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Nuptial pad ("breeding gland") morphology is related to non-random mating in wild male common frogs (*Rana temporaria*)

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Abstract

Androgen levels are closely linked with breeding in male amphibians. Development of the nuptial pad is driven by androgens and is believed to have importance for determining mating success in anurans, but this has not been tested in wild populations. We investigated the association between nuptial pad morphology (length, colour) and mating (amplexus) success in wild male common frogs (*Rana temporaria*) in the UK (Devon in Southern England and central Scotland). Once active breeding had been confirmed, pond water (25 L) was placed in 1–6 replicate mesocosms (66 cm diameter circular, black plastic tubs) *in situ*. Eight male and two female frogs were placed into each mesocosm, and success observed by identifying the male frog(s) in amplexus. The length of nuptial pads for male frogs observed in amplexus was greater than those that did not achieve amplexus. There was no difference in the absolute dark colour of nuptial pads (determined by red/green/blue analysis, Adobe photoshop©) for male frogs observed in amplexus versus those that did not achieve amplexus. However, within each mesocosm, the nuptial pad was relatively darker for winning male frogs compared to losing male frogs. Overall, 91% of winning male frogs from Devon, and 89% winning male frogs from Scotland, possessed either a longer and/or a darker nuptial pad, compared with frogs that did not achieve amplexus. These data suggest that features of nuptial pad morphology are associated with amplexus (and thus mating) success in male common frogs. Given that nuptial pads have been identified in all anuran amphibian species analysed to date, nuptial pad morphology may contribute to non-random mating strategies across a broad range of species.

KEYWORDS
amphibians, anura, reproduction, reproductive behaviour

1　|　INTRODUCTION

Reproduction in amphibians is closely tied to proximate environmental conditions, including temperature and rainfall. For the vast majority of species, anuran amphibians (frogs and toads) rely on freshwater bodies for oviposition and development during larval stages (i.e. tadpoles). Patterns of rainfall and temperature influence the frequency of breeding, with temperate species generally reproducing after a period of prolonged rainfall and increasing temperature, whereas tropical species typically reproduce over more

protracted time periods (Duellman & Trueb, 1994). Temperate species normally begin oviposition between late Winter and early Spring, utilising both permanent and ephemeral water bodies, with temperature and photoperiod acting as signals to regulate the hormonal response to these environmental cues (Delgado et al., 1989; Eikenaar et al., 2012; Varriale et al., 1986).

Breeding in anurans is characterised by "amplexus," the process of males clasping females to facilitate external fertilisation. Reproduction is primarily controlled by androgens in male anurans (Carr, 2011; Rastogi et al., 2011), and higher plasma androgen levels have been reported in cane toads (*Bufo (Rhinella) marinus*) found in amplexus, compared to lone males (Orchinik et al., 1988). As androgens have also been shown to control the development and maintenance of nuptial pads, or "breeding glands" (Brizzi et al., 2003; Epstein & Blackburn, 1997), these are a likely contributor to non-random mating in anurans. Supporting this, amplecting wild male Columbia spotted frogs (*Rana luteiventris*) have been reported to possess larger nuptial pads compared to lone males (Greene & Funk, 2009) and in a laboratory study male, *Silurana tropicalis* that were successful in amplexing females had a darker and longer nuptial pad compared to those that did not achieve amplexus (Orton et al., 2020). In the latter study, nuptial pad morphology was also related with androgen status, evidenced by greater numbers of spermatogonia in the testis, and higher plasma androgen levels. Therefore, larger nuptial pad size and darker colour are suspected to contribute to non-random mating in a range of anuran species.

