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Review

A review of non-destructive biomonitoring techniques to assess the impacts of pollution on reproductive health in frogs and toads

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ABSTRACT

In anuran amphibians (frogs and toads), evidence linking pollution to population declines is limited, in particular through impaired reproduction. Here we review the evidence for pollutant-induced alterations on reproductive endpoints in wild anurans with a particular focus on the application of non-destructive endpoints including on sex ratios, male reproductive phenotypes (data are too scarce for females) and reproductive outputs (reflective of mating success). Data evidencing alterations in sex ratio in wild anurans are scarce, however, both feminisation and masculinisation in response to pollution have been reported (seven studies). Male nuptial pad morphology and calling behaviour display high sensitivity to pollutant-exposure and are important features determining male breeding success, however there is considerable variation in these endpoints and inconsistencies in the responses of them to pollution are reported in wild anurans. Data for clutch size are insufficient to assess sensitivity to pollutants (five studies only). However, hatch success and offspring fitness (tadpole survival/development) are sensitive to pollution, with clear linkages to population stability. In conclusion, there are a wide range of non destructive measures with good potential for application to assess/monitor reproductive health in wild anurans, however, a greater understanding of pollutant effects on these endpoints is needed. There measures deserve wider application as they are relatively simple and inexpensive to implement, and as they can be applied non-destructively are widely applicable to our declining anuran populations.

1. Introduction

Amphibians have been in significant decline for several decades, with ~40% of species classified as endangered (Monastersky, 2014; Stuart et al., 2004). As with many other wildlife phyla, the primary causes of amphibian declines are attributed to habitat loss, fragmentation, and degradation (Díaz et al., 2019). Other major causes of decline include disease, most notably infection with the fungus *Batrachochytrium dendrobatidis*, causing chytridiomycosis disease (Skerratt et al., 2007). Pollution too is cited as being amongst the most important threats to amphibians (Díaz et al., 2019) and to freshwater ecosystems (*e.g.* Lemm et al., 2021), upon which most amphibians rely for completion of their lifecycle.

Amphibians consist of the Anura (frogs and toads), the Caudata (newts and salamanders), and the Gymnophiona (caecilians). This review centres on pollution effects on the Anura (frogs/toads) as there are few data for other Amphibia. Most Anura have a biphasic life cycle, inhabiting both freshwater and terrestrial ecosystems, and occupy a wide range of ecological niches. Reproduction in anurans is closely tied to climatic conditions, including temperature and rainfall and is characterised by 'amplexus', the process of males clasping females to facilitate external fertilisation. Amplexus is likely related to circulating hormone levels, for example, plasma androgen and corticosterone concentrations have been shown to be higher in cane toads (*Rhinella marinus*) found in amplexus, compared to lone males (Orchinik et al., 1988). The vast majority of anuran species rely on freshwater bodies for oviposition and development during their larval stage (*i.e.* tadpoles).

Within the anuran literature, most attention on chemical effects has been focused on the impacts of agrochemicals on reproductive endpoints. Amphibians are especially at high risk of exposure to these chemicals because breeding ponds are often situated in, or in close proximity to, areas of agricultural land-use (*e.g.* Bishop et al., 1999;

Hayes et al., 2010). More recently pharmaceuticals have been reported to affect anurans causing disruptions to reproductive tissues and outputs (Melvin et al., 2014; Säfholm et al., 2014). Many of these types of pollutants are classed as endocrine disrupting chemicals, which elicit alterations to endocrine systems through hypothalamo-pituitary-thyroid, hypothalamo-pituitary-gonadal and hypothalamo-pituitary-adrenal/interrenal axes; the primary centres controlling reproduction and

development in anurans. The majority of publications on chemical effects on reproduction in wild anurans have focused on the condition of intersex (egg cells within testicular tissue) comparing 'reference' and 'polluted' sites and/or other features of gonadal histopathology. These findings have been reviewed recently, both for wild (Orton and Tyler, 2015) and for laboratory exposed (Trudeau et al., 2020) amphibians, and we refer the reader to these papers for these details.This review is

Table 1

Evidence for pollutant effects on reproductive capability and potential in wild anurans (updated taxonomy source: www.amphibiaweb.org).

Common name	Latin name	Pollutant/Land use Type	Reported Effects	Reference
Western toad	*Anaxyrus boreas*	Orchard wetlands	No effect on hatch success (Table S3)	Bishop et al. (2010)
Southern toad	*Anaxyrus terrestris*	Coal combustion waste	Higher circulating androgen	Hopkins et al. (1997)
		Coal combustion waste	No effect on clutch size (Table S2), reduced hatch success (Table S3)	Metts et al. (2013)
		Coal ash	Reduced hatch success (Table S3)	Rowe et al. (2001)
American toads	*Bufo americanus*	Agricultural land	Higher tadpole abnormalities (Table S3)	Bishop et al. (1999)
Common toads	*Bufo bufo*	Estrogenic activity	Feminised sex ratio	Pickford et al. (2015)
		Anthropogenic land cover	No effect on sex reversal	Nemesházi et al. (2022)
		Agricultural land	Higher circulating androgen/no effect on nuptial pad size	Orton et al. (2014)
		Agricultural land	No effect on clutch size (Table S2) nor hatch success (Table S3) Delayed metamorphosis (Table S3)	Bókony et al. (2018)
		Agricultural land	Reduced hatch success, higher larval mortality and delayed growth post-metamorphosis (seeTable S3)	Orton and Routledge (2011)
		Agricultural land	Higher clutch sizeTable S2), reduced hatch success, higher larval mortality and smaller tadpole size (seeTable S3)	Adams et al. (2021)
Mongolian toad	*Bufo raddei*	Heavy metal contamination	Increased forelimb strength/greater nuptial pad size/larger larynx	Guo et al. (2018)
		Heavy metal contamination	No effect on clutch size (Table S2)	Zhang et al. (2018)
Natterjack toad	*Epidalea calamita*	Agroecosystem	Smaller forelimb size/greater nuptial pad size	Zamora-Camacho and Comas (2017)
		Agricultural	Masclinised/delayed gonadal development Smaller size at metamorphosis (Table S3)	Macleod et al. (2023)
Narrow-mouthed toad	*Gastrophryne carolinensis*	Coal combustion	Reduced hatch success (Table S3)	Hopkins et al. (2006)
Cane toad	*Rhinella marinus*	Agricultural land	Smaller forelimb/nuptial pad size	McCoy et al. (2008)
		Agricultural land	Smaller larynx	Zlotnik et al. (2019)
South american common toad	*Rhinella arenarum*	Agriultural land	Smaller size of tadpoles and higher incidence of abnormalities (Table S3)	Babini et al. (2015)
		Agricultural land	Higher mortality and higher incidence of abnormalities (Table S3)	Babini et al. (2016)
Great basin spadefoot toad	*Spea intermontana*	Orchard wetlands	Reduced hatch success (Table S3)	Bishop et al. (2010)
Pacific tree frog	*Pseudacris regilla*	Orchard wetlands	Reduced hatch success (Table S3)	Bishop et al. (2010)
Lesser snouted tree frog	*Scinax nasicus*	Agricultural land	Reduced hatch success (Table S3)	Peltzer et al. (2008)
Stripe snouted tree frog	*Scinax squalirostris*	Agricultural land (rice paddies)	Delayed metamorphosis (Table S3)	Attademo et al. (2014)
Veined tree frog	*Trachycephalus typhonius*	Agricultural/industrial land	No effect on hatch success, higher tadpole abnormality incidence (Table S3)	Peltzer et al. (2013)
Túngara frog	*Engystomops pustulosus*	Agricultural land	Reduced clutch size (Table S2) and reduced hatch success (Table S3)	Orton et al. (2022)
Moustached frog	*Leptodactylus mystacinus*	Agricultural land (rice paddies)	Delayed metamorphosis (Table S3)	Attademo et al. (2014)
No common name	*Physalaemus cuvieri*	Agricultural land	Higher incidence of abnormalities (Table S3)	Borges et al. (2019)
Whistling frog	*Leptodactylus fuscus*	Agricultural land	High incidence of abnormalities (not observed in reference site for comparison:Table S3)	Borges et al. (2019)
African clawed frog	*Xenopus laevis*	Maize *versus* non-maize	No significant effects on larynx size	Smith et al. (2005)
Red legged frogs	*Rana aurora*	Agricultural	Reduced hatch success (Table S3)	de Solla et al. (2002)
Green frog	*Rana clamitans*	Suburban land/estrogenic chemicals	Feminised sex ratio	Lambert et al. (2015)
		In situ exposure to EE2	Reduced hatch success (Table S3)	Park and Kidd (2005)
		Orchard wetlands	No effect on hatch success (Table S3)	Harris et al. (1998)
		Industrial river catchment	Reduced hatch success (Table S3)	Karasov et al. (2005)
		Agricultural	Higher tadpole abnormality incidence (Table S3)	Bishop et al. (1999)
		Metal contamination	Higher tadpole abnormality incidence (Table S3)	McDaniel et al. (2004)
Mink frogs	*Rana septentrionalis*	*In situ* exposure to EE2	No effect on hatch success (Table S3)	Park and Kidd (2005)
Agile frog	*Rana latastei*	Anthropogenic land cover	Sex reversal (masculinisation of genetic females)	Nemesházi et al. (2020)
Columbia spotted frog	*Rana luteiventris*	Orchard wetlands	No effect on hatch success (Table S3)	Bishop et al. (2010)
Northern leopard frogs	*Rana pipiens*	Orchard wetlands	No effect on hatch success nor timing of metamorphosis (Table S3)	Harris et al. (1998)
		Contaminated wetlands	No effect on hatch success (Table S3)	McDaniel et al. (1998)
		Industrial river catchment	Reduced hatch success (Table S3)	Karasov et al. (2005)
		Contaminated wetlands	Higher tadpole abnormality incidence/accelerated metamorphosis (Table S3)	McDaniel et al. (2004)

