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Front cover: Common toad *Bufo Bufo* migrating to breeding site in Inverness. See article on page 204.

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Unusual lack of reproduction in toad populations from agricultural habitats

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Anthropogenic alterations of habitats can have detrimental consequences for biodiversity. Documenting these effects require monitoring in multiple sites that vary in the degree of alterations over long temporal scales, a task that is challenging. Yet, simple naturalist observations can reveal major ongoing events affecting wild populations, and serve as a basis for further investigations. We quantified breeding parameters of spined toad (*Bufo spinosus*) populations from forested (preserved) and agricultural (altered) habitats. We found that reproduction did not occur at the sites surrounded by agriculture, while it occurred successfully in ponds from forests. Males were present at all sites, but females, amplexus, egg strings and tadpoles remained absent from agricultural sites. Observations made at the same sites indicated that breeding occurred during previous years. Our observations of habitat- and sex-specific lack of reproduction may have critical consequences for the persistence of populations of a widespread amphibian species in agricultural areas.

Keywords: Amphibian, *Bufo spinosus*, breeding, conservation, reproductive success

INTRODUCTION

Biodiversity is dramatically affected by human activities leading to an alteration of ecosystems (Chapin et al., 2000; Myers & Knoll, 2001; Brooks et al., 2002). Human activities, such as intensive farming, generate habitat alteration, fragmentation and simplification (e.g. Maron & Fitzsimons, 2007). In addition, agricultural landscapes often suffer from the massive use of pesticides, which contaminate the environment and the wildlife (Schäfer et al., 2007).

As a consequence, these modern agricultural practices can have detrimental impacts on fauna and flora (Myers & Knoll, 2001; Brooks et al., 2002; Fahrig, 2003; Relyea, 2009). In order to persist in these altered habitats, wildlife must adjust to these ongoing changes. However, the ability of a species to persist in agriculture landscapes can be jeopardised when critical elements necessary to perform its life-cycle are missing in the environment. For example, the lack of trees or shrubs can impair the ability of some bird species to breed in simplified landscapes (Newton, 1994; Verhulst et al., 2004). Similarly, amphibian populations will disappear if suitable breeding ponds are missing following habitat simplification (Smith & Green, 2005). In addition to habitat alteration, other effects can be linked to the increasing use of chemical inputs that aim to improve crop productivity in agricultural habitats (McLaughlin & Minneau, 1995; Köhler & Triebskorn, 2013). For instance, pesticides are used to control pests (e.g., weeds, insect, fungi) that negatively impact crop productivity. These pesticides can have toxic effects on non-target components. For example, they have been shown to negatively impact reproduction in wildlife species, through various mechanisms that spans from direct toxic or sublethal effects (Mnif et al., 2011; Cheron & Brischoux, 2020) to alterations of ecosystem functioning (e.g., disruption of the food web, Relyea & Hoverman, 2008).

The direct effects of habitat alteration on population persistence are relatively easy to assess (see above). Yet, assessments of indirect effects of agricultural practices on population persistence are more challenging and require population monitoring in multiple sites that vary in their habitat structure (i.e., degree of alteration and fragmentation). To document these effects, simple naturalist observations can be important because they often help to reveal major ongoing and detrimental events that affect wild populations (Sagarin & Pauchard, 2010; Sagarin & Pauchard, 2012; Mauz & Granjou, 2013).

During the course of a study that aimed to compare toad (*Bufo spinosus*) populations between forested (preserved) areas and agricultural (simplified) habitats, we opportunistically quantified breeding parameters (number of males, presence of amplexus, egg strings and tadpoles) in both types of habitat in Western France (Fig. 1). The toad (*Bufo spinosus*) is a widespread species that can live in a variety of habitats and has been previously shown to persist even in highly modified agricultural areas (Arntzen et al., 2014, Guillot et al., 2016). As in most anuran species, *B. spinosus* have a biphasic life-cycle with an extensive use of terrestrial habitats during

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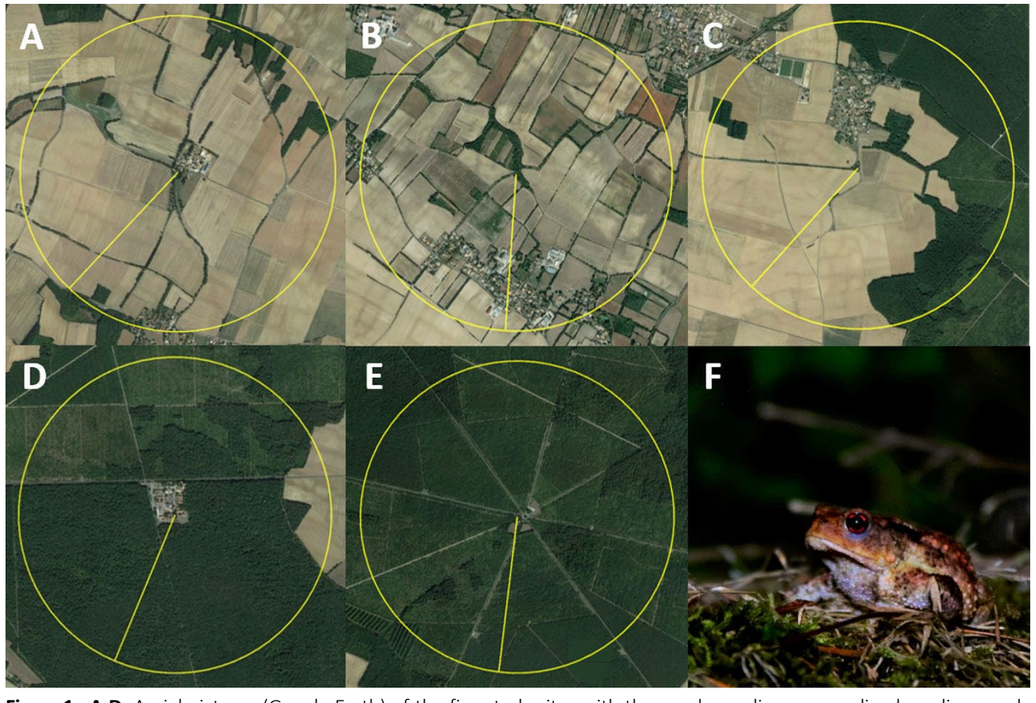

Figure 1. A-D: Aerial pictures (Google Earth) of the five study sites with the one km radius surrounding breeding ponds used to illustrate the contrast between three agricultural sites (A, B and C) and two forested sites (D and E). Letters in the pictures relate to site numbers in Table 1. **F**: Picture of an individual *Bufo spinosus* in the field in South Deux-Sèvres, France .

most of the year, and a short breeding season (~1 month) in aquatic sites (ponds) where mating occurs and eggs and tadpoles develop (Reading, 1998; Kelleher et al., 2018; Brischoux & Cheron, 2019). The breeding season occurs at the end of winter (February – March). During this period, male toads migrate towards aquatic breeding sites where they wait for females (Reading, 1998). Males can remain at the breeding site for several weeks, while females leave shortly after mating and egg-laying (Davies & Halliday, 1977). Eggs and tadpoles develop over three to four months before metamorphosis and subsequent dispersal in nearby terrestrial habitats. Reproductive events can be easily assessed later in the season (when breeders have left the breeding site) by monitoring the presence of egg strings and tadpoles.

The terrestrial part of the annual cycle of toads occurs in various environments usually within one km from the breeding pond (Janin et al., 2011; Guillot et al., 2016). Two of our study sites were located in forested areas where forest cover represented > 95 % within a circle of a one km radius centered on the breeding pond; while three sites were located in agricultural areas (composed mainly of large fields) where forest cover was always < 35 % within the same surface area (Fig. 1). Forest and agricultural sites were situated in close proximity (maximum distance 12 km) in order to avoid diverging climatic conditions that may affect timing of reproduction.

Observations were made from early January (week one) to late June (week 26) 2020. At the onset of the reproductive period (from week one to week 11) all study sites were monitored every night. Observations were stopped from week 12 to week 16 because of the lockdown linked to the COVID-19 pandemic. Observations resumed on week 17 on a monthly basis until late June (week 26) in order to assess the presence of developing tadpoles.

Due to of the diverging reproductive behaviour of males and females (see above), we made the following observations. Males were individually counted when abundances were < 10 individuals and number of individuals was approximated by increment of 10 individuals when abundances were > 10 individuals. Females remain only briefly at the breeding pond, and amplexus occurs in areas where precise quantification is precluded (in highly vegetated areas or deeper water). As a consequence, we assessed female presence through the observation of amplexus and qualified for each site whether amplexus was observed of not (present/absent). When reproduction occurred, large numbers of egg strings and tadpoles precluded direct enumeration and successful reproduction was assessed with the presence/absence of egg strings and tadpoles.

We emphasise that our opportunistic observations are qualitative rather than quantitative for most parameters recorded as they were not directly linked to the primary goal of the surveys we performed (assessment of reproductive success across habitats).

Table 1. Summary of the data collected during our surveys. Male abundances show min-max number of individuals observed for each week. Female presence or absence was assessed through observations of amplexus. The presence of egg strings and developing tadpoles was also documented. "ND" stands for "no data". "NO" refers to absence of individuals at periods during which presence was expected, while "-" refers to absence of individuals at periods when absence was expected.