Anurans are defined as either "explosive" or "prolonged" breeders; however, in reality, these represent two ends of a continuum, rather than exclusive categories (Wells, 1977). Broadly speaking, explosive breeding strategies are characterised by a short breeding period (lasting days to weeks), in which large male aggregations are common. This type of breeding strategy is common in temperate species, due to the short time period when climatic conditions are suitable (i.e. warmer temperatures and rainfall). In most cases of explosive breeding, scramble competitions are common, whereby several males attempt to amplex with a female and dislodge other males. Therefore, male–male competition is considered the primary driver of mate selection, for example, in the common toad (*Bufo bufo*), where "breeding balls" are common (Davies & Halliday, 1977, 1979). Mate choice by males has been demonstrated in common toads whereby males preferentially chose larger females when placed in enclosed breeding areas with a female-biased sex ratio, and female mate choice is assumed to not occur (Arntzen, 1999). In contrast, in prolonged breeding species, breeding may occur over a period of months, with males competing to establish territories, with female mate choice generally considered to be the major factor in determining pairings. This type of breeding strategy is more common in tropical species, when climatic conditions are suitable for breeding over extended time periods, for example, as occurs in the túngara frog (*Engystomops pustolosus*) that breed throughout the year when conditions are suitable (Ryan, 1980, 2010).

The common frog (*Rana temporaria*) is considered to be an explosively breeding species, with the breeding period being reported to last around 10 days (Arak, 1983; Haapanen, 1982; Ryser, 1989). As operational sex ratios in wild populations are heavily male biased, it is unlikely that mate choice by males would occur (Elmberg, 1991). Indeed, male mate choice in this species – whereby preference of male frogs for larger females or those with higher fecundity - has been refuted with random mating being reported in this species (Arak, 1983; Elmberg, 1987, 1991). However, the possibility of non-random mating in common frogs has not been investigated under the male-biased operational sex ratios which mimic those found in wild populations.

In this study, we set out to investigate the contribution of nuptial pad morphology (length and colour) to non-random mating in wild common frogs mimicking the male-biased operational sex ratio found in wild populations (Arak, 1983). We hypothesise that both nuptial pad length and colour contribute to non-random mating, and we tested this in several populations inhabiting two geographically distant regions of the UK (Devon in Southern England and central Scotland).

2 | METHODS

2.1 | Locations

In total, breeding frogs were studied at eight sites in Devon in 2015 (D1–D8), one site in Devon in 2020 (D9) and two sites in Scotland in 2020 (S1–S2). Of the sites visited in Devon, six were located in urban/suburban gardens and two in large rural gardens. In Scotland, both the sites were located in urban parks. All mesocosms were set up on the same night at each site during February–early March (2015, 2020, 2021). See Table 1 for details.

2.2 | Experimental design

On arrival at each site in the early evening, air and water temperatures were recorded. Male and female frogs were caught and placed in separate buckets containing ambient pond water. If amplectant pairs were found, the males and females were gently separated before being placed in their respective buckets. Male frogs were weighed and photographed for identification purposes (via their unique patterning on the dorsal surface), and their nuptial pad length was measured with callipers and photographed (see below for details). In 2015, nuptial pad width was also measured, but as this was highly correlated with nuptial pad length, this measurement was not used in subsequent years to reduce the time needed to take the measurements from each male frog (see Supplemental information, Figure S1). Once a male had been weighed, photographed, and the nuptial pad measured (this took 5–6 min per frog), it was placed in a mesocosm, until it contained eight male frogs. The mesocosms consisted of large tubs (diameter = 66 cm) to which pond water was added (25 L) that were then covered with mesh and placed on the edge of the breeding pond. To recover from handling stress, there followed a 10-min interval from the time the

TABLE 1 Site locations and description in Devon (Southern England) and central Scotland (UK)

