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Artificial reproduction using leuprolide acetate in the frog *Rana pipiens*

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Procuring offspring from captive animals can be important for research and conservation efforts. Yet, reliable methods for obtaining fertilised eggs are unavailable for many species. In this study, we examined the efficacy of one drug, leuprolide acetate, to induce reproduction (i.e. egg production, tadpole hatching) in the northern leopard frog, Rana pipiens. We found that leuprolide acetate successfully induced breeding and larval development in animals that were overwintered in the lab, but not in animals caught during the breeding season. These results indicate that leuprolide acetate can be successful in inducing breeding and fertilisation of frog eggs, but that its effectiveness might be contingent upon length of time in captivity and the animals having undergone an artificial overwintering period in the laboratory prior to induction. Artificial breeding success is species and context dependent; therefore, identifying additional methods effective across taxa will help support species in need of management intervention.

Keywords: Amphibian; Amphibian reproduction; Assisted reproductive technologies; Breeding; Hormone induction; Leuprolide Acetate; Spawning

INTRODUCTION

Assisted reproductive techniques can play an important role in the management of declining or endangered species. Developing and refining protocols for captive breeding can facilitate basic wildlife research, enable effective management of captive populations, and improve reintroduction and management outcomes (Wildt, 2000, Andrabi & Maxwell, 2007). Amphibians are currently experiencing dramatic global declines in biodiversity (Skerratt et al., 2007). Because many species of amphibian do not breed easily in captivity, assisted reproduction methods are being developed to aid in the conservation of some declining species (Kouba et al., 2012, Clulow et al., 2014). Hormone treatment is often required to induce reproduction in captive animals, and although a number of drug treatments have been shown to be effective in inducing fertilisation, their effectiveness can be highly species and environment dependent (Trudeau et al., 2010, Silla & Roberts, 2012).

In vertebrates, ovulation and sperm production requires luteinizing hormone, and gonadotropin-releasing hormone (GnRH) is the primary stimulator of luteinizing hormone production. Artificial reproduction treatments often use GnRH or luteinizing hormone analogues to promote ovulation and sperm release (Clulow et al., 2014, 2018). A luteinizing hormone-like compound called human chorionic gonadotropin (hCG) can be effective in some frog species, but this hormone has been much more variable in its ability to induce ovulation and/or sperm production in

other amphibians (Mann et al., 2010, Clulow et al., 2018). Another commonly used induction method involves injection of a combination of a GnRH-agonist compound and a specific catecholamine dopamine receptor, both of which have been designed to block the inhibitory effects of dopamine that enables the release of gametes in vertebrates. One catecholamine dopamine receptor that has been effective in inducing reproduction in a number of frog species is metoclopramide hydrochloride (Trudeau et al., 2010). However, GnRH agonist compounds alone, without a coupled specific catecholamine dopamine receptor, have also been successful in inducing reproduction (Vu et al., 2017). Leuprolide acetate is a GnRH agonist that has been used successfully to induce reproduction in a number of frog species (Byrne & Silla, 2010; Trumbo, 2015, Clulow et al., 2018). Leuprolide acetate is the medical compound in the drugs Lupron (Tap Pharmaceuticals) and Lucrin (Abbott), which are used in treating prostate cancer and endometriosis, and for in vitro fertilisation in humans (Chetkowski et al., 1989; Dlugi et al., 1990; Persad, 2002).

The purpose of this study was to trial the use of leuprolide acetate as a method of inducing reproduction in *Rana pipiens*, a species that is often maintained in captivity for research and educational purposes (e.g. teaching dissections). Captive animals used in scientific experiments and for education are often wild caught, even if purchased through a supplier (with large scale suppliers shipping animals across the United States). Developing alternative methods for captive breeding would reduce stress on wild populations, and promote

