Preliminary investigation of a one-hundred-year-old population of introduced water frogs in Britain

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ABSTRACT — We investigated the taxonomic composition and habitat selection of the longest surviving introduced water frog population in Britain. Water-frogs were first introduced to Beam Brook (Surrey, southeast England) around 1905. Using RAPD analysis of embryos and larvae, we identified three taxa (*Rana esculenta*, *R. lessonae* and *R. ridibunda*) and detected a fourth for which we had no RAPD reference material. We consider this was likely to be *R. perezi*, since male advertisement calls of this species as well as those of *R. bergeri* (closely related to *R. lessonae*) were recorded recently at Beam Brook. We have no evidence of adult male *R. ridibunda* at Beam Brook, and the embryos of this species were probably produced by matings of *R. esculenta*. The water frogs bred in a subset of the 44 ponds present at the site. There were no sharp distinctions among the different taxa with respect to ponds used for breeding. However, stepwise multiple regression analysis indicated that *Rana lessonae* reproduction was preferentially associated with the warmest ponds, *R. esculenta* spawn and larvae were most abundant in densely vegetated pools, and *R. ridibunda* was associated with relatively low oxygen concentrations.

THE British Isles have an impoverished herpetofauna relative to mainland Europe (Gasc et al., 1997). This is largely due to relatively cool summer temperatures in Britain, but is also in part an accidental consequence of post-glacial colonisation processes. Biogeographical patterns in the recolonisation of Europe are discussed by Taberlet et al. (1998) and Hewitt, (1999). Many species spread north as the climate ameliorated during the postglacial warming, but there was only a limited window of time during which Britain could be colonised. Initially joined to mainland Europe by a land bridge, Britain finally became separated by sea around 7500-8000 years ago (Lambeck, (1995). Evidence suggests that at least nine species of amphibians reached Britain naturally before that time. Six species (*Triturus helveticus*, *T. vulgaris*, *T. cristatus*, *Bufo bufo*, *B. calamita* and *Rana temporaria*) are still extant, while three (*R. dalmatina*, *R. arvalis* and *R. lessonae*) have subsequently become extinct (Gleed-Owen, (1998). A 'northern clade' of *R. lessonae*, found also in Norway and Sweden (but morphologically distinct from pool frogs in mainland Europe), survived in England as a single isolated population until the 1990s (Zeisset & Beebee, 2001; Wycherley et al., 2002).

By contrast there have been many introductions of amphibians into Britain, both accidental and deliberate, over the past 200 years (Lever, 1980). Water frogs have been among the most widespread of these introductions, with the first (probably mixed *R. lessonae* and *R. esculenta*) documented in 1837 (Smith, 1951). Most populations of these frogs became extinct within a few years, but there have been notable exceptions. The most successful amphibian invader in Britain is *R. ridibunda*, which was introduced to south-east England in 1935 and is now widespread in large wetland areas (Menzies, 1962; Beebee & Griffiths, 2000). However, the longest surviving introduced water frog population in Britain exists at Beam Brook in

Surrey. Frogs have been present at this site for almost 100 years (Gillett, 1988) and continue to thrive there. Repeated introductions, into artificial ponds created at a commercial nursery, occurred over many decades until the mid-1960s, when introductions ceased. Although the origins and species were not documented, the water frogs at Beam Brook were certainly of mixed taxa and were probably imported mainly from Belgium, France, the Netherlands, Italy and Spain. Since the late 1980s the frogs have extended their range in the Beam Brook area, coinciding with a general climatic warming (Beebee, 1995; Walther et al., 2002), and now occur over some 150 square kilometres (Wycherley & Anstis, 2001).

The Beam Brook water-frogs have not been studied previously and are of interest for several reasons. Firstly, their taxonomic composition is unusual and, because of their mixed origins, may be more complex than can be found at any natural site in Europe. Analysis of male advertisement calls at Beam Brook has indicated the presence of *R. bergeri*, *R. esculenta*, *R. lessonae* and *R. perezi* (Wycherley et al., 2003). Because of their complex hybridogenetic reproduction mechanisms (Tunner, 1974; Graf & Pols-Pelaz, 1989; Roesli & Reyer, 2000), other forms – such as female *R. ridibunda* and cryptic hybrids – might also occur at the site. In addition, the spread of water frogs associated with climate change might pose threats to native communities and it will be important to evaluate the impact of this highly unusual water frog complex in future years. In this paper we report a preliminary investigation of the water frog community at Beam Brook and its association with pond habitat features.