			Week number											
Observations	**Sites**	**Habitat**	**1-4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12-16**	**17**	**21**	**26**
Number of males	A	Agriculture	0	1-3	1	0	0	0	0	0	ND	-	-	-
	B	Agriculture	0	40	10-40	3-10	1-3	1	1	0	ND	-	-	-
	C	Agriculture	ND	ND	ND	100	70	50	50	50	ND	-	-	-
	D	Forest	0	10-20	20	20	10-20	10-20	10	10	ND	-	-	-
	E	Forest	0	30	30	20	10-20	20	20	10	ND	-	-	-
Presence of amplexus	A	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-
	B	Agriculture	NO	NO	YES	YES	NO	NO	NO	NO	ND	-	-	-
	C	Agriculture	ND	ND	ND	YES	NO	NO	NO	NO	ND	-	-	-
	D	Forest	NO	YES	YES	YES	YES	YES	YES	YES	ND	-	-	-
	E	Forest	NO	YES	YES	YES	YES	YES	YES	YES	ND	-	-	-
Presence of egg strings	A	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-
	B	Agriculture	NO	NO	NO	NO	NO	NO	NO	NO	ND	-	-	-
	C	Agriculture	ND	ND	ND	NO	NO	NO	NO	NO	ND	-	-	-
	D	Forest	NO	NO	YES	YES	YES	YES	YES	YES	ND	-	-	-
	E	Forest	NO	NO	YES	YES	YES	YES	YES	YES	ND	-	-	-
Presence of tadpoles	A	Agriculture	-	-	-	-	-	-	-	-	ND	NO	NO	NO
	B	Agriculture	-	-	-	-	-	-	-	-	ND	NO	NO	NO
	C	Agriculture	-	-	-	-	-	-	-	-	ND	NO	NO	NO
	D	Forest	-	-	-	-	-	-	-	-	ND	YES	YES	NO
	E	Forest	-	-	-	-	-	-	-	-	ND	YES	YES	NO

Observations are summarised in Table 1. Overall, we found that reproduction did not occur at the three sites from agricultural habitats, while it occurred successfully in breeding ponds from forested areas (presence of egg strings and tadpoles, Table 1).

At all of our study sites, breeding males were present, yet with variable abundances (Table 1). Mean number of adult males was 19.0±28.4 (range 0-100) for agricultural sites and 15.6±8.3 (range 0-30) for forest sites (Table 1). These numbers suggest that abundances of reproductive males did not seem to be related to the surrounding habitat structures. Indeed, some sites from agricultural areas displayed numbers of males that equaled or even exceeded those from forested habitats (Table 1). Importantly, the onset of the reproductive period (first observations of males occurring at the study sites) was similar between habitat types (occurring on week 5, Table 1), suggesting that climatic (micro-) conditions did not significantly influence reproduction between sites. These observations tend to further indicate that the lack of reproduction we recorded (see below) may not be linked to a lack of breeding males (although one agricultural site was characterised by lower abundances, Table 1), but rather to a lack of reproductive females.

Indeed, the most clear-cut difference between our study sites was linked to the presence of females (assessed through the presence of visible amplexus, Table 1) and their reproductive success (assessed through the presence of egg strings and developing tadpoles, Table 1). Amplexus was observed on very few nights (one or two nights) at two of the agricultural sites, and was not observed at the other agricultural site. Conversely, amplectant pairs were observed steadily almost every night over six weeks at the sites surrounded by forest. No egg-strings or developing tadpole were observed at all three sites from agricultural habitats, while egg strings and developing tadpoles were present at the two forest sites. Importantly, these observations suggest that females did not migrate to breed in sites surrounded by agricultural areas and, thus, that habitat-specific and sex-specific responses to habitat perturbations occurred in adult females.

It is important to stress that our observations are unreplicated and preliminary and that we have not observed this phenomenon in previous years. Therefore, these observations do not give any strong clue regarding the mechanisms through which habitat-specific and probably sex-specific lack of reproduction has occurred. Yet, previous observations made at the same study sites (Guillot et al., 2016; MC and FB unpublished data) indicate that breeding successfully occurred at some of these agricultural sites at least in 2015 and in 2019; 2 years during which we monitored reproduction at some of those sites and for which egg strings and developing

tadpoles were observed. Although we acknowledge the limitations of our observational study, we believe it is important to document, at least in a qualitative way, a potential problem for the persistence of the populations of a widespread amphibian species in agricultural areas (Guerry & Hunter, 2002, Boissinot et al., 2019); and we urge other researchers to share similar observations.

Author Contributions

F.B. and F.A. proposed the initial idea and together with M.R. and M.C. contributed to its development. M.R., M.C. and F.B. performed field work. M.R. and M.C. tabulated the resulting data. All authors discussed the results, and substantially contributed to the writing.

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SHORT NOTE

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"Reconstructions of the past distribution of *Testudo graeca* mitochondrial lineages in the Middle East and Transcaucasia support multiple refugia since the Last Glacial Maximum": A response to Turkozan et al. (2021)

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Species distribution models (SDMs) are frequently used to characterise current, past or future realised environmental niches. Two recent studies applied different approaches to infer range dynamics in eastern subspecies of the spur-thighed tortoise *Testudo graeca* Linnaeus, 1758. We discuss differences in the conclusions of the two papers and use multivariate environmental similarity surface (MESS) analyses to show that the results of the study by Turkozan et al. (2021), recently published in the Herpetological Journal, are compromised by extrapolation and therefore have to be interpreted with caution.

Keywords: Glacial refugia, multivariate environmental similarity surface (MESS), range shifts, species distribution modelling, spur-thighed tortoise

Understanding how endangered taxa are distributed is a basic prerequisite for conservation planning and, in the face of the sixth mass extinction event during Earth's history affecting vertebrates (cf. Ceballos et al., 2020), of paramount importance. Ever refined approaches for species distribution modelling substantially contribute to a better knowledge of the current, past and future distribution ranges of chelonians (e.g., Ihlow et al., 2012; Rödder et al., 2013), one of the most threatened vertebrate groups (TTWG, 2017). Recently, Turkozan et al. (2021) aimed to clarify the distribution and past range dynamics of spur-thighed tortoises (*Testudo graeca*) harbouring different mitochondrial lineages that are generally identified as distinct subspecies (TTWG, 2017). We appreciate the efforts undertaken by Turkozan et al. (2021) but found some misconceptions in their article that we highlight in this note.

Turkozan et al. (2021) used species distribution models (SDMs) to predict the ranges of the five eastern subspecies of *T. graeca*. SDMs are frequently applied to characterise current realised environmental niches and estimate potential geographic distributions of taxa. By projecting SDMs onto paleoclimatic or putative future conditions, range shifts can be inferred. However, predictor variables are extrapolated when projecting models through space (whenever the projection area is larger than the training range) and time (projecting onto future or past climatic conditions; e.g., Elith et al., 2009, 2010). This requires cautious interpretation of modelling results (Elith et al., 2010; Owens et al., 2013). Extrapolation effects tend to increase when models are trained with geographically restricted data sets (e.g., Elith et al., 2010; Rocchini et al., 2011; Engler & Rödder, 2012; Owens et al., 2013).

Turkozan et al. (2021) inferred environmental niche models for each of the five studied subspecies using ten uncorrelated bioclimatic predictors (seven temperature-related and three precipitation-related variables) and the maximum entropy modelling algorithm MaxEnt (Phillips et al., 2006; Phillips & Dudík, 2008). Another recent study (Javanbakht et al., 2017) examined three of these subspecies using *n*-dimensional hypervolumes based on principal components derived from 19 bioclimatic variables (cf. Blonder et al., 2014; Blonder, 2018). In order to study paleoclimatic range dynamics, both teams projected their resulting models onto reconstructions of climatic conditions of the mid-Holocene (6,000 BP) and the Last Glacial Maximum (LGM, 21,000 BP) but came to different conclusions.

According to Turkozan et al. (2021), the distribution ranges of two subspecies (*T. g. armeniaca* and *T. g. buxtoni*) were almost exclusively shaped by a single precipitation-related variable, respectively (with permutation contributions of 95.8 % and 85 %), while the ranges of *T. g. ibera* and *T. g. zarudnyi* were predominantly affected by a single temperature-related predictor (75 % and 88 %, respectively). The distribution of *T. g. terrestris* was inferred to be shaped by precipitation- and temperature-related predictors. In contrast, Javanbakht et al. (2017) found that the distribution of *T. g. armeniaca*, *T. g. buxtoni*, and *T. g. zarudnyi* was predominantly limited

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by precipitation. For the latter subspecies, this conflicts with the results of Turkozan et al. (2021).

Turkozan et al. (2021) suggested that multiple glacial refugia existed and that since the LGM the potentially suitable geographic space has expanded for *T. g. ibera*, contracted for *T. g. zarudnyi*, and remained stable for *T. g. terrestris*. For *T. g. armeniaca* and *T. g. buxtoni*, the patterns were inconclusive. In contrast, Javanbakht et al. (2017) suggested that the ranges of the three studied subspecies (*T. g. armeniaca*, *T. g. buxtoni*, *T. g. zarudnyi*) experienced only slight shifts and did not expand significantly after the LGM.

The methods used by Turkozan et al. (2021) and Javanbakht et al. (2017) are fundamentally different, having distinct underlying conceptual and computational principles. Correlative SDMs, such as MaxEnt used by Turkozan et al. (2021), are prone to extrapolation errors when projected through space and time (Elith et al., 2010; Owens et al., 2013). In contrast, profiling techniques based on multivariate analyses, such as the non-parametric kernel density estimation (KDE) and *n*-dimensional hypervolumes used by Javanbakht et al. (2017), are more robust and allow the characterisation of realised niches based on delimitation of niche volumes. Especially when calibration areas are small (VanDerWal et al., 2009), projections onto other time slices or geographic areas derived from correlative models are compromised by uncertainty (Rocchini et al., 2011). Unfortunately, Turkozan et al. (2021) did not account for such uncertainties.

To examine the impact of extrapolation on their results, we used multivariate environmental similarity surface (MESS) analyses (Elith et al., 2010). MESS analyses identify areas where one or more predictor variables experience conditions beyond the respective calibration range and, thus, are compromised by extrapolation (cf. Elith et al., 2010). To construct MESS maps, we georeferenced the minimum convex polygons (MCPs) used as model training range by Turkozan et al. (2021) and performed MESS analyses using the packages dismo (Hijmans et al., 2017) and raster (Hijmans, 2020) for Cran R (R Development Core Team, 2020). MESS analyses were computed for each of the ten predictors used by Turkozan et al. (2021) separately, rescaled to 0 (no extrapolation) and 1 (extrapolation) and subsequently summed to show the number of variables affected by extrapolation per geographic region (for R code, see Supplementary Materials).