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the establishment of fully captive colonies for science and educational purposes. Further, *R. pipiens* populations are declining in parts of their native range. Although some populations are stable, captive rearing and reintroduction efforts are employed in areas where populations have declined, with active reintroductions for 35 years, and almost 8,000 animals released per year (Randall et al., 2016). In captive *R. pipiens*, hormone inductions are required, because males and females will not successfully mate without intervention (Trudeau et al., 2010; Vu et al., 2017). For *R. pipiens*, the combination of a GnRH agonist plus metoclopramide hydrochloride, as well GnRH agonist alone, have been shown to induce reproduction with a fertilisation rate of between 70 and 90 %; however, success often varies with the timing of induction and can also depend upon captive conditions prior to induction (Trudeau et al., 2010; Vu et al., 2017). Therefore, in this study we, 1) tested an alternative drug, leuprolide acetate, which has been trialled in many other amphibian species (reviewed in Clulow et al., 2018), but not in *R. pipiens*, and 2) compared the drug's ability to successfully induce reproduction and lead to the hatching of tadpoles in animals that were captured from the wild and housed under different conditions. Wild individuals were caught during the breeding season (in the early spring upon ice melt following hibernation) from three different populations, maintained in captivity for a short period, and injected with leuprolide acetate to induce reproduction at the same time. Next, we tested the efficacy of housing animals in captivity for an extended period of time, artificially overwintering them, and inducing reproduction after emergence from overwintering. The implications of this trial, specifically with using animals from different populations, are to determine 1) if and how animals from different populations and different natural timing of reproduction can be induced at the same time, and 2) to determine if leuprolide acetate is effective at inducing reproduction in *R. pipiens*.

METHODS

Animal Husbandry
The animals were wild-captured by hand during the early spring breeding season just following their natural hibernation in 2017 from three locations: Crosby Township, Ohio (February 24: one female, four males), Linesville, Pennsylvania (March 26 & 28: five females, four males) and Milton, Vermont (May 17: three females, five males). A second group of wild caught animals were collected in the autumn of 2017 from Shelburn, Vermont (October 14, 21 & 22: four females) and Linesville, Pennsylvania (October 11: three females, six males).

After collection and before hormone treatment, animals were housed in the laboratory in individual enclosures (36 x 21 x 25 cm) containing 1 L aged tap water and tilted to provide both aquatic and terrestrial habitats. A hide made of PVC pipe cut in half length wise (8 cm long x 3 cm high) was placed in each enclosure. When not breeding, *R. pipiens* tends to spend more time in the terrestrial environment, and 1L or less per individual is sufficient (Boice & Williams, 1971; Tennessen et al., 2009). Animals were fed vitamin-dusted crickets ad libitum and

water was changed every five days. The animals were maintained at room temperature (18 to 23 °C) with a light:dark cycle of 14:10 h, and monitored daily for general health.

Breeding Mesocosms
Mesocosms (Rubbermaid stock tanks, 189 L capacity) were set up indoors, filled with 70 L of aged tap water, covered with screen and provisioned with a bundle of sticks (for attachment of egg masses). The mesocosms had a step on the inside, which the animals used as a terrestrial habitat, and a floating plastic platform (25 x 17 cm) was also provided. The air temperature was maintained at 16 to 18 °C, which is within the range of temperatures for *R. pipiens* breeding in nature (Gilbert et al., 1994; Trudeau et al., 2010). Half water changes were performed every 10 days, and animals in the mesocosms were fed adult crickets ad libitum every five days. After each trial was complete, animals were returned to their individual enclosures. Number of animals per mesocosm per experiment varied because we wanted to keep populations separate. Mesocosms were checked at least once per day and egg masses were removed as described below.

Hormone Treatment
After acclimation, the animals were treated with leuprolide acetate in the evening, 2 h before sunset. Males and females were injected intraperitoneally using a 26 gauge needle with 0.2 mg of leuprolide acetate (LupronDepot®, Tap Pharmaceuticals), diluted in sterile APBS, for an injection volume of 500 µL per animal (Bowcock et al., 2013; Trumbo, 2015). Twenty-four hours following the first injection, all females were treated a second time with 0.1 mg of leuprolide acetate in APBS for an injection volume of 250 µL per female. The leuprolide acetate stock solution was stored at -20 °C between trials. After injection animals were checked daily, and the egg masses they produced were transferred to new containers (36 x 21 x 25 cm), filled with 5 L aged tap water, and monitored for development for at least seven days. Half water changes were conducted every five days. Egg mass success was determined by observed egg hatching. Animals remained in the breeding mesocosms for seven days.