Code	Name	DD	Characterisation	Size (diameter)	Description	Mesocosm (number)
D1	Twindle Beer	50.60107–3.6095	Suburban garden	Small (~2 m)	Artificial lined pond containing leaf litter and newts	5
D2	Exeter	50.7168–3.51672	Urban garden	Small (<1 m)	Artificial lined pond containing high amount of leaf litter	6
D3	Grenville	50.47177–3.55508	Suburban garden	Small (<1 m)	Artificial lined pond with large newt population	1
D4	Clovelly	51.01238–4.21604	Suburban garden	Small (~2 m)	Artificial lined pond containing little organic matter	4
D5	Silverton	50.81744–3.47724	Suburban garden	Medium (~3 m)	Artificial lined pond containing little organic matter	5
D6	Newton Abbot	50.50655–3.61206	Suburban garden	Small (~2 m)	Artificial lined pond containing leaf litter	1
D7	Kenton	50.63865–3.47543	Rural garden	Medium (~4 m)	Artificial lined pond containing little organic matter	1
D8	Sidmouth	50.6863–3.24799	Rural garden	Large (~20 m)	Unlined pond with natural substrate	4
D9	Exeter (2020)	50.7168–3.51672	Urban garden	Small (<1 m)	Artificial lined pond containing high amount of leaf litter	3
S1	Glasgow	55.874223–4.290995	Urban garden	Small (~2 m)	Unlined pond with natural substrate	2
S2	Robroy.	55.886413–4.194971	Urban park	Large (~50 m)	Unlined pond with natural substrate	3

Abbreviations: DD, decimal degrees; Robroy, Robroyston.

final male frog was added, before adding two females to the mesocosm (i.e. a 4:1 male: female sex ratio). The time at which the two females were added marked the beginning of the breeding experiment (i.e. the start of the 270 min observation period). This process was repeated for each replicate mesocosm. A 4:1 male: female sex ratio (8 males and 2 females) was chosen to mimic natural conditions in common frog populations, which typically range between 6:1 and 2:1, male: female ratio (Arak, 1983). The two male frogs that were observed in amplexus with the two female frogs was recorded for each replicate mesocosm every 45 min, for a maximum of 270 min (i.e. 6 observations). At each observation, the identity of the male frog(s) in amplexus was recorded, as well as the presence of any frog spawn. At the end of the breeding trial, all contents of the mesocosms were returned to the natal ponds. In agreement with previous reports (Elmberg, 1991), takeovers were rarely observed (four times in total out of 35 mesocosms) and these all occurred within the first 90 min, with one male frog only remaining in amplexus for the remaining period – this frog being deemed the winning individual.

2.3 | Morphological measurements

When each set of 8 male and 2 female frogs had been caught, each male was removed from the bucket, and following photography of their dorsal pattern for identification, the body mass was recorded

(Pesola hanging scales, 0.01 g/1 g). The length of the nuptial pad was measured (digital callipers, 0.01 mm/200 mm) and the nuptial pad was photographed in order to quantify the darkness of the colour. To take the photographs, a tripod and clamp was set up, with the camera (Canon digital SLR) clamped 30–40 cm above the frog arm. LED lighting was used to illuminate the arm with photographs taken without flash (night-time sampling). To control for variability in the lighting/colour measurements taken from the nuptial pads, both the absolute dark colour in winning versus losing frogs and the relative difference were analysed (see below).

2.4 | Image analysis

Images were analysed with Adobe Photoshop©. Briefly, the magnetic lasso tool was used to select the nuptial pad area in the photo, and the magic wand tool was used to select the non-reflected areas of the nuptial pad. Once selected, the "histogram" feature was activated and "RGB" selected, giving the mean colour value (i.e. red/green/blue). All photographs were analysed twice without knowledge of the site or whether the frog won or lost the competition for the female. The co-efficient of variation was then analysed for the two measurements taken from each photo, and if the variation was more than 10%, then a third measurement was recorded. The mean of two measurements – either the initial

two or when a third measurement was taken, the two measurements with the smallest difference between them – were used for analysis.

