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Sperm parameters following hormonal induction of spermiation in an endangered frog [the alpine tree frog] (*Litoria verreauxii alpina*)

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ABSTRACT

Context. With global amphibian biodiversity rapidly declining, improving reproductive technology outcomes has become essential. Captive breeding programs have struggled because amphibian breeding physiology often requires specific environmental cues that reproductive technologies can circumvent. **Aims.** This study tests the efficiency of hormonal induction by evaluating sperm quality in the endangered *Litoria verreauxii alpina*. **Methods.** We assessed the effects of exogenous hormones – gonadotrophin-releasing hormone agonist (GnRH-a, Lucrin), and human chorionic gonadotrophin (hCG, Chorulon) – on sperm quality. **Key results.** Hormone induction with hCG showed high efficacy while GnRH-a yielded a low response in producing sperm. Sperm quantity was affected by time post injection, with the greatest quantity at 1 h post injection. Sperm quality was also affected by time, where the sperm head size decreased by 11% at 7 h post injection. **Conclusions.** Based on the results from this study, we recommend that that sperm be collected soon after induction, and not more than 4 h post induction in *L. v. alpina*. More work needs to be completed before recommending an optimal hormone induction method and dose, but 120 IU of hCG per male was successful for inducing spermiation. **Implications.** This study represents a useful starting point for developing assisted reproductive techniques for non-model organisms.

Keywords: amphibian reproduction, artificial reproductive technologies, captive breeding, endangered species, hormonal induction, IVF, sperm viability, spermic urine.

Introduction

Amphibians around the world face a stark future of rapid and continued biodiversity loss due to threats such as human impact, epidemic disease and climate change (Houlahan *et al.* 2000; Stuart *et al.* 2004; Collins 2010). As such, it is imperative that humanity's role in the catastrophic decline of amphibian biodiversity be recognised (Houlahan *et al.* 2000) and that rapid and significant initiatives be taken to ensure the protection of these species from mass extinction. One conservation management option is the establishment of captive breeding programs, and several have been established around the world to mitigate the risk of extinction for critically endangered species. Yet, many programs have faced challenges that limit the breeding success of amphibians in captivity (Kouba *et al.* 2009). Recent advancements in assisted reproductive technologies, such as hormonal induction, have provided a promising avenue towards overcoming ecological and behavioural barriers, and improved the genetic management of biodiversity for amphibian captive breeding colonies (Kouba *et al.* 2013; Clulow *et al.* 2018).

Hormonal induction typically refers to the administration of an exogenous hormone to elicit a specific physiological response. In the context of reproductive technology, this technique aims to induce spermiation and/or ovulation. By employing the use of specific hormones, gamete release can be induced through an interaction with the neuroendocrine hypothalamic–pituitary–gonadal axis (Clulow *et al.* 2018). Under natural breeding, both

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abiotic and biotic environmental stimuli trigger the hypothalamic–pituitary–gonadal axis, which regulates the release of gametes in amphibians. Within the hypothalamic–pituitary–gonadal axis there are several different levels that can be triggered by exogeneous hormones to induce gamete release (Silla and Byrne 2019). Two commonly used hormones to promote the release of gametes in adult frogs are gonadotrophin-releasing hormone agonist (GnRH-a) and human chorionic gonadotrophin (hCG). GnRH-a analogues are synthetic hormones that are structurally similar to GnRH molecules found in the brain. GnRH-a induces gamete release *via* a hypothalamic approach; it activates the pituitary to release luteinising hormone, which then triggers an axis cascade to release gametes (Silla and Byrne 2019). In contrast, purified hCG uses a hypophyseal approach to gamete induction. It activates the axis cascade at a lower level, directly in the gonads, by mimicking the luteinising hormone surge required to stimulate the final stage of gamete maturation and release (Silla and Byrne 2019). GnRH-a is considered to be more effective at inducing gamete release because it acts at a higher level in the hypothalamic–pituitary–gonadal axis and thus provides a more balanced simulation of reproductive events (Zohar and Mylonas 2001; Silla and Roberts 2012). Additionally, GnRH-a requires lower doses of the drug than hCG and is effective in many species (Silla and Roberts 2012).