METHODS

Study site

The Beam Brook Field Station comprises 44 ponds within about 2 ha of low-lying field habitat in Surrey, southeast England. The substrate is clay and all the ponds were created artificially within the past 100 years. Most of the ponds contain abundant growths of macrophytes, especially *Glyceria fluitans*, *Potamogeton natans* and *Nymphaea* species. There were several amphibians in addition to the water frogs considered here. These included both native species (*Triturus vulgaris*, *T. cristatus*, *Rana temporaria*) and other introduced species (*T. alpestris* and *T. carnifex*). Fish, notably *Gasterosteus aculeatus* and *Carassius auratus*, occurred in some – but not all – of the pools. Invertebrates were abundant, especially odonate larvae, dytiscid water beetles, and bugs such as corixids and Ilyocoris cimicoides. A random selection of 12 ponds at Beam Brook was used in the present study.

Water frog observations

The Beam Brook ponds were visited twice weekly between March and July 2000. Numbers of adult frogs visible in the water or on the bank were counted in each pond, on sunny days, at every visit. These numbers were used as minimum estimates of the numbers of water frogs in each pond, and to compare relative numbers of frogs in the 12 study ponds. The onset and completion of calling, and the onset of spawning, were recorded separately for each pond. Spawn was visible among the aquatic vegetation, and its occurrence was recorded at every visit during the breeding season. We did not attempt to quantify the amount of spawn in each pond because female water frogs each deposit several small clumps, some of which are hidden in dense vegetation. From every pond where breeding occurred (n = 7), at least five clumps of spawn were collected at widely separate localities to ensure they came from different females. Spawn samples from each pond were pooled together, 25 embryos were selected at random, and these were allowed to develop for six weeks. The survivors were then stored in ethanol for later genetic analysis. Later in the season (late July/early August) larvae from the same breeding ponds were sampled. We used a sweep net to sample round each pond perimeter, then bulked the catch from each pond into separate buckets, and finally took a random subsample of 10 individuals per pond. These were also stored in ethanol pending genetic identification.

Genetic identification

Entire embryos or small sections of larval tails were digested overnight at 55°C with proteinase K (Hitchings & Beebee, 1998). DNA was extracted using phenol/chloroform, precipitated with ethanol, and then each sample was redissolved in 50 µl distilled water. The concentration of DNA in each sample was determined by measuring absorbance at 260 nm. Water frogs were identified by RAPD analysis as outlined by Zeisset & Beebee (1998). 25 ng of DNA were used in PCR assays with primer PR6, and products were electrophoresed through 1.5% agarose gels together with known reference standards (DNA extracted from individually identified *Rana lessonae*, *R. esculenta* and *R. ridibunda*). After electrophoresis at 60 volts for about three hours, each gel was examined under a UV transilluminator and then photographed using an Eagle-Eye imaging system (Maniatis et al., 1982).

Habitat features

Habitat data were collected at Beam Brook between March and July 2000. We measured the following abiotic factors: the pond dimensions surface area and maximum depth, early and late in the season (March 25 and May 25, respectively); water temperatures on visit days, and maximum and minimum water temperatures (recorded weekly), all at a fixed depth of 15 cm in each pond; oxygen levels (mg/litre), taken weekly in each pond using a portable meter at a fixed depth of 15 cm, always early in the day and away from vegetation to minimise effects of photosynthesis; and pH, using a portable meter away from vegetation on a single sampling date in April. We also measured biotic factors: presence or absence of fish as revealed by observation and sweep netting; the distribution and abundance of macrophytes in each pond on March 25 and May 25; and the nature of macroinvertebrates at a single sampling time in early July. For vegetation cover, each pond was divided into 1 x 1 metre grid squares and the extent of macrophyte growth (submerged, surface and emergent combined) was estimated for each. Data from all the squares in a pond were assembled together to obtain an estimate for the entire pond. The survey methods we used for invertebrates were based on standard three-minute hand-net sampling (National Pond Survey: Pond Action, 1998). Three-minute sub-samples were taken around the entire pond edge. All the main mesohabitats in each pond were sampled. Each mesohabitat was netted vigorously to collect macro-invertebrates. The total pond sample was then placed in a labelled bucket and taken to the laboratory for identifications. In subsequent analysis we used the Biological Monitoring Working Party (BMWP) score system for freshwater macroinvertebrates. Eighty-five macroinvertebrate families or taxa each attract a score from 1-10 reflecting tolerance to pollution or oxygen depletion. High scoring families are sensitive to oxygen depletion. The sum of the BMWP scores for each pond gives the final BMWP score. The Average Score Per Taxon (ASPT) is calculated by dividing the BMWP score by the number of scorable families present. (Williams et al., 1998; Biggs et al., 2000).

