RESEARCH ARTICLE   **Open Access**

Nasal administration of gonadotropin releasing hormone (GnRH) elicits sperm production in Fowler's toads (*Anaxyrus fowleri*)

Allison R. Julien[1], Andrew J. Kouba[2], David Kabelik[3], Jean M. Feugang[4], Scott T. Willard[4] and Carrie K. Kouba[1,5*]

Abstract

Background: Declining amphibian populations around the world necessitate the establishment of captive assurance colonies as a hedge against extinction. For species that are difficult to breed in captivity, assisted reproductive techniques, such as treatment with exogenous hormones, are necessary for successful reproduction. The purpose of this study was to determine whether intranasal administration of a gonadotropin releasing hormone analog (GnRHa) elicits sperm production in anurans.

Methods: Male *Anaxyrus fowleri* ($n = 15$/trt) were nasally administered GnRHa (1, 5, 10, and 20 μg) in phosphate buffered saline (PBS) using a pipette. Spermic urine was collected from animals over an 8 h period. Samples were assessed for sperm presence, motility, and concentration.

Results: Treatment of male toads with a PBS control and 1 μg of GnRHa did not elicit sperm production. Nasal administration of GnRHa resulted in sperm production from 60, 93, and 80% of males in the 5, 10, and 20 μg treatment groups, respectively. Sperm motilities averaged 70, 63, and 52% within the 5, 10, and 20 μg treatment groups, respectively, with the highest ($p < 0.05$) sperm motility observed using 5 μg of GnRHa. Significantly higher sperm concentrations were observed in males treated with 10 μg of GnRHa compared with 5 or 20 μg of GnRHa.

Conclusion: Nasal administration of GnRHa was successful in eliciting spermiation from male *Anaxyrus fowleri*, which typically began less than 3 h after treatment. Nasal administration of GnRHa may provide a novel non-invasive method of hormone delivery for at-risk amphibian species that have low reproductive output.

Keywords: Amphibian, Toad, Captive-breeding, Hormones, Sperm, Reproduction

Background

Like most vertebrates, the neuroendocrine hormone gonadotropin releasing hormone (GnRH) in amphibians mediates gamete production through the hypothalamic-pituitary-gonadal (HPG) axis. Upon onset of favorable environmental conditions, GnRH is released by the hypothalamus to stimulate the anterior pituitary, which then secretes the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These gonadotropins then travel through the bloodstream to the gonads, where they mediate steroid hormone synthesis and gametogenesis [1–3]. Exogenous GnRH treatment has become a powerful tool in stimulating gamete production from a variety of species. For example, GnRH receptor agonists have been successful in inducing spawning for commercial fish farming in species such as snook [4] and bream [5]. In amphibians, GnRH has been found to cause upregulation of GnRH receptors [6], increased production and release of sperm in males [3, 7, 8], and follicular development and ovulation in females [9]. Uteshev et al. [10] found that the effects of exogenous GnRH on amphibian spermiation was similar to that found using pituitary extract, leading to the theory that

* Correspondence: ckv7@msstate.edu
[1]Biochemistry, Molecular Biology, Entomology & Plant Pathology Department, Mississippi State University, Mississippi State, MS 39762, USA
[5]Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, Dorman Hall 402, Starkville, MS 39759, USA
Full list of author information is available at the end of the article

exogenous GnRH stimulates a natural gonadotropin-release cascade.

The successful stimulation of reproductive behaviors and physiological processes in amphibians by exogenous GnRH has led to extensive use of the hormone as a tool for assisted breeding of threatened and endangered species. This is especially the case for species with poor reproductive output where sustainability of the captive collection is at risk. For successful breeding in many amphibians, abiotic and biotic cues are necessary triggers for reproduction and include changes in photoperiod, barometric pressure, humidity, temperature, or food availability, which are sometimes difficult to mimic in a captive environment. Although some threatened amphibian species breed well in captivity (e.g. Panamanian golden frogs, V. Poole, pers. comm.), a large number of captive breeding programs utilize assisted reproductive technologies (ART), such as hormone therapy using GnRH or other exogenous reproductive hormones, to produce animals needed for reintroduction and recovery programs [3, 11].

At present, the most common method of GnRH administration in amphibian ART is an injection near the gonads into the peritoneal cavity [3, 12] or subcutaneously [13]. While intraperitoneal (IP) injections of GnRH and its agonists are known to cause sperm production, there is still some question as to the degree that the hormone acts directly at the level of the testis or indirectly through the anterior pituitary by initiating the endogenous cascade of LH or FSH. Receptors for GnRH in anurans are distributed throughout the body, most notably within the brain, anterior pituitary [14–16], and the gonads [17]. Within the brain and pituitary, there are multiple GnRH receptor types corresponding to multiple GnRH isoforms. The physiological effects of GnRH are thought to be mediated by the location of its receptors as well as the form of the GnRH [18]. Fernald and White [19] determined that anurans express at least two isoforms: GnRH 1 and 2, which are secreted by various GnRH neurons distributed throughout parts of the brain such as the hypothalamus, midbrain, and limbic areas; their release is mediated by environmental cues such as light or moisture [15, 20]. Isoform GnRH 1 mediates hypophyseal function, while the precise function of GnRH 2 remains poorly understood [3, 21].

Exogenous substances administered into the nares of an animal can enter the cranial cavity or reach the pituitary by passive diffusion across the olfactory mucosa through gaps between nerves and into the subarachnoid space and cerebrospinal fluid [22]. From here, possible diffusion into the ventricular or perivascular spaces could circulate the administered substances to various areas of the brain, or around the blood brain barrier to reach the pituitary [23, 24]. In rats, olfactory sensory neuron dendrites are exposed in the nasal passage, and their axons project through the cribriform plate to the olfactory bulb, thus providing a pathway from the external environment directly into the central nervous system [25]. Substances can also actively bind to olfactory receptors and be transported along or within neurons [26, 27]. Within the nasal cavity of amphibians and fish there are both ciliated sensory and non-sensory neurons that extend to the internal nares and onto the olfactory bulb, wherein GnRH receptors are located [22, 28]. For example, in tiger salamanders, GnRH agonists have been found to bind in the epithelium of the nasal cavity and the vomeronasal organ [29]. Indeed, various studies have found that large concentrations of exogenous substances administered nasally bind at the level of the olfactory bulb [23, 24]. However, exogenous substances administered through the nares may also work their way into the throat and could be swallowed; thus, entering the bloodstream via a different route. Hence, multiple intracellular and extracellular pathways from the vertebrate nasal cavity into the cranial cavity, cerebrospinal fluid, or bloodstream are present whereby exogenous hormones could rapidly reach the anterior pituitary, or even bypass the blood-brain barrier and reach other areas of the brain (e.g. hypothalamus) that contain GnRH receptors [25].

