

THE GENETIC DIFFERENTIATION OF COMMON TOADS ON UK FARMLAND:
THE EFFECT OF STRAIGHT-LINE (EUCLIDEAN) DISTANCE AND ISOLATION
BY BARRIERS IN A HETEROGENOUS ENVIRONMENT

David W. Macdonald^{1*}, Rosie D. Salazar¹, Sonia E. Eynard¹, Alex Rogers², Robert S. Coles³,
Robert A. Montgomery^{1,4}

¹ *Wildlife Conservation Research Unit (WildCRU), Department of Zoology, University of Oxford, The Recanati-Kaplan Centre, Tubney House, Abingdon Road, Tubney, Oxfordshire, OX13 5QL. * Author for correspondence: david.macdonald@zoo.ox.ac.uk*

² *Ocean Research & Conservation Group, Department of Zoology, University of Oxford, The Tinbergen Building, South Parks Road, Oxford, OX1 3PS*

³ *Ecosystems and Environment Research Centre, University of Salford, The Crescent, Salford, M5 4WT*

⁴ *Research on the Ecology of Carnivores and their Prey Laboratory (RECaP), Department of Fisheries and Wildlife, Michigan State University, 480 Wilson Road, Room 13 Natural Resources Building, East Lansing, MI 48824, USA*

LRH: D. Macdonald et al.

RRH: Common toad genetic distance

Abstract. - Genetic differentiation plays an integral role in species persistence. However, it remains challenging to quantify the ways in which the degree of isolation affects animal populations. The common toad (*Bufo bufo*) is a species of conservation concern, particularly in the United Kingdom where populations have undergone large-scale declines. There are two types of isolation that are relevant to toad population persistence. These are: *i*) isolation by distance (IBD), where populations that are further apart become more isolated with time, and *ii*) isolation by barrier (IBB), where the presence of barriers to movement can isolate populations. Both IBB and IBD are relevant to common toad survival, and thus conservation, in fragmented landscapes typical of farmlands. We collected common toad genetic material from eight different breeding ponds amongst the rural farmland landscapes of Oxfordshire, England to test the effect of IBD and IBB on toad genetic differentiation. We detected no significant effect of IBD (range 2-22 km between breeding ponds) on common toad genetic differentiation at this scale. We did however, identify a significant and positive relationship between IBB and common toad genetic differentiation. Breeding populations were more genetically different with increasing barrier distance. The lack of a relationship between IBD and toad genetic differentiation could suggest that common toads are not as philopatric as previously thought, with reduced availability of suitable breeding ponds possibly driving more migrants to disperse greater distances and thereby possibly improving genetic mixing of the metapopulation.

Keywords: *Bufo bufo*; Common toad; Genetic distance; IBB; IBD; Isolation by barrier; Isolation by distance.

Genetic differentiation is recognized as a vital component of long-term species persistence as evidenced by the incorporation of genetic research techniques to conservation initiatives (Lynch, 1995; Sunnucks, 2000; Hedrick, 2001). Genetic research now commonly assesses how habitats and landscape configurations may restrict migration, and therefore gene flow (O'Grady et al., 2006; Reed et al., 2007; Sarre & Georges, 2009). Such approaches are particularly relevant for amphibian species that tend to use disparate habitats over large areas, potentially magnifying the effects of genetic isolation (Hanski et al., 2004; Roe & Georges, 2007; Ficetola, Padoa-Schioppa & De Bernardi, 2009). Two common mechanisms of isolation for amphibian species include isolation by distance (IBD) and isolation by barrier (IBB). Isolation by distance suggests that populations that are farther apart should be more genetically dissimilar than populations closer together (Beaumont & Nichols, 1996; Palumbi, 2003). Isolation by barrier implies that populations separated by barriers are more genetically dissimilar because of the increased travel distance necessary to circumvent these obstacles to movement (Keyghobadi et al., 1999; Bush et al., 2011). Identification of the influence of IBD or IBB can be used to better allocate conservation attention toward isolated populations and determine the appropriate course of mitigation action (e.g. translocation or habitat improvement; Reinert, 1991; Cabezas & Moreno, 2007; Stamps & Swaisgood, 2007).