Data analysis

We used the statistical program Statistix 7.0™ (Analytical Software, Tallahassee, USA) for data analysis. All data were checked for normality by the Shapiro-Wilks test and, where necessary, log10- or arcsin-transformed before use. When transformations to normal distributions were not possible, nonparametric tests were employed. Comparisons of ponds with respect to water-frog use were made with Kruskal-Wallis one-way Analysis of Variance (ANOVA). To investigate relationships between pond use and pond features we used correlation and stepwise multiple regression analyses, including the range of biotic and abiotic independent variables described above.

RESULTS

Pond use by all water frogs

A summary of our observations at Beam Brook is provided in Table 1. Examples of how water-frog sightings increased in two of the Beam Brook ponds as spring 2000 progressed are shown in Figure 1. These examples show two extremes, with one heavily used (13) and one relatively little used (28) pond. Frogs first appeared in mid-April, and

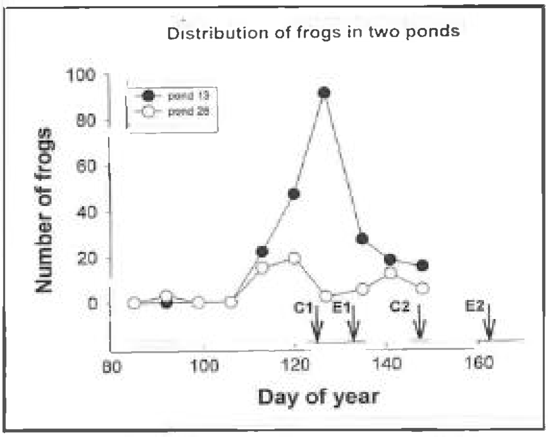

Figure 1. Comparison of frog numbers visiting two sample ponds. C1 = 1st calling day, E1 = time eggs first observed, C2 = 1st calling day, E2 = time eggs first observed.

peak numbers were observed by early May. Excluding the first three weeks of observations before day 110 when very few frogs were seen anywhere, over the subsequent nine weeks there were significant differences in frog numbers among the 12 ponds (Kruskal-Wallis ANOVA statistic = 28.12, P = 0.003). Frogs were seen in all 12 ponds, however, and the highest average number between mid April and July was 32.1 per visit (pond 13) while the lowest was 8.3 per visit (pond 28). Mean numbers of frogs seen per pond over weeks 4-12 inclusive correlated positively with pond surface area (r = 0.862, df = 10, P = 0.0003) but not with any of the other independent variables we measured. Bigger ponds had more frogs than small ponds.

Despite the occurrence of water frogs in all 12 of the study ponds, only seven ponds were used for calling and spawning. There was no significant difference between the numbers of frogs seen in breeding pools (mean 18.6) and non-breeding pools (mean 16.0) between weeks 4 and12 (Kruskal-Wallis statistic = 9.11, P = 0.427). However, breeding pools had higher average oxygen concentrations (6.6 mg/litre) than non-breeding pools (4.7 mg/litre), ANOVA F = 5.44, df = 1,10, P = 0.042. Breeding pools also had lower average macrophyte vegetation in May (61%) than non-breeding pools (96%), ANOVA F = 8.73, df = 1,10, P = 0.014. Fish were present in both non-breeding (one out of four) and breeding (three out of seven) pools.