Our results show that the range estimates of Turkozan et al. (2021) are significantly compromised by extrapolation. This refers to vast areas of the study region, for current conditions as well as reconstructions (mid-Holocene and LGM) across all three used general circulation models (GCMs; Supplementary Materials: Figs. S1-5).

Parenthetically it may be noted that Turkozan et al. (2021) erred when they suggested that factor loadings of a principal component analysis (PCA) have been interpreted erroneously by Javanbakht et al. (2017). In contrast to MaxEnt, the non-parametric multivariate approach used by Javanbakht et al. (2017) requires orthogonal input variables. To ensure orthogonality, input variables are subjected to a PCA prior to modelling (Barros et al., 2016), and the (past) climate reconstructions are projected in the PCA space derived from current climate conditions, resulting in different sets of principal components for each scenario. Thus, Turkozan et al. (2021) apparently misunderstood the matter and misinterpreted data presented by Javanbakht et al. (2017) within the frame of another method (MaxEnt).

In addition to these methodological issues, the study by Turkozan et al. (2021) contains additional flaws. For instance, Turkozan et al. (2021) state in their Abstract that "Since the LGM, we hypothesise that the ranges of lineages have either expanded (*T. g. ibera*), contracted (*T. g. zarudnyi*) or remained stable (*T. g. terrestris*), and for other two taxa (*T. g. armeniaca* and *T. g. buxtoni*) the pattern remains unclear." This contradicts the Discussion section (p. 15), where the authors state that "the distribution model of *T. graeca* clades in the present work are in line with the classical glacial range contraction and interglacial range expansion model (Stewart et al., 2010) except the *zarudyni* [sic!] clade which contracted during the interglacial period." However, the authors did not present any convincing evidence for the latter statement. Turkozan et al. (2021: p. 15) explained that their "analysis supports multiple potential refugia during LGM, namely Caucasus, Anatolia, and Balkans" and that "this is in line with the concept that temperate adapted taxa are confined to southern refugia (Stewart et al., 2010)." Stewart et al. (2010) define refugia as the geographical regions that correspond to the species' maximally contracted geographical range during a glacial period. This is in line with the general understanding of glacial refugia (e.g., Hewitt, 2000; Joger et al., 2007; Schmitt, 2007). Neither Javanbakht et al. (2017) nor Turkozan et al. (2021) inferred massive range restrictions during the last glacial cycle. Instead, it seems that climatically suitable space for *T. g. armeniaca*, *T. g. buxtoni* and *T. g. zarudnyi* experienced only slight shifts since the LGM, what contrasts with the massive Holocene range expansions of thermophilic species in more northern latitudes (Hewitt, 2000; Joger et al., 2007; Schmitt et al., 2007) and the classical refugia model. This situation has been discussed in detail in Javanbakht et al. (2017) and the interested reader is referred to this publication.

Another misinterpretation of the results of Javanbakht et al. (2017) concerns bioclimatic variables shaping the distribution of *T. graeca*. Turkozan et al. (2021: p. 15) state that Javanbakht et al. (2017) ignored temperature-related factors delimiting the species distribution. However, Javanbakht et al. (2017: p. 635) stated that, besides precipitation as the main variable, "other environmental variables shaping the distribution of tortoises in Iran and Transcaucasia are the seasonal variation in temperature expressed as 'temperature seasonality' and 'annual temperature range'" [and that] "seasonal temperature variation seems to be a limiting factor for tortoises in the Middle East, since this region is characterised by a continental climate with hot summers

and cold winters. Hence, the combination of precipitation and high temperature seasonality appear to shape the distributional pattern of *T. graeca* in the eastern part of its range."

Our Short Note revealed that the results of Turkozan et al. (2021) are compromised by misconceptions and misunderstandings. Therefore, they should be interpreted with caution.

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How did the toad get over the sea to Skye? Tracing the colonisation of Scottish inshore islands by common toads (*Bufo bufo*)

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Processes of island colonisation have long been of interest to biologists. Colonisation events themselves are rarely observed, but the processes involved may be inferred using genetic approaches. We investigated possible means of island colonisation by common toads (*Bufo bufo*) in western Scotland (the Isle of Skye and five neighbouring small islands), using evidence derived from nuclear microsatellites and mitochondrial (mt) DNA. Levels of microsatellite allelic richness for populations on Skye were high and comparable to adjacent mainland populations, but lower for populations on small islands. Pairwise measures of genetic distances between populations and a clustering algorithm were both suggestive of frequent gene flow between Skye and the mainland. For small islands the levels of genetic differentiation were higher, implying stronger isolation and no evidence for inbreeding. The distribution of mtDNA haplotypes broadly mirrored the genetic structure revealed by microsatellites. Reconciled with existing palaeoclimatological evidence, since the last glaciation, our findings rule out the possibility that the *B. bufo* populations stem from glacial refugia, or that recent anthropogenic transfer of toads is responsible for their current distribution. The most parsimonious explanation of our data is that the studied inshore islands have been repeatedly colonised via rafting from the mainland or neighbouring islands. This may give us insights into the processes likely to take place when ice sheets retreat poleward as a result of climate change. It also has implications for the colonisation of both native and invasive non-native species, and hence the biosecurity of island refugia.

Keywords: Island biogeography, glaciation, amphibians, rafting

INTRODUCTION

Island populations of widespread species have long attracted the attention of natural scientists (e.g. Wallace, 1880; Whittaker & Fernández-Palacios, 2007). Populations on islands are also of interest to geneticists, due to restrictions on gene flow and the influence of founder effects which can both impact on population viability (Frankham, 1997; Reed & Frankham, 2003). Modes of island colonisation and persistence are further of relevance for studies into how species might adapt to future rapid environmental change (Courchamp et al., 2014).

As one of the most severely threatened groups of those whose status has been assessed (IPBES, 2019), amphibians are a global conservation priority. Amphibians are also suitable subjects for island biogeographical studies, as they have limited powers of dispersal compared to flying animals such as birds, insects and bats, or organisms that drift by wind or zoochory (Cushman, 2006; Allentoft & O'Brien, 2010). Their low to moderate salinity tolerance (reviewed in Hopkins & Brodie, 2015) further implies difficulty particularly when colonising oceanic islands, as suggested by early authors including Darwin (1859, p. 393). For example, archipelagos such as the Canaries, Galapagos and Mauritius are occupied by reptiles, but do not harbour native amphibians. Amphibians have, however, colonised other islands by both anthropogenic and natural means. Human introduction may be accidental (Kuraishi, Matsui & Ota, 2009) or deliberate (e.g. Shine, 2018). In some cases, amphibians arrived naturally before islands were cut off due to sea level rise (e.g. Wang et al., 2014), and in others they colonised islands after their formation. Natural colonisation of islands is assumed to take place for example by rafting upon floating vegetation or debris (Vences et al., 2003; Measey et al., 2007; reviewed in Marin da Fonte et al., 2019; see also Schiesari et al., 2003 for the frequent occurrence of rafting by amphibians in large tropical river basins). In inshore situations, dispersal by swimming could also be assisted by conditions of low-salinity, for example when a lack of wind allows a layer of less dense freshwater from river outflow to lie on top of sea water (discussed in Seppä & Laurila, 1999). Other proposed mechanisms for dispersal of amphibians include tornados (Elsom, 1988) and transport of eggs by waterbirds (for an example on fish see Lovas-Kiss et al., 2020), although documented evidence is largely lacking.

Glaciation has been a principle geomorphological and biogeographic shaper of lands beyond 45° latitude. In Europe, this has led to a pattern of biodiversity richness in central and southern Europe, with reduced diversity linked to post-glacial recolonisation in the north (e.g. Hewitt, 2000). The glacial history of Scotland is similar to that of other European high latitudes, and its fauna is well-studied. Interestingly, the melting of the main glaciers at the end of the last glacial c. 15,000 years before present (ybp) (Mayle et al., 1F999) in Scotland was also followed by a cold period between c. 12,900 and 11,700 ybp, which led to the temporary re-forming of glaciers ranging from Loch Lomond in the central belt northward to Torridon in the western Highlands (the Younger Dryas or Loch Lomond Stadial; Bradwell et al., 2008; Ballantyne, 2019).

The western Scottish Highlands are characterised by low human population density and low levels of intensive agriculture, and are home to three species of amphibians (the common toad *B. bufo*, the common frog *Rana temporaria* and the palmate newt *Lissotriton helveticus*). These species are recorded regularly not only on the mainland but also on a range of inshore islands (McInerny & Minting, 2016; Fiegna et al., 2017; NBN, 2019), which were already separated from the mainland when Britain was still connected to mainland Europe up to 8000 ybp (Lambeck, 1995; Ballantyne, 2019). In the present study we focus on *B. bufo*, a widespread species which has previously served for population genetic investigations in northern European archipelagos (Seppä & Laurila, 1999; Roth & Jehle, 2016). We employ information derived from nuclear and mitochondrial DNA markers to (i) document spatial patterns of genetic variation across the Isle of Skye, adjacent mainland and small islands of the Inner Sound, and (ii) use these data to infer possible means of island colonisation. More specifically, we reconcile the obtained genetic data with existing evidence from palaeoclimatology, and ask whether the islands under study became colonised prior to the Loch Lomond Stadial, for example via land bridges, or after this period when meltwater would have temporarily reduced the salinity of inshore waters. Alternatively, *B. bufo* may also have reached these islands more recently through human introductions or natural means. Our study complements similar local investigations for example on small mammals (White & Searle, 2007; 2008), and provides information on the origin of the westernmost natural populations of a widespread European anuran.

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Table 1. Genetic variability parameters for 11 *Bufo bufo* populations characterised at 8 microsatellite loci.

