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A hormone priming regimen and hibernation affect oviposition in the boreal toad (*Anaxyrus boreas boreas*)

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ABSTRACT

Declines of the southern Rocky Mountain population of boreal toad *(Anaxyrus boreas boreas)* have led to the establishment of a captive assurance population and reintroduction program, in an attempt to preserve and propagate this geographically isolated population. One of the unique adaptations of this species is its ability to survive in cold environments by undergoing long periods of hibernation. In captivity, hibernation can be avoided altogether, decreasing morbidity caused by compromised immune systems. However, it is not entirely clear how essential hibernation is to reproductive success. In this study, the effects of hibernation versus nonhibernation, and exogenous hormones on oviposition, were examined in boreal toad females in the absence of males. In the summers of 2011 and 2012, 20 females housed at Mississippi State University were treated with a double priming dose of hCG and various ovulatory doses of hCG and LH-releasing hormone analog but denied hibernation. Exogenous hormones, in the absence of hibernation, could not induce oviposition over two breeding seasons (2011–2012). In contrast, during the summer of 2012 and 2013, 17 of 22 females (77%) housed at the Native Aquatic Species Restoration Facility (Alamosa, CO, USA) oviposited after they were treated with two priming doses of hCG (3.7 IU/g each) and a single ovulation dose of hCG (13.5 IU/g) and LH-releasing hormone analog (0.4 μg/g) after hibernation. There was a significant difference in oviposition between females that were hibernated and received hormones (2012, $P < 0.05$ and 2013, $P < 0.01$) compared to hibernated control females. In 2013, 12 of 16 remaining Mississippi State University females from the same group used in 2011 and 2012 were hibernated for 1, 3, and 6 months, respectively and then treated with the same hormone regimen administered to females at the Native Aquatic Species Restoration Facility. Together, hibernation and hormone treatments significantly increased oviposition ($P < 0.05$), with 33% of females ovipositing. These results suggest that (1) hibernation is a key factor influencing oviposition that cannot be exclusively circumvented by exogenous hormones; (2) females do not require the presence of a male to oviposit after hormone treatments; and (3) longer hibernation periods are not beneficial for oviposition. The hormonal induction of oviposition in the absence of males and shorter hibernation periods could have important captive management implications for the boreal toad. Furthermore,

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the production of viable offspring by IVF where natural mating is limited could become an important tool for genetic management of this boreal toad captive population.

1. Introduction

The southern Rocky Mountain population of boreal toad *(Anaxyrus boreas boreas*) is a geographically isolated population within the eastern clade of the boreal toad (*Anaxyrus boreas*) and is limited in distribution to high elevations of montane wetland in Colorado, New Mexico, Utah, and southeastern Wyoming [1–5]. The current conservation status of this species is endangered in the state of Colorado and New Mexico and is listed as near threatened by the International Union of Conservation of Nature (IUCN) [6,7]. Significant declines in the southern Rocky Mountain boreal toad population led to the establishment of a captive assurance colony at the Colorado Parks and Wildlife's Native Aquatic Species Restoration Facility (NASRF) in Alamosa, CO, USA [8]. More than 100,000 tadpoles have been reintroduced into the wild over the past 10 years (T. Smith, B.S., pers. comm., 2013).

Survival and hatching rates of egg clutches produced by captive boreal toads are low and variable when compared to egg clutches collected in the wild and raised in captivity (T. Smith, B.S., pers. comm., 2013). For example, in 2012, the yearly reproductive output (number of surviving tadpoles) of this captive population was estimated to be less than or equal to 5%, with either limited or no natural breeding occurring (T. Smith, B.S., pers. comm., 2013). There are many challenges in maintaining a sustainable population of boreal toads in captivity, including the long generation interval to reach sexual maturity (females, 6–7 years; males, 3–4 years), a potential obligate hibernation period during the winter months [6,9], and poorly understood environmental cues these animals require to initiate reproductive behaviors.

Assisted reproductive techniques, such as exogenous hormone administration to induce gamete release and IVF can also be used to increase reproduction and genetic diversity in the absence of natural breeding [8]. Administration of exogenous hormones can stimulate gamete maturation and release in captive amphibians when these processes will not occur naturally [10–20]. Human chorionic gonadotropin (hCG) and an LH-releasing hormone analog (LHRH) are commonly used to induce oviposition in a number of female amphibian species including the boreal toad, *Anaxyrus boreas boreas* [20]; Wyoming toad, *Anaxyrus baxteri* [15]; Bullfrog, *Rana catesbeiana* [21]; American toad, *Anaxyrus americanus* [11]; grass frog, *Limnodynastes tasmaniensis* [12]; coqui, *Eleutherodactylus coqui* [13]; Xenopus, *Xenopus laevis* [22]; and the Günther's toadlet, *Pseudophryne guentheri* [18].

