1-1-2018

Hormone-induced spawning of the critically endangered northern corroboree frog Pseudophryne pengilleyi

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Abstract

Fundamental knowledge of the optimal hormone concentrations required to stimulate amplexus and spawning in breeding pairs of amphibians is currently lacking, hindering our understanding of the proximate mechanisms underpinning mating behaviour. The present study investigated the effects of: (1) the dose of a gonadotropin-releasing hormone analogue (GnRH-A) administered; (2) male-female hormone administration interval; and (3) topical application of GnRH-A, on spawning success in the northern corroboree frog. Administration of GnRH-A at doses of 0.5, 1.0 and 2.0 µg g-1were highly successful, with a significantly greater proportion of hormone-Treated pairs ovipositing (89-100%) compared with the 0 µg g-1treatment (22%). Of the hormone-Treated pairs, those receiving 0.5 µg g-1GnRH-A exhibited the highest fertilisation success (61%). Administration of GnRH-A to males and females simultaneously (0 h) was more effective than injecting males either 48 or 24 h before the injection of females. Overall, administration of GnRH-A was highly successful at inducing spawning in northern corroboree frogs. For the first time, we also effectively induced spawning following the topical application of GnRH-A to the ventral pelvic region. Topical application of GnRH-A eliminates the need for specialised training in amphibian injection, and will allow assisted reproductive technologies to be adopted by a greater number of captive facilities globally.

Publication Details

Silla, A. J., McFadden, M. & Byrne, P. G. (2018). Hormone-induced spawning of the critically endangered northern corroboree frog Pseudophryne pengilleyi. Reproduction, Fertility and Development, 30 (10), 1352-1358.

Hormone-induced spawning of the critically endangered Northern Corroboree Frog, *Pseudophryne pengilleyi*

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Abstract

Fundamental knowledge of the optimal hormone concentrations required to stimulate amplexus and spawning in breeding pairs of amphibians is currently lacking, hindering our understanding of the proximate mechanisms underpinning mating behaviour. The present study investigated the effects of: 1) GnRH-a dose, 2) male:female hormone administration interval, and 3) topical application of Gonadotropin-releasing hormone analogue (GnRH-a), on spawning success in the Northern Corroboree Frog. Administration of GnRH-a at doses of 0.5, 1.0, and 2.0 μg/g were highly successful, with a significantly greater proportion of hormone-treated pairs ovipositing (89 -100%) compared to the 0 μg/g treatment (22%). Of the hormone-treated pairs, those receiving 0.5 μg/g exhibited the highest fertilisation success (61%). Administration of GnRH-a to males and females simultaneously (0hrs) was more effective than injecting males at either 48 or 24 hrs prior to the injection of females. Overall, administration of GnRH-a was highly successful at inducing spawning in Northern Corroboree Frogs. For the first time, we also effectively induced spawning following the topical application of GnRH-a to the ventral pelvic region. Topical application of GnRH-a eliminates the need for specialised training in amphibian injection, and will allow assisted reproductive technologies to be adopted by a greater number of captive facilities globally.

Keywords: amphibian, assisted reproductive technology, captive breeding, conservation, gamete-release, GnRH, LHRH, mating, reproduction, spawning.

Introduction

Advances in assisted reproductive technologies (ARTs) have markedly improved the efficiency and sustainability of agricultural animal production. More recently, the value of ARTs for endangered species recovery has been recognised in order to overcome the behavioural impediments to natural mating and fertilisation that captive animals often encounter (Durrant 2009). Recent reports confirm that we are already amidst a sixth mass extinction (Ceballos *et al.* 2015), with numerous conservation breeding programs established for threatened species globally. These programs aim to maintain genetically viable insurance colonies *ex situ*, while also providing individuals for population augmentation, translocation and reintroduction *in situ*. Despite considerable efforts to mimic natural environmental cues, many amphibian conservation breeding programs continue to face difficulties reliably and predictably initiating breeding behaviour in captivity (Kouba *et al.* 2009). This deficiency threatens the genetic viability of insurance colonies and has limited the generation of large-numbers of individuals for release. Assisted reproductive technologies, such as the hormonal induction of spawning, gamete-release and artificial fertilisation, have the potential to contribute to amphibian conservation by enhancing species propagation, synchronizing breeding events and permitting greater control over the genetic management of insurance colonies.