No hormone negative controls were included in this study because in *R. pipiens*, it is generally understood that under captive conditions animals will not successfully mate without a hormone induction (Trudeau et al., 2010,;2013, Vu et al., 2017).

Induction in Animals Caught During the Breeding Season
Animals were captured from the wild during their spring breeding season (February-May), transported to the lab, and maintained in an environmental chamber (Conviron) at 8 °C prior to hormone treatment (Ohio animals for 12 wk, Pennsylvania animals for 9 wk, and Vermont animals for 1 wk). The purpose of the delay for some populations was to induce all animals together, for experimental purposes, such as a common garden experiment of several populations. Animals were warmed slowly to 16 °C over seven days (1.1 °C per day), and placed inside the breeding mesocosms on May 23, 2017. Animals were housed communally by population with a maximum of 6

animals per mesocosm (similar to male to female ratios of Trudeau et al., 2010; Vu et al., 2017): Ohio animals in one mesocosm, Pennsylvania animals in two mesocosms, Vermont in one mesocosm (Supp. Table 1). The animals were allowed to acclimate inside the mesocosms for at least 36 h before hormone treatments began. Animals were then injected with leuprolide acetate, following the protocol described above.

Artificial Overwintering and Induction

Animals collected from Pennsylvania and Vermont were used in this experiment. Some animals were collected in the autumn of 2017 (n = 10), and the others were from the previous experiment (collected in the spring and held in captivity for 10 to 12 mo, n = 16). All animals were housed individually, as described above, prior to mesocosm acclimation and hormone treatment. Animals were overwintered at 4 ˚C for 8 wk (December to February) in the lab, similar to what has been described previously for captive breeding in *R. pipiens* (Trudeau et al., 2010; 2013). For overwintering, animals were placed in an environmental chamber (Conviron) and the air temperature in the chamber was slowly decreased from 18 ˚C to 4 ˚C over a period of nine days (1.5 ˚C / day). During this period, the water was changed once but the animals were no longer fed, with feeding ending 3 days before cooling began. Once the temperature reached 4 ˚C, the animals were moved to smaller enclosures (25 x 17 x 10 cm) filled with 2 L of pre-chilled aged tap water, which was enough to ensure that the animals remained submerged. The animals were kept in the dark, and half water changes were conducted with pre-chilled aged tap water every seven to 10 days (Lillo, 1980). Immediately before water changes, oxygen levels in three haphazardly chosen enclosures were monitored (using a YSI ProODO Handheld Oxygen Meter) to ensure levels remained above 70 % dissolved oxygen, near the optimum pressure for O^2 absorption efficiency of ranid frogs and well above hypoxic conditions (Tattersall & Ultsch, 2008). After 8 wk of overwintering, the air temperature in the environmental chamber was gradually increased from 4 ˚C to 16 ˚C over nine days (1.5 ˚C / day). During this time the animals remained in the same enclosures, but with 0.5 L of water, and with the enclosures tilted to provide aquatic and terrestrial habitats. We began feeding again on day five of this gradual warming period. After the warming period, animals were transferred to breeding mesocosms (with ratio as close as to 1 female and 1 male per mesocosm (e.g. Silla et al., 2018) as possible while maintaining population and collection time separation), and induced with leuprolide acetate following a three day acclimation period, as described above.

Ethics Approval

This research was conducted under the University of Pittsburgh Institutional Animal Care and Use Committee protocol IML-17091291-9 "Effects of climate change on host-pathogen interactions in chytridiomycosis." The animal collection was conducted under permits from Pennsylvania Fish and Boat scientific collection permit number 2017-01-0177, Ohio Division of Wildlife Wild animal permit number 18-149, and Vermont Fish and Wildlife Department scientific collection permit number SR-2016-17.