Unlike northern leopard frogs (*Rana pipiens*) and chorus frogs (*Pseudacris triseriata maculata*), boreal toads cannot tolerate freezing and do not undergo true hibernation [6]. Despite lowered metabolic functions of respiration and behavior, temperate species such as *Anaxyrus bufo* can still show periods of activity during the winter months [23,24]. Similarly, artificially hibernated boreal toads also show some level of activity, moving within the hibernacula (albeit slowly), retaining water, fasting, and occasionally defecating (pers. observ). Hibernation not only provides a survival mechanism for winter, it is also linked to processes that occur during the active parts of the year, such as reproduction [23,25–27]. Early studies by Jorgensen [25] and Pinder et al. [24] showed that, in several anuran species, individuals denied hibernation undergo continuous vitellogenic development of oocytes that become atretic and are never deposited. In contrast, there is some evidence that sexual maturation is somewhat malleable under laboratory conditions: In captive *Bufo bufo*, individuals denied hibernation in the first year of life attained sexual maturity at a younger age and had slower growth rates compared to their hibernated counterparts [27].

Roth et al. [20] reported that hCG and LHRH can elicit oviposition in both hibernated and nonhibernated boreal toads in conjunction with amplexus. Although more nonhibernated females oviposited than hibernated females, fertilization rates in the study remained low. Therefore, it remains unclear how important hibernation is to gamete release and quality in this species. Initially, Roth et al. [20] injected animals with a single dose of LHRH. In some cases where females failed to oviposit after a single injection, eggs were obtained after a second dose of LHRH administered 48 hours later [20]. Priming with exogenous hormones can induce oviposition in a number of amphibian species [14,15,18]. In the Wyoming toad, oviposition can be induced by an hCG priming regimen followed by a combination dose of hCG and LHRH [15]. In the Günther's toadlet, a priming regimen of LHRH can also induce oviposition [18].

Despite the apparent importance of hibernation to amphibian physiological function, mortality during this period can be significant [28]. Low temperatures can compromise ectothermic immune systems, making individuals more susceptible to disease [28]. Morbidity is particularly undesirable when conserving endangered species and has often raised the question: Is it better to hibernate animals or can this adaptation be overridden using exogenous hormones?

The purpose of our study was to (1) compare hormone-induced oviposition (oviposition without amplexus) in female boreal toads, either with or without hibernation; and (2) evaluate a priming regimen of hCG for oocyte maturation and a combination dose of hCG and LHRH for ovulation and oviposition. Parameters measured included the number of animals ovipositing, time taken to oviposit, and the effect of weight and hibernation on reproductive success. Because the relationship between hormone priming, hibernation, and gamete production is unknown, this study will help answer questions about how these factors can be manipulated to maximize reproductive success in the boreal toad.

2. Materials and methods

2.1. Animals and housing

2.1.1. Native Aquatic Species Restoration Facility, Alamosa, CO, USA

Male and female boreal toads held at NASRF (number of individuals in 2012; 769 and 2013, 707) were housed together in populations according to the region from which the original founders were obtained. Only sexually mature females (8–12 years, n = 45) were included in our studies (2012–2013), ranging in weight from 30 to 84.3 g. For the breeding season, approximately 10 to 20 toads were kept in rectangular fiberglass tanks (121 × 60 × 30 cm) with free flowing groundwater and tilted at a 20° angle to allow constant drainage. The water temperature in tanks during the breeding season ranged from 15 °C to 18.3 °C. Tank lighting consisted of a single 60-W Daylight Blue Reptile Bulb (Cat# 232288; Drs. Foster and Smith, WI, USA), a single ReptiSun 5.0 UVB linear fluorescent lamp (Cat# 82899; Drs. Foster and Smith), and a photosensor to provide UVA, UVB, and light and dark cycles that mimicked the natural photoperiod (Alamosa, Colorado, 37.4667° N, 105.8667° W). Food items offered included waxworms, red wiggler worms, mealworms, and crickets (three times per week). Crickets were dusted with ReptiVite powder (Zoo Med Laboratories, Inc., Costa Mesa, CA, USA). Crickets and mealworms were gut loaded with Bug Burger (Allen's Repashy, La Jolla, CA, USA), and red wigglers were gut loaded with fruit and vegetables before feeding. Native Aquatic Species Restoration Facility toads were hibernated between December and May in plastic boxes (33 × 13 × 15 cm) lined with a layer of activated carbon, moistened sand (3.81-cm deep), and moistened sphagnum moss. Toads were hibernated in groups (3–8/box) representing specific populations, and temperatures were maintained between 2 °C and 6 °C in an EcoPro G2 1350 Liter Upright Refrigerated Cabinet (Foster Refrigerator Corp., Hudson, NY, USA).