Exogenous reproductive hormones have been used to successfully induce spawning and gamete-release in a number of anuran (frog and toad) and urodele (newt and salamander) species (Byrne and Silla 2010; Mansour *et al.* 2011; Silla 2011; Trudeau *et al.* 2013; Calatayud *et al.* 2015; Uteshev *et al.* 2015; Della Togna *et al.* 2017). The two hormones most commonly employed are human chorionic gonadotropin (hCG) and gonadotropin- releasing hormone (GnRH, also known as luteinizing hormone- releasing hormone; LHRH). The administration of purified hCG mimics the luteinizing hormone (LH) surge required to stimulate final gamete maturation and release (hypophyseal approach; Vu and Trudeau 2016). For most amphibian species, hCG is less effective, and may reflect species specificity in LH-receptor affinities (Silla and Roberts 2012). In contrast, synthetic GnRH-a is structurally similar to the GnRH-1 molecule found in the anterior pre-optic area of the hypothalamus and the median eminence of the amphibian brain (Vu and Trudeau 2016). GnRH-a acts by stimulating the anterior pituitary gland to synthesise and release natural LH (hypothalamic approach; Vu and Trudeau 2016). Administration of GnRH-a has been shown to effectively

stimulate ovulation and spermiation in a diversity of anurans in the absence of a mating partner (Michael *et al.* 2004; Silla 2010; Silla 2011; Silla and Roberts 2012; Jacobs *et al.* 2016). By contrast, the ability of GnRH-a to elicit mating behaviour, and in particular the optimal doses required to stimulate amplexus and spawning, has seldom been tested empirically, hindering our understanding of the proximate mechanisms underpinning mating behaviour in amphibians.

The administration of GnRH-a is typically achieved via intraperitoneal or subcutaneous injection. However, amphibians also afford a unique opportunity to develop methods for the topical application of exogenous hormones (epicutaneous administration) due to their highly permeable, hypervascularised skin surfaces. The ability to induce spermiation through the topical application of GnRH-a has previously been tested in American Toads (*Bufo americanus*) and Gulf Coast Toads (*Incilius valliceps*), with varying degrees of success (Obringer *et al.* 2000; Rowson *et al.* 2001). To date, no attempt has been made to employ these protocols to induce ovulation or spawning in amphibians. Refining protocols for the topical application of GnRH-a would be of enormous benefit to amphibian conservation because it would eliminate the need for specialised training in amphibian injection and allow ARTs to be adopted by a greater number of captive facilities.

The northern corroboree frog (*Pseudophryne pengilleyi*) is considered one of Australia's most threatened vertebrates, listed as Critically Endangered by state and federal governments, and Endangered by the IUCN (McFadden *et al.* 2016). The species has been the focus of an intensive captive breeding and reintroduction program since 2003, established as a partnership between the Taronga Conservation Society Australia (TCSA), Tidbinbilla Nature Reserve and the NSW Office of Environment and Heritage (OEH) (McFadden *et al.* 2016). Although northern corroboree frogs have been bred successfully in captivity for a number of years, a proportion of gravid females fail to spawn annually, reducing the reproductive potential of captive colonies. Additionally, captive populations display strong mating biases with less than a third of available males contributing to mating success. Over time, such captive mating biases may lead to a loss of genetic variation and adaptive potential that could compromise re-introduction success.