In this study, we hypothesized that small concentrations of nasally-administered GnRH would be successful in eliciting spermiation in male Fowler's toads (*Anaxyrus fowleri*), a common model species, due to the close proximity of nasal entry routes to neuroendocrine pathways mediating gamete production and development. To test this hypothesis, we administered four different concentrations of a GnRH agonist intranasally and monitored quantity and quality of sperm produced over 8 h, compared to controls.

Results

Treatment with 1 μg of GnRHa did not elicit sperm production in any male and was therefore not included in further analyses of treatments. In addition, control males, administered phosphate-buffered saline (PBS), did not produce sperm, nor were any animals producing sperm prior to hormone administration at T0. The average sample volume (mL), sperm concentration (sperm/mL), total sperm, percent motility (M), percent forward progresssive motility (FPM), and total motile sperm of each treatment group across all time points is summarized in Table 1.

Number of responders and latency to sperm production

Figure 1 shows the percentage of males responding to hormone treatment over time and the average latencies to sperm release. Following nasal administration of GnRHa, 60% of males responded to 5 μg treatments, 93% of males responded to 10 μg treatments, and 80% of males responded to 20 μg treatments (Fig. 1a). There

Table 1 Sperm characteristics for Fowler toads treated intranasally with three different concentrations of GnRH

GnRHa (µg) Treatment	Responding Males	Mean Sperm Characteristics					
		Spermic Urine Volume (mL)	Concentration Sperm/mL ($\times 10^5$)	Total Sperm ($\times 10^5$)	Motility (%)	Forward Progressive Motility (%)	Total Motile Sperm ($\times 10^5$) (Total sperm x % Motility)
5	9/15	0.25 ± 0.03	2.39 ± 0.67[a]	0.94 ± 0.34[a]	72.0 ± 4.0[a]	48.0 ± 4.5[a]	0.52 ± 0.17[a]
10	14/15	0.30 ± 0.04	5.68 ± 0.80[b]	2.41 ± 0.49[b]	63.0 ± 3.6[a]	35.0 ± 3.4[b]	1.66 ± 0.37[b]
20	12/15	0.42 ± 0.05	3.33 ± 0.64[ab]	1.66 ± 0.45[ab]	49.0 ± 4.0[b]	29.0 ± 3.5[b]	0.74 ± 0.20[ab]

Data are shown as mean ± SEM. Significant differences within columns are indicated by lettered superscripts
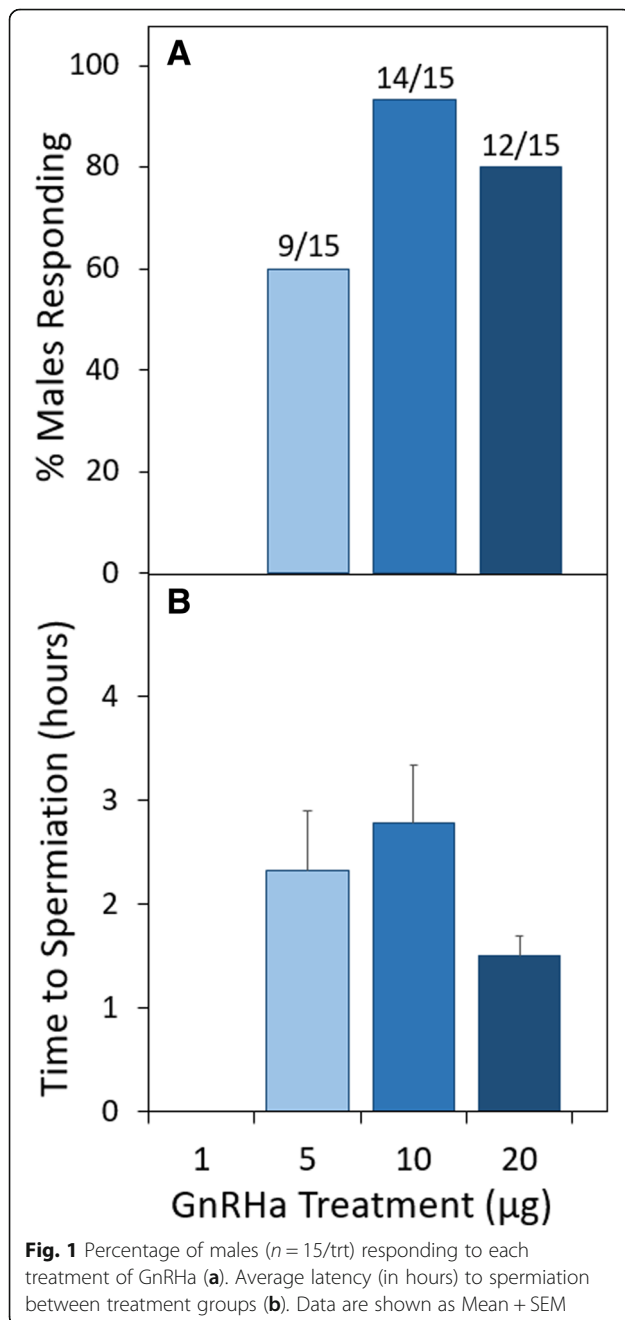
Fig. 1 Percentage of males ($n = 15$/trt) responding to each treatment of GnRHa (**a**). Average latency (in hours) to spermiation between treatment groups (**b**). Data are shown as Mean + SEM