EE2 = Ethinylestradiol

centred on non destructive methods for assessing the role of pollutants on amphibian reproductive health as such approaches that can be applied to all wild anuran populations including those of high conservation concern. Furthermore intersex is likely a poor indicator of reproductive toxicity in anuran amphibians, not least due to the fact that it occur naturally in some anuran species and can also vary between populations of conspecifics (Orton and Tyler, 2015).

In this review, we assess the biological underpinning for biomarkers of reproductive potential, including male reproductive morphology (nuptial pad, forelimb) and behaviour (calling), as well as presenting the evidence for effects of pollution on these endpoints. In turn we provide a perspective on their potential utility for indicating pollutant-induced reproductive toxicity in anurans. We also assess the evidence for pollutant effects on reproductive outcomes (clutch size, hatching success, offspring fitness) and explore evidence for linkages with population level effects and sex ratios in wild anurans. Differences in breeding strategy may influence the applicability of reproductive biomarkers across the Anura, in particular for temperate *versus* tropical species, and we make reference to these differences throughout the manuscript as appropriate. Briefly, temperate species begin oviposition between late winter and early spring, using 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). In contrast, tropical species are generally not limited by the availability of water bodies, and their time tends to be partitioned between foraging, searching for a mate and, in some species, providing for their offspring. Males of species inhabiting warm/tropical climates, therefore, often compete to acquire high quality territories, characterised by high food availability and/or high quality breeding pools (*e.g.* túngara frog, *Engystomops pustulosus*: Ryan, 1985).

The taxonomic classifications of anurans has changed over the period of the literature reviewed and here we use the currently accepted names (common and Latin names for the species when first mentioned, and common names only thereafter – a full list of all species included in this review is provided in Table S1). Table 1 provides an overview of all species with existing data on the effects of pollutants on the measures of sex ratio, reproductive potential or reproductive outputs and Fig. 1 provides an overview of the endpoints analysed and reviewed in this manuscript.

2. Non-destructive measures of reproductive potential in individuals and chemical pollutant effects on these measures

2.1. Mate acquisition strategies

Mate acquisition strategies and biomarkers of reproductive potential are intimately linked, as high quality reproductive morphology, physiology and behaviour are central to achieving breeding success. Anurans generally reproduce after a period of prolonged rainfall, with temperate species commonly reproducing over shorter timescales and tropical species typically reproducing over more protracted time periods. The length of breeding period is defined as either 'explosive' or 'prolonged' in anurans; however, in reality, these are two ends of a continuum, rather than exclusive categories. There is no clear distinction between 'explosive' and 'prolonged' species, with many species defined as 'explosive' employing longer breeding periods (weeks as opposed to days) such as the common frog, *Rana temporaria* (Haapanen, 1982). These differing breeding dynamics likely affect the applicability of reproductive biomarkers, though this is not well understood. For example, explosive breeding strategies are characterised by a short breeding period (lasting days to weeks), where there are often large male aggregations and the operational sex ratio is heavily male biased and take-over attempts are common, for example, with common toads, *Bufo bufo* (Davies and
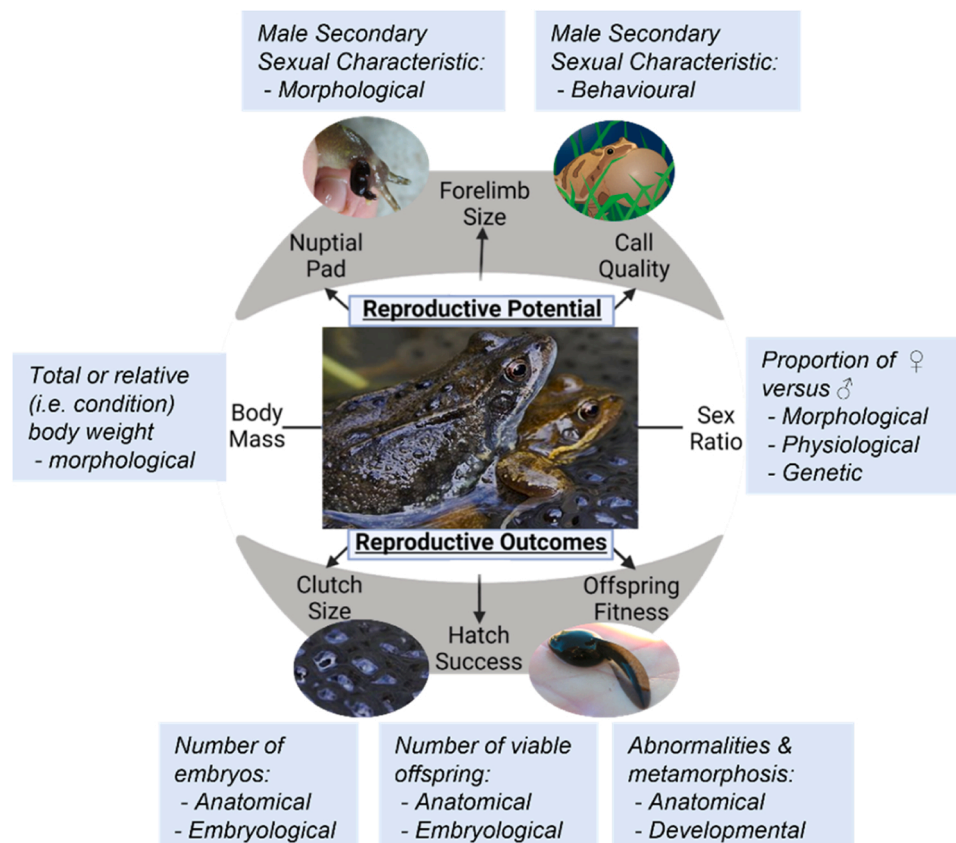