The present study aimed to empirically test protocols to hormonally induce spawning behaviour in the critically endangered northern corroboree frog, *Pseudophryne pengilleyi*. Specific objectives were to investigate: 1) the effect of GnRH-a dose, 2) the effect of male:female hormone administration interval, and 3) the effect of topical application of

GnRH-a, on spawning success. The percentage of pairs ovipositing, number of eggs oviposited, and percentage fertilisation were determined for each experiment.

Methods

Ethics Statement

All procedures were conducted following evaluation and approval by the Taronga Conservation Society Australia's Animal Ethics Committee (protocol numbers 3b/08/14 and 3a/11/16).

Study Species

The northern corroboree frog (*Pseudophryne pengilleyi*) is a small (25-30mm snout-vent length), terrestrial frog easily recognised by longitudinal black and lime-green/yellow dorsal colouration (Figure 1a). The species is restricted to areas above 850m altitude in the Brindabella and Fiery ranges of New South Wales and the Australian Capital Territory in south-eastern Australia. The region experiences an average annual rainfall of 1,200 mm and snowfall at higher elevations during winter. Breeding in this species commences in late austral summer and continues until early autumn. Male *P.pengilleyi* construct shallow terrestrial nests in isolated frost-hollow grasslands, narrow seeps, and open bogs that are subject to seasonal inundation (Osborne 1991; Scheele *et al.* 2017). Females oviposit a small clutch of between 16 - 40 eggs (mean = 24.0, Osborne 1991), though captive females have been recorded ovipositing up to 59 eggs (range = 17 - 59, mean = 35.90 ± 1.01, n=79; unpublished data). Fertilised eggs undergo intracapsular embryonic development, which is typically suspended at Gosner Stage 26–28. In the field, terrestrial embryos may remain in suspended development for several weeks until heavy autumn rainfall floods the nest and hypoxia triggers tadpoles to hatch into temporary pools (Osborne 1991). This reproductive mode (terrestrial egg mass with aquatic free-living larvae) is characteristic of the majority of species in the genus *Pseudophryne* (Watson and Martin 1973).

Animal husbandry

Northern corroboree frogs were maintained in an isolated, biosecurity facility located at Taronga Zoo, Mosman, NSW, Australia. Internal lighting within the facility was controlled using a weatherproof light sensitive switch (HPM, NSW, Australia) set to simulate local

photoperiod. Lighting was provided using fluorescent tubes (10.0 UV-B, Reptisun, Germany) suspended approximately 36cm above each shelf, resulting in 20–30 μW / cm^2 UV-B at the substrate floor of each enclosure. Ambient temperature within the facility was cycled annually to reflect seasonal changes in the average climatic conditions experienced in the subalpine areas where the species naturally occurs. Temperatures ranged from 5 - 20℃, including a 6-week hibernation period. Programmed temperatures were at a maximum during the breeding season, when frogs were maintained on a 20℃/ 17℃ day/ night temperature cycle. Outside of the breeding season, male and female northern corroboree frogs were communally housed in same sex groups in ventilated, clear plastic terraria (28cm L × 17cm W × 18cm H; 4-6 individuals per. terrarium). Each terrarium contained a layer of aquarium gravel (particle size ~4 mm) approximately two cm deep, in addition to a layer of hydrated sphagnum moss approximately 5 cm deep covering half of the enclosure floor. Holes (3 mm D) were drilled in the base of each terrarium for drainage. Enclosure substrates were sprayed with reverse osmosis (RO) water twice weekly to break down and remove excrement and detritus. Frogs were fed a diet of 6-9 day old hatchling crickets (*Acheta domestica*; 15–20 crickets per. individual) once every five days. Crickets were dusted with calcium powder (Calcium with Vit. D_3, Rep-Cal Research Labs, United States) prior to every feed and a multivitamin supplement (Herptivite, Rep-Cal Research Labs, United States) every alternate feed.