Tadpoles were held in tanks (121 × 60 × 30 cm) fitted with continuous running water and fed a diet of VetOne Vitamin B complex HP (cat# 510216; 1 mL/gallon; VetOne, Boise, ID, USA) daily, Hikari Tropical Algae Wafers (Hikari Co., Ltd, Himeji, Japan) two to three times per day (feeding was adjusted according to the previous days consumption), Mazuri Amphibian and Carnivorous Reptile Gel (PMI Nutrition International LLC's, St. Louis, MO, USA) diet (2–3 times daily), and Repashy Soilent Green tadpole mix (Allen's Repashy, La Jolla, CA, USA).

2.1.2. Mississippi State University Amphibian Conservation Lab, Starkville, MS, USA

Fifty-four boreal toads (34 males and 20 females) were housed in same sex groups of four to five individuals in plastic polycarbonate containers (46 × 66 × 30 cm; Habitat Systems Limited [Des Moines, IA, USA]) with a constant water supply. Toads were offered various prey items including waxworms, red wiggler worms, mealworms, and crickets (three times per week). Crickets were dusted with ReptiVite powder (Zoo Med Laboratories, Inc.) and waxworms and mealworms were gut loaded with fruit and vegetables before feeding as well as a powdered combination of algae wafers, spirulina, dried fish flakes, and dried river shrimp. Toads were kept on a natural light cycle (Starkville, MS, USA; 33.4625° N, 88.8200° W), and temperatures were maintained between 20 °C and 23 °C and 49% to 55% humidity throughout the year. Mississippi State University (MSU) toads were not hibernated for the first two consecutive seasons (winter 2011 and 2012). Starting in December 2012, male and female toads were hibernated in individual hibernacula, which consisted of a plastic box (18 × 32 × 11 cm) containing a combination of moistened soil, sand, and peat moss substrate (four females per tub per group). Toads were fasted 2 weeks before hibernation and were acclimated to a reduction in temperature by slow cooling in a refrigerator at 8 °C for 1 week and then transferred to a refrigerator set at 4 °C. Humidity and temperature inside the refrigerator were monitored using an electronic thermostat (AcuRite, 00611RX; Chaney Instruments, Co., Lake Geneva, WI, USA), and females were checked daily and sprayed with aged tap water every 4 to 5 days to ensure they remained hydrated. Animal management and research studies reported here were reviewed and approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC # 10-082).

2.2. Hormones

Two exogenous hormones were used during this study: an LHRH analog ([des-Gly10], D-Ala6 ethylamide acetate, cat#: L4513; Sigma–Aldrich, St. Louis, MO, USA) and hCG (cat# C1063; Sigma–Aldrich). The powdered hormones were dissolved in sterile PBS (1X PBS) to the appropriate concentrations. Priming and ovulatory doses are described in the following. All doses were administered as gram per body weight (g/BW). Hormone stocks were stored at −20 °C and thawed on the day of use. All hormone injections were administered by intraperitoneal injection using a 27-gauge needle. The injections, two priming doses of hCG and one ovulatory dose of hCG + LHRH, were administered over a series of days, as described in the following, for each experiment. Control animals received injections of PBS alone in a similar volume.