Location	Site	N	A/L	AR	Ho	He	F_{IS}	ML	PA
Loch Iain Oig, Kyle of Lochalsh, mainland	MAK	10	5.38	3.73	0.54	0.65	0.18	0	7
Toscaig, nr Applecross, mainland	MAT	10	4.50	3.14	0.51	0.51	0.00	0	2
Lochan Dubh, Broadford, Skye	SKB	11	3.88	3.33	0.36	0.41	0.13	0	2
Loch a Mhuilinn, Portree, Skye	SKP	20	4.25	2.73	0.49	0.51	0.03	0	2
Pabay	PAB	19	1.63	1.57	0.26	0.20	-0.27	4	0
Loch Beag, Raasay	RAB	9	3.38	2.72	0.49	0.50	0.027	1	1
Oskaig, Raasay	RAO	10	4.00	2.86	0.45	0.47	0.053	1	2
Loch na h Iolaire, Rona	ROI	11	3.25	2.49	0.34	0.39	0.12	0	1
Township reservoir, Rona	ROT	13	2.88	2.33	0.37	0.36	0.00	3	1
East of Loch Dubh, Scalpay	SCD	10	4.50	3.30	0.56	0.58	0.024	0	3
Loch nan Leac, Crowlin	CRO	31	4.73	2.90	0.56	0.61	0.12	0	6

n, number of individuals sampled; A/L, mean number of alleles per locus; AR, allelic richness; Ho, observed heterozygosity; He, expected heterozygosity; ML, number of monomorphic loci; PA, number of private alleles.

MATERIALS & METHODS

Field sampling

This study took place in the western Scottish Highlands (UK), and encompassed two waterbodies on the Isle of Skye (1 656 km² in area, connected to the mainland by a ca. 500 m long bridge erected in 1995), two waterbodies on the adjacent mainland, and seven waterbodies across all islands with standing freshwater in the Inner Sound (Rona and Raasay, two waterbodies each; Scalpay, Pabay and Crowlin, one waterbody each; see Table 1 and Figure 1). Crowlin is seldom visited and like Pabay has no permanent human population, while Scalpay and Rona each have fewer than five inhabitants. The isolated islands range in size from 1.3 km² (Pabay) to 53.4 km² (Raasay) and have been separated by sea since the last period of glacial activity in the area ended approximately 9500 years BP (Lambeck, 1995), although it is possible to cross from Skye to Scalpay during extreme low tides. A total of 157 samples were collected between 2013 and 2015, as eggs derived from ten spawn strings at each site, or tadpoles taken at least 10 m apart to reduce the risk of sampling siblings (n = 9-31 individuals per population, Table 1). Samples were stored in 1.5 ml Eppendorf tubes filled with absolute ethanol.

Genetic analyses

DNA was extracted from whole eggs or tadpole tail tissue using the Qiagen DNEasy Blood and Tissue extraction kit (Qiagen, UK) following the manufacturer's protocol. Concentration of extracted DNA was quantified using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA) and standardised to approximately 10 ng/µl.

For analyses of mitochondrial DNA, a 722bp long fragment of the *cytb* region was amplified for three individuals from all 11 population using PCR primers described in Recuero et al. (2012; F: ATCTACCTTCACATCGGACGAG, R: AGTTTRTTTTCTGTGAGTCC), and a 10 µl PCR reaction mix containing 10–50 ng DNA, 5 pmol (5 mmol/L) of each primer, 0.15 mmol/L of each dNTP, 1.5 mmol/L MgCl2, and 0.5–1.0 U Taq polymerase (GoTaq) in the manufacturer's buffer. The PCR reaction was carried out at the following amplification conditions: 2 min at 96 °C, followed by 37 cycles of 30 s at 94 °C, 45 s annealing at 53 °C and 1 min 30 s at 72 °C, and a final 5 mins at 72 °C. In total, 154 samples from all but one population (SCM) were also genotyped at eight existing *B. bufo* microsatellite loci (Bbuf11, Bbuf15, Bbuf24, Bbuf39, Bbuf46, Bbuf54, Bbuf62, and Bbuf65; Brede et al. 2001). PCRs contained 10–50 ng DNA, 5 pmol (5 mmol/L) of each primer, 0.15 mmol/L of each dNTP, 1.5 mmol/L MgCl2, and 0.5–1.0 U Taq polymerase (Advanced Biotechnologies, Columbia, MD) in the manufacturer's buffer, at a total volume of 10 µl. The PCR profiles were 94 °C for 2 min, followed by 39 cycles of 94 °C for 30 s, the primer-specific annealing temperatures as in Brede et al. (2001) for 30 s, and 72 °C for 30 s. We used PCR primer-specific annealing temperatures as described in Brede et al. (2001), with the exception of Bbuf11 which was found to yield more PCR product at an annealing temperature of 56 °C. Primers were labelled with fluorochromes PCR products, and were separated by capillary electrophoresis using an ABI 3130 Genetic Analyser (Applied Biosystems), and sized using Peak Scanner Software v1.0 (Applied Biosystems).

Statistical analyses

Haplotype sequences derived from the mtDNA analysis were aligned using Clustal W (Thompson et al., 1994) in BioEdit ver 7.1.3.0 (Hall, 1999). Obtained sequences were compared with existing data in GenBank, with haplotype designations following the terminology of Tuncay et al. (2018). To illustrate the population share across haplotypes, and to distinguish between ancestral and derived haplotypes, NETWORK 10 (Fluxus Technology Ltd., www.fluxus-engineering.com/sharenet.htm) was used to compile a median-joining (MJ) network. Due to the limited number of samples available for each population we refrained from detailed statistical analyses. For microsatellites, observed (H_o) and expected (H_e) heterozygosities, deviations from Hardy-Weinberg equilibrium and pairwise F_{ST} values between populations were calculated using the software GENEPOP 4.4 (Rousset, 2008). Allelic richness values for each population were calculated using FSTAT (Goudet, 1995). Following Rousset (1997), a pattern of isolation by distance was evaluated using Mantel tests (10000 permutations) comparing linearised F_{ST} values F_{ST} /(1- F_{ST}) with log-transformed pairwise geographic distances carried out using the *R* package VEGAN (Oksanen et al., 2018). A Kruskal-Wallis test was carried out in *R* version 3.5.0 (*R* Core Team, 2018) to compare F_{ST} values calculated for populations separated by sea and those separated by land. Spearman rank correlations between the mean of a population's F_{ST} values and both its allelic richness and expected heterozygosity were also calculated using *R*. STRUCTURE 2.3.4 (Pritchard et al., 2000) was used to identify the most likely number of genetic clusters (*K*) within the dataset. STRUCTURE uses a Bayesian iterative algorithm to assign the membership of each sample probabilities to a pre-defined number of clusters. Largely following Porras-Hurtado et al. (2013), 20 independent runs were performed for each value of *K* from 1 to 13, with 200 000 Markov Chain Monte Carlo iterations after a burn-in of 200,000 iterations. The best-supported value of *K* was determined using *ΔK*, related to the rate of change in log probability between successive *K* values (Evanno et al., 2005), using STRUCTURE HARVESTER (Earl & von Holdt, 2012). Replicates for each level of *K* were aligned using CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) and graphical output was produced using DISTRUCT 1.1 (Rosenberg, 2004).

RESULTS

The mtDNA analysis revealed a total of five haplotypes (Fig. 1). H_9 (32 % of all individuals, present in six populations) is commonly reported in the western distribution of *B. bufo*, and has been previously found in the UK (Recuero et al., 2012; Arntzen et al., 2017); all other haplotypes have not previously been reported for *B. bufo*. The most frequent haplotype (H_62) represented 56 % of individuals across nine populations, and differed from H_9 by a single base substitution. Three further haplotypes were represented by one (H_65) or two (H_63 and H_64) individuals (Fig. 2). The new sequences have been deposited in Genbank (accession numbers: MZ318468 – MZ318490).

For microsatellites, the PCR success rate was 91 %. Mean number of alleles per locus ranged between 1.63 for an island population (PAB) and 5.38 for a mainland population (MAK). Both mainland populations and one Skye population (SKB) showed higher levels of allelic richness (3.14-3.73) than all but one island population (SCD, on Scalpay which is connected to the Isle of Skye at low water spring tides (Admiralty Chart 2498, 2018). Four populations revealed monomorphic loci, all of which were situated on small islands (Table 1). F_{ST} values for populations on the mainland and on the large Isle of Skye, or on the same small island ranged from -0.01 to 0.13, whereas those between populations on the smaller islands ranged from 0.07 to 0.50 (Table 2), with highly significant differences between these two groups (Kruskal-Wallis test, p <0.001). The allelic richness of a population was strongly negatively associated with its degree of isolation (defined by the mean of the pairwise F_{ST} values with all other populations; Spearman rank correlation coefficient -0.84, p = 0.001). Similarly, expected mean heterozygosity showed a marginally significant tendency towards a negative correlation with mean F_{ST} (Spearman rank correlation coefficient -0.58, p = 0.062). A weak, but significant, isolation-by-distance effect was present (Mantel test, r = 0.23, *p* = 0.04).