Fig. 1. Overview of non-destructive measures included in this review article.

Halliday, 1977; Davies and Halliday, 1979). In contrast, species with prolonged breeding seasons may breed over a period of months, whereby males compete to establish territories, and female choice is generally the major factor in determining pairings. As female choice in prolonged breeding species is typically based on energetically costly male vocalisations, calling characteristics may have more value as biomarkers of reproductive health in these species. For example, it is well-known that female choice drives mate acquisition in the túngara frog, a tropical species employing a prolonged breeding mode (Ryan, 1980; Ryan, 2010). Further, in Peter's dwarf frog (*Physalaemus petersi),* female choice has been shown to lead to divergence in male call types and strong female preference for local call patterns has driven behavioural isolation between populations leading to speciation (Boul et al., 2007). These differing breeding dynamics likely impact the applicability of biomarkers of reproductive health, though this is not well understood. In this review, where information on breeding strategy is available, these are incorporated into the discussion in the individual biomarkers sections as appropriate.

In addition to specific biomarkers discussed in this review, it is generally assumed that a larger male body size is an advantage to better enable males to dislodge competitors or to maintain their grip and hold a female in amplexus – which has been shown to be the case in toads (Bókony et al., 2018; common toads: Davies and Halliday, 1977; Davies and Halliday, 1979; cane toads: Lee and Price, 2001; Orton et al., 2014). Although these generalisations on the advantage conferred by body size are applicable to many species, they are by no means universal, and exceptions have been documented in species employing both explosive and prolonged breeding modes. For example, in the explosively breeding moor frog (*Rana arvalis*), in female choice experiments, females did not show preference for larger males and instead their selection was based on some other (undefined) male traits (Sherman et al., 2010). Similarly, male size was not shown to be important in achieving success for amplexus in the prolonged breeding tropical clawed frog, *Silurana tropicalis* (Orton et al., 2020) or in the common frog, *Rana temporaria* (Haapanen, 1982); a species employing an 'explosive' breeding mode, but which is more prolonged than that of some Bufonid species (*i.e.* weeks rather than days). Nevertheless, body size may be an important confounding variable in some cases and therefore should be an included measurement for the analyses of reproductive biomarkers.

2.2. Male forelimb and nuptial pad ('breeding gland')

Aspects of forelimb morphology play fundamental roles in breeding in many anuran species. Two of these features in males that can be measured non-destructively (and non-invasively) are forelimb musculature and size and colour of the nuptial pad. Males of many anuran species display hypertrophied forelimb musculature (*e.g.* Mi, 2012) and muscles directly involved with amplexus are sexually dimorphic (*e.g.* Peters and Aulner, 2000); with obvious advantages during breeding. Forelimb muscle development is dependent on circulating androgen levels and castrated males do not develop enlarged forelimbs (*e.g.* Dorlochter et al., 1994; Muller et al., 1969). Nuptial pads are protrusions on the forelimbs, which are typically present in male anurans on the first digit, but can extend onto the toe surface, and to other digits, or on to the forearm. Their development and maintenance are under androgenic control, they possess a high concentration of androgen receptors, and as they develop during the breeding season nuptial pads increase in size/number and become darkened in colour (for review see: Brizzi et al., 2003; Luna et al., 2018). Nuptial pads appear to be an ancestral feature and have been identified in all amphibian families analysed to date, and the variation in their morphology has recently been described across 507 species (Luna et al., 2018). Additionally, so called 'amplexins' are small proteins released from the nuptial pads during amplexus, which may serve as a means of chemical communication from the male to the female, *via* wounds on the ventral side of the female (Willaert et al., 2013).

In three species employing explosive breeding modes, forelimb size has been found to be positively associated with reproductive success. Successful male cane toads (those found in amplexus) had significantly larger forelimb muscle mass compared with unsuccessful males, which was irrespective of body size (Lee and Corrales, 2002). Similarly, total muscle mass and specific forelimb muscle size were larger in male Asiatic toads (*Bufo gargarizans*) found in amplexus, which was independent of body size (Mi, 2012). In the explosively breeding Columbia spotted frog (*Rana luteiventris*), males found in amplexus had wider forelimbs compared to unpaired males, again irrespective of body size (Greene and Funk, 2009). Interestingly, in the quacking frog (*Crinia georgiana*), which employs direct male-male combat breeding strategy, it was reported that forelimb width did not correlate with offspring paternity (Buzatto et al., 2017).

Similarly as the case with forelimb musculature, for the nuptial pad, frogs found in amplexus had larger/darked nuptial pads compared to those that did not achieve amplexus. For example, wild male Columbia spotted frogs (*Rana luteiventris*) found in amplexus had larger nuptial pads than those males found unpaired, again irrespective of body size (Greene and Funk, 2009). As takeover attempts by unpaired males were observed, but none were successful, it was hypothesised that larger nuptial pads increased the likelihood of maintaining amplexus. Similarly, in wild common frogs, nuptial pad length and colour were larger and darker in frogs that achieved amplexus compared to those that did not upon competitive breeding trials (UK: Orton et al., 2023). In a laboratory study, Orton et al. (2020) reported that male nuptial pad size and colour were the best predictors of achieving amplexus in competitive breeding trials with tropical clawed frogs, whereas forelimb width did not appear to predict breeding success. In the only study found analysing whether achieving breeding success (*i.e.* amplexus) was reflected in an enhanced reproductive outcomes, the size of the nuptial pad was positively correlated with fertility rate in tropical clawed frogs exposed to the anti-androgenic herbicide linuron (Orton et al., 2020). Somewhat confusingly, however, this was not found in the control frogs in that study; which was explained by the authors to be potentially explained by lower overall variability in fertility levels in control frogs.