Common toads (*Bufo bufo* L.) are a highly philopatric (tending to return to or remain near a particular site or area) species (e.g. Reading, Loman & Madsen, 1991) with limited mobility (e.g. Moore, 1954; Sinsch, 1988), and so are likely to be susceptible to isolation of their breeding populations by distance. Further, where the species has undergone population declines (e.g. Beebee, 2012), common toads tend to be rare and particularly vulnerable to the effects of habitat change and land fragmentation which can increase isolation (Ficetola & De Bernardi, 2004). Large urban areas and roads can be particularly costly for migrating common toads, presenting barriers to movement and leading to direct mortality from vehicle

77 traffic (Gibbs & Shriver, 2005; Puky, 2006; Elzanowski *et al.*, 2009). Populations that are
78 isolated exchange fewer individuals, and thereby less genetic material, reducing their
79 genetically similarity over time.

80 Common toads are now a species of conservation concern in the United Kingdom
81 following the large and unexplained local population declines occurring over the last 30 years
82 (Carrier & Beebee, 2003; Petrovan & Schmidt, 2016). Common toads tend to have lower
83 genetic diversity in small urban ponds compared with larger, more rural ponds (Hitchings &
84 Beebee, 1998). Further, common toads inhabiting more fragmented landscapes have higher
85 levels of stress hormones and poorer body condition than toads residing in more contiguous
86 habitats (Janin, Léna & Joly, 2011). Thus, these dynamics may affect fecundity of individual
87 animals with scaling consequences to the population level. Despite these conservation issues,
88 the principles of IBD and IBB, relevant to many anuran species, have not yet been applied to
89 the study of common toads. Natterjack toads (*Bufo calamita*), for example, in Denmark were
90 found to have no detectable pattern of IBD, supporting the notion that the remaining distinct
91 populations are genetically isolated by a different mechanism (Allentoft *et al.* 2009; but see
92 Rowe & Beebee, 2007). Studies of the common frog (*Rana temporaria*) showed reduced
93 population size, genetic differentiation and fitness of populations in fragmented habitats when
94 compared with those from continuous habitats (Johansson, Primmer & Merilä, 2007). Finally,
95 moor frogs (*Rana arvalis*) are especially sensitive to road and railway barriers, the presence
96 of which better explained genetic distance than isolation by distance alone (Vos *et al.*, 2001).

97 To apply conservation action in timely and efficient ways that can prevent future
98 population declines, it is crucial that we determine the current threats to species persistence.
99 This should include threats to the genetic differentiation of species of conservation concern.
100 Here we examine the mechanistic connections between common toad genetic differentiation

and IBB and IBD among eight breeding populations of common toads in Oxfordshire, England, hypothesising that both IBB and IBD significantly influence common toad genetic differentiation.

Materials and Methods

Study Area and Sampling. – We positioned our study among eight common toad breeding ponds in West Oxfordshire, England (Fig. 1). To identify our study ponds we collaborated with the Thames Valley Environmental Records Centre (www.tverc.org), which holds all available information about the plants, animals, wildlife habitats and important wildlife and geological sites in Berkshire and Oxfordshire. In 2011, we visited ponds to detect evidence of common toad breeding, to select our study ponds and to collect samples for screening and optimisation of the genetic extraction and amplification protocol.

We collected genetic samples, in the form of common toad tadpoles, from a sample of ponds at each of these eight toad breeding ponds (Fig.1) between 18th June and 4th July 2012. All animal capture and handling protocols were reviewed and approved by the Oxford Local Ethical Review Committee. Access to private land for collection of samples was granted by each individual landowner. The breeding ponds in our sample averaged an area of 4,484 m² (range 795 m² – 11,436 m²) and were between 3.8 km and 21.5 km (Euclidean distance) apart.

Ponds were selected where toad tadpoles were considered to be present in sufficient numbers so that collection of a sample (26-48 tadpoles, Table 1) would not detrimentally impact the breeding success of that pond in the year of collection (i.e. sample size <5% population size).

124 Sibling tadpoles from *Bufo spp.* tend to shoal together (Blaustein *et al.*, 1990), so we
125 stratified our sampling by shoals so as to avoid sampling closely-related individuals. Where
126 shoals were not easily visible because of dense vegetation or poor water clarity, we spaced
127 collections a minimum of two metres apart. Via these sampling efforts we minimized the
128 potential negative impacts of our collection efforts on the breeding success of any one
129 common toad breeding pair and maximized the potential genetic differentiation of the
130 samples collected from each pond. We controlled sampling effort between ponds by
131 completing each collection during a single visit to each pond. Within this context we had two
132 surveyors complete a circuit of the pond and collect tadpoles from each shoal or net sweep at
133 2 m intervals as appropriate.