Analyses

We compared the number of females that produced egg masses that successfully hatched tadpoles to those that produced either no egg mass or an inviable egg mass across two treatment groups using a 2 x 2 Pearson's chi-squared test in SPSS (v21, IBM Corp. Armonk, NY). Egg mass success was operationally defined by the production of tadpoles. Egg masses that did not develop to hatching were considered unsuccessful.

The first analysis compared the production of viable egg masses between the groups of animals that were caught in the spring and induced directly after the breeding season with those animals that were induced after being artificially overwintered. In this analysis we compared all females that produced completely viable or partially viable (if some but not all of the eggs in the egg mass hatched) egg masses to those that produced either a completely inviable egg mass or no egg mass at all.

The second analysis involved only the animals that experienced an artificial overwintering in the lab, and we compared the proportion of females that produced completely viable egg masses (the whole egg mass hatched) between animals captured during the spring (breeding season) or autumn. Population of origin was not accounted for in our statistical analyses due to insufficient sample size.

RESULTS

The results of this experiment demonstrate that *R. pipiens* can be successfully bred in captivity using leuprolide acetate, but only under certain conditions. In the animals caught during their breeding seasons (February, March and April respectively), they appeared reproductively active (n = 21), with all females (n = 9) gravid (in that they visibly looked wider in their midsection than the males) at the time of capture. Following induction, one female (11.1 % of females; Fig. 1a; Supp. Table 1; had been held in captivity for 12 weeks) produced a small, partial clutch, which was partially successful (only some eggs hatched), and no female produced a fully successful egg mass. Some females (n =5) released their eggs over a period of days/weeks during captivity prior to induction (all females collected from Pennsylvania).

In animals that were overwintered in the lab (n =29, 15 females, 14 males), 60.0 % of the females produced egg masses (9/15), and all produced some tadpoles, but 66 % of those (6 of 9 total egg masses) were successful at producing a large proportion of tadpoles (Supp. Table 1). The proportion of females that produced a successful egg mass after hormone treatment was significantly higher when the animals were artificially overwintered in the lab compared with those collected during their spring breeding season (Fig. 1a; Pearson's: n = 24, χ^2_1 = 5.531, P = 0.033).

When considering only animals that were artificially overwintered in the lab prior to hormone treatment, there was no significant difference in the proportion of females producing a viable egg mass between autumn and spring collected animals (Fig. 1b; Pearson's: n = 15, χ^2_1 = 3.616, P = 0.119). 75 % of the females (6/8) that were collected in the spring and overwintered produced an egg mass,
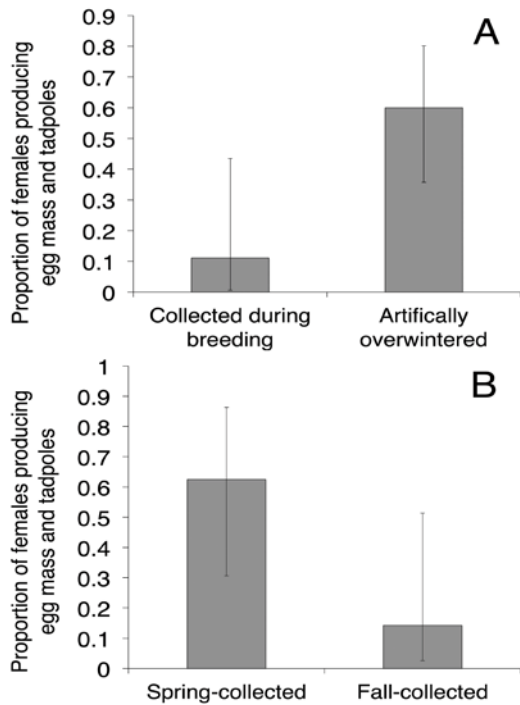
Figure 1. The proportion of females that produced viable egg masses across experiments. Comparisons include A) the proportion of egg masses that were fully or partially viable collected during the breeding season versus those that were artificially overwintered and B) the proportion of fully viable eggs laid from frogs collected in the spring versus autumn and overwintered in the lab. The error bars represent 95 % confidence intervals.

while of the autumn collected and overwintered animals, 43 % (3/7) of the females produced an egg mass. Only one of the egg masses from the autumn-collected animals hatched tadpoles (Supp. Table 1, Fig 1b).