was no significant difference ($p = 0.17$) in the number of males responding to the three treatments. On average, 5 µg of GnRHa resulted in spermiation within 2.3 h, while 10 and 20 µg treatments of GnRHa resulted in spermiation within 2.8 h and 1.5 h, respectively (Fig. 1b). Across all treatment groups, nearly 50% of the males began producing sperm by the 1 h collection period and 73% of the males were producing sperm by the 3 h collection period (Fig. 2). There was no significant difference ($p = 0.32$) in latency to sperm production between treatment groups.
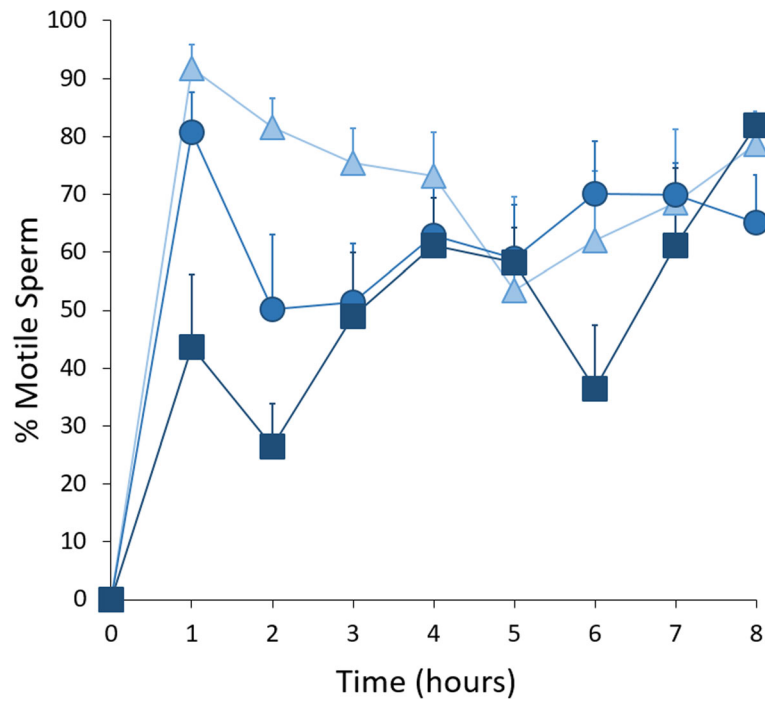
Sperm motility over time

Figure 3 shows the sperm motility over time for the three responding treatments and the variation that occurs between animals and across time points. Peak sperm motility for the 5 µg treatment (92%) and the 10 µg treatment (81%) occurred within 1 h, in contrast to the 20 µg treatment (82%) which peaked at 8 h (Fig. 3).

The generalized linear mixed models analysis showed there was a significant effect of hormone treatment ($p < 0.01$) on motility, but no significant effect of time ($p > 0.05$) or treatment x time interaction ($p > 0.05$). Sperm motility across time points averaged 72, 63, and 49% for the 5, 10 and 20 µg groups, respectively. Average sperm motility was significantly higher with the 5 µg ($p < 0.01$) and 10 µg ($p < 0.04$) treatmentes compared to the 20 µg GnRHa (Table 1; Fig. 3). Otherwise, sperm motility induced by 5 µg verses 10 µg GnRHa was not different ($p = 0.07$).

We also analyzed separately the FPM of sperm samples as an additional metric for quality of sperm. The average FPM of sperm in the 5 µg treatment was 50%, which was significantly higher ($p < 0.05$) than the 10 and 20 µg treatments of GnRHa at 35 and 33% FPM, respectively (Table 1). Figure 4 shows the variation in FPM over time for the three treatment groups. The generalized linear mixed models analysis showed there was a significant effect of time ($p < 0.01$) but not treatment ($p > 0.05$) on sperm FPM, nor was there a treatment x time interaction ($p > 0.05$). The highest FPM occurred at the last 8 h time point collection for all three treatment groups (Fig. 4) in contrast to the highest total motility, which occurred much earlier (Fig. 3). These data indicate that a higher proportion of non-progressive motile sperm are
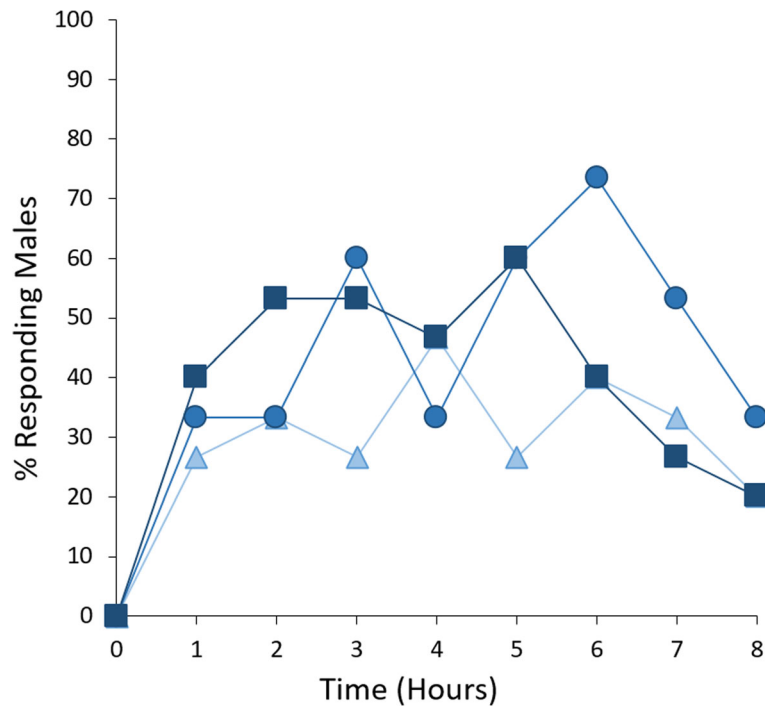
Fig. 2 Percentage of responding males (*n* = 15/trt), as measured through sperm production, over time between GnRHa treatment groups: 5 μ g (▲), 10 μg (●), and 20 μg (■)