In wild anurans, differences in forelimb size and nuptial pad morphology have been reported in frogs/toads collected from polluted *versus* 'reference' environments. For example, Mongolian toads (*Bufo raddei*) collected in China from heavy metal contaminated sites were reported to possess increased forelimb width and ability to exert a contraction force, as well as greater size of nuptial pads compared to those collected from reference sites (Guo et al., 2018). In this publication, a novel method termed 'forelimb force' was reported, which presumably was non-destructive, thereby having potential as a good marker – in particular as previous studies measuring muscular force in anuran forelimbs used approaches that required the termination of the organism to dissect and isolate the muscle fibres (Peters and Aulner, 2000). However, unfortunately this measure was not defined or explained within the reporting publication and so it is unknown how it was measured/quantified. Interestingly, a similar scenario for an enhanced nuptial pad size and also greater forearm thickness has been reported in natterjack toad (*Epidalea calamita*) populations collected from an agroecosystem compared with a reference site in Spain (Zamora-Camacho and Comas, 2017). The authors hypothesised that toads from the polluted site may employ a strategy of higher reproductive investment in the short-term, as these toads were also shown to have a reduced lifespan compared to toads from the control population. In support of these findings, higher levels of circulating androgens were reported in male Southern toads (*Anaxyrus terrestris*) from a coal combustion waste polluted site compared to a reference site (Hopkins et al., 1997) and in common toads from an agricultural site in the UK (Orton et al., 2014). In the latter study, however, there was no difference in nuptial pad size in toads collected from the reference compared to the agricultural site and no correlations between the two variables were observed (Orton et al., 2014). Contrasting with these findings, forelimb width and the number

of nuptial pads were found to be reduced in male cane toads along a gradient of increased agricultural land use (USA) which correlated with feminised colouration (McCoy et al., 2008). In the laboratory we have observed exposure of tropical clawed frogs (*Silurana tropicalis*) to high levels of anti-androgens (linuron or flutamide) results in larger and darker nuptial pads compared to the control, however, forelimb width was reduced (Orton et al., 2018). A smaller nuptial pad size has also been reported in response to ethinylestradiol (EE2) in tropical clawed frogs for exposure during the larval period (Iguchi, 2011).

Concluding this section, due to their role in breeding dynamics and their ubiquity across genera, forelimb width and muscular force, and nuptial pad morphology, have potential as non-destructive biomarkers for measures of breeding capability in male anurans. However, current information does not provide a clear consistent picture on how these endpoints are impacted by different pollutants and their mixtures. The differences in the responses reported for field studies may be related to the different types of types of chemical exposure scenarios, or inter-species differences, or a combination thereof. For laboratory studies, the data are also somewhat contradictory, with enhancements of nuptial pad morphology observed in response to anti-androgenic chemicals (flutamide and linuron) applied at high concentrations (50 or 45 µg/L; though not observed at the lower linuron concentration of 9 µg/L: Orton et al., 2018).

2.3. Calling acoustics and larynx physiology

Calling acoustics are common for communication in the vast majority of anurans, however, acoustic signalling is generally less important in explosively breeding species, where males often either do not call, or have weak mating calls. Both males and females possess larynges that are required for respiration; however, the laryngeal muscle mass is far greater in males (*e.g.* McClelland and Wilczynski, 1989; McClelland et al., 1998). Male vocalisations can determine social spacing in species with prolonged breeding, both for those that establish distinct territories and those that form leks, and calling can be induced by conspecific vocalisations and movements (reviewed in: Wells, 1977). Similarly, to forelimb/nuptial pad morphology, larynx development is controlled by androgens and levels of endogenous steroid hormone concentrations are closely tied to calling behaviour. For example, in wild populations of the Bombay night frog (*Nyctibatrachus humayuni*: Joshi et al., 2017), American green tree frog (*Hyla cinera*: Leary and Harris, 2013) and túngara frog (Marler and Ryan, 1996), calling males had higher androgen levels than non-calling or 'satellite' males.

The purpose of calling is to attract a mate and outcompete rivals, and links between calling behaviour and breeding success have been well demonstrated. Call quality, as measured by rate, dominant frequency and/or pulse rate have been related to male mating success in a range of anuran species (*e.g.* strawberry poison dart frogs, *Dendrobates pumilio*: Pröhl, 2003). Associations between calling characteristics and reproductive outcome (*i.e.* clutch size, hatch success, offspring fitness) have also been reported. One hypothesis for female preference of particular call characteristics is the 'good genes' hypothesis, where increases in energetically costly signals indicate male quality and thus, an increased quality of offspring (*e.g.* in two species of poison frogs, *Dendrobates leucomelas* and *Epipedobates tricolor*: Forsman and Hagman, 2006). Calling has also been shown to positively influence to the number and size of egg clutches, as demonstrated in agile frogs (*Rana latastei*), whereby both outcomes were higher in the territories of males with calls at a lower fundamental frequency (Lesbarrères et al., 2008). However, an absence of this relationship has also been reported in American green tree frogs, whereby the fundamental frequency of an advertisement call did not affect fertilisation success (Gerhardt et al., 1987). Thus, although calling characteristics have often been demonstrated to be linked to positive reproductive outcomes, data are too scarce to make definitive conclusions, and this relationship is likely influenced by species-specific differences.

Given the central role of androgens in the development of the larynx this organ is vulnerable to the effects of exposure to endocrine disrupting chemicals that are anti-androgenic/oestrogenic (Boyd et al., 1999; Tobias and Kelley, 1987). This has been shown for exposure of African clawed frogs to the androgen 17α-methyl dihydrotestosterone that resulted in an increase in advertisement calls in males and female increased receptivity to this effect (time spent close to the speaker: Hoffmann and Kloas, 2012). In contrast exposure to oestrogen and androgen receptor antagonists (*e.g.* flutamide) decreases proportion of advertisement calls compared to rasping noises, the latter of which are non-sexual communication cues (Hoffmann and Kloas, 2012; Hoffmann and Kloas, 2016). In breeding trials with these animals, females preferred advertising calls of control males over estrogen-exposed males (Hoffmann and Kloas, 2012). Contrasting with this, exposure to 17alpha-estradiol increased arm waving and calling in male tropical clawed frogs, both of which are sexual behaviours (Schwendiman and Propper, 2012). Unfortunately, there are no published literature for field studies reporting effects of pollutants on calling behaviour or characteristics, rather effects on calling in wild populations has been inferred through effects on larynx physiology – a destructive endpoint, as the animals must be sacrificed to assess physiological endpoints. Male larynge sizes have been reported as being larger in populations of Mongolian toads at a site with higher heavy metal pollution in China (Guo et al., 2018), reduced in male cane toads from an agricultural area compared with urban areas in Florida, USA (Zlotnik et al., 2019) and to not differ in atrazine sprayed maize growing regions *versus* non-maize growing regions in African clawed frogs from South Africa (Smith et al., 2005). Other types of pollution have also been demosntrated to have the potential to impact calling characteristics. Using a field-to-laboratory experimental design, Smit et al. (2022) reported that laboratory exposure to light and noise pollution resulted in similar call profiles to wild túngara frogs recorded from urban environments in Panama, indicating that these frogs were capable of altering calling patterns in response to environmental conditions.