2.5 | Statistical analysis

Data were analysed using R Software (version 3.6.1). Variation in body mass, nuptial pad length and nuptial pad colour between sites (9 in Devon and 2 in Scotland) was analysed using a Kruskal–Wallis test (data not normally distributed). Generalised Linear Mixed-Effects Models (GLMMs) were used to investigate the difference in nuptial pad length, absolute colour values and male frog mass between winning and losing frogs with winning status as a fixed effect and mesocosm nested within site included as a random factor to account for replicate mesocosms and sites. An interaction term between winning frogs and locations was also included to account for any differences between the Devon and Scotland locations. Data were analysed using a Gamma distribution with log link (glmer; R Package lme4: Pinheiro & Bates, 2000). Due to variability in the lighting used across sites and years, the relative difference in nuptial pad colour was also analysed. To normalise these data between field sites/mesocosms/years, the difference in nuptial pad colour between winning and losing frogs within each mesocosm was calculated. This was done by subtracting the value of the nuptial pad colour for individual winning frogs from the mean value for the losing frogs within the same mesocosm ($n = 2$ winning frogs *per* mesocosm). For nuptial pad colour, as a lower number indicates a darker colour, a negative number demonstrated that the winning male frogs possessed a darker nuptial pad. The difference values calculated as described above were pooled within geographical area (Devon, Scotland) before being analysed for their deviation from zero using a one sample Wilcoxon signed rank test (data were not normally distributed). To investigate the potential interplay between nuptial pad length and colour, a chi-squared test was used to investigate whether the winning frogs possessed either feature, or both, more often than would occur by chance (i.e. 1. Longer nuptial pad combined with darker nuptial pad; 2. Longer nuptial pad only; 3. Darker nuptial pad only; 4. Neither a longer nor darker nuptial pad; compared against a hypothetical equal distribution, 25% likelihood for each of the 4 conditions). *p* values of <.05 were deemed significant.

3 | RESULTS

3.1 | Morphological measurements

Body mass of male frogs across sites generally ranged between 40 and 80g (Figure 1, top) and differed between sites (Kruskal–Wallis, H = 55.91, df = 279, $p < .001$). These differences lay between D1 and D9 versus S2, with the Devon sites being larger than S2 ($p < .001$). Nuptial pad length was similar between sites, generally ranging
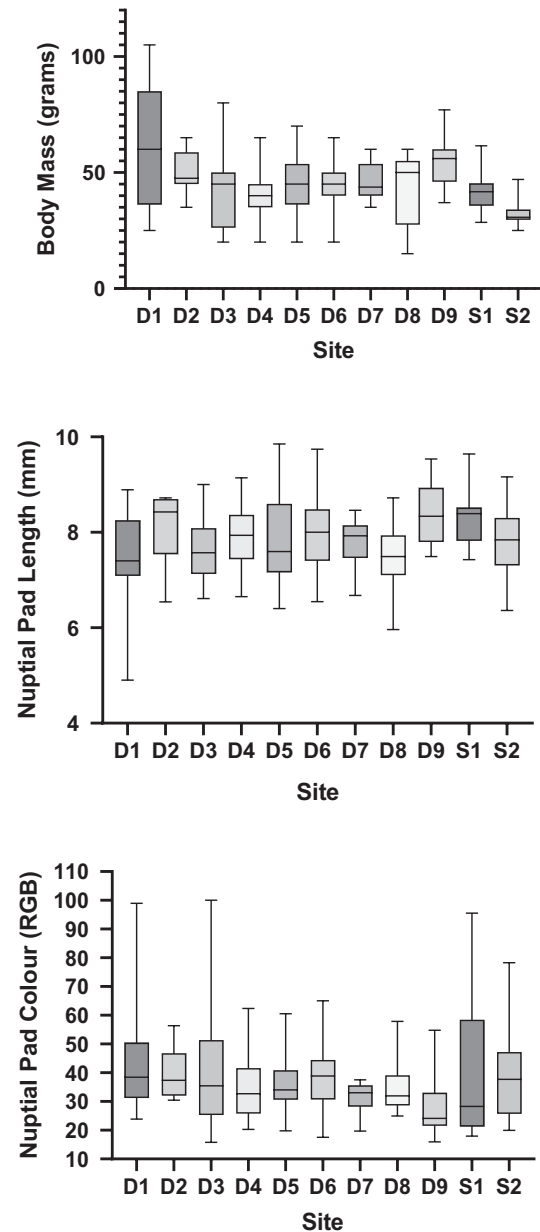