Experiment 1: The effect of GnRH-a dose on spawning success

To determine the effect of GnRH-a dose on spawning success, 36 male-female pairs were allocated to one of four experimental treatments; 0.0, 0.5, 1.0, or 2.0 μg/gram body weight GnRH-a (Leuprorelin acetate; Lucrin®)(n=9 pairs per treatment). All frogs were sexually mature (5-11 years of age) and ranged in weight from 1.39 – 4.00 grams. The male/female body mass ratio of each pair ranged from 53.82 – 74.52 % (mean ± sem = 61.12 ± 0.89) and the body mass of males and females did not differ significantly between treatment groups (one-way ANOVA male mass: $F_{3,32} = 0.042$, $p = 0.988$; female mass: $F_{3,32} = 0.676$, $p = 0.573$).

One week prior to hormone administration, males were removed from communal housing and moved into individual terraria (28cm L × 17cm W × 18cm H) containing substrates as described above (see section *2.3 Animal husbandry*). This was done in order to provide each

male with the opportunity to establish a nest site prior to introducing the female. Individuals within each male-female pair were weighed and administered a single hormone dose corresponding to their experimental treatment. Hormones were diluted in 100 μL of Simplified Amphibian Ringer (SAR; 113 mM NaCl, 2 mM KCl, 1.35 mM CaCl2, 1.2 mM NaHCO3) and administered via subcutaneous injection into the dorsal lymph sac (Figure 1b). Three weeks after hormone administration, terraria were searched for the presence of eggs, and the number of eggs oviposited, and fertilisation success, scored. In the absence of eggs, the male-female pair was categorised as unresponsive. Experiment 1 was conducted from April 10 to May 8, 2014.

Experiment 2: The effect of male:female hormone administration interval on spawning success

To determine the effect of hormone administration interval on spawning success, males were administered GnRH-a at one of three time periods (48, 24, or 0 hrs) prior to the administration of GnRH-a to females (27 male-female pairs, n=9 per treatment). All frogs were sexually mature (5-12 years of age) and ranged in weight from 1.34 – 3.90 grams. The male/female body mass ratio of each pair ranged from 48.20 – 68.83 % (mean ± sem = 56.56 ± 0.87) and the body mass of males and females did not differ significantly between treatment groups (one-way ANOVA male mass: $F_{2,24} = 0.021$, $p = 0.979$; female mass: $F_{2,24} = 0.191$, $p = 0.827$).

One week prior to hormone administration, males were removed from communal housing and moved into individual terraria (28cm L × 17cm W × 18cm H) containing substrates as described above (see section *2.3 Animal husbandry*). Males were removed from their enclosures, weighed and administered a single dose of 0.5 μg/g GnRH-a either 48, 24, or 0 hrs prior to the introduction of freshly injected females. As with the males, all females received a single injection of 0.5 μg/g GnRH-a (diluted in 100 μL of SAR) administered via subcutaneous injection into the dorsal lymph sac (Figure 1b). Three weeks after hormone administration, terraria were searched for the presence of eggs, and the number of eggs oviposited, and fertilisation success, scored. In the absence of eggs, the male-female pair was categorised as unresponsive. Experiment 2 was conducted from March 31 to April 27, 2015.

Experiment 3: The effect of topical application of GnRH-a on spawning success

To determine the effect of topical application of GnRH-a on spawning success, 37 male-female pairs were allocated to one of three experimental treatments (n=11-13 pairs per treatment; 0, 25 or 50 μg/g GnRH-a). All frogs were sexually mature (5-14 years of age) and ranged in weight from 1.45 – 4.39 grams. The male/female body mass ratio of each pair ranged from 46.47 – 72.09 % (mean ± sem = 54.54 ± 0.84) and the body mass of males and females did not differ significantly between treatment groups (one-way ANOVA male mass: $F_{2,33} = 0.177$, $p = 0.839$; female mass: $F_{2,33} = 0.215$, $p = 0.808$).