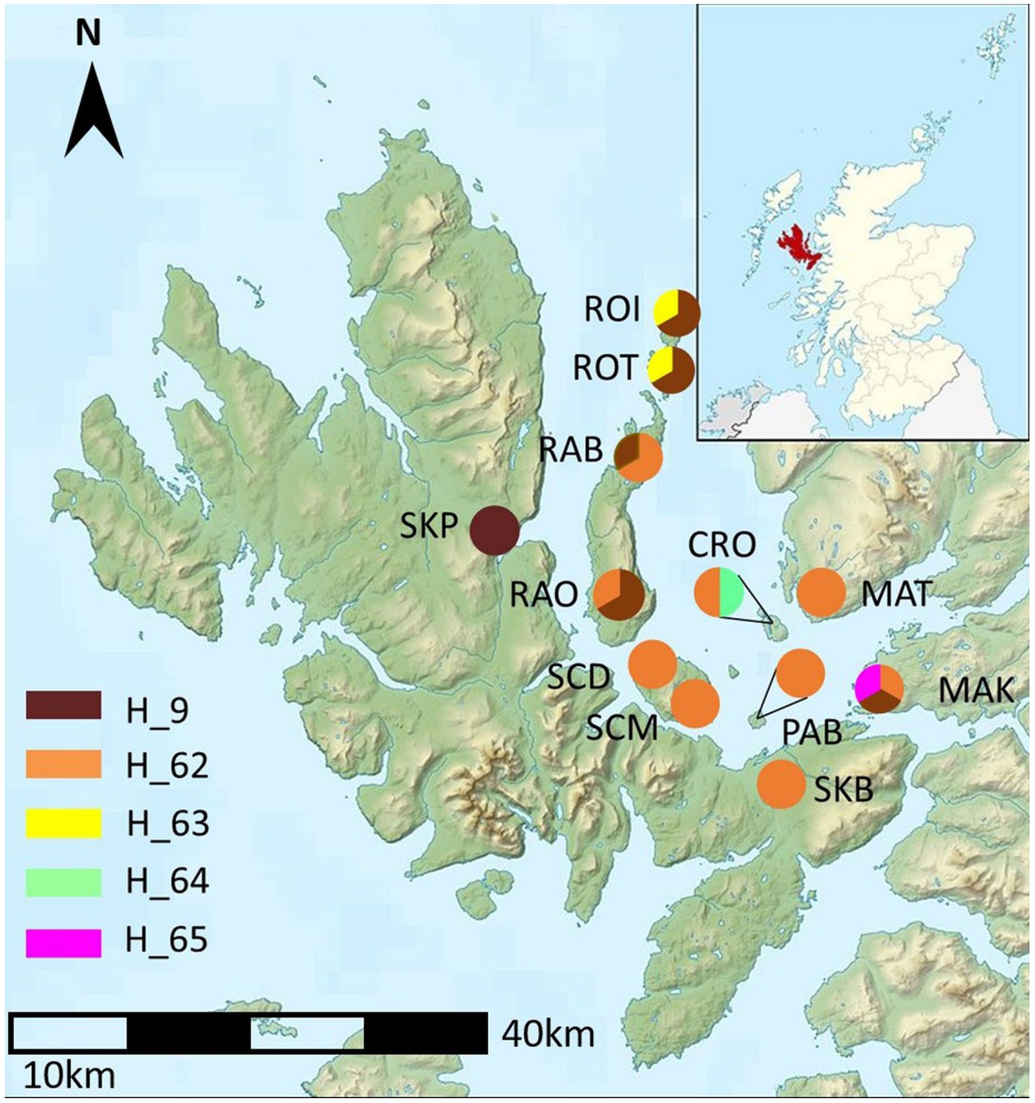

Figure 1. Locations of *Bufo bufo* breeding sites sampled and haplotype distribution. Inset shows position of study area in Scotland. See Table 1 for more detail of sampling sites. Contains Ordnance Survey data © Crown copyright and database right.

The log probability of numbers of clusters according to the STRUCTURE analysis increased from *K* = 1 through *K* = 7, with a modal value of *ΔK* at *K* = 4. The genetic clusters reflected their geographic context. All mainland and Skye populations as well as the populations on Raasay and Scalpay were predominantly assigned to a single cluster, whereas each of the remaining three small islands represented a distinct genetic unit (Fig. 3).

DISCUSSION

The present paper sought to combine new information drawn from DNA with existing geographic and palaeoclimatological evidence to infer the most likely colonisation history of inshore islands in the western Scottish Highlands by *B. bufo*. Below, we first discuss the spatial patterns of genetic variation. We then consider possible mechanisms of island colonisation which could have led to the observed island distribution: deliberate

Table 2. Pairwise F_{ST} values for 11 *Bufo bufo* populations. Continuous box borders denote populations separated by land, the broken border denotes populations separated by land and the Skye Bridge, and the remaining values show populations separated by sea.

	MAK	MAT	SKB	SKP	PAB	RAB	RAO	ROI	ROT	SCD
MAT	0.04									
SKB	-0.01	0.05								
SKP	0.10	0.13	0.06							
PAB	0.32	0.38	0.34	0.32						
RAB	0.11	0.20	0.09	0.17	0.35					
RAO	0.15	0.30	0.18	0.23	0.37	0.07				
ROI	0.24	0.27	0.21	0.28	0.49	0.29	0.32			
ROT	0.30	0.36	0.30	0.37	0.50	0.33	0.32	0.05		
SCD	0.07	0.13	0.09	0.18	0.21	0.13	0.15	0.25	0.27	
CRO	0.13	0.22	0.11	0.20	0.40	0.17	0.24	0.28	0.32	0.20

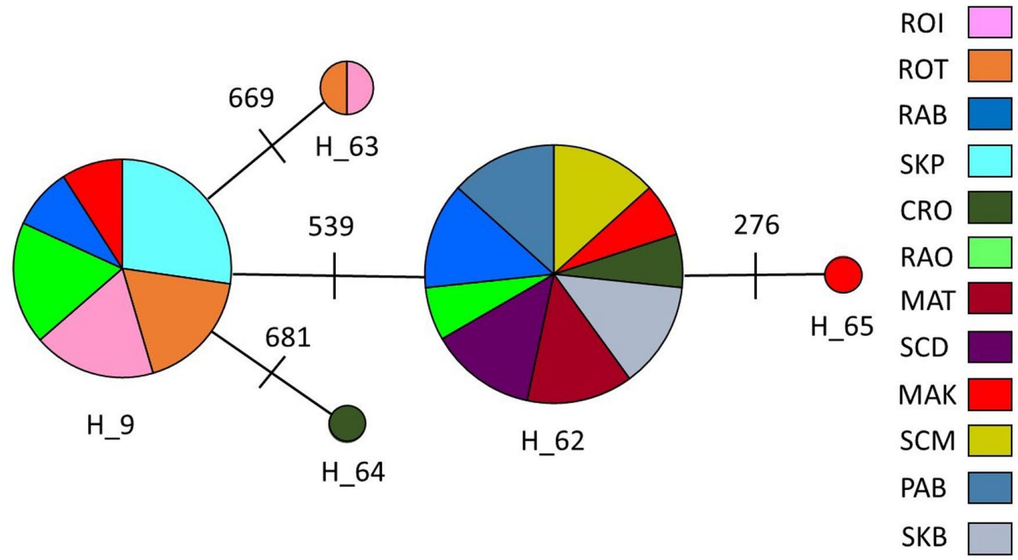

Figure 2. Median Joining Haplotype Network of *B. bufo cytb* sequences from the study area. Nucleotide positions of mutated sites are shown as numbers; shared haplotypes are divided into colours representing the populations shown in Figure 1.

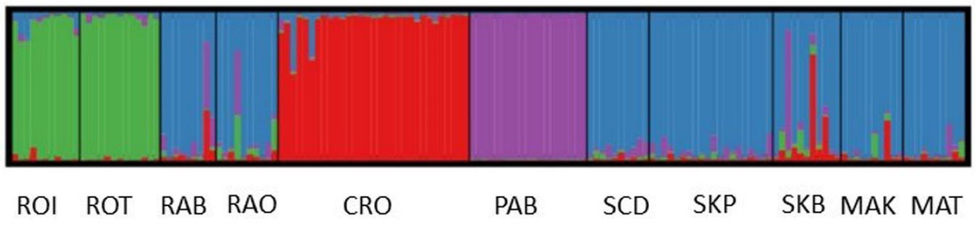

Figure 3. Bar plot showing assignment of all sampled individuals to the 4 genetic clusters determined by STRUCTURE. Each horizontal line denotes an individual, with the size of each colour bar corresponding to the probability of membership of each of four clusters. Three letter codes refer to breeding sites, as shown in Figure 1 and Table 1.

or accidental human introduction, colonisation of islands before their isolation, colonisation of islands by swimming before salinification of the Inner Sound, and colonisation by rafting. Whilst not conclusively supporting a single mechanism, our findings strongly suggest that the last is most likely.

Population genetic structure

Previous studies of *B. bufo* populations on northern European inshore islands have revealed significant levels of differentiation between islands (Seppä & Laurila, 1999; Roth & Jehle, 2016), a finding which is mirrored in our study. We also revealed an overall concordance between the two genetic markers we employed. For example, the two populations on the island of Rona (ROI and ROT) showed unique mtDNA haplotype signatures combined with representing a distinct microsatellite-based cluster. This suggests that the colonisation did not take place through 'island hopping' after a single colonisation event from the mainland, but, for example, multiple times from different mainland sources. Haplotype (H_9) has previously been found in the UK as well as in central and northern Europe (Recuero et al., 2012; Arntzen et al., 2017). Its ancestral position in our study area is indeed confirmed by the haplotype network, which also illustrates that all other haplotypes are separated from each other by a single base substitution. Haplotypes which were previously unrecorded in other parts of the species' range) were also for example found on the western coast of Norway (Tuncay et al., 2018; see also Thörn et al., 2021 for multiple recolonisation routes of *B. bufo* in Scandinavia), and more extended sampling is required to assess their wider distribution and possible relevance for biogeographic patterns. It also needs to be borne in mind that, at a sample size of three individuals per population and with at least five haplotypes present in the area, our sampling regime does not allow us to fully capture the spatial distribution of existing diversity. Based on microsatellites, F_{ST} values between populations of the Inner Sound islands are markedly higher than found in previous studies of *B. bufo* in study areas which are uninterrupted by seawater (Brede & Beebee, 2004; Luquet et al., 2015); or for the closely related *B. spinosus* (Wilkinson et al., 2007; Martinez-Solano & Gonzalez, 2008), suggesting their rather strong isolation. Levels of differentiation between the two populations on Skye and the two populations on the mainland were however markedly lower, suggesting recent gene flow and coinciding with data obtained for small mammals also on Skye (White & Searle, 2008). That population on the nearby islands Raasay and Scalpay contained the same two mtDNA haplotypes and formed a single microsatellite-based cluster with Skye and the mainland suggests that Skye serves as a stepping stone for their colonisation. The distinctiveness of microsatellite genotypes of PAB on the small island of Pabay is paralleled by an excess of heterozygotes, likely reflecting that the local population consists of a very small number of individuals (no eggs or tadpoles were found in the single known available waterbody during later surveys, unpublished).

The degree of physical isolation of given islands from the mainland and the northward direction of prevailing currents paralleled the observed standing amounts of genetic diversity. Monomorphic loci were only found on smaller islands (four out of the seven populations), and are suggestive of genetic drift under a scenario of isolation. Possibly linked to island size, the overall level of genetic differentiation was higher than previously recorded for other populations of this species that were also separated by seawater (Seppä & Laurila, 1999; Roth & Jehle, 2016).