2.3. Experiment 1: Testing of various hormone protocols for oviposition in nonhibernated female toads

This experiment was designed to test if (1) boreal toad females that had not been hibernated could be stimulated to ovulate and oviposit eggs after exogenous hormone stimulation; and (2) determine which of four different hormone regimens proved more effective at inducing oviposition. In 2011, females were randomly divided into four treatment groups (five/treatment) and injected with PBS 2 weeks before hormone administration, such that each animal served as its own control. Once the control test was completed, the females were all administered the same priming regimen, which included administration of 3.7 IU/g hCG (time 0), followed by a second priming hormone dose 96 hours later at the same concentration (3.7 IU/g hCG). Twenty-four hours after the last priming dose, female toads received an ovulatory dose of hCG and LHRH that was administered as follows: group 1: 13.5 IU/g hCG + 0.4 μg/g

LHRH; group 2: 22.5 IU/g hCG + 0.4 μg/g; group 3: 13.5 IU/g hCG + 0.9 μg/g; and group 4: 22.5 IU/g hCG + 0.9 μg/g. The hCG hormone concentrations were derived by calculating the average weight of the females of the colony (37 g) and calculating a dose per body weight equivalent to 500-IU hCG and 830-IU hCG for a 37-g toad. The 500-IU hCG dose was chosen from previous work conducted on the Wyoming toad [13]. The higher dose of hCG was a 1.6-fold increase over the lower dose and was what we deemed a safe level for administration. The concentration of LHRH used was derived from doses previously tested and found safe on the boreal toad at NASRF, where toads were injected with concentrations of 15 and 40 μg per animal (Kevin Thompson, M.S., pers. comm., 2011). The LHRH concentrations divided by average weights of 37 g were converted to a μg/g measure, resulting in 0.4 μg/g and 0.9 μg/g. During early spring and summer (March–July), this experiment was replicated twice, with the first 20 females randomly administered hormones from the four treatment groups mentioned previously. Because all females failed to respond to the treatment, a second replicate was conducted. The second series of treatments were given to each female 5 months (±7 days) after the first treatments, between August and December. During hormone experiments, females were individually housed in small plastic tubs (33 × 13 × 15 cm) containing water and were observed for signs of oviposition for up to 120 hours. All females were treated in the absence of males. If eggs failed to appear during the 120-hour period, animals were returned to their permanent housing and monitored daily for another 144 hours. In the absence of eggs, the experiment was terminated and the animal categorized as nonresponsive. Data collected included weight, time to egg laying, duration of egg laying, and number of eggs oviposited. Because of a lack of oviposition in the two replicates during 2011, experiment 1 was repeated in 2012 using the same hCG priming hormone concentrations as described previously and the ovulatory concentration provided for treatment 1 (13.5 IU/g hCG + 0.4 μg/μL LHRH). A modification in the timing of the hormone administration was incorporated into this third hormone trial with the first priming at time 0, second priming 72 hours later, and the ovulatory dose 24 hours after the last priming dose (96 hours). Data were collected as previously mentioned for any females that may have oviposited.

2.4. Experiment 2: Effect of hibernation and hormone priming on oviposition in boreal toads

Experiment 2 was designed to test whether hibernated female boreal toads could be stimulated to oviposit eggs using the protocol described for group 1 in Section 2.3. All the boreal female toads in this experiment were housed at NASRF (Alamosa, CO, USA) and were hibernated for 6 months (December 2011–May 2012) before hormone treatment. At the end of hibernation, toads were gently warmed by bringing the temperature of the refrigerators up from 4 °C to 8 °C for an acclimation period of 1 week before being returned to summer housing tanks (15 °C–18 °C).

In 2012, female boreal toads (seven treatment and eight control) at NASRF were administered a set of priming and oviposition hormone doses as described in Section 2.3 using the following timeline: first priming at time 0, second priming 72 hours later, and the ovulatory dose 24 hours after the last priming dose (96 hours). The initial priming injection began within 72 hours of the toads' return to summer tanks. After the final ovulatory dose, females were monitored for oviposition for up to 10 days. Data collected included weight (g), time to oviposition (h), and the number of eggs oviposited. A second replicate of this experiment was conducted the following year in 2013 (15 treatment and 15 control). The results from 2012 and 2013 were combined for data analysis.

After successfully inducing oviposition in the hibernated NASRF female boreal toads, a new experiment was designed to (1) test the effects of hibernation and exogenous hormones on the previously nonhibernated population located at MSU and (2) examine the effect of hibernation length on hormone-induced oviposition. To test the effect of hibernation length on oviposition, 12 females were divided into three treatment groups (four per treatment), hibernated for 1 month (treatment 1), 3 months (treatment 2), or 6 months (treatment 3), respectively. Females were treated with hormones following the same protocol described for group 1 in Section 2.3. Data collected included weight, time to oviposition, and number of eggs oviposited.