FIGURE 1 Weight (top), nuptial pad length (middle) and nuptial pad colour (bottom) measured across nine sampling campaigns in Devon (D) and two sampling campaigns in Scotland (S). Data comprise both winning and losing frogs within each site. Lines in boxes are medians, boxes are 25%/75% confidence intervals, whiskers show the data range. *N* values = D1 (32), D2 (8), D3 (40), D4 (32), D5 (48), D6 (40), D7 (8), D8 (12), D9 (24), S1 (16), S2 (20). RGB = red/green/blue.

between 6 and 9mm (Figure 1, middle) and differed between sites (Kruskal–Wallis, H = 30.55, df = 279 $p = .007$). The only major difference observed was between D1 and D9, with a longer nuptial pad observed in frogs sampled from D9 ($p < .001$). More differences between sites were observed for nuptial pad colour (Figure 1, bottom; Kruskal–Wallis, H = 26.50, df = 277, $p = .003$), with frogs from site D9 possessing a darker nuptial pad than those from D1, D2, D3, D5, D6 and S2 ($p < .01$).

3.2 | Differences between winning and losing frogs in Devon (southern England) and Central Scotland

The median weight of winning and losing male frogs was zero, and therefore, no statistical differences were observed s (GLMM: $p = .5$, Figure 2 top) and no differences between Devon versus Scotland were observed (GLMM: $p = .8$, Figure 2 top). For nuptial pad length, there was a difference between winning and losing male frogs, with the median length for winning frogs being 0.22 mm for Devon and 0.19 mm for Scotland (GLMM: $p = .001$, Figure 2 middle) – no differences between the two areas were observed (GLMM: $p = .2$, Figure 2 middle). For nuptial pad colour, no difference in absolute dark colour was observed between winning and losing male frogs (GLMM:
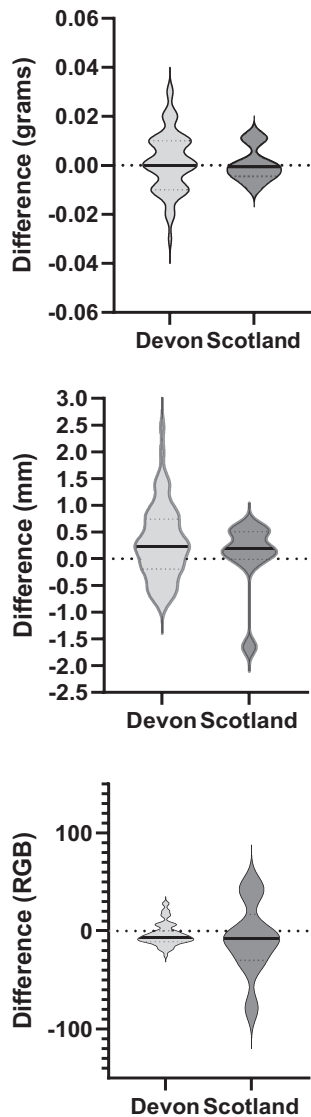
FIGURE 2 Difference in weight (top), nuptial pad length (middle) and nuptial pad colour (bottom) between frogs that achieved amplexus (winning frogs) and those that did not achieve amplexus (losing frogs). Black lines are the median values. Significant differences were observed for nuptial pad length (GLMM: $p = .003$) and for nuptial pad colour for Devon only (Wilcoxon test $p = .004$). N values = Devon (60), Scotland (9). RGB = red/green/blue.