There are several different subtypes of both GnRH-a and hCG, derived from non-frog and frog species, that have been trialled to induce gamete release and promote successful spawning in a wide variety of amphibian species (Trudeau *et al.* 2010; Silla and Roberts 2012; Clulow *et al.* 2018; Brannelly *et al.* 2019; Silla and Byrne 2019). While protocols have been refined and the results are consistent in model amphibian species like *Xenopus laevis* and *X. tropicalis* (Mansour *et al.* 2009; Wlizla *et al.* 2017), there have been varying levels of success and consistency in the gamete induction of non-model species (Silla and Roberts 2012; Clulow *et al.* 2018; Silla and Byrne 2019). Interestingly, induction of Australian tree frogs from the amphibian family Pelodryadidae appears to be difficult and inconsistent using the currently available exogeneous hormones (Clulow *et al.* 2018). Female induction has been unsuccessful thus far, and the reason is unknown; however, it might be due to the GnRH-a and hCG molecules not mimicking the endogenic equivalent molecule closely enough to elicit a robust response (Clulow *et al.* 2018; Silla and Byrne 2019).

The aim of this study was to test the hormonal induction of spermiation in a non-model endangered amphibian species, the alpine tree frog, *Litoria verreauxii alpina*. The alpine tree frog is endemic to the alpine regions of Australia and listed as critically endangered in Victoria and New South Wales. We tested two commonly used exogenous hormones, GnRH-a (Lucrin) and hCG (Chorulon) for their success in inducing spermiation. We collected spermic urine at 1, 4 and 7 h post injection and assessed sperm quantity and quality *via* concentration and total count of sperm, motility, and morphology of sperm cells. Captive breeding is challenging in this species, where hormone induction has not been successful to date and captive breeding events have occurred only in outdoor facilities (Brannelly, unpubl. data). The success of hormonal induction for spermiation had not been tested the alpine tree frog, and testing induction protocols are critical for the establishment of captive breeding colonies.

Methods

Husbandry

L. v. alpina were overwintered for 3 months at 4°C in the dark in a 14 cm × 9 cm × 6 cm enclosure with a damp moss substrate. The air temperature slowly decreased from 17°C to 4°C at a rate of no faster than 1°C per day. When the animals reached 7°C, the enclosures were cleaned, and the substrate was replaced. During the overwinter period, the enclosures were cleaned every 5 weeks. Animals were misted with aged tap water (at temperature) daily, but animals were not fed during the overwintering period. After 3 months we increased the temperature to 11°C at a rate of no faster than 1°C per day, and the animals were moved to outdoor enclosures in the morning, when the air temperature was 11°C. Animals were placed in 64 cm × 42 cm × 30 cm half terrestrial half aquatic enclosures containing 20 L of aquatic habitat, fitted with artificial plants. Animals were monitored and tanks were flushed with 10 L of aged tap water daily, and animals were fed gut loaded and vitamin dusted crickets once per week. Animals were maintained in outdoor enclosures in Werribee, Victoria, Australia, for 3 months (June–September) before the experiment began. Daily temperatures during this period were an average monthly minimum of 8°C and an average monthly maximum of 18°C, which is similar to the weather in their natural habitat during the breeding season (Brannelly *et al.* 2015).

Experimental procedure

This experiment consisted of three treatment groups: no hormone, GnRH-a, and hCG. In this study we used the pharmaceuticals Lucrin (a GnRH-a hormone, leuprolide acetate) and Chorulon (a purified hCG). To determine the appropriate dosage of the pharmaceuticals used in this study, we conducted a small pilot study where we induced sperm production in one animal using an injection dose of 3 μg of Lucrin (GnRH-a, approximately 1 μg/g) in 100 μL of filter sterilised amphibian ringers solution, and a second animal induced using a dose of 24 IU of Chorulon (hCG, approximately 8 IU/g) diluted in 100 μL of filter sterilised amphibian ringers solution into the peritoneal cavity using a 27 g needle. These dosages were chosen because they are

commonly successful in the literature across a wide variety of species (Silla and Roberts 2012; Silla *et al.* 2019). Neither of these two induction dosages produced any visible sperm at 1, 4 or 7 h post injection. Therefore, we adjusted the hormone dosages used in this trial. Male animals in this study ranged in size from 2.80–4.41 g with a mean of 3.30 ± 0.47 g. We induced spermiation here using a set concentration dose of 1.5 μg GnRH-a diluted in 100 μL of filter sterilised amphibian ringers' solution, or 120 IU hCG, undiluted at an injection volume of 120 μL, into the peritoneal cavity of each individual using a 27 g needle based on our failed pilot trial and the success in other *Litoria* species (Silla *et al.* 2019).