2.5. IVF using eggs obtained by hormonal induction

To determine the viability of eggs oviposited after hormone treatment, a subset of eggs from five females at NASRF were chosen at random for an IVF trial as part of the NASRF tadpole reintroduction program. In brief, spermic urine was obtained by injecting males with a combination dose of 10 IU/g BW hCG and 0.4 μg/g BW LHRH. Sperm samples were collected in a Petri dish between 3 and 7 hours after injection. Spermic urine was stored for less than 24 hours at 4 °C before carrying out IVF on freshly oviposited eggs. After oviposition, 50 to 100 eggs were placed in a Petri dish (100 × 90 cm) and approximately 100,000 sperm/100 μL was pipetted evenly across the egg mass within each dish. After a 5-minute dry fertilization period, eggs were flooded with aged tap water. Fertilized eggs were transferred to large holding tanks (18 × 32 × 11 cm) 1 to 2 hours after fertilization, and embryonic development was recorded at 96 hours (Gosner stage 17) and again at the time of reintroduction into the wild (Gosner stages 28–36 [27]).

2.6. Statistical analysis

Data are expressed as the mean ± standard error. All statistical analyses were carried out in SAS 9.3 statistical software (Cary, NC, USA), and significance was set at $P < 0.05$. The difference in mean boreal toad oviposition rates for treatments (1) hibernation versus no hibernation, (2) hormone treated versus control, and (3) year 2012 versus year 2013 was evaluated by Student's *t* test. Using the folded-F statistic in the equality of variance test, we found the assumption of equal variances to be violated for both sets of treatments. Therefore, we used an approximate

Table 1
A comparison of data from breeding colonies at Mississippi State University (MSU) and at the Native Aquatic Species Restoration Facility (NASRF) for hibernated toads.

Results summary	Hibernated	
	NASRF	MSU
Total number (n) of animals	45	16
Hormone versus control (n)	22/23	12/4
Number (n) of animals ovipositing (2012 and 2013)	17/0	4/0
Weight (g) before hibernation	n/a[a]	42.9 ± 2[b]
Weight (g) after hibernation	58.86 ± 4[b]	50.7 ± 2[b]
Time (h) to abdominal contraction	5 ± 2[b]	n/a
Average time (h) to oviposition	35.5 ± 5.64[b]	60 ± 12[b]
Percentage number of animals ovipositing	77.3%	33.3%
Total number of eggs	37,064[c]	4787
Average number of eggs/treatment group	2415.60 ± 239[b]	1659 ± 131[b]

Results are presented as averages across the 2012 and 2013 breeding seasons with ±standard error of the mean (SEM) for weights, abdominal contractions, spawning time, and number of eggs.
Abbreviation: n/a, not applicable.
[a] Weight not recorded prehibernation at NASRF.
[b] Results given are for hibernated toads and are presented as average ± SEM of weights, abdominal contractions, spawning time, and number of eggs.
[c] Total number of eggs reflect numbers collected from 15 of the 17 females that oviposited. In two cases, some eggs were lost from egg masses so these were not included as part of the totals in this table.

version, the Satterthwaite adjusted *t* test, to compensate for unequal variances. These results were verified using the Wilcoxon exact test.

To determine if animal weight significantly affects oviposition, mean time to egg deposition, or mean number of eggs oviposited, animals were grouped according to their weights (1: 34.5–44.4 g, 2: 44.5–54.4 g, 3: 54.5–64.4 g, 4: 64.5–74.5 g, and 5: 74.5–84.4 g) and analyzed by the general linearized model. The general linearized model was also used to determine if weight varied over time because of total hibernation period. The repeated-measures procedure explored interactions between time (five instances: prehibernation, posthibernation, priming #1, priming #2, and ovulatory dose) and hibernation periods (three groups: 1, 3, and 6 months) to determine their effects on change in weight. Significant hibernation by time interactions were explored further by the least-square means procedure using the SLICE function to examine simple effects in the time by hibernation interaction.

3. Results

3.1. Experiment 1: Testing of various hormone protocols for oviposition in nonhibernated female toads

In 2011, none of the 20 nonhibernated female boreal toads housed at MSU oviposited irrespective of hormone treatment regimen administered or the time of year treatment took place. In 2012, after the success of our hormone protocol on NASRF female boreal toads, 12 of 16 nonhibernated females housed at MSU were treated with the same protocol. None of the nonhibernated females oviposited eggs after hormone treatment.