As with experiments 1 and 2 detailed above, male frogs were removed from communal housing and moved into individual terraria (28cm L × 17cm W × 18cm H) one week prior to the introduction of females. Individuals within each male-female pair were weighed and administered a single hormone dose corresponding to their experimental treatment (0, 25 or 50 μg/g GnRH-a; n= 11, 13 and 13, respectively). Hormones were diluted in 100 μL of distilled water and administered dermally via drop-wise topical application onto the ventral abdominal surface (Figure 1c). Three weeks after hormone administration, terraria were searched for the presence of eggs, and the number of eggs oviposited, and fertilisation success, scored. In the absence of eggs, the male-female pair was categorised as unresponsive. Experiment 3 was conducted from March 23 to April 20, 2017.

Statistical Analyses

The numbers of male-female pairs ovipositing were compared between treatment groups in each experiment using two-tailed Fisher's exact tests. One-way analyses of variance (ANOVAs) were used to test for statistical differences in the mean number of eggs oviposited or percent fertilisation, between experimental treatments. Comparisons among treatment means were conducted using Tukey-Kramer Honestly Significant Difference (HSD) post hoc tests. To verify homogeneity of variances, Levene's tests were performed. If variances were unequal, Kruskal-Wallis tests (KW) were conducted, and post hoc treatment comparisons were made using Wilcoxon matched-pair tests. All statistical analyses were performed using JMP Pro 11.0.0 software package (SAS Institute Inc. North Carolina, USE). For all analyses, statistical significance was accepted at $P < 0.05$.

Results

Experiment 1: The effect of GnRH-a dose on spawning success

The number of male-female pairs ovipositing in response to the administration of 0.5, 1.0, or 2.0 μg/g GnRH-a was significantly greater than the number of pairs ovipositing in response to the 0 μg/g GnRH-a treatment (no hormone stimulation)(Fisher's Exact Tests, $P < 0.05$; Table 1). Similarly, the total number of eggs laid in response to GnRH-a differed significantly among dose treatments (one-way ANOVA, $F_{3,32} = 7.186$, $p = 0.0008$), with pairs receiving 0.5, 1.0, or 2.0 μg/g GnRH-a producing a significantly greater number of eggs than pairs receiving 0.0 μg/g GnRH-a (Tukey-Kramer HSD, $P < 0.05$; Table 1). Percent fertilisation was calculated for all pairs that oviposited. Overall, mean percent fertilisation also differed significantly among treatment groups (Kruskal Wallice test, $\chi^2 = 9.051$, $p = 0.0286$), with percent fertilisation significantly higher in the 0.0 μg/g GnRH-a dose treatment compared with the 1.0 and 2.0 μg/g dose treatments (Tukey-Kramer HSD, $P < 0.05$; Table 1). Mean percent fertilisation of clutches in the 0.5 μg/g GnRH-a dose treatment was not significantly different from any of the remaining doses (0.0, 1.0 & 2.0 μg/g GnRH-a; Tukey-Kramer HSD, $P > 0.05$; Table 1).

Experiment 2: The effect of male:female hormone administration interval on spawning success

Hormone administration interval did not significantly effect the number of pairs that oviposited (Fisher's Exact Tests, $P > 0.05$), or the number of eggs laid (Kruskal Wallice test, $\chi^2 = 0.621$, $p = 0.733$; Table 2). Similarly, mean percent fertilisation did not differ significantly among treatment groups (Kruskal Wallice test, $\chi^2 = 5.584$, $p = 0.0613$; Table 2). However, Wilcoxon matched-pair post-hoc tests indicated that mean percent fertilisation was significantly higher in the 0-hr treatment compared to 24-hrs (64% and 25%, respectively; Table 2).