134 To facilitate genetic analysis, we euthanized tadpoles using an approved *Schedule 1*
135 technique (therefore not a regulated procedure under the Animals Scientific Procedures Act
136 1986). We administered a lethal dose of anaesthetic using Benzocaine (Orajel®; Cecala,
137 Price & Dorcas, 2007), followed by pithing before storing in the freezer in 90% ethanol.
138

139 *DNA extraction and genotyping.* – We extracted DNA from tadpole tails (remainder
140 of the tissue retained for future use) using QIAGEN DNeasy blood and tissues extraction kit.
141 We amplified the samples by multiplex polymerase chain reaction (PCR) by following the
142 QIAGEN Type-it kit protocol (details in Table 2). We randomly selected three individuals for
143 use in the PCR protocol optimisation: Ga21, Hi23 and St4. We used primers for seven
144 microsatellite loci previously developed for this species (Brede *et al.*, 2001) (Table 2). Due to
145 the overlapping size of the loci analysed we created two separate multiplex associations and
146 one singleplex (Table 2). The labels used for multiplex were 6FAM: blue, PET: red, NED:
147 yellow and VIC: green. The details of the PCR cycles are provided in Table 2.

Using GENEPOP 4.2 (Raymond & Rousset, 1995; Rousset, 2008) and FSTAT 2.9.3 (Goudet, 1995) software we measured the number of alleles, observed and unbiased expected heterozygosity H_e (Nei, 1978), genetic differentiation, allelic richness and inbreeding coefficient (F_{IS}) for each breeding pond and each locus. We used program FreeNA (Chapuis & Estoup, 2007) to perform checking for genotyping errors such as allelic dropout, stuttering and null alleles so as to infer their possible impact on the population analysis.

Using the exact test in GENEPOP we tested for departure from Hardy-Weinberg equilibrium for each breeding pond at each locus and tested for linkage disequilibrium with probability tests across pairs of loci. When necessary, we adjusted significance levels using the sequential Bonferroni correction (Rice, 1989) in the R statistical software built in function p.adjust (R Core Team, 2008).

Genetic differentiation. – We measured genetic differentiation, in the form of F_{ST} values, among all possible combinations (28 pairs) of the toad breeding pond pairs from the eight ponds using seven microsatellite loci. As microsatellites are neutral markers (not under selection) we assume that genetic differentiation (change in these traits) between populations has occurred through genetic drift (LD was tested using GENEPOP and none of the tests was significant). Calculation and significance of genetic differentiation, using permutation test, between pairs of breeding ponds was estimated using FSTAT 2.9.3 (Goudet, 1995).

IBD and IBB. – To measure IBD, we quantified Euclidean distance in ArcMap v10.0 (ESRI, 2011) using the Point Distance tool, with each pond paired with all others to produce 28 distances. Next, we mapped IBB using cost distance estimation. Cost distance (in metres) was calculated around natural barriers to toad movement. We described these barriers by mapping the predicted relative probability of toad occurrence in the Oxfordshire pond cluster

based on a resource selection function (RSF) developed in a previous study (Salazar *et al.*, 2016). We fit the RSF as a function of environmental features including proximity to; *i*) wooded habitat, *ii*) urban areas, and *iii*) water bodies. Barriers in the cost distance prediction (Fig. 2) were areas of 0% relative probability of toad occurrence, further from woodland and water bodies and closer to urban areas (Salazar *et al.*, 2016). The spatial prediction, based on the environmental parameter estimates of the RSF, displayed the habitat units within the study area that were suitable for toads. We used the cost distance tool in ArcMap to calculate the least cost distance between all possible pairs of ponds within the configuration of habitat within this RSF prediction (Fig. 2).

Statistical analyses. – We created matrices describing the pairwise physical separation between ponds using two metrics. We used both the simple Euclidian distance between them and the metric intended to incorporate the cost of movement across the landscape between them (i.e., cost distance described above). These matrices were then compared with the matrix describing the pairwise genetic similarity between toad populations inhabiting the sampled ponds. We measured the association between the genetic and separation matrices using Spearman's correlation coefficient. The significance of this statistic was estimated by randomly reallocating the order of the elements in one of the matrices. We did this with the *zt* software (Bonnet & Van de Peer, 2002). We also performed the same procedure to derive a partial correlation between genetic distance and cost distance controlling for Euclidean distance with partial Mantel test using the same software. This tests a null hypothesis that the association between genetic similarity and cost distance is not accounted for by Euclidean distance.