The animals we collected in the spring and held in captivity for nearly one year were large, reproductively mature adults (n = 17; range = 35 to 63 g, mean ± sd = 47.65 ± 9.06 g) at the time of induction; females were gravid after overwintering, and males had visibly defined secondary sexual characteristics that indicate breeding status such as enlarged vocal sacs and darkened nuptial pads. However, most animals collected in the autumn (11 of 13) were small (likely young-of the year from 2017; 11 to 27 g, mean ± sd = 18.73 ± 4.41 g at time of induction). The small females did not appear gravid at the time of induction, but the males had darkened nuptial pads and visible vocal sacs. Two of the females collected in the autumn were large (60 and 64 g) and appeared gravid.

All but two (80 %) of the large adult females that were overwintered (> 35 g at time of induction) produced a full egg mass, and one of those two females produced a handful of viable eggs but not a whole egg mass (see footnote on Supp. Table 1). It should be noted that one small female (19 g at time of induction, collected in the autumn) produced a full egg mass that successfully hatched tadpoles, although the size of the mass was much smaller than those produced by the larger females.

One animal died during overwintering in the lab (a male that was caught in the spring). This male frog survived three weeks after entering overwintering conditions. Another male that was collected in the spring died during the acclimation period inside the breeding mesocosms (two days after entering the breeding mesocosm). The causes of these deaths are unknown.

DISCUSSION

Assisted reproductive techniques can be useful for wildlife conservation, particularly in collaboration with captive breeding programs for declining species (Wildt, 2000; Andrabi & Maxwell, 2007). They can also be useful in basic scientific research for applications that require the production of viable embryos at a specific time. While there are a number of hormone induction methods published for use in amphibian species, no one method works for all species. The use of leuprolide acetate had not been reported previously in *R. pipiens*, but this treatment has been used with success in many other amphibians (Silla & Roberts, 2012; Trumbo, 2015, Clulow et al., 2018). In this study we compared the number of females producing egg masses that develop to tadpoles among groups of animals that had experienced different durations of captivity prior to treatment with leuprolide acetate. We were unsuccessful in procuring viable egg masses from animals collected during their spring breeding season (when females were naturally gravid) and maintained for a period of one to 12 weeks in the lab, but achieved higher success in induction of animals after they had been in captivity for an extended period of time.

We had little success in procuring tadpoles from animals we collected during their breeding season. Interestingly, other published accounts describe higher success rates for same-season induction using other hormone treatments. *Rana pipiens* have previously been collected upon emergence from their overwintering habitat, and maintained under laboratory conditions for up to one month prior to induction using a GnRH-agonist compound and a specific catecholamine dopamine receptor antagonist, with high reproduction success after induction, between 70 and 90 % fertilisation success (Trudeau et al., 2010; Vu et al., 2017). One of the aims of this study was to investigate if induction could be delayed in animals caught during their breeding season so that individuals from different populations could be induced together, a useful tool for certain experimental designs. Our study demonstrates that delayed induction using leuprolide acetate was unsuccessful if animals were collected and induced while naturally gravid.

The failure for induced breeding in the spring-collected animals could be due to the extended stay in captivity prior to hormonal induction. In two of the three populations tested we housed the animals in captivity for up to eight weeks longer than previously published accounts (Trudeau et al., 2010; Vu et al., 2017), and the females had been gravid for much longer than they would have been in nature in those populations (Zenisek, 1963). Some of the gravid females we collected began to deposit eggs in their individual enclosures prior to hormone induction treatment. When a healthy gravid female is unable to successfully mate, she might either arrest egg development or reabsorb the eggs (Kouba et al., 2012).