Experiment 3: The effect of topical application of GnRH-a on spawning success

The number of male-female pairs ovipositing in response to the topical administration of 25 μg/g GnRH-a was significantly greater than the number of pairs ovipositing in response to the 0 μg/g GnRH-a dose treatment (Fisher's Exact Test, $P = 0.0377$). In contrast, the number of pairs ovipositing in response to 50 μg/g GnRH-a, was not significantly different from either the 0 μg/g or 25 μg/g dose treatments (Fisher's Exact Tests, $P < 0.05$; Table 3). Similarly, the number of eggs laid in response to GnRH-a differed significantly among dose treatments (one-way ANOVA, $F_{2,34} = 3.540$, $p = 0.040$), with pairs receiving 25 μg/g GnRH-a producing a significantly greater number of eggs than the 0 μg/g GnRH-a dose treatment (Tukey-Kramer HSD, $P < 0.05$; Table 3). The number of eggs laid in response to the topical administration of 50 μg/g GnRH-a was not significantly different from either the 0 μg/g or 25 μg/g dose treatments (Tukey-Kramer HSD, $P > 0.05$; Table 3). Percent fertilisation was calculated for all pairs that oviposited, with mean percent fertilisation statistically similar among treatment groups (one-way ANOVA, $F_{2, 18} = 0.517$, $p = 0.605$; Table 3).

Discussion

Assisted reproductive technologies have enormous potential to enhance captive breeding and reintroduction programs by improving species propagation and permitting greater control over the genetic management of insurance colonies. To date empirical studies have predominantly focused on quantifying the effects of exogenous hormone administration on gamete-release in individuals (in the absence of a mating partner), as a precursor to gamete-storage and artificial fertilisation (AF, also known as in vitro fertilisation, IVF) (Browne *et al.* 2006; Byrne and Silla 2010; Silla 2011; Silla 2013; Uteshev *et al.* 2015; Della Togna *et al.* 2017). Fundamental knowledge of the optimal hormone concentrations required to stimulate amplexus and spawning in breeding pairs of amphibians is substantially lacking by comparison, hindering our understanding of the proximate mechanisms underpinning mating behaviour in amphibians. In the present study we aimed to empirically test protocols to hormonally induce spawning behaviour in the critically endangered northern corroboree frog. Specifically, we investigated the effects of 1) GnRH-a dose, 2) male:female hormone administration interval, and 3) topical application of GnRH-a, on spawning success.

Results from this study showed that the administration of GnRH-a at doses of 0.5 μg/g, 1 μg/g, and 2 μg/g body weight were highly successful at inducing spawning, with a significantly greater proportion of hormone treated pairs ovipositing (72 -100%) compared to

pairs in the 0 μg/g GnRH-a dose treatment (22%). Our results are consistent with those of previous studies on northern leopard frogs (*Lithobates pipiens*) that report a significant increase in the spawning success of hormone treated frogs (42 -100% and 88 -89%, respectively) compared with untreated animals, which failed to spawn (Trudeau *et al.* 2010; Trudeau *et al.* 2013). Similar findings have also been reported in captive rocky mountain boreal toads (*Anaxryus boreas boreas*), where hormone administration effectively doubled the proportion of spawning pairs (from 17% to 33%) (Calatayud *et al.* 2017). Interestingly, previous studies inducing spawning in amphibians have all administered GnRH-a in combination with other reproductive hormones, including hCG and dopamine antagonists (metoclopramide, pimozide & domperidone) (Trudeau *et al.* 2010; Trudeau *et al.* 2013; Calatayud *et al.* 2017). The present study is the first to demonstrate that GnRH-a alone can induce 100% of male-female pairs to spawn at doses of either 0.5 or 2 μg/g and highlights the importance of establishing dose- response curves for individual hormones.