Both calling and spawning started around April 20th, within a week of frogs appearing in the ponds in significant numbers. There were differences among the breeding ponds with respect to the start and end of breeding activity. As shown in Figure 1, calling started 20 days and spawning 26 days earlier in pond 13 relative to pond 28. Calling onset was associated with minimum water temperatures as revealed by stepwise multiple regression:

First calling day = 7.329-2.866 (log_{10} mean minimum water temperature, °C)

In this regression, adjusted r^2 = 0.8143, P = 0.0034. Ponds that warmed up earliest in the season were used first. Duration of calling in a particular pond was significantly related to its depth in July, with deeper ponds sustaining calling for longer periods than shallow:

Calling duration (days) = 1.00 + 11.60 (July depth, cm).

In this case, adjusted r^2 = 0.941, P = <0.001. First spawn date was also strongly correlated with temperature – in this case, the actual water temperature measured on the day:

First spawn day = -0.385 + 0.214 (water temperature, °C).

This relationship was highly significant (adjusted r^2 = 0.944, P = < 0.0001) and shows that ponds where spawning started late were, by that time, warmer than ponds where it started early.

Use of ponds by different water-frog taxa

Table 2 summarises the genetic identification, by RAPD analysis, of embryos (April) and well-grown larvae (July/early August) in the seven ponds used by water frogs for breeding. Spawn samples varied between ponds with respect to embryo viability, and in particular this was relatively low (<70% overall) in ponds 28 and 30. Water frogs clearly identified at Beam Brook by the genetic analysis were *R. lessonae*, *R. ridibunda* and *R. esculenta*. However, there was also a fourth taxon with a distinctive RAPD profile for which

Pond biotic and abiotic characteristics												
Pond	**1**	**3**	**5**	**8**	**13**	**14**	**16**	**17**	**28**	**30**	**36**	**37**
Biotic features												
Mean no. Frogs/pond	16	13	14	12	19	13	8	5	5	7	6	7
Spawn present	-	+	-	+	+	+	+	-	+	+	-	-
Fish present	+	+	-	+	+	-	-	-	-	-	-	-
No. predatory invertebrate taxa	5	5	5	3	5	5	4	4	3	4	4	2
Diversity ASPT index	4.47	5.0	4.58	4.12	4.47	4.73	4.79	4.5	4.59	4.19	4.43	4.0
% vegetation cover (March)	40	35	78	34	46	29	79	86	33	30	83	15
% vegetation cover (May)	90	46	97	42	84	58	100	93	67	30	100	100
Abiotic features												
Surface area (m^2)	133	190	105	166	185	174	47.6	59.6	51.1	6.6	21.7	19.8
Maximum depth in m (March)	0.5	0.8	0.12	0.58	0.56	0.47	0.39	0.32	0.22	0.44	0.53	0.35
Maximum depth in m (May)	0.81	0.92	0.08	0.62	0.8	0.59	0.2	0.31	0.32	0.41	0	0
Water temperature at spawning onset (C)	-	17.1	-	19.2	19.5	28.0	22.2	-	21.3	24.0	-	-
Mean maximum temperature (C)	18.1	23.5	25.4	23.2	21.1	22.8	21.8	22.1	19.9	20.5	23.7	21.4
Mean minimum temperature (C)	9.8	10.9	8.6	11.4	10.8	10.9	9.6	9.6	8.7	11.0	9.6	10.4
Mean oxygen concentration (mg/l)	5.09	5.09	4.85	7.18	7.87	8.43	6.31	6.25	6.76	6.87	3.83	3.72
pH	7.2	7.0	6.7	7.3	7.2	6.7	6.7	6.7	6.8	7.3	7.2	7.3

Table 1. Biotic and abiotic characteristics of study ponds.

we did not have a reference marker. We have assumed that this was probably *R. perezi* because the presence of this frog at Beam Brook, in addition to other water frogs, was demonstrated by analysis of male advertisement calls (Wycherley et al., 2003).