$p = .17$) and no difference between Devon/Scotland was observed (GLMM: $p = .06$). However, a relative difference in colour between winning and losing frogs within each mesocosm was observed and the median colour value for winning frogs was −6.1 (Devon) or −7.1 (Scotland) darker than for losing frogs (one sample Wilcoxon test; Devon $p = .004$; Scotland $p = .4$). See supplemental information text S1 for details of the models.

3.3 | Interplay between nuptial pad length and colour to predict breeding outcomes

In an analysis where hypothetically an equal distribution of each of the four conditions (darker colour and longer nuptial pad, darker colour only, longer only or neither) was assumed (and assigned a value of 25%), comparing our dataset against this, we found a significant difference for amplexus success in frogs sampled from Devon (chi-square $p < .001$, Figure 3). For Scotland, although proportions were similar, this was not significantly different from the hypothetical equal distribution (chi-square, $p = .19$). In total, 91% of male winning in amplexus possessed either a longer or a darker nuptial pad (or both) in frogs sampled from Devon and 89% of winning frogs possessed either a longer or a darker nuptial pad (or both) in frogs sampled from Scotland (Figure 3).

4 | DISCUSSION

Here we demonstrate that nuptial pad morphology has a key role in male mating success in the anuran amphibian species, *Rana temporaria*. Our findings indicate non-random mating in common frogs that previously have been reported to employ a random mating strategy (Arak, 1983; Elmberg, 1987, 1991).

Nuptial pad length and colour (darkness) differed between those male frogs that achieved amplexus with the female compared with those that did not upon competitive breeding trials. The findings reported here are perhaps unsurprising – as nuptial pad growth and maintenance is known to be under the control of androgens, and these sex steroids perform a central role in breeding capability in anurans (see introduction). However, although males of wild Columbia frogs found in amplexus possessed a larger nuptial pad than lone males (Greene & Funk, 2009), the importance of this feature for breeding success has not previously been experimentally demonstrated in wild anurans. In laboratory reared *Silurana tropicalis*, nuptial pad morphology was shown to be central to breeding success (Orton et al., 2020). Thus, the importance of this feature to breeding success has now been demonstrated in three anuran species, which employ a range of breeding modes (i.e. *S. tropicalis* - prolonged, common frog/Columbia frog – "explosive"; Duellman & Trueb, 1994). It is also possible that nuptial pad morphology may be important for breeding success in female moor frogs (*Rana arvalis*) – another species employing an explosive breeding mode – which were reported to base their mate preference on "undefined phenotypic male traits"
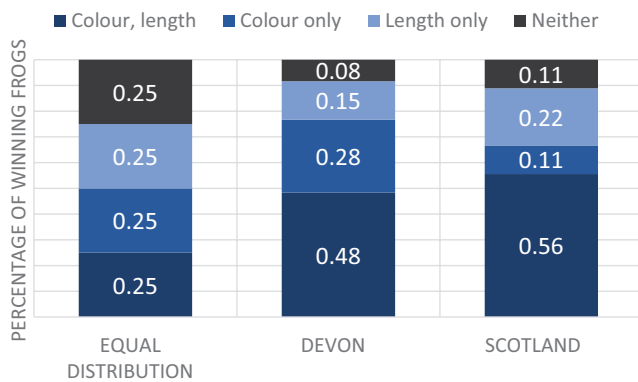
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FIGURE 3 Comparison of winning frogs sampled from Devon and Scotland which possessed a darker and longer nuptial pad, a darker nuptial pad only, a longer nuptial pad only or neither a darker nor longer nuptial pad, to a hypothetical equal distribution. A significant difference was observed for the Devon frogs (chi-square, $p < .001$). N = Devon (60), Scotland (9).

(Sherman et al., 2010). Thus, in combination with the known physiological features of the nuptial pad, we thereby hypothesise that nuptial pad morphology may be important for non-random mating more widely across the Anura.