As there is evidence that calling characteristics are related to offspring fitness and reproductive success, there is good potential for the use of calling as a measure to assess pollutant-induced adverse effects on reproductive health. However, calling is also influenced by many environmental factors, in addition to chemicals, for example temperature and ambient noise. It is furthermore also heavily influenced by the number of other males calling in the surrounding area. These factors therefore make it difficult to standardise measures of calling both within and between populations. The importance of calling also varies between species and reproductive strategies. Given that prolonged breeders rely more on acoustic communications to advertise quality for female choice, whereas explosive breeders are considered to rely more direct male competition, such as scramble competitions (Davies and Halliday, 1979), calling as a biomarker may be of greater potential value in species employing prolonged breeding modes.

2.4. Reproductive output and chemical pollutant effects

Reproductive outputs in the context of this article focuses on clutch size (number of eggs spawned), hatching success (fertilisation percentage or the number of viable offspring) and offspring fitness (factors affecting the likelihood of survival to adulthood and successful reproduction).

2.5. Clutch size

Clutch size relates to the number of eggs laid by the female and is synonymous with fecundity. A positive relationship between female size and clutch size has been reported in a range of species (common toad: Arntzen, 1999; wood frog, *Rana sylvatica*: Berven, 1988; emerald glass frog, *Espadarana prosoblepon*: Goyes Vallejos et al., 2021; Hylidae spp.: Prado and Haddad, 2005), however, this is not the case in all species, as

seen in tropical Leptodactylid frogs (Orton et al., 2022; Prado and Haddad, 2005). Therefore the generally held assumption that body size in females and fecundity are positively correlated across all the Anura is not correct, and instead this relationship difers between species, and is likely connected to their breeding mode (*i.e.* prolonged, explosive).

For the five available studies that have reported on the effects of pollutants on clutch size in wild anurans, three found no differences (all on toad species from the Bufonid family (common toad, Hungary: Bókony et al., 2018; Southern toad, Canada: Metts et al., 2013; Mongolian toad, China: Zhang et al., 2018). In two of these studies, collected amplecting pairs were maintainted in clean water for the reproductive assessments, with the number of deposited eggs determined either directly by counting the number of eggs laid in the case for Southern toads (agricultural/coal combustion: Metts et al., 2013) or indirectly by recording pre- and post- spawning weight for female common toads (agricultural/urban: Bókony et al., 2018). In the third study, Mongolian toads were caged *in situ*, and the number of eggs deposited by the female were counted (metal contamination, Mongolian toads: Zhang et al., 2018). For the studies that reported effects on clutch size, also using a caged *in situ* design, fecundity (corrected for female body mass) was positively correlated with estimated pesticide levels in an agricultural landscape in Germany for common toads (Adams et al., 2021). Contrasting with these findings, severely reduced clutch size has been reported in túngara frogs inhabiting agricultural sites in Trinidad, where the number of eggs laid by collected amplectant pairs was reduced by 60% compared to those collected from reference sites (Orton et al., 2022).

Laboratory based investigations into the effects of pollution on clutch size are scarce (three known publications: Gyllenhammar et al., 2009; Kvarnryd et al., 2011; Porter et al., 2011 - see Table S2) which is likely related to the labour-intensive experimental design required for such studies. In these studies the basic experimental design involved developmental exposure to the pollutant, followed by a grow-out period and then a subsequent assessment of breeding of the adult frogs (Gyllenhammar et al., 2009; Kvarnryd et al., 2011; Porter et al., 2011). These studies tested the effects of three endocrine disrupting chemicals, specifically, EE2, levonorgestrol (a progestin) or octylphenol (a weakly estrogenic chemical) at moderate to high exposure levels. No effects were seen for exposure to EE2 (6–600 pM) (Gyllenhammar et al., 2009) nor octylphenol (1–36 ug/L: Porter et al., 2011), however the study with levonorgestrel (50 pM), found reduced egg laying by 90%. This effect was found to be due to the lack of oviducts, rather than fewer eggs being produced (no effects were observed at the lower test concentrations of 6 pM: Kvarnryd et al., 2011). From the very limited data reported on clutch size (see Table S2), it is not possible to draw any firm conclusion regarding the sensitivity of egg production to pollutant exposure.

It is surprising that clutch size has not been investigated more in anurans, particularly since it is easy to measure, it is non-destructive endpoint, requires no specialist equipment, and furthermore is a regularly measured endpoint for toxicant effects in fish (*e.g.* Benejam et al., 2010). More data on this endpoint are much needed, particularly as females are generally considered the 'limiting sex' in vertebrate populations due to the high investment needed for producing eggs. Although there will be direct link between clutch size and number of offspring, the level of redundancy to which point a population might be impacted is not known for any anuran species, and likely varies hugely between species depending on their life history and breeding dynamics.

2.6. Hatching success

Hatching success is defined here as the number of viable offspring produced immediately after hatching. This endpoint for anurans is nearly always reported as a percentage from a sub-sample of eggs collected, rather than the total number of offspring produced from an amplectant pair (but see: Orton et al., 2022). Hatching success in response to pollution exposure in laboratory settings has also been reported, however, this is relatively rare due to the most common experimental design for ecotoxicology experiments with Anura beginning exposures at the free-swimming phase.

Hatching success has been investigated by various authors in temperate amphibian species *via* rearing collected spawn in 'clean' laboratory water (see Table S3). In four of these studies (Adams et al., 2021; Bókony et al., 2018; Metts et al., 2013; Orton and Routledge, 2011), spawn from common toads or Southern toads were reared in laboratory 'clean' water either *via* the collection of wild breeding adults or *via* the collection of toadspawn from natural spawning events. Orton and Routledge (2011), Metts et al. (2013) and Adams et al. (2021) reported reduced hatch success in response to the pollution levels that were detected in natal pond water (agricultural/coal combustion (metal) contamination), whereas Bókony et al. (2018) reported no effects of agricultural pollution. It should be recognised that the laboratory exposure environment for the developing offspring was not synonymous with their natal water bodies (though maternal transfer of pollutants still likely occurred, *e.g.*, Metts et al., 2013). This type of experimental design likely occurs due to the practicalities of recording these data for species with a long hatch time as rearing subsamples of the spawn in natal site water, would necessitate natal sites to be re-visited several times *per* week to collect water to perform water changes.