Island colonisation

Human introduction, both accidental and deliberate, are well documented for islands of western Scotland. For example, wood mice *Apodemus sylvaticus* on islands of the Outer Hebrides appear more closely related to populations from Scandinavia than to those from the Scottish mainland or Skye, possibly through accidental transport in Viking cargoes, although their parasites do not show the same pattern (Berry, 1979; Angus, 2001). The islands of the Inner Sound have been visited by boat since the days of the first settlers, and current settlement patterns or island sizes are rather uninformative for tracing releases (for example, the vole *Microtus agrestis* is common on the island of Uist but absent from the larger, more inhabited neighbouring island of Lewis and Harris; Angus, 1980).

Herpetofauna, including bufonids, are well known to be accidently transported though human activity (White & Shine, 2009; Tingley et al., 2017). All of the islands have been used for rearing livestock, leading to opportunities for stowaways. However, interviews with a family of local graziers suggested that, for the example of Crowlin, there has been no transport of fodder in at least the last 100 years. Since prior to construction of roads and railways the main means of transport to the area was by boat, larger islands on the Outer Hebrides would be more likely to hold *B. bufo* than the relatively unimportant islands of the Inner Sound if accidental transport is common. However, there are no records of toads from the Outer Hebrides prior to the 21st century (NBN 2019), whereas the New Statistical Account (1845) already reported the "islands of the parish abound with them" in Kilmuir in the north of Skye. This suggests that accidental transport is an unlikely means of colonisation.

Intentional amphibian introductions have generally been of edible species (e.g. *Lithobates catesbeanus* to the Philippines; Pili et al., 2019), for pest control (e.g. Rhinella marina; Shine, 2018) and for ornamental purposes and the release of pets (e.g. *Ichthyosaura alpestris* to France, New Zealand and mainland Britain; Arntzen et al., 2016). It seems unlikely that *B. bufo* would have been deliberately introduced for the above reasons, although releases are known from similar habitats in Norway (Dolmen & Seland, 2016). Deliberate small-scale introductions elsewhere in Scotland have been documented for the great crested newt *Triturus cristatus*, the smooth newt *L. vulgaris* and the alpine newt *I. alpestris* (McInerny & Minting, 2016), in addition

to extra-limital releases of *R. temporaria* and *L. helveticus* which served as demonstration animals from schools in the Outer Hebrides (Stewart Angus, unpublished data). Such small-scale introductions would however be reflected in the genetic make-up of populations (e.g. low allelic richness or lack of haplotype diversity, see Arntzen et al., 2010; Tingley et al., 2015), and would not explain the population on the uninhabited Crowlin Island.

Natural colonisation therefore seems the most likely explanation of the presence of toads on Skye and the Inner Sound. Toads may have colonised these islands for example via land bridges prior to the Loch Lomond Stadial and have persisted since, as has been argued for shrews (White & Searle 2008). *B. bufo* reaches latitudes of 68° and occurs within 1 km of glaciers elsewhere in Europe (Sillero et al., 2014). However, at the time of the Loch Lomond re-advance, ice sheets were present over much of Scotland (Ballantyne, 2019). While other amphibians can reproduce in waterbodies on permafrost (*Salamandrella keyserlingii*, Alfimov & Berman, 2010), we have found no similar records for anurans such as *B. bufo*. Notwithstanding phylogeographic evidence from other taxa for persistence in glaciated areas (e.g. King et al., 2020; Taylor, 1983), we therefore consider the hypothesis of relict populations surviving during the Loch Lomond Stadial to be rather unlikely.

A further hypothesis is that *B. bufo* could have colonised Skye and surrounding islands following the Loch Lomond re-advance and related rising temperatures, but before the islands became cut off by salt water. The current salinity of Inner Sound is slightly lower than that of the open sea (34 – 34.5 ‰ salt c.f. 35 ‰ in the nearby Atlantic; Barne et al., 1997) but well above the tolerance level for *B. bufo* (Beebee, 1983). Due to isostatic uplift of islands offsetting eustatic sea level rises, the relative sea level in the region remained roughly the same over the last 9000 years (Shennan et al., 2000), meaning that the islands were not joined to the mainland. However, the waters of the largely landlocked Inner Sound would have been mainly composed of meltwater from retreating glaciers. Meltwater from surviving glaciers in the mountains would probably have been at its peak in spring, coinciding with amphibian movements and spawning, and the boreal toad *Anaxyrus boreas* has been recorded swimming in glacial runoff (Taylor, 1983). Salt tolerance in amphibians may not be as rare as previously assumed, with coastal populations showing strong evidence of increased saltwater tolerance (Hopkins & Brodie, 2015; Albecker et al., 2021); indeed anecdotally *B. bufo* has been described swimming in the Baltic Sea at a salinity of 5-8 ‰ (Thulin & Andrushaitis, 2003). Under a scenario of colonisation exclusively by swimming, each of the islands would however have become isolated broadly simultaneously by the increasing salinity, as a hypothesis leading to long-term isolation associated with significant genetic erosion (effective population sizes in *B. bufo* are low; Brede & Beebee, 2004, Coles et al., 2019). This is however not reflected in our genetic data, which show that small islands such as Crowlin have substantial levels of genetic variation.

Although amphibians likely show lower propensity for colonisation by rafting than more desiccation-resistant taxa such as reptiles or arthropods, this mode of dispersal appears possible across the relatively short inshore distances involved in this study (for a review see Marin da Fonte, 2019). The melting of glaciers is also associated with frequent spates, whereby sections of riverbank detach and float downstream and out to sea, along with biota they contain (washouts of pools adjacent to rivers, possibly containing amphibian spawn or larvae). Such processes might have been paralleled by 'rock slope failures' arising from seismic activity on shorelines associated with release from glacial loading (Ballantyne et al., 2014). The general occurrence of occasional rafting would leave a genetic signature in which islands with larger coasts and those closest to the mainland river outflows are characterised by the highest levels of genetic diversity due to repeated arrivals of new colonists.

We found clear evidence of strong isolation between small island and mainland populations, with the most northerly islands showing the lowest levels of allelic richness (with the exception of the very small population on Pabay), whereas genetic differentiation between Skye and the mainland was less pronounced. Skye is connected to the mainland by a ca. 500 m long bridge completed in 1995, although we do not assume that it is an important means of colonisation and gene flow for *B. bufo* (the highly mobile pine marten *Martes martes* have been able to colonise Skye over the bridge, but by 2010 were not known from further north than Broadford in the south of Skye; Cottis, 2011). Given the large *B. bufo* populations, their long documented history on the island and their wide spatial distribution on Skye before and after the bridge's construction (New Statistical Account, 1845; NBN, 2019), it seems highly unlikely that enough toads to affect population genetics could have crossed the bridge. Skye is also connected to Scalpay by land at extreme low tides (Admiralty Chart 2498, 2018), which likely explains the low F_{ST} values between these sites. Occasional zoochory by birds would result in similar spatial genetic patterns but is generally deemed less likely, although birds have locally been found to carry snails (Evans, 1915) and might have led to colonisation of the nearby Uists by a further two molluscs species (Angus, 2001). Taken together, our evidence suggests that rafting is the most likely means of colonisation, and there is clearly an opportunity for future studies of amphibians and other low-mobility salt-intolerant taxa through systematic examination of rafts and debris.

The coasts of Scotland have much in common with other high latitude post-glacial marine-influenced areas such as Norway, Canada, Alaska, Chile and Southern New Zealand. The range of colonisation modes may differ in non-glacial settings, however. From a conservation perspective, our findings may offer some hope for unassisted range expansion of amphibians. The lack of evidence of inbreeding is particularly positive, and may be applicable for isolated mainland coastal populations as well as those on islands. On a more negative note, the ability of amphibians to cross seemingly impassable barriers means that island populations may not be safe from the spread of disease (e.g. chytrid) or non-native species (e.g. risk of colonisation of Maude Island New Zealand stronghold of *Leiopelma hamiltoni*, by invasive *Litoria* spp.).

Ethical statement

All aspects of fieldwork, including biosecurity, collection of specimens and handling of animals adhered to Scottish Natural Heritage's (the Scottish Government's statutory nature agency) guidelines.

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Rhinella icterica and *Rhinella ornata* (Anura: Bufonidae) tadpoles do not recognise siblings

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Benefits conferred to animals living in groups may be greater if groups are formed by relatives rather than non-relatives, because cooperating with relatives increases the probability of their own genes being passed on to group offspring (inclusive fitness). Non-social aggregations are formed in response to environmental characteristics, while social aggregations are formed from the attraction among individuals. The attraction or repulsion between individuals is mediated by recognition mechanisms, which mediate important ecological processes and behaviours. Here, we conducted laboratory experiments to test if tadpoles of two sympatric bufonids, *Rhinella icterica* and *R. ornata*, are able to recognise siblings. We collected eggs of the two species in the field and raised them in laboratory settings, according to three different methods: siblings and non-siblings reared in separated containers; siblings and non-siblings reared in the same container separated by a plastic net; and eggs from the same spawn reared separately, each one in an individual container. Later, we tested if tadpoles could choose between groups of siblings and non-siblings. The results indicate that tadpoles of neither species were able to discriminate between siblings and non-siblings, regardless of the rearing methods. Therefore, kinship is less important than environmental factors in tadpole aggregation behaviour of these species, and it may be dependent on the balance between costs and benefits. Our results can be used as a start point to better understand tadpole aggregation behaviour and recognition mechanisms in these species.

Keywords: kin recognition, aggregation behaviour, chemical communication, Atlantic Forest

INTRODUCTION

Tadpoles of many anuran species live in groups, which increases individual survival by decreasing predation rate, and increasing foraging and thermoregulation efficiency (Watt et al., 1997; Hoff et al., 1999; Eterovick, 2000; Hero et al., 2001). However, when resources are limited, there are some costs of group formation, as increasing competition, cannibalism, predation, disease susceptibility, and inbreeding (Hamilton & May, 1977; Shykoff & Schmid-Hempel, 1991; Pfennig et al., 1993; Goater et al., 1994).