Rana lessonae and *R. esculenta* embryos were present in all seven ponds. *R. ridibunda* were found in six ponds, and '*R. perezi*' in five. *Rana lessonae* and *R. esculenta* genotypes were almost equally common, constituting 36% and 38% respectively of the embryos surviving to hatch. *Rana ridibunda* and *R. perezi* were both much rarer, with the former at 14% and the latter at 11% of the total. However, since *R. ridibunda* at this site were probably all derived from the matings of *R. esculenta* (see Discussion), their viability is expected to be low (Graf & Pols-Pelaz, 1989). Two of the ponds with relatively high numbers of *R. ridibunda* (28 and 30) were also those with

Table 2. RAPD Identification of Water frog eggs and larvae. The percentage occurrence of each taxon is listed for individual ponds.

Pond	**3**	**8**	**13**	**14**	**16**	**28**	**30**
% egg survival	100	100	100	100	100	64	64
Percentage taxon composition of embryos							
R .lessonae	44	60	36	40	24	31	36
R. esculenta	28	40	32	40	60	50	25
R. ridibunda	24	0	8	8	4	19	21
"*R. perezi*"	4	0	24	12	12	0	17
Percentage taxon composition of larvae							
R. lessonae	30	40	30	50	0	10	0
R. esculenta	60	40	40	30	60	20	0
R. ridibunda	10	10	0	0	20	30	0
"*R. perezi*"	0	10	30	20	20	40	0
Percentage taxon composition of pooled embryo and larval samples							
R. lessonae	40	54	34	43	17	23	36
R. esculenta	37	40	34	37	60	38	25
R. ridibunda	20	3	6	6	9	23	21
"*R. perezi*"	3	3	26	14	14	15	17

substantial embryonic mortality prior to analysis, and it may be that at fertilisation the percentage of *R. ridibunda* was higher in these ponds than our data suggest.

No larvae were later found in pond 30. *Rana lessonae* larvae were not found in pond 16, but otherwise both *R. lessonae* and *R. esculenta* larvae were detected wherever they occurred earlier as spawn. *Rana ridibunda* larvae were not found in two ponds where they occurred in spawn (13 and 14) but were caught in one pond (8) where they were not detected in the spawn sampling. '*Rana perezi*' was absent from one pond were its genotype was seen in spawn (3), but was found in two where it was not seen in spawn (8 and 28). Indeed, in pond 28 '*R. perezi*' larvae occurred at relatively high abundance. These differences between spawn and larval samples indicated a substantial sampling error, and necessitated caution when analysing the taxon-specific data. Combining both the embryonic and larval genotype identifications, it was evident that all four taxa occurred in all seven ponds.

Despite the proportional changes among species between two sampling times, we considered it safest to use the average proportions (embryos + larvae) as indicators of pond suitability for each species. This should reduce the effects of sampling error at each of the two separate sampling times and give some idea as to which features were, over the entire breeding period, most associated with each taxon. After pooling data for the two sampling times, arcsin transformations of proportional use by each taxon were normally distributed. We therefore investigated by regression analysis whether any biotic or abiotic factors were associated with pond use by each taxon, as judged by proportional representation in each pond. Since all the breeding ponds were heavily used, proportionality data were not likely to be strongly biased by (for example) a high proportional value in one pond representing only a very small amount of spawn altogether. For *R. lessonae*, stepwise multiple regression identified just minimum average temperature as an associated variable:

Arcsin [proportion of R. lessonae] = -0.866 + 0.103 (average minimum temperature, C)

In this case adjusted r^2 = 66.0% and P = 0.016. *Rana lessonae* therefore showed a preference for relatively warm ponds. Multiple regression for *R. esculenta* yielded only pond vegetation cover later in the season as a significant predictor:

Arcsin [proportion R. esculenta] = 0.233 + 0.228 arcsin (proportion vegetation cover).

For this regression, adjusted r^2= 62.8% and P = 0.021. *R. esculenta* therefore showed a preference for the more vegetated ponds. In the case of *R. ridibunda*, only average oxygen concentration was significant:

Arcsin [proportion of R. ridibunda] = 0.417 - 0.044 (average oxygen concentration, mg/litre).

In this case adjusted r^2 = 52.5% and P = 0.040. *Rana ridibunda* was therefore associated with the less-well-oxygenated pools. No significant predictor was found for '*R. perezi*'.