Other approaches to assess hatch success have included an *in situ* experimental design, where a known number of eggs are placed in cages at natal spawning sites. Several studies employing this type of design have been reported from Canada with a range of Ranid species, with mixed results regarding the impacts of pollutants. In a couple of studies, no effects were observed (green frog/northern leopard frog, *Rana pipiens/Rana clamitans*: McDaniel et al., 2008; mink frog, *Rana septentrionalis*: Park and Kidd, 2005). In other studies, reduced hatching success was reported – for example in green frogs upon *in situ* exposure to 5 ng/L EE2 exposure in the great lakes, Ontario (Park and Kidd, 2005) and red legged frogs (*Rana aurora*) from agricultural sites in British Columbia (2002). Other studies also reported associations between pollution and reduced hatch success, but with less robust statistical outcomes. For example, some – but not all – populations of northern leopard frogs/green frogs located in apple orchard sites displayed reduced hatch success (Ontario), but this was not correlated with any of the pesticides measured nor with site type (Harris et al., 1998). Also, although reduced hatching success was reported in great basin spadefoot toads (*Spea intermontana*), Pacific treefrogs (*Pseudacris regilla*), Western toads (*Anaxyrus boreas*) and Columbia spotted frogs inhabiting conventionally pesticide sprayed fruit orchards (17 pesticides at ng/L levels), many of the reported results were not in fact statistically significant (Bishop et al., 2010).

Employing a slightly different experimental design, embryos have sometimes been collected from reference sites and placed in caged enclosures *in situ*. In these studies, therefore, the assumption is that the experimental embryos have no prior experience of polluted conditions. In general, results from these studies are more convincing for negative effects of pollution on hatch success indicating that transplanted organisms may be relatively more sensitive compared to native individuals. For example, a negative correlation between hatching success and polychlorinated biphenyl contamination was observed for green frog and leopard frog embryos placed along a contaminated river in Wisconsin, USA (hatching success ranged between 0% and 100%: Karasov et al., 2005). Also, hatch success of the lesser snouted tree frog (*Scinax nasicus*) placed in cages at agricultural sites in Argentina reported hatching rates of between 0 or 40% success compared with 100% for a reference site (Peltzer et al., 2008). Reduced hatch success (11–15% reduction) of transplanted embryos has also been reported in Southern toads (2001) and for narrow-mouthed toads (*Gastrophryne carolinensis*) (Hopkins et al., 2006) in response to metal contamination.

These data demonstrate the high variability inherent in these types of field-based studies, and the difficulties in attempting to draw conclusions from findings across such a wide range of different anuran species,

contaminant types, countries and habitats (see Table S3). It should also be emphasised that in many cases collected eggs are reared in clean laboratory water, which differs from their natal ponds and the chemical content of laboratory water is rarely reported upon, complicating interpretations on some of the reported findings. In addition, for studies employing a caged experimental design, as these are not standardised, the cage construction/feeding regime *in situ* exposures are highly heterogenous between studies (or undefined). Further, care must be taken when attributing pollution as the causal factor driving observed reductions in hatch success as demonstrated in red legged frogs, where no differences in hatching success were reported in the embryos reared in site water *ex situ* (in the laboratory), whereas reduced success was observed in the caged *in situ* embryos (de Solla et al., 2002). Similarly, no effects on hatching success were reported in veined treefrogs (*Trachycephalus typhonius*) exposed to metal contaminated sediments (collected in Argentina) that were maintained in outdoor microcosms (Peltzer et al., 2013).

For many tropical species due to higher temperature, the hatch time is typically much reduced, reducing the need for water changes which more easily allow for studies to employ the use of natal site water. In a study reporting the total number of hatched tadpoles as a proportion of the total number of eggs laid, a small but statistically significant reduction (5% lower) was reported from pairs of túngara frogs collected from agricultural sites compared to reference sites in Trinidad (Orton et al., 2022). There are indications from the Amphibia (Yu et al., 2013), that hatch success may be relatively insensitive compared to larval life stages, presumably due to the protective barrier surrounding embryos, such as the jelly coat in amphibians.

Overall, although there is evidence for negative impacts of pollution on hatch success and this will likely be associated with and population vigour/stability, the level of redundancy is not established for any Anuran population making it difficult to assess for the impact of pollution on this endpoint at the population level. In a Ranid frog (red-legged frog), for example, survival under 'natural' conditions may be as low as 5% in a stable population (Calef, 1973). Hatching success, however, has been shown to be related to embryonic development duration and hatchling body size of successfully hatched individuals (spined toad, *Bufo spinosus*: Cheron et al., 2021); perhaps supporting this measure as having importance for population stability. Nevertheless, the relationship between hatching success and population stability will likely vary hugely across different species depending on individual species life history and breeding/survivorship dynamics. Thus, although hatching success has been sometimes reported in the literature, due to the huge range in reported effects – likely due to the range of different species, methodologies and pollutant types investigated – it is not possible at this time to making any firm conclusions on the sensitivity of this endpoint to the effects pollution, or provide numeracy against its importance to anuran population stability.

2.7. Offspring fitness

For anurans, offspring fitness here is defined (admittedly in a rather limited way) as timing of metamorphosis and body size at metamorphosis, as these factors are known to influence the likelihood of a juvenile surviving to reproductive maturity (Berven, 1990; Rose, 2005) and there are data demonstrating effects of pollutants on these endpoints. In addition, tadpole abnormalities are included under the 'fitness' remit as data also exist for pollutant effects on this endpoint; however links between this endpoint with population stability are unknown. There have been many studies where embryos and larvae have been directly exposed to pollutants in the laboratory – albeit often at high (and environmentally irrelevant) exposure levels – and which have reported reduced body mass at metamorphosis, alterations in developmental time and increased frequency of abnormalities in the offspring (reviewed in: Egea-Serrano et al., 2012). In that review a meta-analysis of 48 publications reported an overall negative impact of pollutants on

body mass at metamorphosis (−7.5%) and a considerable increase in abnormality frequency (+ 535%); though time to metamorphosis was not significantly impacted. A more recent review assessing for effects of pollution on growth and development in laboratory exposed amphibians reported broadly similar findings, with metals and pesticides both reported to increase the frequency of abnormalities in exposed tadpoles in a range of species (Slaby et al., 2019), albeit exposure levels were often unrealistically high.

Similarly to hatch success, studies into the effects of pollutants on offspring fitness have employed either a caged *in situ* experimental design or a laboratory reared *ex situ* approach; either with laboratory 'clean' water, or with water collected from the natal environment. In relation to development, both delayed and accelerated metamorphosis has been reported, as well as no effects. Using an *ex situ* experimental design, Bókony et al. (2018) reported increased time to complete metamorphosis in common toad larvae derived from spawn collected from agricultural sites whereas Orton and Routledge (2011) – using a similar experimental design – reported no effects on this endpoint. Native leopard frog tadpoles caged *in situ* in metal contaminated sites completed metamorphosis more quickly than those from reference sites (McDaniel et al., 2004), but for leopard frogs and green frogs maintained in orchard sites, no alterations in development time were observed (Harris et al., 1998). For transplanted embryos, slower development of tadpoles of stripe snouted tree frogs (*Scinax squalirostris*) and moustached frogs (*Leptodactylus mystacinus*) was reported in rice growing regions in Argentina (Attademo et al., 2014). In general, the data are too sparse and varied to be able to make any conclusions on the effects of pollutants on development.