Non-social groupings are formed in response to environmental characteristics (e.g., feeding microhabitats and temperature gradients), while social groups are formed from attraction between individuals (Wassersug, 1973; Hoff et al., 1999). An aggregation can be formed by genetically related or unrelated individuals (Waldman, 1982; Glos et al., 2007), but benefits conferred to animals living in groups may be greater if groups are formed by relatives than non-relatives, because cooperating with relatives increases the probability of their own genes being passed on to group offspring (inclusive fitness; Hamilton, 1964).

In this context, species that live in groups of related individuals tend to show adaptations that allow kin recognition (Blaustein & O'Hara, 1983; Waldman, 1988). Thus, association between siblings may act in aggregation maintenance through sharing spatial and temporal distribution (indirect recognition), through phenotypic matching (direct recognition), or both (Blaustein & O'Hara, 1983; Waldman, 1988).

Tadpoles of some anuran species discriminate between siblings and non-siblings (reviewed in Blaustein & Waldman, 1992). This discrimination consists of behaviour differences toward relatives of different kinship levels and non-relatives (Waldman, 1988). The adaptive values of this behaviour may be related to increasing and developing the tadpoles' coexistence in related groups (Waldman, 1988; Blaustein & Waldman, 1992). Mechanisms that allow siblings recognition in tadpoles can give them adaptive advantages, as in tadpoles of some species that have more rapid development when living among relatives (Jasienski, 1988; Twomey et al., 2008), and as some cannibalistic tadpoles that prevent predation of relatives (Pfennig et al., 1993).

Recognition mechanisms in tadpoles are developed

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during the embryonic phase or shortly after hatching (Waldman, 1981, 1882; Blaustein & O'Hara, 1982) and it may persist following metamorphosis (Blaustein et al., 1984; Waldman, 1989; Graves et al., 1993). There are three basic types of kin recognition mechanisms (Blaustein & O'Hara, 1983). First, recognition may originate from social or familiar learning mechanisms, a process by which individuals from some familiar groups learn to recognise others from early development stages, even if they have not developed a mechanism to identify their siblings (Waldman, 1982). Second is phenotypic matching, which occurs when an individual learns and remembers a specific characteristic of their own or their relatives (e.g., odour, colour, or particular mark), which may be a similar feature or a noticeable difference. Phenotypic matching is fundamentally different from familiar recognition because they provide recognition of unfamiliar individuals (Blaustein & O'Hara, 1983). The third one relies on specific genes recognition, also provides kin and non-kin recognition. However, this mechanism is innate and is expressed by a phenotypic characteristic (e.g., odour) and different mechanisms can operate isolated or simultaneously (Blaustein & O'Hara, 1983).

Here, we conducted laboratory experiments to test if tadpoles of two toad species, *Rhinella icterica* and *R. ornata* can recognise siblings. *Rhinella icterica* belongs to the *R. marina* group (Maciel et al., 2010), while *R. ornata* is a member of the *R. crucifer* group (Baldissera Jr. et al., 2004). These species have schooling behaviour (Eterovick, 2000; Simon, 2010; pers. obs.), likely living in groups of siblings, because spawn consists of thousands of eggs. They often co-occur in sites within the Atlantic Forest of south-eastern Brazil, where they have a well-known reproductive season, laying eggs in shallow waters (Bertoluci, 1992, 1998; Bertoluci & Rodrigues, 2002; Narvaes et al., 2009). We addressed the following questions: (1) do tadpoles prefer to associate with siblings than non-siblings (kin recognition)?; and (2) does familiarity (prior social contact with non-siblings tadpoles) influence recognition mechanisms?

METHODS

We collected eggs of both species between July and August 2017 at the Boracéia Biological Station (23˚38' S, 45˚52' W), an Atlantic Forest reserve, São Paulo, south-eastern Brazil. We collected two spawns each of both *Rhinella icterica* and *R. ornata* (ca. 600 eggs of each spawn). Spawn could be easily assigned to species in the field because *R. ornata* has smaller eggs arranged in a single string, while *R. icterica* deposits larger eggs arranged in a double string (Simon, 2010; pers. obs.). We transported eggs to the Laboratório de Zoologia de Vertebrados, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, in plastic pots containing water from ponds where spawns were collected.

Spawn were raised in the laboratory at room temperature, with a natural photoperiod, and with aeration by aquarium pumps. Embryos were between

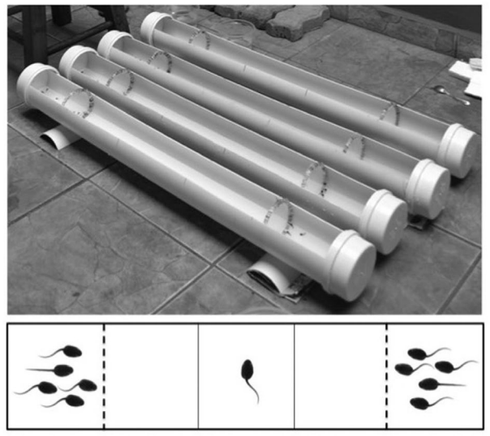

Figure 1. Test arena. Bottom: it is represented, in the right and left extremities, the stimulus groups (20 tadpoles in each group) and, in central area, the test tadpole. Dashed line represents a net, which delimits stimulus groups areas, but allows chemical and visual stimuli flow to central region. Vertical grey lines represent demarcation of areas close to each stimulus group. Each division is 20 cm long, totalling 100 cm of arena total length.

stages 16 and 18 (Gosner, 1960) when they were separated from the rest of the spawn. We used three different rearing methods: (1) tadpoles without prior contact with non-siblings: 300 eggs reared with siblings only, from the same spawn allocated in two 50 L opaque container (one container for each spawn) (2) tadpoles of two different spawns reared in the same container, enabling contact with chemical and visual cues of non-siblings: 150 eggs from each spawn in an opaque 50 L container and each group of tadpoles separated by a plastic net (0.5 mm mesh); and (3) eggs from the same spawn reared separately (n=120), each in a 0.5 L opaque container. Tadpoles were fed once daily with ornamental fish food. Water in each container was changed twice a week to keep the water clean. After metamorphosis, we kept the juveniles in a terrarium, and prior to release at the locations where the eggs were collected.

Experimental trials were conducted between August and September 2017 between 0800 and 1800, using tadpoles between stages 25 and 38. Trials were carried out in four plastic containers (100 × 15 × 10 cm) filled with spring water (pH = 6.3; Fig. 1). At each end of a container a 0.5 mm mesh plastic net was placed, delimiting the stimulus groups areas (20 tadpoles in each group). The central part of each container was marked with a permanent pen, dividing it into three equal-sized areas.

At the beginning of each trial, one tadpole was placed at the centre of each of the four containers (see similar designs in O'Hara & Blaustein, 1981, 1982; Blaustein & O'Hara, 1982, 1986; Cornell et al., 1989; Saidapur & Girish, 2000; Leu et al., 2013; Rajput et al., 2014; and Pizzato et al., 2016). After 10 minutes of acclimation, we observed tadpole behaviour using a video recording

Table 1. Synthesis of association and recognition tests. Familiar = tadpoles reared in contact with tested tadpole; non-familiar = tadpoles reared without contact with tested tadpole

Experiment	Stimulus group 1	Tested tadpole	Stimulus group 2
Control	familiar siblings	1st rearing method	familiar siblings
1	familiar siblings	1st rearing method	non-familiar non-siblings
2	familiar siblings	2nd rearing method	familiar non-siblings
3	non-familiar siblings	3rd rearing method	non-familiar non-siblings
4	Familiar siblings	1st rearing method	non-familiar siblings

camera (Kodak z990) for 29 minutes, and then measured the amount of time each tadpole remained in the region next to each stimulus group. Each tadpole was tested only once and after each test containers were cleaned and water changed. At each test we turned containers at 90° and inverted the side of each stimulus group, in order to avoid possible environmental influences. Each trial was replicated 32 times during the daytime period on successive days. Four replicates were filmed at a time. The same procedures were repeated for both species.

For each trial, both stimulus groups were chosen considering kinship and familiarity (prior contact) with test-tadpole (Table 1; familiar = reared in contact with test-tadpole; unfamiliar = reared without contact with test-tadpole):

Control: siblings with prior contact vs. siblings with prior contact. All tadpoles from the same spawn and reared together in one container. We expect no difference in tadpole preference to aggregate with either group.

Experiment 1: siblings with prior contact vs. non-siblings without prior contact. Test tadpoles reared without prior contact with non-siblings. One stimulus group formed by tadpoles from the same spawn reared together with test-tadpoles. The other stimulus group is formed by non-siblings of the test tadpole. Through this experiment we tested if the tadpoles of these species prefer to associate with siblings than non-siblings.

Experiment 2: siblings with prior contact vs. non-siblings with prior contact. Siblings and non-siblings reared in the same container, separated by a plastic net. One stimulus group formed by siblings reared together with test-tadpoles. The other stimulus group formed by non-siblings reared with chemical and visual contact of test tadpole. Through this experiment we tested if the contact between siblings and non-siblings during development influences association choice to one of the groups by test tadpoles.

Experiment 3: siblings without prior contact vs. non-siblings without prior contact. Test tadpoles from the same spawn reared separately (isolated). One stimulus group formed by tadpoles from the same spawn as test-tadpoles. The other stimulus group formed by tadpoles from a different spawn of test-tadpole. Through this experiment we tested if the lack of prior contact with other tadpoles influences in test-tadpole choice.

Experiment 4: siblings with prior contact vs. siblings without prior contact. Test tadpoles reared without prior contact with non-siblings. One stimulus group formed by tadpoles reared together with test-tadpoles. The other stimulus group formed by siblings of test tadpole reared in another container. Through this experiment we tested if familiarity is required to sibling association.