DISCUSSION

The Beam Brook ponds have supported introduced water frogs since 1905 (Gillett, 1988). Our combined studies of male advertisement calls and genetics (RAPD analyses) suggest that at least five different taxa were present in 2000, notably *R. bergeri*, *R. esculenta*, *R. lessonae*, *R. perezi* and *R. ridibunda*. It is likely that our genetic methods would not distinguish *R. lessonae* from its close relative *R. bergeri*, both of which were detected on the basis of male advertisement calls (Wycherley et al., 2003). We do not have separate RAPD reference material for these two taxa, but microsatellite analysis of *R. lessonae* at Beam Brook revealed allelic phenotypes subtly different from those seen elsewhere (I. Zeisset, pers. com.). It may be that there is currently some type of hybrid *lessonae/bergeri* population at Beam Brook, and this clearly requires further study. We detected *R. ridibunda* embryos and larvae by RAPD analysis but did not record *R. ridibunda* male advertisement calls at Beam Brook. This lack of *ridibunda* calls is explicable because *ridibunda* progeny in a LE system would all be females. Although *ridibunda* genomes normally have low viability in this situation due the accumulation of deleterious mutations, there may be many different *ridibunda* clones at Beambrook consequent on the multiple importations from widely different geographical locations in the early twentieth century. Such 'distant' *ridibunda* clones often generate viable progeny when *esculenta* interbreed (Guex et al., 2002). It is extremely unlikely that *R. bergeri* (from Italy) and *R. perezi* (from Spain) coexist anywhere under natural conditions. The fact that they are present together at Beam Brook, together with *R. lessonae*, *R. esculenta* and *R. ridibunda*, probably make this a unique situation. Lode & Pagano (2000) found smaller differences between the male advertisement calls of *R. esculenta* and *R. perezi* than between the calls of *R. ridibunda* and *R. perezi*. They suggested that the former mating combination was the most likely for the origin of the hybrid *R. kl. grafi*, so the possibility exists for this taxon also to occur at Beam Brook, though we have not detected it. *Rana lessonae* and *R. esculenta* together constituted the great majority (> 70%) of the water frog embryos and larvae we sampled at Beam Brook, and the site therefore supports what is primarily a LE-like breeding system.

We also found some evidence of the ecological factors at Beam Brook that may influence the success of water frogs there. Breeding ponds generally had high oxygen concentrations and low amounts of aquatic vegetation relative to non-breeding ponds. These factors may be relevant to the survival of spawn and larvae. Spawning was not synchronous, and late spawning was associated with higher temperatures rather than with ponds which simply warmed more slowly than early ponds. Although not statistically significant, it was notable that our "fourth" species (putatively *R. perezi*) seemed to be associated with late spawning in very warm ponds such as pool 28 (where 40% of larvae were identified as the 'fourth' species). *Rana lessonae* was also associated with reproduction in relatively warm ponds. This accords with previous studies of *R. lessonae* elsewhere in Europe (Negovetic et al., 2001). *Rana esculenta* embryos and larvae correlated most strongly with the extent of aquatic vegetation during the summer months. This in turn was negatively associated with pond depth (multiple regression adjusted r^2 = 37.2%, P = 0.021). Larvae of this hybrid have a fitness advantage in temporary or otherwise unpredictable habitats (Semlitsch & Reyer, 1992), in keeping with the Beam Brook results. *Rana ridibunda* larvae were associated with ponds relatively low in oxygen. This was unexpected, because *R. ridibunda* larvae are less tolerant of anoxia than those of either *R. esculenta* or *R. lessonae* (Plenet et al., 2000). It is probably unlikely, however, that any of the breeding ponds at Beam Brook become dangerously anoxic during the summer months. All are rich in macrophytes and invertebrates, and ASPT scores at Beam Brook were all relatively high (>4.0). Ponds rarely have ASPT values above a score of 5 (J. Biggs, pers.com.). All ponds at Beam Brook scored in the very good category. Between 12 and 19 invertebrate groups occurred in each pond.

Evidently there is still much to discover about the water frog community at Beam Brook. It would be interesting to know in detail how mating systems operate between the various taxa present, and whether hybrids such as *R. grafi* also occur. Indeed, the taxa identified separately by male advertisement calls and RAPD analyses still require complete formal reconciliation using a wider range of RAPD reference material. It will also be important to determine whether just some or all of these taxa are involved in the colonisation of surrounding ponds and watercourses that has accelerated in recent years.

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