In addition to the timing of metamorphosis, the body mass at the completion of metamorphosis also has importance as a measure of offspring fitness. This has not been measured often, and again, there is a wide variety of experimental designs and results reported. Three studies employing an *ex situ* design reported a lower body mass in metamorphs or juveniles in relation to agricultural environments (common toads, reared in 'clean' laboratory water: Bókony et al., 2018; Orton and Routledge, 2011; natterjack toads, reared in natal agricultural water: Macleod et al., 2023). The body mass of tadpoles prior to the completion of metamorphosis in response to pollution has also been reported. For wild tadpoles collected from agricultural *versus* non-agricultural sites in Argentina, a smaller length and lower body mass was reported for South American common toad (*Rhinella arenarum*) tadpoles (Babini et al., 2015). For transplanted embryos caged *in situ*, negative effects of agricultural environments on tadpole body mass was reported in stripe snouted treefrogs and moustached frogs in Argentina (Attademo et al., 2014). Again with transplanted embryos, reduced body mass was reported for veined treefrogs exposed to contaminated sediment in outdoor mesocosms compared to control sediments (Peltzer et al., 2013) and higher mortality, along with reduced body condition of the surviving tadpoles, was reported for South American common toads exposed *ex situ* to pond water from agricultural areas (Babini et al., 2016). Finally, reduced body size (tadpole body length) was reported in tadpoles deriving from embryos collected from agricultural areas in Germany and reared in laboratory water *ex situ* (Adams et al., 2021). Overall, similarly to that reported for hatch success, there appears to be potentially greater sensitivity of anurans to pollutant impacts for transplanted rather than native embryos (see Table S3 for details).

Considering tadpole abnormality frequency, in Canada, Bishop et al. (1999) reported higher tadpole abnormalities in American toads, *Bufo americanus* and green frogs exposed to water from agricultural sites through both *in situ* caged field site exposures and using a field to laboratory experimental design. Similarly, high tadpole abnormalities were reported in caged green and northern leopard frogs placed in metal contaminated sites compared to reference sites (McDaniel et al., 2004). In Argentina, higher tadpole abnormalities were reported in veined treefrogs held in outdoor mesocosms and exposed to metal contaminated sediments (Peltzer et al., 2013) Abnormalities of the oral disc have

also been reported in wild tadpoles, with potential negaitve impacts on feeding and thereby growth and survival. For tadpoles collected from agricultural *versus* non-agricultural sites in Argentina, a higher frequency of abnormal labial teeth rows were reported in South American common toad tadpoles (Babini et al., 2015) as well as in the Leptodactylid frog, *Physalaemus cuvieri* (Borges et al., 2019).

Similarly to hatching success, due to the paucity of data and large range of species, methodologies and pollutant types investigated, it is not possible at this time to making any firm conclusions on the sensitivity of endpoints related to offpsring fitness to the effects pollution, or provide numeracy against its importance to anuran population stability. However, in general, alterations to development and tadpole abnormalities appear to be relatively common, which is in line with what is observed in laboratory exposures of anurans to chemicals.

2.8. Measurement of sex ratio in populations and effects of chemical polluntion

Sex ratio is defined as the proportional numbers of males and females in a population. The phenotypic sex of individuals is determined for wild populations through either their external morphology, or *via* gonadal physiology (morphology/histopathology), the latter for which requires destructive sampling. Complicating the use of gonadal physiology to determine sex, gonadal physiology does not always align with genetic sex in larval/juvenile anurans, as inter- and intra- specific differences in the mode of gonadal differentiation employed exist across the Anura (*i.e.* differentiated, undifferentiated or semi-differentiated – with undifferentiated races having undefined sex potentially through juvenile stages: Witschi, 1929). A comprehensive understanding of the type of gonadal differentiation that occurs in the study species and individual populations is therefore essential to determine deviations from the 'normal' 1:1 male:female ratio when assessing sex ratio in early life stages. Population sex ratios however can be confidently assessed through sampling sexually mature adults thus avoiding biases that may be created through artefacts of gonadal ontogeny. In explosively breeding anuran species (those that breed over a period of days or weeks), the operational sex ratio is highly male biased, as large aggregations of males typically arrive at the breeding site prior to the females. Furthermore, males tend to remain at the site for prolonged intervals during the breeding period, whereas females leave directly following mating and spawning. Thus, the operational sex ratio measured during the breeding season in species employing the explosive breeding mode does not necessarily represent the adult sex ratio in the population; but if the breeding system for the selected species is known, this can be accounted for. It has been suggested that sex-reversals have evolutionary significance and that they may be a 'naturally' occurring phenomenon (Rodrigues et al., 2018). Perhaps in support of this perspective, no differences in fitness of offspring (size at metamorphosis completion, development) were observed in agile frogs between males, females or sex-reversed offspring, with sex reversal induced by exposure to a heat stress (Bókony et al., 2021).

Alterations in sex ratio assessed *via* the gonadal phenotype have been reported often in response to pollutant treatments in laboratory exposed amphibians, including for oestrogenic chemicals (reviewed in: Orton and Tyler, 2015). However, field studies reporting altered phenotypic sex ratios in response to pollution in wild anurans are scarce. Two field studies, however, have provided evidence for a positive relationship between estrogen exposure and gonadal phenotypic feminisation. In the USA, assessment of the gross gonadal morphology of green frog tadpoles indicated correlations between feminised sex ratios and suburban land use, and levels of oestrogens (estrone, phytoestrogens) were detected at higher levels in ponds containing higher proportions of phenotypic females (Lambert et al., 2015). Similarly, a positive relationship has been shown between oestrogenic activity in the water and the proportion of offspring developing as females in caged common toad tadpoles, as assessed *via* gonadal histopathology, at pond sites in the UK (Pickford

et al., 2015). In a further field study, feminised phenotypic sex ratios found in wild populations of common frogs (Alho et al., 2008) was proposed to be due to oestrogenic pollution, however this was not corroborated with any analytical analyses of the exposure environment. Two studies have reported alterations to sex ratio of metamorphs, with feminisation reported in the offspring of common toads for spawn collected from an agricultural environment and reared in laboratory water (Orton and Routledge, 2011) and masculinised/delayed gonadal development reported in natterjack toads where spawn derived from a reference site were reared in water collected from agricultural sites (Macleod et al., 2022).

To avoid destructive sampling for identifying anuran sex, approaches have been adopted to identify sex-determining genes in somatic tissue (*e.g.* via buccal swab, toe clip, tadpole tail tip) although this is challenging with anurans, due to the prevalence of homomorphy and polyploidy across anuran lineages (Eggert, 2004). A master sex determining gene was identified for African clawed frogs (*Xenopus laevis*) more than a decade ago, were *dm-w* promotes the development of the ovary (Yoshimoto et al., 2008). Sex-determining genes have more recently been reported also for common frogs (Alho et al., 2010), agile frogs (Nemesházi et al., 2020) and for common toads (Nemesházi et al., 2022). Using these markers, sex-reversal has been reported in wild common frogs, which displayed feminisation of phenotypic sex (Alho et al., 2010) and in agile frogs, which displayed masculinisation of phenotypic sex (Nemesházi et al., 2020). For the latter study, a positive correlation between anthropogenic land cover and sex-reversal was reported (XX/male ratio increased on average from 12.8% to 29.3% as total anthropogenic land cover increased from zero to 50%). However, as the selected populations were distributed unequally and clustered either within anthropogenic or 'reference' areas, the possibility of this finding being due to distinct population phylogeny could not be discounted.