RESULTS

We extracted biological material from a total of 259 tadpoles collected from the eight studied populations. All seven microsatellite loci were successfully amplified and polymorphic. The number of alleles detected varied from nine (*Bbufμ14*) to 31 (*Bbufμ65*), indicating new alleles in all loci compared to Brede *et al.* (2001). Expected heterozygosity ranged from 0.57 to 0.89 while observed heterozygosity varied from 0.55 to 0.83. The allelic richness values per breeding pond varied from 7.9 (Cumnor) to 9.5 (Bagley Wood). After correction using the ENA tool provided by FreeNa (Chapuis & Estoup, 2007), null alleles were present on average 2.4% over all breeding ponds and loci and between 1.1 % over all ponds for the locus *Bbufμ62* and 5.3 % over all ponds for the locus *Bbufμ15*.

Genetic differentiation across ponds was not significantly different from zero for three pairs of ponds: Fawler-Bagley Wood, Cothill-Garford, Ducklington-Standlake (Table 3) and therefore these pairs were considered genetically homogenous. Ducklington-Standlake (~ 6 km apart) were the most genetically similar breeding ponds pair and were notable for the high density of ponds between the two locations.

We detected no significant relationship between Euclidean distance and genetic differentiation (Mantel test: $r = -0.17$, $p = 0.237$). The relationship between cost distance and genetic differentiation was positive and significant both with and without control of Euclidean distance (simple Mantel test: $r = 0.48$, $p < 0.05$, partial Mantel test (Euclidean controlled): $r = 0.48$, $p < 0.05$).

DISCUSSION

We assessed the impacts of IBB and IBD on common toad genetic differentiation among eight breeding ponds in Oxfordshire, England. The number of alleles, their size ranges, as well as expected and observed heterozygosity were mostly higher than in Brede *et al.* (2001), possibly as a result of the larger number of alleles identified in our study. The genetic differentiation that we detected between toad breeding ponds was possibly best explained by IBB. We suspect that the size and strength of these barriers determined how much the cost distance would need to increase to circumvent the barrier. This result demonstrates the effect of urban areas with reduced density of more hospitable habitats (woodland, water bodies) as barriers to migration, affecting the ability of toads to move around the landscape to breed away from their natal pond (Hitchings & Beebee, 1997; Hamer & McDonnell, 2008; Goldberg & Waits, 2010). These results are consistent with established studies that identified lower genetic differentiation in toad ponds in urban areas (Hitchings & Beebee, 1998).

We detected the least genetic differentiation in a breeding pond pair that were ~ 6 km apart (Ducklington-Standlake). Despite being separated by this distance these two ponds were genetically homogenous as inferred from our examination of the seven microsatellite markers. This result may suggest a greater level of movement of individuals between these ponds than was possibly expected considering their reported philopatry (Reading *et al.*, 1991) and the maximum migration distance recorded for toads of 3.6 km (Moore, 1954; Heusser, 1958). Common toads can colonise new ponds when the nearest breeding pond is within 950 m (Baker & Halliday, 1999). Considering the high density of ponds between Standlake and Ducklington (Fig. 1), we hypothesize that the abundance of ponds in this landscape matrix function as stepping stones, facilitating movement of individuals or their genetic material over several generations. Though it is impossible to know the extent to which artificial toad translocation has affected the genetic structure of these breeding populations, it is unlikely to

have been commonplace as most of the ponds in this study were relatively isolated. Possible exceptions were the ponds at Fowler (accessible and closely monitored by the public as part of a toad crossing scheme) and Bagley Wood (in the grounds of a school), which were genetically homogenous despite the relatively large Euclidean and cost distances separating them (Fig. 1).

We failed to detect a significant relationship between common toad genetic differentiation and Euclidean distance at the local landscape scale. These findings may suggest that despite concern over recent dramatic population-level declines (Carrier & Beebee, 2003; Petrovan & Schmidt, 2016), connectivity between rural common toad populations at the local landscape scale might be maintained with appropriate management. To encourage a greater level of movement between breeding populations we suggest the creation of more ponds and woody habitat. Creation of ponds would go some way to redress the losses experienced in the last century (Williams *et al.*, 2010) and creation of nearby woody habitat would improve the relative probability of toads using these habitats during the terrestrial phase. Increasing the number of ponds increases pond density and so reduces distances between ponds that, though not shown to be limiting at the local landscape scale, may become more important at a larger scale.