To prepare for hormonal injection, each frog was removed from their outdoor enclosure and placed in individual 14 cm × 9 cm × 6 cm enclosures in a temperature-controlled laboratory maintained at 17°C with 2 cm of aged tap water substrate to ensure hydration and improve spermic urine collection efficiency. Animals were allowed to acclimatise for 3 h, and then injected with their assigned treatment (no hormone control, 1.5 μg of GnRH-a, or 120 IU of hCG).

This experiment was limited by the small number of *L. v. alpina* males (n = 7) available for this trial. To ensure a sufficient sample size that was statistically robust, we completed this experiment using the same individuals over two treatment rounds. In each sampling round two males were assigned haphazardly to receive a no-hormone control (100 μL of filter sterilised amphibian ringers' solution injected into the peritoneal cavity using a 27 g needle). At least 2 days later all animals were randomly assigned to either the hCG (n = 4) or the GnRH-a (n = 3) treatment and spermic urine samples were collected. The second round took place 14 days later, where two different males were haphazardly chosen to receive the no-hormone control, and 2 days after that, all animals were randomly assigned to either the hCG (n = 3) or the GnRH-a (n = 4) treatment.

To ensure that sperm quality remained over the course of repeated hormonal injections, we waited at least 2 weeks between hormonal induction injections to ensure that sperm quality and quantity was consistent across sampling. It is understood that repeated hormonal inductions can lower fertility in males (Swerdloff *et al.* 1985); however, sperm quality is not affected in amphibians by hormonal injections that are >1 week apart (Roth and Obringer 2003; McDonough *et al.* 2016; Arregui *et al.* 2019). No sperm was found in the no-hormone control samples nor in the small pilot study; therefore, we assumed that no sperm was present in the urine prior to hormonal induction. We chose to sample a subset of animals (n = 2) in each collection round as our no-hormone controls, rather than collect urine from each animal prior to induction at every sampling period. We chose this sampling procedure in order to reduce the handling stress of the spermic urine collection in line with animal ethics guidelines to reduce cumulative burdens. We accounted for the potential effects of induction round on spermiation and the uneven handling procedure for control animals in our statistical analyses (Supplemental materials).

Spermic urine collection

Following hormone injection, animals were then placed back in their enclosure for 1 h before the first spermic urine collection. Spermic urine was collected at 1, 4 and 7 h post injection. During the sample collection, the cloacal region of the animal was lightly dried using a Kim wipe and the animals were given a gentle abdominal massage for 3 min to encourage urine excretion. Following the abdominal massage, we inserted a fire-polished glass Drummond microcapillary tube (25 μL) into the cloaca a maximum of 5 mm and gently oscillated the tube to encourage urine excretion in all animals. Each frog was handled a maximum of 5 min in total at each sample collection timepoint.

Spermic urine quantity and quality assessment

Spermic urine samples were assessed by the volume of urine produced at each sampling point, the concentration of the sperm within the spermic urine, and percent mobility of the sperm cells, and morphology of the sperm cells. Sperm cell concentration was assessed by diluting the sample 1:10 in amphibian ringers' solution and measuring the concentration of cells using a hemocytometer at 200× magnification, where at least 100 sperm cells were counted in at least two aliquots of each sample. Sperm motility was measured as a binary (motile or non-motile sperm cells). A motile sperm cell was one that demonstrated movement within 30 s of observation at 200× magnification. We assessed motility of at least 70 sperm cells per sample *via* non-overlapping video footage at several different fields of view taken across the sample. We assessed morphology using a sperm sample smear that was air dried, flame fixed, and stained using Coomassie Blue R-250 (Bio Rad Laboratories Pty Ltd) (Larson and Miller 1999; Huo *et al.* 2002) for 1 min and rinsed three times with sterile water. Stained slides were dried and mounted using Permamount Mounting Medium (Fisher Scientific). We made sperm smears for all spermic urine samples, even if no or few sperm cells were observed to *via* hemocytometer assessment. We photographed sperm cells at 400× magnification at several field of view across each sample. We measured sperm head length and sperm tail length to the nearest 0.01 μm of 30 cells per sample using ImageJ software (Wayne Rasvan, NIMH). Head and tail length as a form of morphological assessment is common following hormone induction of spermiation in a wide variety of species (Scarano *et al.* 2006; Maria *et al.* 2010; Villaverde-Morcillo *et al.* 2017) including amphibians (Della Togna *et al.* 2017).