In summary, as yet the significance of sex-reversals for populations of wild anurans is unknown. Sex ratios nevertheless have the potential for application to studies for assessing pollutant-induced reproductive toxicity in wild anuran populations, and even to predict population trajectories (Nemesházi et al., 2021). However, the use of sex ratio as an effective indicator of pollution effects requires a better understanding of natural sex ratios in wild populations and specifies-specific sensitivity to sex-reversal. For example, marked interspecific variability in response to estrogen-induced feminisation has been reported for laboratory exposures of the African clawed frog, the European treefrog (*Hyla arborea*) and the green toad (*Bufo viridis*) for exposure to ethinylestradiol (EE2: Tamschick et al., 2016). Further, in contrast to agile frogs collected from similar areas in Hungary, in a recent study on common toads, no relationship between anthropogenic land use and genetic sex-reversal was observed (Nemesházi et al., 2022). The development of sex markers that can be measured in somatic tissue (*e.g. via* buccal swab, toe clip, tadpole tail tip), such as those optimised for common frogs, agile frogs and common toads, allows the quantification of sex non-destructively and thereby facilitates wider investigation into this question.

3. Conclusions

Given the rapid global declines in amphibian populations and with increasingly links of some of these declines with pollution, there is an urgent need for non-destructive methods for assessing for pollutant-induced impacts on reproductive health in wild anurans. Research effort in this regard also needs to be widened to more tropical anuran species, which are generally more highly threatened than temperate species, but for which very few data exist. In most cases data are still relatively scarce to allow firm conclusions to be drawn on the robustness of non-destructive methods, in terms of their reliability to indicate adverse effects on reproductive biomarkers and/or their respective sensitivities to pollution (see Fig. 2). Some male secondary sexual characteristics, offspring fitness and somatic genetic markers for detecting sex reversal however offer good potential candidates for
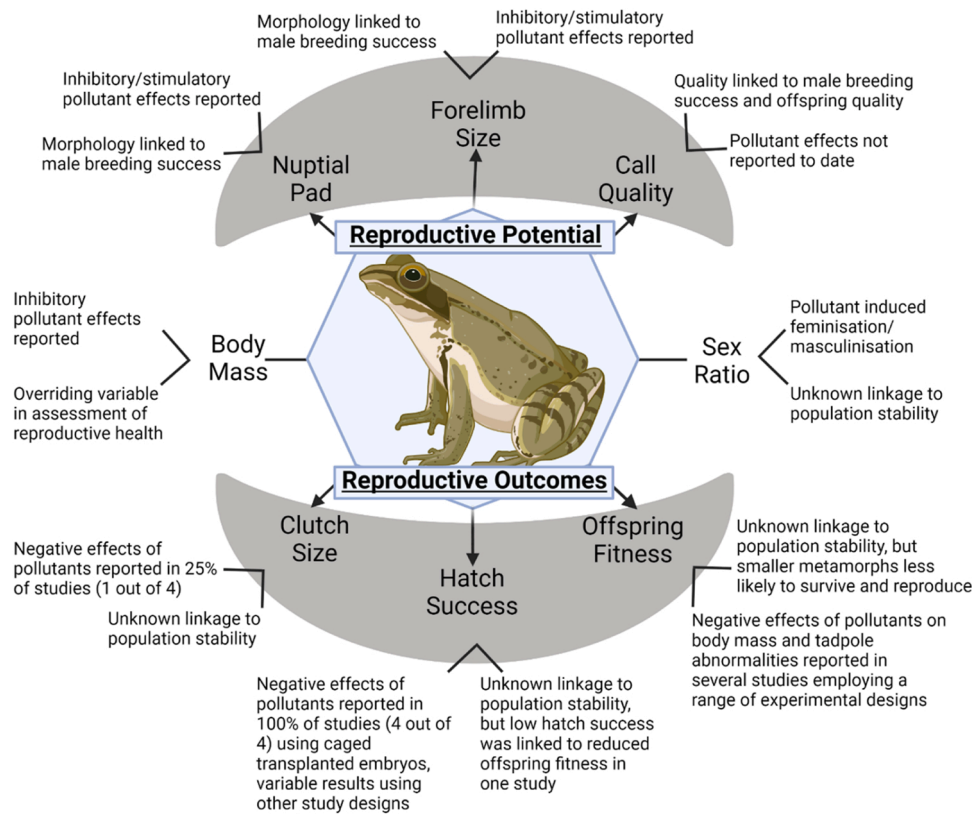
Fig. 2. Overview for effects of pollutants on features of reproductive potential and outcomes, and potential linkage to adverse population effects.

application to wild anuran populations and warrant further investigation. For biomarkers of reproductive capability, nuptial pad size and colour appear to be predictive of reproductive success. However, using these endpoints as markers of pollutant-induced adverse effects on reproductive health is especially complex, as the effects of pollutants are variable, with both stimulation (larger/darker nuptial pads) and inhibition (smaller/lighter nuptial pads) being reported in response to pollutants.

For endpoints related to reproductive outcomes, offspring fitness appears to be more sensitive to pollutant exposure compared to hatching success. With obvious links with maintaining viable/stable populations, measures of offspring fitness need to be measured more extensively in wild anuran amphibian populations. Clutch size has only been measured a handful of times and is of direct relevance to population stability as females are typically regarded as the 'limiting sex' in vertebrate populations. As clutch size can be measured non-destructively and does not require specialist equipment/training, this endpoint deserves wider application in studies on wild anurans to assess pollutant-induced impacts on reproductive health.

The use of body size as a specific measurement related to reproductive outputs is limited due to its overarching role in every aspect of the individual's life. Nevertheless, for this reason, body size needs consideration in studies investigating reproduction, particularly as female body size has sometimes been positively correlated with fecundity (*e.g.* Davies and Halliday, 1977; Prado and Haddad, 2005). In addition, there are many reports of smaller size in frogs collected from degraded/polluted *versus* reference/unpolluted environments. For example, Mexican/plains spadefoot toads (*Spea multiplicata/bombifrons*) and the great plains toad (*Anaxyrus cognatus*) were smaller in cultivated *versus* uncultivated wetlands in the USA (Gray and Smith, 2005), male common toads were smaller in an agricultural *versus* a non-agricultural site in the UK (Orton et al., 2014) and male and female túngara frogs were smaller in agricultural compared to suburban or reference sites (Orton et al., 2022).

In studies on other vertebrates, epigenetically mediated changes in phenotype, for example in response to exposure to EDCs, have been shown to persist over multiple generations and adaptive evolutionary responses to pollution toxicity have been reported in fishes (*e.g.* Whitehead et al., 2012). The offspring of these polluation adapted fish however had reduced growth rates (in clean water) and were be more susceptible to other stressors compared with the offspring of conspecifics from non-contaminated sites (Meyer and Di Giulio, 2002). Research on anurans is lacking in these areas (but see: Karlsson et al., 2021), however, it is likely that such effects also occur in anuran amphibians and this need more attention for the assessment of pollutant-induced impacts on reproductive health and how this may affect non-destructive measures of reproductive health in anurans.

Declaration of Competing Interest

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115163.

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