Nuptial pads have been identified in all amphibian families analysed to date (including Ranids, Leptodactylids, Bufonids, Pipids and Hylids: Duellman & Trueb, 1994; Epstein & Blackburn, 1997; Luna et al., 2018; Thomas et al., 1993), suggesting evolutionary selection for the preservation of this morphology. Interestingly, measurements of the nuptial pad were similar across all sites across both Devon and Scotland (all winning frogs nuptial pad length were between 7.5–9.5 mm and nuptial pad colour between 15.82 and 84.26 RGB), despite the small sample size in Scotland, indicating that these features may be subject to stabilising selective pressure, though this is a hypothesis only. At present little is understood regarding the underlying mechanisms that drive growth or darkening of nuptial pads and this did not form part of this study. Here, approximately 35% of winning frogs in both Devon and Scotland possessed either a longer nuptial pad or a darker nuptial pad (approximately 50% with both), indicating that size and colour may be only partially underpinned via the same mechanisms. Further research in this area would be beneficial to help unravel the importance of this morphology in driving non-random mating strategies across the Anura. Our experimental design does not allow us to delineate the relative importance of mate choice versus male competition, and it would be interesting to understand the relative importance of nuptial pad morphology for these two mechanisms that contribute to breeding dynamics.

In agreement with the findings in this study, it has been reported several times that body mass/snout-vent length of male frogs is not associated with breeding success in wild populations of common frogs (Elmberg, 1991; Haapanen, 1982; Ryser, 1989). It is not surprising, therefore, that here no correlation between body mass and nuptial pad length/colour was observed (see Figures S2 and S3). Similarly, male size was unimportant in achieving success for amplexus in the prolonged breeding tropical clawed frog *Silurana*

tropicalis (Orton et al., 2020). This is in contrast to many Bufonid (toad) species, where a larger male body size has been reported as an advantage to better enable males to dislodge competitors or to maintain their grip in amplexus (*Bufo bufo*: Bókony et al., 2018; *Bufo bufo*: Davies & Halliday, 1977; *Bufo bufo*: Davies & Halliday, 1979; *Bufo (rhinella) marinus*: Lee & Price, 2001; *Bufo bufo*: Orton et al., 2014). This difference may be due to the mis-identification of breeding strategy, as although both common frogs and common toads are defined as explosive breeding anurans, in fact, the breeding period for common frogs is longer than that for common toads (10–14 days compared to 2–4 days). Thus, this should not be considered to be the same breeding strategy, taking for example that common toads are found in "breeding balls" and "scramble competition" strategy, whereas common frogs are almost always found in pairs. Although, as previously mentioned, breeding strategy is recognised as a continuum (prolonged-explosive), species are often commonly characterised as either "prolonged" or "explosive" within the published literature, which is too simplistic to describe the inter-specific range of breeding strategies employed.

Finally, the identification of a feature that contributes to non-random mating may have importance for assessing reproductive capability in amphibian populations. To date, little is understood regarding the potential role of reproductive impairment to population declines (Orton & Tyler, 2015) with the majority of research addressing adult mortality, for example, as a result of chytridiomycosis disease (Skerratt et al., 2007). As amphibians are the fastest declining vertebrate group, with 40% of species threatened with extinction (Monastersky, 2014; Stuart et al., 2004), understanding the mechanisms driving these declines is urgently required. In male-biased populations, it is likely that only a small proportion of the male population contributes to subsequent generations. Where nuptial pad morphology is shown to contribute to non-random mating, this feature could perhaps be used as a tool for the selection of the contributing male frogs within a population in order to assess the potential role of male reproductive dysfunction in driving declines in anurans. Further, nuptial pad morphology could potentially be used as a biomarker to assess the internal physiology and individual quality of anurans, though this would require validation, and in particular for wild anurans.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT
The datasets used and/or analysed during the current study are available in the supplementary information and from the corresponding author on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.