Statistical analyses

The effect of hormonal induction on spermiation, sperm cell quantity and quality was determined using mixed effects models. All analyses were conducted in R in the RStudio interface (RStudio Team 2016; R Core Team 2017). Effect size was calculated as Cohen's *d* statistic where appropriate. To determine if mass was affected by hormone injection round, a linear mixed effects model (LMM) was conducted using the package 'lme4' (Bates *et al.* 2015), where mass was the dependent variable, the fixed effect was the treatment round (where round two occurred 2 weeks after round one) and individual was the random effect. The treatment round was included as a random effect in the remaining statistical analyses where it improved model fit, and variance was greater than zero (see Supplemental materials).

To determine the effect of hormone type and time after injection on sperm concentration and number of cells per sample, LMMs were conducted where concentration (sperm cells per μL of spermic urine collected, $\log_{10}$ transformed) and total sperm ($\log_{10}$ transformed) within the sample were dependent variables, the fixed effects were drug type (120 IU of Chorulon per frog of hCG; 1.5 μg of Lucrin per frog of GnRH), time since injection (1, 4, and 7 h), and drug × time, and the random effects were individual and treatment round. Analyses for sperm cell quality were only conducted on animals injected with hCG. A generalised linear mixed effects model (GLMM) was conducted using a beta distribution in the package 'glmmTMB' (Magnusson *et al.* 2020) to determine the effect of time after injection on sperm motility. The proportion of motile sperm cells within the sample was the dependent variable, the fixed effect was time after injection and the random effect was individual. We assessed the effect of time after injection on sperm morphology using LMMs where sperm head length and tail length were dependent variables, time after injection was a fixed effect and the random effects were individual and treatment round. Full model results are presented in Supplementary materials Table S1.

Animal ethics statement

All work was conducted under approval of the University of Melbourne's Animal Ethics Committee (Application 10267), and Wildlife Act 1975 Research Authorisation permit number 10010126.

Results

Volume of spermic urine

The spermic urine samples from no-hormone control samples did not contain sperm cells, and 5.08 ± 0.79 μL of urine was collected per sample. The animals treated with 120 IU of hCG on average produced 11.5 ± 6.9 μL of spermic urine and all animals produced sperm across the collection period; however, one animal produced a low concentration of sperm 7 h after injection. The animals treated with 1.5 μg GnRH-a produced an average of 5.8 ± 4.0 μL of spermic urine and only two of the seven animals produced sperm cells. These two animals produced sperm in 1 and 4 h after injection; no samples collected 7 h after injection with GnRH-a contained sperm cells. The mass of the males in this study averaged 3.30 ± 0.47 g, and there was no effect of the treatment round observed on animal mass (LMM: $P = 0.336$, Table S1).

Sperm quantity assessment

Animals produced a higher concentration of sperm when injected with hCG compared to GnRH-a (LMM: $P > 0.001$), and time after injection affected the quantity of sperm cells that were released (LMM: $P > 0.001$; Fig. 1*a*; Table S1). There was a 205-fold difference in sperm concentration between hCG and GnRH-a at 1 h after injection: the median sperm concentration for animals injected with hCG 1 h after injection was 1.55×10^4 cells per μL (interquartile range, IQR: 1.68×10^4), and 75 cells per μL (IQR: 4.71×10^3) for animals injected with GnRH-a ($d = 2.15$; Fig. 1*a*). There was 4.60-fold difference in sperm concentration in animals injected with hCG from 1 to 7 h after injection: the median sperm concentration for animals injected with hCG 7 h after injection was 2.76×10^3 cells per μL (IQR: 3.21×10^3; $d = 2.85$; Fig. 1*a*). Animals injected with GnRH-a did not produce sperm cells 7 h after injection.