The fertilisation success of clutches oviposited in the present study also differed significantly according to the GnRH-a dose administered. Male-female pairs in the 0 μg/g GnRH-a dose treatment exhibited the greatest percentage fertilisation (97%), however it is important to note that only two pairs oviposited and the fertilisation success of untreated animals may vary with additional replication. Of the hormone treated pairs ovipositing (n=8-9 per treatment), those injected with 0.5 μg/g GnRH-a exhibited the greatest percentage fertilisation (61%), while frogs in the higher dose treatments (1 & 2 μg/g GnRH-a) displayed low mean fertilisation success (< 22%). One explanation for the reduced fertilisation success observed in the higher dose treatments is that oocytes underwent a process of over-ripening. Over-ripening results from the aging and deterioration of oocytes retained for an extended period within the coelomic cavity of a female post ovulation (Bromage *et al.* 1994; Silla 2011). Oocyte over-ripening may have occurred in the higher dose treatments if oocyte maturation and ovulation was stimulated too quickly, resulting in the retention of oocytes prior to amplexus and fertilisation. This explanation is consistent with the observation that embryos oviposited within the higher dose treatments were often scattered or clumped in small groups rather than deposited in a discrete, well-defined nest.

Interestingly, amphibian species appear to vary considerably in their sensitivity to GnRH-a administration (Silla and Roberts 2012), despite the general conservation of the structure and function of GnRH among vertebrates (Gore 2002). If doses of GnRH-a administered are too low for a given species, they may induce the upregulation of GnRH -receptors without a corresponding change in LH synthesis and release (Conn 1986; Gore

2002), resulting in incomplete oocyte maturation, reduced spawning rates and/or fertilisation success. As GnRH-a doses administered are increased, nearing optimal concentrations, up-regulation of GnRH receptors continues, receptor numbers are elevated and the tissue responds with the LH surge required to stimulate final oocyte maturation and ovulation (Conn 1986; Gore 2002). If optimal GnRH-a doses are exceeded, oocyte over-ripening may occur resulting in a reduction of oocyte quality and diminished fertilisation success (Silla 2011). Interspecific variation in the comparative efficacy of GnRH administration, and therefore optimal dose, is unlikely to be driven by species-specific differences in the structure of natural GnRH, as the amino acid sequences of these molecules are highly conserved across all vertebrate species (Gore 2002). However, given the phylogenetic diversity of anurans and the diversity of reproductive modes they exhibit, it is reasonable to expect interspecific variance, not in the GnRH molecules themselves, but in the timing and concentration of GnRH released. Gaining knowledge of the optimal GnRH doses required to stimulate amplexus and spawning across a diversity of species will therefore further our understanding of the evolution of the proximate mechanisms controlling mating behaviour in amphibians.

An alternative approach to the injection of GnRH-a is the epicutaneous administration of the hormone directly to the ventral abdominal skin surface (topical application). The topical application of GnRH-a was initially tested in male American toads to induce spermiation with poor success (22% spermiation response)(Obringer *et al.* 2000). However, further protocol refinement using higher hormone doses led to the successful induction of spermiation in both American toads and gulf coast toads (75% spermiation response) (Rowson *et al.* 2001). The present study tested for the first time the efficacy of GnRH-a applied topically to the ventral abdominal surface of male and female frogs to induce spawning. Topical administration protocols were highly successful, with 77% of male-female pairs ovipositing in response to a dose of 25 μg/g GnRH-a. This is the first demonstration that topical application of reproductive hormones can induce spawning in an amphibian. Amphibians possess highly vascularised, permeable ventral pelvic surfaces that enable the rapid absorption of water and low molecular weight compounds (Toledo and Jared 1993). This is particularly true for terrestrial amphibians, which exhibit a greater intensity of cutaneous vascularisation in the pelvic region compared with aquatic species (Toledo and Jared 1993), making them ideal candidates for the topical application of reproductive hormones.