Fig. 3 Average total sperm motility over time for males (*n* = 15/trt) between GnRHa treatment groups: 5 μ g (▲), 10 μg (●), and 20 μg (■). Total motility is defined as motile (M) sperm + forward progressive motile (FPM) sperm. Data are shown as mean + SEM
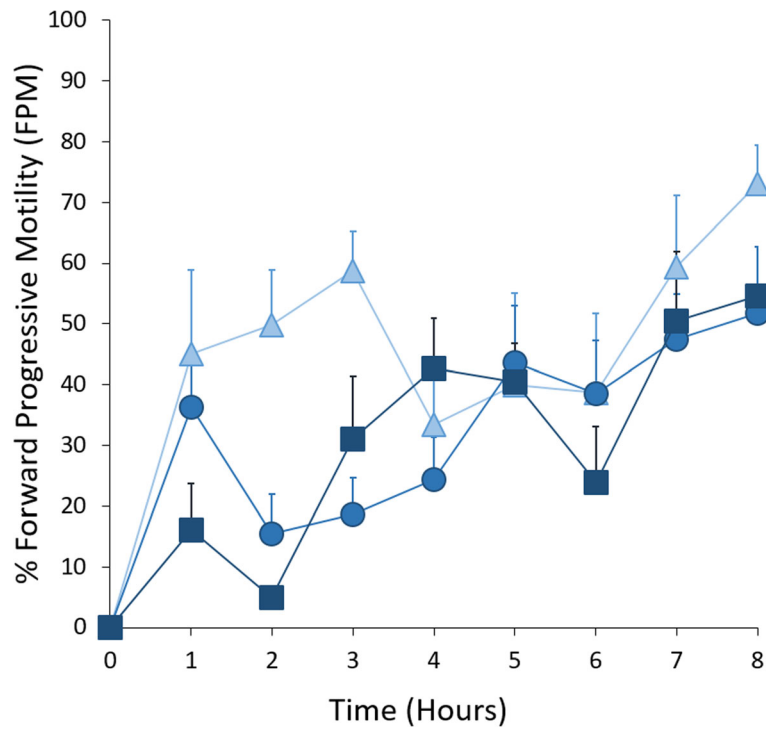
Fig. 4 Average sperm forward progressive motility (FPM) over time for males ($n = 15$/trt) between GnRHa treatment groups: 5 μg (▲), 10 µg (●), and 20 µg (■). Data are shown as mean + SEM
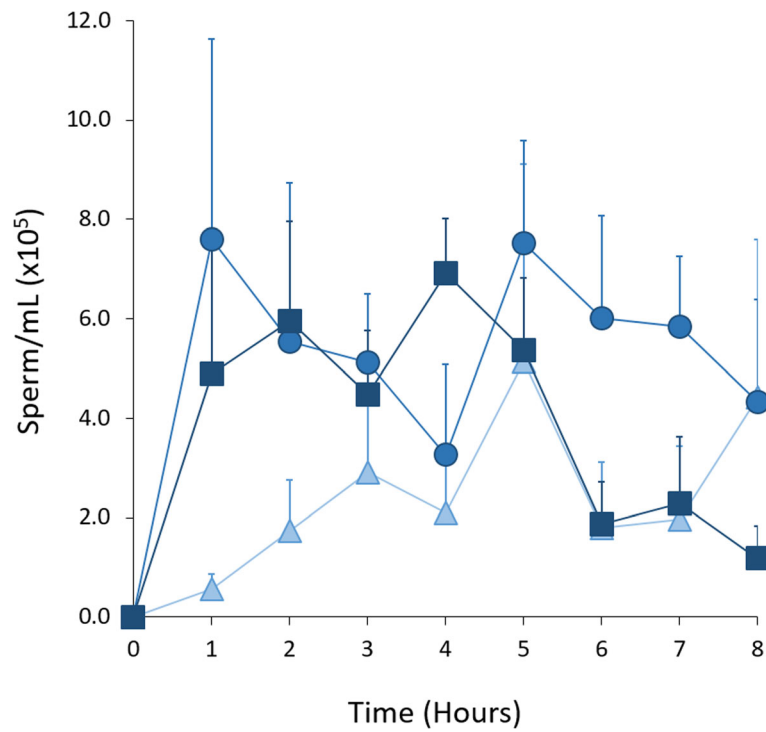
Fig. 5 Average amount of sperm/mL produced over time by males ($n = 15$/trt) between GnRHa treatment groups: 5 μg (▲), 10 µg (●), and 20 µg (■). Data are shown as mean + SEM

produced earlier and that quality of the sperm collections may increase over time to an unknown point before declining again.

Sperm concentration over time

Males administered 10 µg of GnRHa produced significantly greater ($p < 0.01$) average sperm concentrations than males administered 5 µg GnRHa, though concentration was not different between the 5 and 20 µg ($p = 0.07$) or between the 10 and 20 µg GnRHa groups ($p = 0.06$) (Table 1). Figure 5 shows the average sperm concentration (sperm/mL) over time for the three responding treatments and the variation that occurs across animals and time points. The generalized linear mixed models analysis showed there was a significant effect of hormone treatment ($p < 0.01$) on sperm concentration (sperm/mL), but no significant effect of time ($p > 0.05$) or treatment x time interaction ($p > 0.05$). However, peak sperm concentration (sperm/mL) occurred at 1 h following 10 µg (7.64×10^5 sperm/mL) of GnRHa administration, whereas 5 µg (5.14×10^5 sperm/mL) and 20 µg (6.91×10^5 sperm/mL) treatments had peak sperm concentration 4 and 5 h following hormone administration, respectively (Fig. 5). We also calculated total sperm in a sample and subsequently the total number of motile sperm, with samples averaged across treatment groups (Table 1; Fig. 6). Overall, more animals (93%) produced sperm in response to the 10 µg GnRHa treatment group and had a higher concentration of sperm.

The highest sperm/mL (5.68×10^5), total sperm (2.41×10^5), and total motile sperm concentration (1.66×10^5) resulted from treatment with 10 µg of GnRHa (Fig. 6). Because percent motility was typically between 50 and 70%, total motile sperm concentration may be a more valuable reflection of the quality of a sample and its fertilization potential than total sperm/mL.