3.2. Experiment 2: Effect of hibernation and hormone priming on oviposition

In 2012 and 2013, 22 control females were injected with PBS and failed to oviposit. However, oviposition occurred in 86% (6 of 7, 2012) and 73% (11 of 15, 2013) of females when treated with a priming regimen of hCG followed by a combination ovulatory dose of hCG and LHRH. Significant differences were observed between control and hormone-treated females in 2012 ($P < 0.05$) and 2013 ($P < 0.01$), with respect to oviposition. Overall, 77% (17 of 22) of NASRF hibernated females treated with hormones in 2012 and 2013 oviposited, with hormone treatment being the only factor to significantly affect oviposition (control vs. hormone treatment, $P < 0.01$) and no significant effect of year (2012 and 2013) on oviposition ($P > 0.05$). A total of 11,462 and 25,602 eggs were obtained from NASRF females by oviposition after hormone treatment in 2012 and 2013, respectively (average egg clutch numbers are shown in Table 1). In 2013, 6 of 15 females had been previously treated with hormones in 2012. Fifty percent (3 of 6) of the

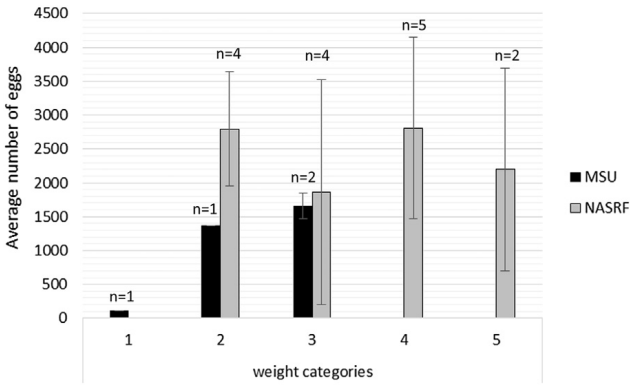

Fig. 1. Total number of eggs oviposited in 2012 and 2013 after hCG and LH-releasing hormone analog treatments. Egg numbers were counted individually and pooled for 2012 and 2013, results are presented with ±standard error of the mean. Weights did not significantly affect oviposition ($P > 0.05$) or the number of eggs per clutch ($P > 0.05$). Females at Mississippi State University (MSU) and at the Native Aquatic Species Restoration Facility (NASRF) were classified into the following weight categories, group 1: 34.5 to 44.4 g; group 2: 44.5 to 54.4 g; group 3: 54.5 to 64.4 g; group 4: 64.5 to 74.4 g; group 5: 74.5 to 84.4 g.

females injected with hormones in two consecutive seasons oviposited. Out of the remaining nine hibernated females injected in the summer of 2013, which had not been previously treated in 2012, 88% (8 of 9) oviposited after hormone treatment. In total, 23% of NASRF females failed to oviposit between the two breeding seasons despite hormone treatment.

In 2012, distinct abdominal contractions were observed in NASRF females before oviposition, but the time between the initiation of contractions and oviposition was not recorded. In 2013, the average time from the initiation of contractions to egg deposition was recorded and found to be approximately 5 ± 2 hours (Table 1). There was no significant difference in the number of females that oviposited in 2012 compared to 2013 ($P > 0.05$) or the total number of eggs deposited ($P > 0.05$) between years. Weight did not significantly affect oviposition ($P > 0.05$) or the number of eggs per clutch ($P > 0.05$) (Fig. 1). Furthermore, time taken to oviposit did not significantly differ between weight categories (Fig. 2).

Oviposition was observed in hormone-treated female toads housed at MSU after hibernation periods of 1, 3, or 6 months. This is in stark contrast to the results observed in 2011 and 2012 in which all nonhibernated females treated with hormones failed to oviposit. There was no significant effect of hibernation ($P > 0.05$) or the interaction between the hibernation period and weight on oviposition ($P > 0.05$) or the egg clutch size ($P > 0.05$) (Fig. 1). It must be noted that few hibernated MSU females oviposited with one female from each in group 1 (1 month), one female from group 2 (3 months), and two females in group 3 (6 months; Fig. 1) ovipositing. Time to oviposition did not significantly differ between weight categories 2 to 5 ($P > 0.05$); however, one MSU female in category 1 had a longer ovipositing time (96 hours) and deposited a smaller egg clutch than that observed in the other categories (Fig. 2). Length of hibernation (1, 3, and 6 months) had a significant effect on weight fluctuations ($P < 0.01$). However, no significant differences were observed in weight fluctuations between priming doses (1 and 2) and the ovulatory dose ($P > 0.05$).