Summary. – We collected common toad genetic material from eight different breeding populations in rural England to test the effect of isolation by distance and isolation by barrier on toad genetic differentiation. We detected no significant effect of distance but did identify a significant and positive relationship between barriers and common toad genetic differentiation, with breeding populations being more genetically different with increasing barrier cost distance.

268

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409

410 **TABLES**

411 **Table 1.** Tissue sample numbers, source ponds, and sampling dates for the collection of
 412 common toad (*Bufo bufo*) samples in Oxfordshire, England.

Pond name	No. tadpoles	Code	Sampling date
Bagley Wood	26	BW 1-26	25/06/12
Combe	48	Cb 1-48	18/06/12
Cumnor	30	Cu 1-30	27/06/12
Fawler	45	Fa 1-45	27/06/12
Garford	26	Ga 1-26	22/06/12
Standlake	31	St 1-31	29/06/12
Cothill	27	Hi 1-27	04/07/12
Ducklington	26	RC 1-26	26/06/12

413

414 **Table 2.** The seven microsatellite loci and their associated primers used for amplification of the common toad (*Bufo bufo*) samples (Brede et al.
415 2001) in Oxfordshire, England, together with the associated Multiplex design, labelling and Polymerase Chain Reaction conditions.

Microsatellite loci (GenBank code)		Primer sequences (5'-3')
Bbuf54 (AY037820)		FAM-CATTGCGCTGCTGTCAGATTACAC TTAGGGATTGCCGTCCAGTTGTC
Bbuf14 (AY037811)		NED-CGTGCATGCAAGTGTACCTAACC ATGGAGAGTGAAGGGGAAAGAGTG
Bbuf47 (AY037818)		VIC-GGATCAAGCCCTCAGACAACTC CACAGCAGCAGAAATTTGACCAG
Bbuf62 (AY037821)		PET-GCACATTCCTGTGTCCGTGTATAG ATTCCGAAAACGAAAAGAAAAGAG
Bbuf15 (AY037812)		FAM-TCAATATAGGAGTCCCAGAATGTC AATCCCCTAGCGTACACAAGATAC
Bbuf11 (AY037809)		GTCACATGGATAATAAATGAGACC TCTAATATTGATGACCAGACAACC
Bbuf65 (AY037823)		PET-GGATCTAAGCGCTGTGAGAGTGA CGGTCCGTGTTACCACTGATGC
PCR Name	Gene amplified/ Flourescent label	Reaction cycles
Multiplex4	Bbuf μ 15/Blue, Bbuf μ 14/Yellow, Bbuf μ 47/Red, Bbuf μ 65/Green	95°C for 5min denaturation, 94°C 30sec, 55°C 30sec and 70°C 30sec x25 cycles, 70°C for 30min final elongation step.
Multiplex6	Bbuf μ 54/Blue, Bbuf μ 62/Red	95°C for 5min denaturation, 94°C 30sec, 57°C 30sec and 70°C 30sec x23 cycles, 70°C for 45min final elongation step.
Singleplex11	Bbuf μ 11	94°C for 3min denaturation, 94°C 30sec, 51°C 1min and 72°C 1min x25 cycles, 72°C for 30min final elongation step.

416 **Table 3.** Pairwise Fst values between pairs of breeding patches.

Breeding patches	Bagley Wood	Combe	Cothill	Cumnor	Ducklington	Fawler	Garford	Standlake
Bagley Wood	-							
Combe	0.0280	-						
Cothill	0.0389	0.0305	-					
Cumnor	0.0639	0.0298	0.0576	-				
Ducklington	0.0605	0.0182	0.0410	0.0305	-			
Fawler	0.0137	0.0122	0.0343	0.0453	0.0206	-		
Garford	0.0364	0.0146	0.0078	0.0416	0.0264	0.0229	-	
Standlake	0.0373	0.0181	0.0387	0.0442	0.0033	0.0107	0.0237	-

417 Significant values are indicated in bold.

418

419 **FIGURE LEGENDS**

420 **Figure 1** Locations of eight ponds in Oxfordshire, England from which common toad (*Bufo*
421 *bufo*) genetic material was collected.