Discussion

To the author's knowledge, this is the first time intranasal GnRHa administration has been used to elicit spermiation in an anuran. Of the five treatments tested, GnRHa concentrations of 5–20 µg resulted in sperm production for 60–93% of the animals. The lowest concentration of GnRHa we used, 1 µg, was not effective at stimulating sperm production and may indicate that the concentration of hormone was too low to adequately trigger a downstream cascade of gonadotropin signaling. We found that spermiation occurred within 2 h of hormone treatment and sperm quantity and quality remained elevated for up to 8 h, allowing for multiple collections of sperm from individuals. Moreover, the use of a pipette to administer hormone intranasally, compared to traditional methods of IP injections, is a novel, minimally invasive delivery method that should be easily transferable to captive breeding programs. We speculate that, given its proximity and route of transfer, nasal administration of GnRHa targeted its receptors within the pituitary to modulate LH/FSH secretion and subsequent steroidogenesis and gamete production in the testes.
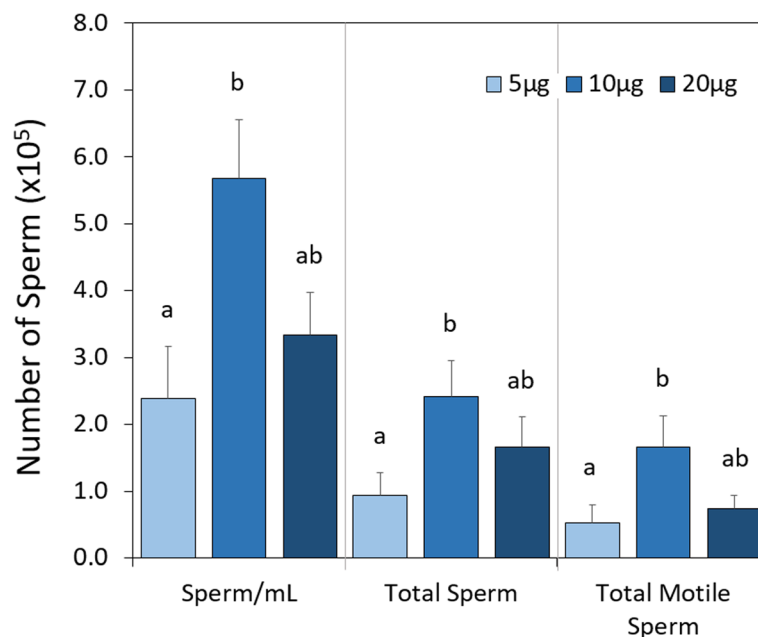
Fig. 6 Average number of sperm in sample following treatment of GnRHa (5, 10, and 20 µg) calculated as sperm/mL, total number of sperm, and total motile sperm. Different letters indicate significant differences ($p < 0.05$) within a category. Data are shown as mean + SEM

For amphibians, the most common hormone delivery method to stimulate gamete production is intraperitoneal (IP) injections; thus, we know much more about the physiological responses of animals receiving hormones via this delivery route. We surmise that IP injections of GnRHa may act systemically on GnRH receptors located throughout the body including on the gonads and pituitary; thus, diluting the overall effect on the HPG axis. Bambino, Schreiber and Hsueh [30] found that treatments of GnRH in rats decreased testicular LH receptors as a result of repeated exposure, thereby reducing modulation of testicular function by pituitary-released gonadotropins resulting in a decrease in spermatogenesis. We suggest that nasal administration of GnRHa would target receptors within the pituitary or even brain, thereby directing and reducing the amount of exogenous hormone needed compared to IP injections, reducing the risk of receptor downregulation. There are three receptors for GnRH that are located throughout the amphibian brain and pituitary: GnRH1R, GnRH2R, and GnRH3R [21]. Various forms of GnRH bind to these different types of receptors with differential affinity and likely play different roles in reproductive functions [31]. For example, GnRH 2 is thought to primarily act as a neuromodulator within the midbrain while GnRH 1 is suggested to control gonadotropin release [32–34]. Hence, our nasal administration of GnRHa, a homolog of GnRH 1, and subsequent sperm production in the toads is likely acting through GnRH 1 receptors in the anterior pituitary, although there may be other receptor binding occurring as well.

Kouba et al. [8] reported that in *Anaxyrus americanus*, IP injections of GnRHa (1–32 μg) resulted in only 35% of the males producing sperm. In comparison, the current study showed an increased number of males (maximum 93%) produced sperm following nasally administered GnRHa treatments. While both *A. fowleri* and *A. americanus* are from the same family bufonidae, species differences may impact the efficiency of hormone treatments, regardless of administrative route. Moreover, the study by Kouba et al. [8] was performed outside of the breeding season for *A. americanus*, while the current study was performed within the breeding season of *A. fowleri*. Comparing the effectiveness of intranasally administered GnRHa in eliciting spermiation outside of the breeding season in *A. fowleri* would give insight into the dependence of spermatogenesis on abiotic factors.

Here, we report a sperm motility over time ranging between 49 and 72%, depending on GnRHa concentration, which is slightly lower than other studies using IP injections of GnRHa. In *Atelopus zeteki*, average motility was approximately 85% for treatments of 1–4 μg per gram of toad body weight [35] while in *A. americanus*, GnRHa treatments resulted in an average motility of approximately 80% [12]. Peak sperm production, where sperm/ mL concentration and motility coincide, was observed at 1 h post-administration of 10 μg of GnRHa in the current study. The other two treatments did not generate a discernable peak in sperm production. In *A. americanus*, a clear peak in combined optimal sperm motility and concentration was not observed across 24 h post GnRHa IP injection [12]. In the current study, the average observed latency was 2.2 h across all three responding GnRHa treatments reported. In the bufonid *A. zeteki*, peak sperm production following IP GnRHa administration was found at 3.5 h post-treatment [35], although sperm presence was first observed at 1.5 h, which is similar to the average latency to spermiation we found with nasal administration in *A. fowleri*. However, in the study with *A. americanus*, sperm collections were not initiated until 3 h post-hormone treatment [12], which makes the comparison of sperm production latency to the current study difficult to assess. In this study, we ceased spermic urine collections after 8 h, and did not perform a 24 h collection. Previous studies in bufonids have shown a high amount of variability in the duration of sperm release, with spermic urine production ranging from 3 h to up to 24 h post-hormone administration [12, 34]. However, sperm concentrations began to significantly decline by 9 h post-administration, and reached negligible concentration by 24 h. Therefore, sperm collected within the first 8–9 h post-administration would likely be of the highest quality and concentration for in-vitro fertilization.