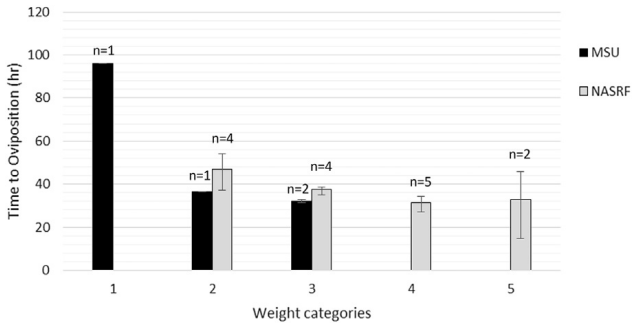

Fig. 2. Average time taken in hours for females to oviposit after hCG and LH-releasing hormone analog treatments relative to weight categories. Females housed at Mississippi State University (MSU) and at the Native Aquatic Species Restoration Facility (NASRF) showed no significant difference in the time taken to oviposit with respect to their weight categories (2–5; $P > 0.05$). Results are presented as average ± standard error of the mean for time (h). Females at MSU and NASRF were classified into the following weight categories: group 1: 34.5 to 44.4 g; group 2: 44.5 to 54.4 g; group 3: 54.5 to 64.4 g; group 4: 64.5 to 74.4 g; group 5: 74.5 to 84.4 g.

Finally, hibernation and hormone treatments interacted to significantly affect oviposition ($P < 0.05$).

3.3. IVF using eggs obtained by hormonal induction

In 2013, 5 of the 15 female boreal toads at NASRF that oviposited after treatment with hCG and LHRH were used in IVF experiments. A total of 5003 out of 10,006 eggs were manually fertilized. Although initial fertilization and cleavage rates could not be determined because of husbandry protocols for animals designated for reintroduction, a total of 927 (18.5%) embryos were counted 96 hours after IVF (Gosner stages 17–19 [29]). After 2 months, a total of 406 tadpoles (8.12%) were still surviving. Tadpoles were reintroduced into the wild once back limb development had begun, between Gosner stages 28 to 38 [29].

4. Discussion

The captive boreal toad colony at NASRF rarely displays naturally occurring reproductive behaviors or spawning (NASRF staff pers. comm. and pers. observ.). Hence, the administration of exogenous hormones to stimulate reproductive behaviors and the use of IVF when eggs are oviposited after hormonal induction have become essential in managing reproduction in this species. In this study, administration of two priming doses of hCG, followed by a combination dose of hCG and LHRH, resulted in 77% of the females ovipositing. In contrast, female toads that were not hibernated did not oviposit eggs, regardless of treatment or priming regimen. Interestingly, neither oviposition rate nor the average number of eggs per clutch was different between females hibernated for 1, 3, or 6 months. In the future, we will test the strength of these observations by increasing the sample size for these experimental treatments.

Although the effects of exogenous hormones on oocyte maturation have not been studied in the boreal toad, our results indicate that hibernation in combination with exogenous hormones showed a significant correlation with oviposition. In the hibernating northern leopard frog (*R pipiens*), oocytes undergo synchronous meiotic maturation after hormonal induction, resulting in a synergistic increase in steroid production [30]. In *Xenopus*, hCG can induce oocyte maturation *via* increased production of progesterone, but ovulation requires a higher level of progesterone than egg maturation [31]. Administration of hCG in humans has a synergistic effect on progesterone production by binding to LH/hCG receptor formations in more differentiated granulosa cells [32]. Initial induction of progesterone *in vitro* can take up to 48 hours; however, synergistic accumulation of the hormone can be observed 3 to 5 days later [32]. The responses observed in our study after two hCG priming doses may indicate a similar synergistic increase in progesterone in boreal females to that described previously. The administration of hCG and LHRH, as a final combination dose, may provide the LH surge required to elevate progesterone to an ovulation-inducing threshold.