Data consisted of differences between the time spent by the test-tadpole in the compartments located near stimulus groups 1 and 2. The differences between time spent by test-tadpoles near each stimulus group, as well as the mean of differences and the pseudo median of differences, when negative, indicate a longer time spent by tadpoles near stimulus group 2, whereas, when positive, they indicate a longer time spent by tadpoles near stimulus group 1. We verified if data of each experiment corresponded to normal distribution by Shapiro-Wilk test. We used a paired t-test to analyse data of Control, and experiments 1 and 4 with *R. icterica* tadpoles and in Control, experiments 2, 3 and 4 with *R. ornata*; and Wilcoxon signed-rank test to analyse data of experiments 2 and 3 with *R. icterica* and experiment 1 with *R. ornata*. Tests were two-tailed. Analyses were performed in R platform (R Core Team, 2017).

RESULTS

Data varied more for *R. ornata* than *R. icterica*, but all experiments for both species exhibited random pattern or non-significant differences between the time spent by tadpoles close to siblings or non-siblings (Figs. 2 and 3). In Experiment 1 with *R. ornata*, tadpoles remained considerably longer near non-siblings, but the difference was not significant. Results of experiments 2 and 3 further confirm this pattern.

In the Control, experiments 1 and 4 with *R. icterica* and in the Control, experiments 2, 3 and 4, with *R. ornata*, the mean of differences did not differ (Tables 2 and 3). Similarly, in experiments 2 and 3 with *R. icterica* and in experiment 1 with *R. ornata*, the pseudomedian of differences did not differ (Tables 2 and 3). These results indicate that regardless of previous contact with siblings the tadpoles of *Rhinella icterica* and *Rhinella ornata* do not exhibit spatial attraction to siblings. This suggests that kinship in these tadpoles is not relevant for aggregation behaviour.

DISCUSSION

Tadpoles of *Rhinella icterica* and *R. ornata* may aggregate in response to factors other than sibling association. Other stimuli to aggregate can be related with reduction of

Table 2. Statitstic tests results for each experiment with *Rhinella icterica* tadpoles. df = degrees of freedom; CI = confidence interval

Experiment	Shapiro-Wilk test	Paired-t test (t) or Wilcoxon (V)	Mean of differences	Pseudomedian of differences	CI (95 %)	
					Inf. Lim	Sup. Lim.
Control	W = 0.97 p = 0.58	t = -0.98 ; df = 31; p= 0.33	-159,68	-	-490.68	171.3
1	W = 0.97 p = 0.72	t = 0.44 ; df = 31; p= 0.66	44,8	-	-162.26	252.01
2	W = 0.89 p = 0.003	t = 348 ; df = 31; p= 0.12	-	111	-36	235
3	W = 0.88 p = 0.002	t = 295 ; df = 30; p= 0.36	-	87.63	-110	273
4	W = 0.96 p = 0.37	t = 0.11 ; df = 31; p= 0.91	11.31	-	-195.11	217.73

Table 3. Statitstic tests results for each experiment with *Rhinella ornata* tadpoles. df = degrees of freedom; CI = confidance interval

Experiment	Shapiro-Wilk test	Paired-t test (t) or Wilcoxon (V)	Mean of differences	Pseudomedian of differences	CI (95 %)	
					Inf. Lim	Sup. Lim.
Control	W = 0.96 p = 0.47	t = 1.08; df = 31; p = 0.28	154.37	-	-135.44	444.19
1	W = 0.91 p = 0.01	V = 160; df = 31; p = 0.052	-	-408.5	-853	1
2	W = 0.93 p = 0.059	t = 1.49; df = 31; p = 0.14	270.68	-	-97.81	639.19
3	W = 0.95 p = 0.18	t = -0.60; df = 31; p = 0.55	-111.31	-	-488.86	266.24
4	W = 0.94 p = 0.07	t = 1.09; df = 31; p = 0.28	164.68	-	-141.92	471.30

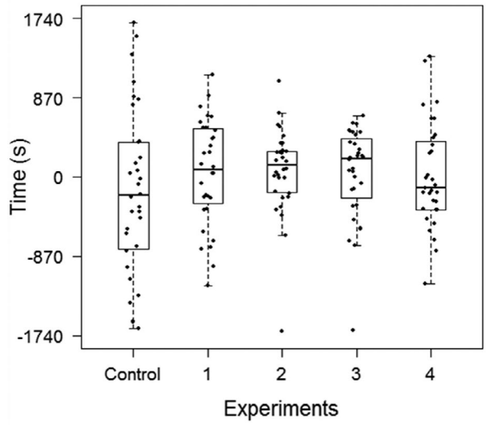

Figure 2. Box plot with dots, representing experiments 1, 2, 3, 4 and control executed with *Rhinella icterica* tadpoles. In each plot, points correspond to the difference between time spent by tested tadpole in each trial close to stimulus group 1 and 2. Positive values correspond to a longer time spent by test-tadpole close to stimulus group 1, while negative values correspond to a longer time spent by the test-tadpole close to stimulus group 2.

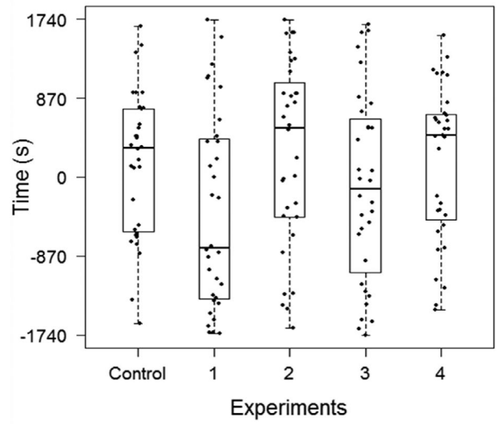

Figure 3. Box plot with dots, representing experiments 1, 2, 3, 4 and control executed with *Rhinella ornata* tapoles. In each plot, points correspond to the difference between time spent by tested tadpole in each trial close to stimulus group 1 and 2. Positive values correspond to a longer time spent by the test-tadpole close to stimulus group 1, while negative values correspond to a longer time spent by the test-tadpole close to stimulus group 2.

predation risk and response to predator cues (Watt et al., 1997), thermotaxy (Wassersug, 1973), facilitating access to food particles (as in tadpoles of *Rhinella pombali*; Eterovick, 2000), and reinforcement of aposematism (Wassersug, 1981).

Because there is very little information about larval ecology and schooling of these species, information from genetically similar species may help explain the absence of sibling attraction in these species. In *Rhinella marina*, which belongs to the same group as *R. icterica* (Maciel et al., 2010), there was a weak tendency of association with siblings (Raven et al., 2017). In tests of choice between a siblings group and an empty compartment, tested tadpoles spent significantly more time near sibling group, whereas when submitted to choice between non-siblings and an empty compartment, tested tadpoles exhibited a random distribution. However, in a third test tadpoles failed to discriminate between siblings and non-siblings. In combination with the results of other experiments, they conclude that tadpoles of *R. marina* aggregate in response to abiotic factors such as light levels, temperature and structural complexity.

Although kin recognition among tadpoles occurs in several bufonids (e.g., Waldman, 1981, 1982; O'Hara & Blaustein, 1982; Saidapur & Girish, 2000; Gramapurohit et al., 2006; Eluvathingal et al., 2009), species of *Rhinella* do not discriminate kin (Raven et al., 2017; present study). In tadpoles of other anuran families, presence of this behaviour is also variable even within the same genus, such as *Lithobates* (Ranidae; Waldman, 1984; Fishwild et al., 1990) and *Spea* (Scaphiopodidae; Pfennig, 1990; Hall et al., 1995).

Tadpoles of two bufonid species, (*Anaxyrus americanus* and *A. boreas*) recognise siblings when it was reared only with siblings, but not when it was reared with siblings and non-siblings together (Waldman, 1981; O'Hara & Blaustein, 1982). In the present study the results were similar for both *R. icterica* and *R. ornata* even with different rearing methods, indicating that previous contact does not influence the choice of aggregation with more or less related tadpoles. In Experiment 4, results were also similar for both species: tadpoles were randomly distributed, indicating that prior contact is not an important factor to sibling association in tadpoles of these species.

The absence of kin recognition in tadpoles of *R. icterica* and *R. ornata* suggests that kinship is less important than environmental factors in the aggregation behaviour. However, even with the presence of recognition, the decision of which action to take is often context-dependent, in other words, it is expected that an action (attraction or repulsion) will only occur whether its cost does not exceed the benefits (Waldman, 1987, 1988; Reeve, 1989).

For some authors the absence of sibling discrimination among tadpoles in laboratory tests is due to absence of stimuli to aggregation behaviour (Blaustein et al., 1993). When there are few selective pressures that lead to aggregation, sibling association tend to be weak, because tadpoles get few benefits from this behaviour (Blaustein and O'Hara, 1986).

Both recognition processes and schooling may vary within the same species depending on some factors, such as presence and density of predators (Wrona, 1991; Fitzgerald, 1992; Watt et al., 1997), diets (Gamboa et al., 1990; Pfennig, 1990), development stage (Blaustein & O'Hara, 1986; Rautio et al., 1991; Blaustein et al., 1993; Nicieza et al., 1999), resource distribution, and temperature variation (Hokit & Blaustein, 1997). For example, *Lithobates sylvaticus* tadpoles recognised and were attracted to relatives in laboratory experiments, but in natural environments they demonstrated both attraction and repulsion to relatives in different ponds (Waldman, 1984; Halverson et al., 2006).

Our experiments controlled most environmental variables that could influence spatial preference by tadpoles, thus focusing only on presence or absence of kin recognition traits. Therefore, the lack of attraction to siblings by these tadpoles could be due to a lack of stimulus and selective pressures for schooling behaviour. Another explanation could be that the recognition mechanisms in these species act in high levels, as conspecifics groups. Polettini Neto & Bertoluci (2021) found that tadpoles of *Rhinella icterica* have preference to associate with conspecifics, while tadpoles of *R. ornata* do not show any discrimination between conspecifics and heterospecifics. Our results can be used as a start point to better understand tadpole aggregation behaviour and recognition mechanisms in these species, and more information on larval ecology of these species will contribute for more accurate interpretations of these behaviours.

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