In the study of *A. americanus*, Kouba et al. [12] also tested the effectiveness of human chorionic gonadotropin (hCG) on sperm production. Unlike the hypophyseal functions of GnRH, hCG mimics the downstream effects of luteinizing hormone (LH) and acts directly on either the testes or the ovaries [9, 36] to induce gamete maturation and release. Following the highest treatment of hCG (300 IU), 100% of males produced sperm with a peak of 12×10^6 sperm/mL. While the concentration achieved from hCG was higher than in the current study, motilities ranged from 40 to 80%, similar to motilities reported here. Compared to GnRHa, however, hCG is more expensive, must be used at higher concentrations, and stock availability can fluctuate. Therefore, despite its increased ability to produce higher concentrations of sperm, the relative cost and comparable motilities may make nasally administered GnRHa a viable substitute for IP-administered hCG, especially for small amphibians that are difficult to inject.

In order for nasal administration of exogenous hormone to be considered a useful tool for assisted reproductive technologies (ART), it is imperative that sperm be collected in concentrations that are useful for in-vitro fertilization (IVF) or cryopreservation. To achieve high rates of fertilization in anurans, sperm concentrations (sperm/mL) should be between 1 and 8×10^5 [11,

Langhorne et al., unpublished]. As noted in Table 1, each treatment group of GnRHa above 1 μg resulted in an average sperm/mL concentration of 2.6×10^5 or above, though the 10 μg treatment group resulted in the highest sperm concentrations overall. Adequate numbers of sperm could be collected over time by pooling samples across 8 h (or longer), helping to meet the criteria for IVF of a useful tool for amphibian ART ([11], Langhorne et al., unpublished). As previously mentioned, the 10 μg treatment groups also elicited the highest sperm concentrations overall compared to the other two treatment groups. It may be that males were more responsive to the 10 μg compared to the 20 μg GnRHa treatment due to higher concentrations of hormone eliciting negative feedback responses or causing a desensitization and/or downregulation of receptors. Taken together, these data suggest that administrations of 10 μg of GnRHa could be used to collect high enough amounts of sperm for assisted breeding following nasal hormone administration for this species.

The implementation of nasal hormone stimulation in captive amphibian breeding may have several advantages over IP injections. For example, IP injections require larger hormone concentrations calculated on a per gram body weight basis, which can be expensive or pose a risk to animal health and reproductive output. By contrast, nasal administration uses lower concentrations of hormone, smaller volumes of delivery vehicle, and is less invasive than IP injections. In addition, many zoological institutions require trained veterinarians to administer injections to animals in collections, whereas non-invasive hormone treatment by pipetting through the nares would not require this level of expertise. Furthermore, eliminating the need for IP injections increases the safety factor for animals that are too small to safely inject with a needle. Thus, nasal administration is a viable alternative hormone delivery route, owing to its similarity in sperm production variables to that of previously published results from IP injections of GnRHa.

Conclusions
This study demonstrates for the first time that exogenous GnRH can be delivered intranasally in an anuran species to stimulate a downstream physiological effects of gamete production and release. Moreover, GnRHa concentrations between 5 and 20 μg successfully elicited sperm production in up to 93% of male *A. fowleri* and across at least 8 h. The results of these experiments demonstrate that non-invasive nasal administration of hormone is a viable substitute for IP injections, which may be particularly helpful in other, smaller amphibians.

Methods
Animals
Sexually mature male *A. fowleri* toads were collected from Oktibbeha County in Mississippi during the

breeding season from April–July 2016 and 2017 (Permit #0728161); all experiments were conducted within the breeding season. Toads were collected from the wild as adults, so it was not possible to assign age classes. Animals were housed in groups of four to six in ventilated polycarbonate tanks (30 cm H × 46 cm W × 66 cm L). Average temperatures were maintained at 21°C and animals were kept at 12 h night/day cycles. Crickets and mealworms were provided three times a week, and water was provided ad libitum. Insects were gut-loaded with Repashy SuperLoad® (Repashy Ventures Inc., CA, USA) and dusted with a vitamin D supplement prior to feeding. Toads were housed in accordance with IACUC protocols at Mississippi State University (IACUC #16–406). Following this study, animals were housed at Mississippi State University until natural death.

Hormone treatment comparison on sperm production
In the present study, a commercial GnRH analog (GnRHa) with the trade name luteinizing hormone releasing hormone analog (LHRHa; Sigma-Aldrich, Product #L4513) was used for all hormone treatments. Toads (n = 15/treatment) were intranasally administered four treatment concentrations of GnRHa (1, 5, 10, or 20 μg) or phosphate buffered saline (PBS) as a control. Hormone was suspended in PBS to achieve the desired concentration in 20 μl, with 10 μl administered to each nare via pipette. Animals were treated in the morning, and then placed in temporary holding tubs for the duration of sperm collections. Due to very small concentrations of hormone and volumes delivered into the nares, males were given a fixed amount of GnRHa per treatment group rather than adjusting the GnRHa amount on a per gram body weight basis. Average male weight was 24.0 ± 0.8 g.