The total number of sperm cells produced within a spermic urine sample followed a similar pattern to sperm cell concentration: there was a significant effect of both the drug administered (LMM: $P > 0.001$) and sample collection time (LMM: $P > 0.001$) in the number of sperm produced per sample (Fig. 1*b*, Table S1). There was a 1235-fold difference in the total cells produced with hCG injection compared to GnRH-a at 1 h post injection, where animals injected with hCG produced a median of 2.78×10^6 sperm cells per sample (IQR: 2.80×10^6) and animals injected with GnRH-a produced a median of 2.25×10^2 sperm cells per sample (IQR: 8.53×10^5; $d = 1.93$; Fig. 1*b*). There was a 6.66-fold difference of the total cells produced per samples collected between 1 and 7 h after injection with hCG: a median of 3.63×10^4 sperm cells per sample (IQR: 3.44×10^4) 7 h after injection ($d = 2.05$; Fig. 1*b*). There was a significant interactive effect of drug type and time after injection (LMM: $P > 0.001$), where animal injected with GnRH-a experienced a more dramatic decline in total sperm present within the sample between 1 and 7 h after injection compared to the animals injected with hCG (Fig. 1*b*; Table S1).

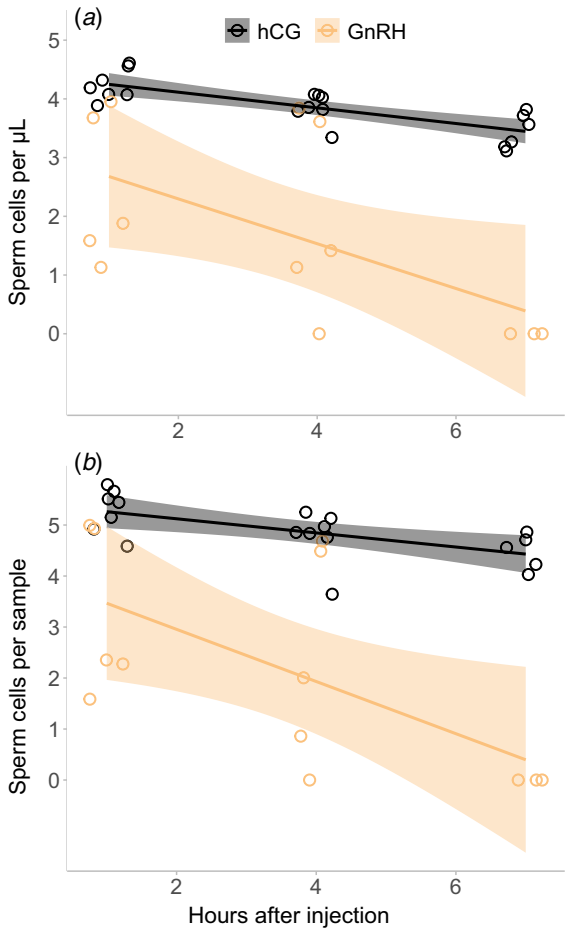

Fig. 1. Sperm cell quantity per sample: (*a*) sperm cell concentration per µL per sample and (*b*) number of cells within the whole sample. Concentration and total number of cells are $\log_{10}$ transformed. The points represent individual samples, and the lines represent the smoothed conditional means. The shading around the lines represents standard error.

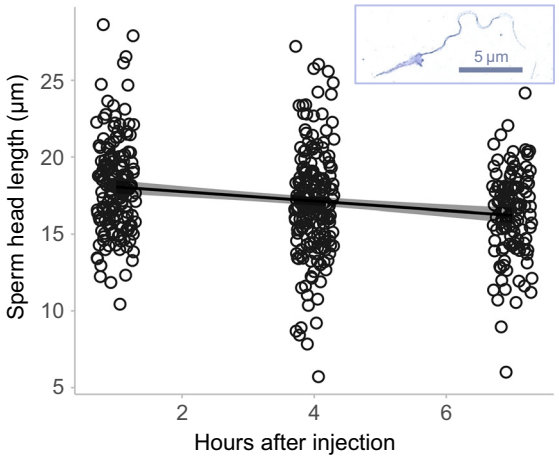

Fig. 2. Length of individual sperm head (µm) collected at three different timepoints (1, 4 and 7 h) after injection with 120 IU of hCG. Per sample we measured 30 sperm heads at 400× magnification. Only samples from animals that were injected with hCG were included in this figure because induction was highly repeatable using this method (compared to GnRH-a induction in this species). The points represent individual sperm cells measured within a sample, and the lines represent the smoothed conditional means. The shading around the lines represents standard error. The image embedded into the top right is a sperm cell at 400× magnification. The scale bar represents 5 µm.

