchwald 2001) have been shown to be barriers to amphibian migration (but see also Safner et al. 2011). Thus, we would expect urban populations to be relatively isolated, leading to higher genetic differentiation between these populations than between populations in continuous habitats. In our results the most urban population (HG) displayed the highest F_{ST} -s, which could be an indication for isolation induced by urbanisation. When Hitchings and Beebee (1997) compared urban and rural populations of *R. temporaria* (and *Bufo bufo*) in the UK, they found that the urban populations were on average smaller and more isolated, and there were substantial genetic differences between urban sites separated by an average of only 2.3 km (F_{ST} among urban populations 0.388, F_{ST} among rural populations 0.145). Also, for the eastern red-backed salamander (*Plethodon cinereus*), a higher genetic differentiation between urban as compared to natural populations has been shown (Noël et al. 2007). However, in a recent follow-up study by Zeisset and Beebee (2010) using partially the same populations in the UK, they found no difference between rural and urban populations in terms of genetic

Table 3 Pairwise F_{ST} (lower matrix) and geographic distances (km, upper matrix) between *R. temporaria* populations used in this study

	VG	VN	SG1	SN1	SG2	SN2	EG1	EN1	EG2	EN2	HG	HN
VG		2.1	15.3	14.0	14.5	15.8	21.2	20.4	20.5	21.4	8.8	5.2
VN	0.02		14.9	13.7	14.2	15.6	19.4	18.7	19.2	20.3	8.4	5.1
SG1	0.015	0.015		21.4	1.2	1.1	30.0	29.4	32.0	33.5	23.3	20.0
SN1	0.025	0.019	0.021		0.8	1.9	29.2	28.6	31.3	32.5	22.1	18.8
SG2	0.019	0.024	0.012	−0.001		1.4	29.9	29.3	31.7	33.1	22.6	19.2
SN2	0.017	0.013	0.009	0.019	0.012		31.3	30.5	33.1	34.4	24.0	20.6
EG1	0.008	0.015	0.015	0.013	0.010	0.014		0.8	6.0	7.7	15.6	17.5
EN1	0.018	0.013	0.015	0.016	0.014	0.022	0.017		5.6	7.5	14.9	16.7
EG2	0.012	0.011	0.011	0.016	0.019	0.017	0.013	0.008		1.9	13.1	15.9
EN2	0.014	0.024	0.020	0.018	0.016	0.018	0.005	0.022	0.011		13.6	16.6
HG	0.017	0.009	0.036	0.023	0.027	0.025	0.014	0.027	0.014	0.034		3.5
HN	0.021	0.013	0.018	0.002	0.010	0.016	0.004	0.027	0.013	0.015	0.015	

diversities or average differentiation based on seven microsatellite loci. Similarly, Vos et al. (2001) found low genetic differentiation ($F_{ST} = 0.052$) between moor frog (*R. arvalis*) populations in a fragmented landscape in The Netherlands. Small to moderate ($F_{ST} = 0.022$ – 0.060) urban-induced genetic differentiations were also encountered by Arens et al. (2007) for the same species. When these results are combined with observations showing increased amphibian species richness in urban and suburban habitats (Lane and Burgin 2008), it has been suggested that urban habitats may in fact, benefit some amphibian species, particularly those that can take advantage of the new habitats created by human alterations (Tyler and Watson 1998; Castro et al. 2005). As the effects of habitat loss and constraints on dispersal have profound impacts on amphibian population sizes (Mitchell and Jung Brown 2008), the exchange of even a few individuals between patches each generation may suffice to create a panmictic population, although the dynamics of each patch may still be fairly independent (Safner et al. 2011).

Hamer and McDonnell (2008) in a recent review expressed the need for more studies focusing on the ongoing processes and complex habitat use of amphibians in urban environments. With the extreme decline of natural ponds and other small water bodies in urban areas, golf course ponds can today represent a significant proportion of the water habitats available for aquatic species. For example, over a quarter of all available permanent, freshwater ponds in the greater Stockholm area are on golf courses (Colding et al. 2009). If managed and designed properly, also taking into consideration surrounding environmental factors, such as the quality and connectivity of the terrestrial habitat, golf courses may thus present valuable opportunities for biodiversity conservation (Hodgkinson et al. 2007; Semlitsch et al. 2007). In northern latitudes, golf course ponds may be favorable to frogs, as wintertime

freezing naturally eliminates or considerably reduces predation by fish and insects, such as dragonfly larvae, at least in shallow ponds which freeze completely (Brönmark and Hansson 2005).

Our results suggest that golf courses may have an important role in maintaining viable amphibian populations in urban and suburban surroundings. Within the greater Helsinki region, golf course ponds supported populations of common frogs of similar size as the natural sites. Additionally, even though golf course landscapes, which mainly comprise open lawn, do not seem favorable for frogs, they may have some elements, such as ditches or meadows that can serve as green corridors to nearby surrounding environments. The positive outcome of this study, regarding the lack of genetic isolation, indicates that it possibly takes several frog generations for processes of fragmentation to lead to genetic isolation within the area studied.

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