Spermic urine collection over time and sperm analysis
Hormone treatment is designated as time zero (T0). Urine collections were conducted immediately prior to T0 and every hour afterwards for 8 h. Urine was collected at each time point by gently suspending the males above a petri dish. Using a thumb and index finger, the hind limbs were spread until urination occurred (≤ 1 min) into the dish. The samples were then collected and placed in 1.5 mL Eppendorf tubes for immediate analysis. If sperm was observed in the urine, samples were categorized as a response and spermic urine samples were evaluated for volume, motility parameters and concentration. Sperm motility was further divided into the categories of forward progressive motile sperm (FPM; sperm exhibiting flagellar movement and progressing forward), non-progressively motile sperm (NPM; sperm exhibiting flagellar movement, but not progressing forward), and non-motile sperm (NM; sperm with no flagellar movement). Motility (M) was calculated as the

sum of FPM and NPM. We also used motility, concentration, and total volume to calculate motile sperm/mL, total sperm produced, and total motile sperm in a given sample. Sperm motility was assessed using a phase contrast microscope (Olympus CX43), while sperm concentration was assessed using a hemocytometer counting chamber (Hausser Scientific #3200).

Data evaluation and statistical analysis

If a male did not release sperm in a urine sample at a given time point, that male was not included in the analysis of that time point. Responders to each treatment group were analyzed with a chi-squared test. Males were considered "responders" if they released sperm following GnRHa administration. All data were assessed for normality and homogeneity of variance using the Shapiro-Wilke's test and Levene's test, respectively. Percentage data were arcsine transformed prior to analysis. Data that did not meet the parameters for normality or homogeneity of variances (latency to spermiation, total motility, forward progressive motility, concentration, total sperm and total motile sperm) were analyzed using a Kruskal-Wallis one-way analysis of variance followed by the Dwass, Steel, Critchlow-Fligner method for multiple comparison analysis. In order to determine interaction effects between time and treatment we performed a generalized linear mixed models procedure for each sperm parameter. Statistical analyses were performed in SAS version 9.4 (Cary, North Carolina, USA). Values are expressed as mean ± SEM and differences were considered to be significant at $p \leq 0.05$.

Abbreviations
ART: Assisted Reproductive Technologies; FPM: Forward progressive motile; FSH: Follicle-Stimulating Hormone; GnRH: Gonadotropin Releasing Hormone; GnRH1R, GnRH2R, GnRH3R: Gonadotropin Releasing Hormone Receptor (1, 2 or 3); GnRHa: Gonadotropin Releasing Hormone agonist; hCG: human Chorionic Gonadotropin; HPG: Hypothalamic-pituitary-gonadal (axis); IACUC: Institutional Animal Care and Use Committee; IP: Intraperitoneal; IVF: In-vitro Fertilization; LH: Luteinizing Hormone; LHRHa: Luteinizing Hormone Releasing Hormone analog; M: Motility; NM: Non-motile sperm; NPM: Non-progressively motile sperm; PBS: Phosphate Buffered Saline; SAS: Statistical Analysis System; SEM: Standard Error of the Mean

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Permission for animal collection and use was provided by Mississippi Department of Wildlife, Fisheries and Parks under permit #0728161. Animal husbandry and institutional use was performed in accordance with IACUC protocols at Mississippi State University (IACUC #16–406).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
[1]Biochemistry, Molecular Biology, Entomology & Plant Pathology Department, Mississippi State University, Mississippi State, MS 39762, USA. [2]Wildlife, Fisheries, and Aquaculture Department, Mississippi State University, Mississippi State, MS 39762, USA. [3]Neuroscience Program and Biology Department, Rhodes College, Memphis, TN 38112, USA. [4]Animal and Dairy Sciences Department, Mississippi State University, Mississippi State, MS 39762, USA. [5]Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, Dorman Hall 402, Starkville, MS 39759, USA.

References
1. Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, et al. Gonadotropin-Releasing Hormone: One Polypeptide Regulates Secretion of Luteinizing and Follicle-Stimulating Hormones. Science (80-). 1971;173(4001): 1036 LP–1038.
2. King JA, Millar RP. Evolution of gonadotropin-releasing hormones. Trends Endocrinol Metab. 1992;3(9):339–46.
3. Vu M, Trudeau VL. Neuroendocrine control of spawning in amphibians and its practical applications. Gen Comp Endocrinol. 2016;234:28–39. Available from. https://doi.org/10.1016/j.ygcen.2016.03.024.
4. Contreras-García MJ, Contreras-Sanchez WM, Hernández-Vidal U, Mcdonald-Vera A. Induced spawning of the common Snook (Centropomus undecimalis) in captivity using GnRH-a implants. Ecosistemas y Recur Agropecu. 2015;2(6):357–62 Available from: https://www.researchgate.net/publication/281149419_Induced_spawning_of_the_common_snook_Centropomus_undecimalis_in_captivity_using_GnRH-a_implants .
5. Kucharczyk D, Kujawa R, Mamcarz A, Targońtska-Dietrich K, Wyszomirska E, Glogowski J, et al. Induced spawning in bream (Abramis brama L.) using pellets containing GnRH. Czech J Anim Sci. 2005;50(3):89–95.
6. Vu M, Weiler B, Trudeau VL. Time- and dose-related effects of a gonadotropin-releasing hormone agonist and dopamine antagonist on reproduction in the northern leopard frog (Lithobates pipiens). Gen Comp Endocrinol. 2017;254:86–96. Available from. https://doi.org/10.1016/j.ygcen.2017.09.023.

7.   Waggener WL, Carroll EJ. A method for hormonal induction of sperm release in anurans (eight species) and in vitro fertilization in *Lepidobatrachus* species. Develop Growth Differ. 1998;40:19–25.

8.   Kouba A, Vance C, Calatayud N, Rowlison T, Langhorne C, Willard S. Assisted reproductive technologies (ART) for amphibians. Amphib Husb Resour Guid. 2012;2(March 2018):1–47.

9.   Browne RK, Seratt J, Vance C, Kouba A. Hormonal priming, induction of ovulation and in-vitro fertilization of the endangered Wyoming toad (*Bufo baxteri*). Reprod Biol Endocrinol. 2006;4:1–11.

10. Uteshev VK, Shishova NV, Kaurova SA, Browne RK, Gakhova EN. Hormonal induction of spermatozoa from amphibians with *Rana temporaria* and *Bufo bufo* as anuran models. Reprod Fertil Dev. 2012;24(4):599–607.