Incorporating ARTs into existing conservation breeding programs has the potential to enhance species propagation, allow the synchronisation of breeding events, and increase

genetic diversity and adaptive potential of the offspring generated. Furthermore, incorporating ARTs enables better control over breeding designs and provides an opportunity for selective breeding of particular genotypes. As a result, ARTs are being increasingly employed by captive facilities to compliment traditional breeding methods and enhance species recovery (Silla *et al.* 2015). At present, a vast number of CBPs for threatened amphibians are yet to benefit from the implementation of ARTs. One reason for this is that a disproportionate number of threatened amphibian species originate from developing countries within Neotropical, Afrotropical and Indomalayan regions (collectively harbouring >82% of rapidly declining amphibians)(Stuart *et al.* 2004). CBPs in these locations often have limited resources and lack veterinary capacity or personnel with expertise in amphibian injection. The topical hormone administration protocols developed in the present study have enormous potential to increase the number of captive facilities globally adopting ARTs. Such cost effective protocols that eliminate the need for specialised training (such as animal injection or gamete-collection) are urgently needed to assist amphibian species recovery.

Conclusions

Assisted reproductive technologies have enormous potential to contribute to amphibian conservation breeding programs by increasing species propagation, synchronizing breeding events and permitting greater control over the genetic management of insurance colonies. Here we demonstrate that GnRH-a can be effectively used to induce spawning in the critically endangered northern corroboree frog, with 100% of male-female pairs ovipositing in response to an optimal dose of 0.5 μg/g. In a world first, we also effectively induced spawning following the topical application of GnRH-a to the ventral pelvic region. Topical application of reproductive hormones eliminates the need for specialised training in amphibian injection. Refinement of these protocols will therefore allow ARTs to be adopted by a greater number of captive facilities globally to enhance threatened species recovery.

Acknowledgements

We acknowledge staff from Taronga Conservation Society Australia's Herpetofauna division, in particular Caitlin Gilchrist, Cara Anger and Emma Bembrick, for assistance with amphibian husbandry. We also acknowledge the support of NSW Office of Environment and Heritage (OEH) Threatened Species Officer, Dr David Hunter and members of the Corroboree Frog Recovery Team. This study was funded by the NSW Environmental Trust (grant no. 2012/RD/0105) and Australian Research Council (Linkage Grant LP140100808).

Authors' contributions

AS, PB and MM designed the experiment and collected the data. AS and PB performed the statistical analyses. AS wrote the manuscript with input from all authors.

Competing interests

The authors declare that there are no competing interests.

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Figure and table captions

Figure one: A) adult northern corroboree frog, *P. pengilleyi*; B) subcutaneous injection of reproductive hormones into the dorsal lymph sac; C) topical administration of reproductive hormones onto the ventral abdominal surface.

Table one: The effect of GnRHa dose on spawning success.

Data shown are the number of pairs ovipositing/total number of pairs, or mean ± SEM (n=9 per. treatment). Data were analysed using Fisher's Exact Tests (pairs ovipositing), one-way ANOVA (total eggs) or Kruskal Wallice Test (percent fertilisation). Letters displayed are the result of post-hoc tests. Within a row, treatments that share a letter are not significantly different (P>0.05). See methods for details of all statistical analyses.

Table two: The effect of injection interval between the administration of GnRHa to males and females on spawning success.

Data shown are the number of pairs ovipositing/total number of pairs, or mean ± SEM (n=9 per. treatment). Data were analysed using Fisher's Exact Tests (pairs ovipositing), or Kruskal Wallice Tests (total eggs, percent fertilisation). Letters displayed are the result of post-hoc tests. Within a row, treatments that share a letter are not significantly different (P>0.05). See methods for details of all statistical analyses.

Table three: The effect of topical application of GnRHa on spawning success.

Data shown are the number of pairs ovipositing/total number of pairs, or mean ± SEM (n=11-13 per. treatment). Data were analysed using Fisher's Exact Tests (pairs ovipositing), or one-way ANOVAs (total eggs, percent fertilisation). Letters displayed are the result of post-hoc tests. Within a row, treatments that share a letter are not significantly different (P>0.05). See methods for details of all statistical analyses.