Sperm quality assessment

Sperm cell quality was assessed only in hCG treated animals because all animals produced a high number of sperm at each sampling point. Sperm cell motility was unaffected by time (GLMM: $P = 0.188$; Table S1); sperm samples across all collection timepoints had a median motility of 25.37% (IQR: 2.21%) following injection with hCG.

Sperm cell head length significantly decreased with time after hCG injection (LMM: $P < 0.001$; Table S1; Fig. 2). Sperm cells collected 7 h post injection were 11.0% shorter in length than sperm cells collected 1 h post injection; the mean ± s.d. of sperm head length in samples collected 1 h following injection was 18.1 ± 3.0 µm, and 16.3 ± 2.8 µm in samples collected 7 h following injection ($d = 0.62$). There was no effect of time of sample collection on sperm tail length (LMM: $P < 0.001$; Table S1): the average sperm tail length was 34.06 ± 7.22 µm across all samples collected from frogs injected with hCG (Fig. 2).

Discussion

Sperm concentration and quality

We found that induction using exogenous hormones can successfully promote spermiation in the critically endangered *L. v. alpina*. We found that a 120 IU dose of hCG has high success at inducing spermiation, with all individuals producing a high quantity of sperm cells at 1, 4 and 7 h following injection. In contrast, we found that a 1.5 µg dose of GnRH-a was not a reliable method for inducing spermiation. Only 29% of males produced sperm cells, and the sperm quantity was substantially lower than in animals induced using hCG.

Sperm cell quantity and concentration decreased at each collection point following induction using both GnRH-a and hCG. We found that the peak of sperm cell quantity was 1 h after injection. However, in other species, including others within the *Litoria* genus, the optimum collection time is between 2 and 5 h after injection (Silla and Roberts 2012; Clulow *et al.* 2018; Silla *et al.* 2019). Peak sperm concentrations and total sperm cells recovered in *L. v. alpina* were higher than in other species: approximately 15% higher sperm concentration than *Bufo baxteri* at peak spermiation (Browne *et al.* 2006), 3× higher concentration than

Atelopus zeteki (Della Togna *et al.* 2017), and 4–250× more sperm cells collected over the collection period than other Australian species (Silla and Roberts 2012; Silla *et al.* 2019). In fact, the number of sperm released 1 h following induction using GnRH-a is consistent with total sperm production in other Australian amphibian species (Silla and Roberts 2012), although this method is not as reliable in *L. v. alpina* compared to other Australian species. The differences between our results and results from other species indicates that sperm induction patterns are species specific; therefore, troubleshooting spermiation techniques are imperative before designing an experiment.

Using hCG induction of spermiation we found that while sperm motility and sperm tail length remained consistent across timepoints, sperm head length decreased with time. While time after hormone induction often affects sperm concentration, the quality of the sperm is typically unaffected by collection timepoint (Obringer *et al.* 2000; Silla and Roberts 2012; Uteshev *et al.* 2012; Della Togna *et al.* 2017; Langhorne *et al.* 2021). However, in this study we found a decrease in sperm head length over time, which might indicate decreasing fitness of the sperm. The capsule of the sperm head plays a critical role in penetrating the jelly capsule of the amphibian egg, and a longer sperm head length might have a penetrative advantage (Byrne *et al.* 2003). Smaller sperm head size can be a result of decreasing fertility or sperm quality (Guo *et al.* 2018), and multiple sperm releases can lead to a decrease in sperm quality with each release (Cornwallis and Birkhead 2007). It is possible that the high-quality sperm were depleted within the peak of sperm production following hormonal induction, and the sperm released later were of lower quality or contained a higher proportion of immature sperm (Villaverde-Morcillo *et al.* 2017; Arregui *et al.* 2020). Repeated mating events are known to deplete sperm stores (Rubolini *et al.* 2007; Hettyey *et al.* 2009), and reduce sperm quality (Mayorga-Torres *et al.* 2016). If sperm quality reduces after the peak of spermiation following exogenous hormone induction in *L. v. alpina*, it will be critical to collect sperm during the peak time of release to ensure success in captive breeding.