A previous study in the boreal toad suggests that exogenous hormones can be used to induce oviposition even in the absence of a hibernation period [20]. However, in our study, sexually mature nonhibernated female boreal toads

(8–10 years old) treated with hormones failed to oviposit over two consecutive breeding seasons. The differences in our results compared to those of Roth et al., [20] may be due to several factors including differences in hormone protocols, age of the animals at the time of treatments, reproductive history, and the captive environment. For example, in the study by Roth et al. [20], females that were not hibernated were significantly heavier than females that were. A lack of hibernation and constant access to food may have resulted in these females reaching sexual maturity at an earlier age than those that were hibernated, which is similar to earlier findings on the common European toad, *A bufo* [27]. Consequently, hibernation may not be the only factor influencing reproduction but part of a more complex system. Our mature females were closer in weight to the hibernated females in the previous boreal toad study [20]. However, in our study, weight did not significantly affect the likelihood of oviposition. The lack of significance regarding the influence of weight in our study compared with the results of Roth et al. remains unclear, and the small sample size of this study cannot be discounted as a factor influencing our results [20]. Furthermore, observations indicated that weight changes observed during hibernation were due to increased water retention. Urine samples obtained from females first emerging from hibernation often accounted for 2% to 18% of their total weight. Retention of water during the hibernation period could be an adaptation for managing hydration over long periods of time. Furthermore, this would support previous evidence indicating that egg masses reach maximum growth (and weight) before females enter hibernation [24].

Although animals at both locations in this study were housed indoors on a seasonal light–dark cycle, NASRF animals were exposed to different aquatic and environmental temperature fluctuations than animals housed at MSU. Temperature and light have a pronounced and often interlinked effect on endocrine–ocular function. Light and thermally induced changes to photopigment molecules are indistinguishable from each other but are both associated with increased ovarian weights and increased number of vitellogenic oocytes, which are the major source of ovarian steroid hormone [33–37]. Females at MSU did not oviposit for two breeding seasons in the absence of a hibernation period despite naturally varying light cycles. Therefore, light alone did not influence reproductive response. In our study, the reestablishment of hibernation was positively correlated with oviposition and could be the result of cold affecting the half-life of LH. As suggested in previous studies, exposure to prolonged periods of time at lowered temperatures results in persistent accumulation of LH, which in turn leads to an LH surge and oviposition [38,39]. However, it is unclear what, if any, temperature threshold is required to initiate hibernation or metabolic depression [26], or if there is an optimum range of temperatures which promote egg maturation. Furthermore, a 2- to 4-year obligate refractory period after oviposition has been suggested as a more efficient reproductive strategy in this species and may also account for the lack of oviposition observed during this study [40].

In addition to the effects of hibernation and hormone treatments, our results indicate that the duration of hibernation, 1, 3, or 6 months, did not significantly affect oviposition. Although hibernation provides an energy-efficient way to manage survival during winter, it is unclear how long the period of cold exposure must last to induce the biochemical changes in metabolism, genetics, and protein synthesis required for oocytes to undergo cytoplasmic and nuclear maturation [41,42]. The duration of cold periods required for egg maturation may be more malleable than previously believed and is likely species specific and environmentally dependent. This study provides preliminary results on the effects of hibernation length on reproductive viability in this species. Further studies should include a larger sample size and exploration of different temperatures that would allow the determination of a temperature threshold for initiation of hibernation. At a captive management level, the use of short hibernation periods could be useful for a number of reasons. For instance, shorter hibernation periods could mean better manipulation of the time at which animals are bred and reduced stress caused by fasting which could result in compromised immune systems and morbidity [28].

The absence of natural mating in captive amphibian colonies also presents obvious obstacles for the management and preservation of endangered species. At the NASRF, natural breeding is rarely observed and often results in poor fertility (<5%). In this study, a small-scale experiment, using eggs obtained from five females, resulted in the reintroduction of 406 tadpoles. This is a promising and realistic application of IVF that could be expanded to operate on a much larger scale. The potential application of this technique, from a research and management perspective, is invaluable.

4.1. Conclusions

Hibernation may be a key component of a complex system involved in boreal toad reproduction. Exogenous hormones in conjunction with hibernation remain essential to the success of the boreal toad captive breeding program; however, shortened hibernation periods that still result in oviposition could have important management implications for its future. The induction of ovulation and the application of IVF to obtain embryos will provide an important additional tool for the management of this and other amphibian species. Future studies, using a larger sample size, should examine temperature requirements to initiate hibernation and the effects of hibernation length on oviposition.

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