422

423 **Figure 2** The spatial prediction of the resource selection function (RSF) depicting common
424 toad (*Bufo bufo*) habitat in Oxfordshire, England. The permeable habitat (grey) among eight
425 Oxfordshire ponds (black dots). The areas in white are those predicted to have a 0%
426 probability of common toad occurrence

427

Figure 1

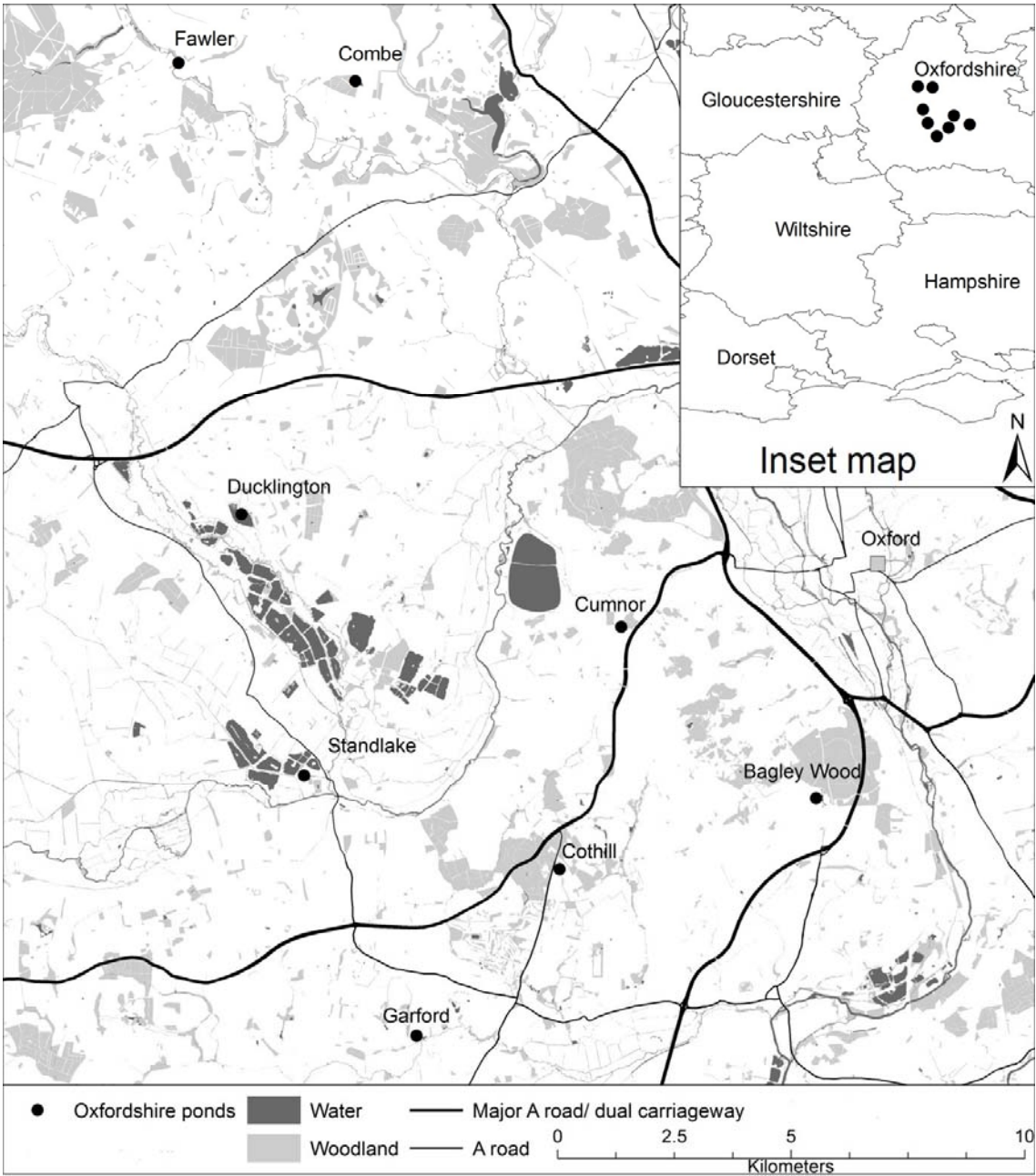


Figure 2