Overall motility in this species was low, with a median motility across collection points at approximately 25%. While this proportion of motile sperm cells is low compared to other amphibian species such as *Bufo baxteri* with 95% sperm motility, *Atelopus zeteki* with >86% motility, *Litoria booroolongensis* with 58–62% motility and *Rana* [*Lithobates*] *sevosa* with 49% motility following hormonal induction of spermiation (Browne *et al.* 2006; Della Togna *et al.* 2017; Silla *et al.* 2019; Hinkson and Poo 2020). However, there are some species with lower reported motility, such as 10.5% motility in *Dendrobates auratus* (Lipke *et al.* 2009). Sperm motility in some amphibian species declines rapidly over time (Wolf and Hedrick 1971; Browne *et al.* 2015) and the low motility observed here might be due to the time between collection and assessment. The decline in sperm motility over time for *L. v. alpina* is unknown, but we made sure to assess the sperm motility within 15 min of sample collection. However, the low motility observed here across samples might not affect fertility; motility might not be as important for external fertilisers as it is for species that reproduce using internal fertilisation (Dziminski *et al.* 2010).

Hormone induction methods

Our findings that hCG induction of spermiation was more efficient than using GnRH-a was interesting and unexpected. In many other species, GnRH-a induction has higher consistency and success in spermiation, where males have a stronger response to Lucrin compared to Chorulon (Byrne and Silla 2010; Silla and Roberts 2012). It is possible that the low efficiency of GnRH-a induction was due to the incorrect dosage, even though our dosages were within the range that were successful in other species (Della Togna *et al.* 2017). We did not assess dose efficiency in this study, and because optimal dosages vary among species, more work will need to be conducted to determine the optimal dose of either hormone for this species (Clulow *et al.* 2018; Silla *et al.* 2019).

GnRH-a inductions often have a latency period and stimulate gamete production over a longer time frame than hCG induction (Zohar and Mylonas 2001). Yet, we found that GnRH-a induction led to a fast response as well as a sharp decline in the quantity of sperm produced over time compared to hCG induction. The poor response to GnRH-a might be because the GnRH-a analogue in Lucrin is not an ideal match to the GnRH sub-types found in *L. v. alpina*. There are over 20 subtypes of GnRH molecules found across vertebrate species, and for most amphibians the GnRH molecules have not been directly characterised (Clulow *et al.* 2018). Gamete induction using GnRH-a has been suggested as a more favourable and balanced simulation of reproduction than hCG because it acts on a higher level in the hypothalamic–pituitary–gonadal axis (Zohar and Mylonas 2001; Silla and Roberts 2012). Furthermore, effective induction using GnRH-a is often sustained for a longer period of time (Zohar and Mylonas 2001), which can be ideal for species that are difficult to breed in captivity. Therefore, it would be useful to explore the efficacy of different GnRH-a molecules in *L. v. alpina* as well as assessing appropriate dosages to determine the optimal method of hormonal induction of spermination.

Conclusions

Assisted reproductive technologies are critical to the success of many captive colonies of endangered amphibian species. Assisted reproductive technologies can reduce the need for specific environmental stimuli and help maintain genetic diversity within the population by ensuring most

individuals are able to breed. However, when developing optimum protocols for the husbandry and maintenance of critically endangered species, finding the appropriate sample size for robust testing of procedures can be a challenge. This study serves as a valuable starting point for the critically endangered *L. v. alpina* and can aid in the conservation efforts for this species. While our samples sizes were small, we were able to optimise the use of few animals to effectively compare two methods of hormonal induction of spermiation. We used a carefully planned study design and statistical analyses to account for the potential confounding effects of repeated measures on this small number of individuals.

Based on the information presented in this study, we recommend that sperm collection occur within the 1 h after hormonal induction in *L. v. alpina* to optimise sperm quantity and quality. The high success in sperm production with the use of hCG is promising, while more fundamental work is needed before we can suggest a GnRH-a analogue recommendation. Our results highlight the species-specific differences in the effectiveness of assisted reproductive technologies among amphibians. Prior to adopting an exogenous induction protocol for a novel species, we recommend troubleshooting the protocol to ensure success in collecting spermic urine.

Supplementary material

Supplementary material is available online.

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