Perhaps these females' eggs had become too developed to be successfully arrested or reabsorbed, causing the females to release them in order to avoid becoming egg-bound, which can be fatal (Kouba et al., 2012). Perhaps these animals would be successful at producing offspring if they had been induced earlier, rather than waiting for the last population to emerge for breeding. However, it should be noted that none of the animals from the last population we collected (Vermont) successfully produced egg masses during induction, and these animals were induced within two weeks of capture, which is similar to other previously conducted studies (Trudeau et al., 2010; Vu et al., 2017). One possible explanation for this failed induction attempt is that these animals might have appeared gravid, but might not have been gravid enough to reproduce. We did not perform an ultrasound to verify the gravid status of the females (Calatayud et al., 2018; Graham et al., 2018), but because they were captured during active aggregate breeding it was assumed that females were present in the pond for the purpose of laying eggs.

Further, the male to female ratio within the mesocosms could have limited success of induction, as the only mesocosm with one female to four males was the one that laid a partial egg mass. We had tried to follow the ratio of males to females as had been successful for this species previously (Trudeau et al., 2010; Vu et al., 2017), but it is possible that fewer females to a mesocosm or a higher male to female ratio might have improved success. While the sample sizes are small within this experiment, our results indicate that animals collected in the spring and induced with leuprolide acetate soon after are less likely to successfully reproduce. To limit the effects of sex specific competition, we reduced the density for our next induction trial.

After our attempts to breed spring-collected animals failed, we maintained those animals in captivity and captured more animals to test for an effect of collection time and overwintering on induced breeding success. We were more successful in obtaining egg masses that hatched tadpoles from animals that had been artificially overwintered in captivity prior to hormone treatment than from animals that had been collected during the breeding season, and animals from both populations (Pennsylvania & Vermont) successfully produced tadpoles (Supp. Table 1). Nine egg masses were produced by animals that were overwintered in the lab (60 % of the females produced an egg mass), and 66 % of those egg masses produced a large proportion of tadpoles. Production of egg masses that are only partially successful at hatching tadpoles is a common problem in captive breeding colonies. Failed development/fertilisation of some or all eggs in a clutch can be caused by a number of factors, including poor adult nutrition, lack of environmental stimuli, and stress associated with captivity (Kouba et al., 2012). Captive animals are often less reproductively successful than their wild counterparts and some reproductive failure is to be expected (Kouba et al., 2012).

Some of our autumn-collected animals were likely young of the year, which might explain why we saw fewer egg masses produced following our induction with leuprolide acetate than other trials have reported for induction in *R. pipiens* (Trudeau et al., 2010; Vu et al., 2017). In those young animals we found that the male *R. pipiens* exhibit all the signs of sexual maturity (i.e., have dark thumbs, large forearms and vocal sacs). However, female *R. pipiens* do not typically breed until their second year of life (Gilbert et al., 1994). In our study we found that one small female (19 g) produced an egg mass that successfully hatched tadpoles, indicating that some females might become sexually mature in their first year of life. Perhaps in an effort to optimise breeding success (both egg size and number of eggs), *R. pipiens* females wait until they reach a larger size to begin breeding (Tavecchia et al., 2001). Female size is correlated with reproductive success (i.e. number of viable offspring) in a wide range of taxa, including frogs (Tejedo, 1992; Jorgenson et al., 1993). Artificial reproduction of wild caught animals housed under captive conditions is important for a wide variety of scientific pursuits. *Rana pipiens* is a species that is declining in parts of its range, and therefore, artificial reproduction in this species might be important for conservation purposes (Randall et al., 2016). Trialling a new induction method for *R. pipiens*, we found that adults maintained in captivity and artificially overwintered prior to leuprolide acetate treatment were more successful in producing offspring compared to those caught during the breeding season, held in captivity and induced soon after. Using this combination of leuprolide acetate and captive management, we were able to stimulate reproduction in the majority of females, and the majority of the egg masses they produced developed to hatching. While there are other hormone therapies that have reported success in inducing reproduction in amphibians, it is important to explore multiple methods, because as our study shows, timing of induction and captive conditions might affect success. Although our study trialled hormonal induction procedures only in *R. pipiens*, the success of leuprolide acetate in this and other species (Silla & Roberts, 2012; Clulow et al., 2018) indicates that use of this hormonal induction method could be beneficial to other amphibian taxa in need of artificial reproductive technologies for conservation intervention as well.

ACKNOWLEDGEMENTS

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