11. Kouba A, Vance CK. Applied reproductive technologies and genetic resource banking for amphibian conservation. Reprod Fertil Dev. 2009;21:20.

12. Kouba AJ, del Barco-Trillo J, Vance CK, Milam C, Carr M. A comparison of human chorionic gonadotropin and luteinizing hormone releasing hormone on the induction of spermiation and amplexus in the American toad (*Anaxyrus americanus*). Reprod Biol Endocrinol. 2012;10(1):59. Available from. https://doi.org/10.1186/1477-7827-10-59.

13. Silla AJ, McFadden M, Byrne PG. Hormone-induced spawning of the critically endangered northern corroboree frog *Pseudophryne pengilleyi*. Reprod Fertil Dev. 2018;30(10):1352–8.

14. Muske LE, Moore FL. The nervous terminalis in amphibians: anatomy, chemistry relationship with the hypothalamic gonadotropin-releasing hormone system. Brain Behav. 1988;32(3):141–50.

15. Millar RP. GnRH II and type II GnRH receptors. Trends Endocrinol Metab. 2003;14(1):35–43.

16. Troskie BE, Hapgood JP, Millar RP, Illing N. Complementary deoxyribonucleic acid cloning, gene expression, and ligand selectivity of a novel gonadotropin-releasing hormone receptor expressed in the pituitary and midbrain of *Xenopus laevis*. Endocrinology. 2000;141(5):1764–71.

17. Meccariello R, Franzoni MF, Chianese R, Cottone E, Scarpa D, Donna D, et al. Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian *Rana esculenta*. Endocrinology. 2008;149(5):2149–58.

18. Millar RP. GnRHs and GnRH receptors. Anim Reprod Sci. 2005;88(1–2 SPEC. ISS):5–28.

19. Fernald RD, White RB. Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front Neuroendocrinol. 1999;240:224–40.

20. Quiroga L, Sanabria E, Jahn G, Fornés M. Spermatogenesis and plasma testosterone levels under field conditions, in males of the common toad (*Rhinella arenarum* Hensel, 1867), from the Monte Desert, vol. 148. Argentina; Belgian J Zool. 2018;148(1):105–18.

21. Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. Endocr Rev. 2004;25(2):235–75.

22. Lochhead JJ, Thorne RG. Intranasal delivery of biologics to the central nervous system. Adv Drug Deliv Rev. 2012;64(7):614–28. Available from. https://doi.org/10.1016/j.addr.2011.11.002.

23. Charlton ST, Whetstone J, Fayinka ST, Read KD, Illum L, Davis SS. Evaluation of direct transport pathways of glycine receptor antagonists and an angiotensin antagonist from the nasal cavity to the central nervous system in the rat model. Pharm Res. 2008;25(7):1531–43.

24. Falcone JA, Salameh TS, Yi X, Cordy BJ, Mortell WG, Kabanov AV, et al. Intranasal administration as a route for drug delivery to the brain: evidence for a unique pathway for albumin. J Pharmacol Exp Ther. 2014;351(1):54–60. Available from. https://doi.org/10.1124/jpet.114.216705.

25. Thorne RG, Pronk GJ. Delivery of insulin-like growth factor-i to the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration. Neuroscience. 2004;127(2):481–96. Available from. https://doi.org/10.1016/j.neuroscience.2004.05.029.

26. Jennes L, Dalati B, Michael Conn P. Distribution of gonadrotropin releasing hormone agonist binding sites in the rat central nervous system. Brain Res. 1988;452(1–2):156–64.

27. Petrovich GD, Canteras NS, Swanson LW. Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. Brain Res Rev. 2001;38(1–2):247–89.

28. Maruska KP, Fernald RD. Social regulation of gene expression in the hypothalamic-pituitary-gonadal Axis. Physiology. 2011;26(6):412–23. Available from. https://doi.org/10.1152/physiol.00032.2011.

29. Wirsig-Wiechmann CR, Jennes L. Gonadotropin-releasing hormone agonist binding in tiger salamander nasal cavity. Neurosci Lett. 1993;160(2):201–4. Available from: https://www.sciencedirect.com/science/article/pii/030439409390413F?via%3Dihub .

30. Bambino TH, Schreiber JR, Hsueh AJW. Gonadotropin-releasing hormone and its agonist inhibit testicular luteinizing hormone receptor and steroidogenesis in immature and adult hypophysectomized rats. Endocrinology. 1980;107(4):908–17.

31. King JK, Millar RP. Identification of his 5, Trp 7, Tyr8-GnRH (chicken GnRH II) in amphibian brain. Peptides. 1986;7:827–34. Available from: https://www.sciencedirect.com/science/article/pii/0196978186901026.

32. Amano M, Oka Y, Yamanome T, Okuzawa K, Yamamori K. Three GnRH systems in the brain and pituitary of a pleuronectiform fish, the barfin flounder *Verasper moseri*. Cell Tissue Res. 2002;309(2):323–9. Available from. https://doi.org/10.1007/s00441-002-0594-z.

33. Licht P, McCreery BR, Barnes R, Pang R. Seasonal and stress related changes in plasma gonadotropins, sex steroids and corticosterone in the bullfrog, *Rana catesbeiana*. Gen Comp Endocrinol. 1983;50:124–45.

34. Della Togna G, Trudeau VL, Gratwicke B, Evans M, Augustine L, Chia H, et al. Effects of hormonal stimulation on the concentration and quality of excreted spermatozoa in the critically endangered Panamanian golden frog (*Atelopus zeteki*). Theriogenology. 2017;91:27–35. Available from. https://doi.org/10.1016/j.theriogenology.2016.12.033.

35. Silla AJ, Roberts JD. Investigating patterns in the spermiation response of eight Australian frogs administered human chorionic gonadotropin (hCG) and luteinizing hormone-releasing hormone (LHRHa). Gen Comp Endocrinol. 2012;179(1):128–36. Available from. https://doi.org/10.1016/j.ygcen.2012.08.009.

36. Humaidan P, Bredkjær HE, Bungum L, Bungum M, Grøndahl ML, Westergaard L, et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: a prospective randomized study. Hum Reprod. 2005;20(5):1213–20.