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Corrigendum

Sperm output and body condition are maintained independently of hibernation in an endangered temperate amphibian

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Sperm output and body condition are maintained independently of hibernation in an endangered temperate amphibian

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Abstract. Hibernation is an integral part of the life history of species living in seasonal environments. However, our knowledge about the link between hibernation and reproductive success in amphibians remains limited, which poses an obstacle for critical conservation efforts. To fill this gap, we quantified the effects of captive hibernation on sperm quality, sperm quantity and body condition in an endangered anuran, the Wyoming toad (*Anaxyrus baxteri*), and used naturally hibernated wild toads as a standard for comparison. We hypothesised that hibernation is essential for optimal sperm output but is detrimental to body condition. Sperm collection was performed using assisted reproductive technologies for both captive and wild toads. Contrary to our hypotheses, no differences were observed in sperm metrics (total number of cells, concentration, motility and viability) or in body condition across captive treatment groups (0, 30 or 60 days of hibernation). Moreover, no difference was found between sperm metrics of captive toads and wild toads. These unexpected findings suggest that hibernation may not be an essential process for spermiation in *A. baxteri* while using exogenous hormones, and illustrate the potential of temperate amphibians to adapt to varying environmental conditions during winter months.

Additional keywords: Anura, *Anaxyrus baxteri*, assisted reproductive technology, bufonidae, captive breeding, conservation.

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Introduction

Seasonal exposure to low temperatures represents a physiological challenge but is an integral part of the breeding cycle for some species, including insects (Norling 1984), amphibians (Wells 2007), reptiles (Licht *et al.* 1985), birds (Ruf and Geiser 2015) and mammals (Ruf and Geiser 2015). For animals living in temperate regions, hibernation reduces metabolic energy requirements (Geiser 2004; Heldmaier *et al.* 2004) and is an important adaptation that increases survivorship by reducing the threats of harsh environmental conditions, low food availability and predation (Bieber *et al.* 2012). In both endotherms and ectotherms, temperatures can provide seasonal cues to stimulate gonadal function (Jørgensen 1986; Parua *et al.* 1998; Calatayud *et al.* 2015; Morrow *et al.* 2016) and trigger reproductive behaviour after emergence from hibernation (Dark 2005; Roth *et al.* 2010).

Stored energy reserves in hibernating animals are primarily used for physiological maintenance (Donohoe *et al.* 1998; Villecco *et al.* 1999), gamete production (Fitzpatrick 1976; Girish and Saidapur 2000) and post-emergence breeding (Pope and Matthews 2002). However, despite its potentially critical role in the reproductive cycle, our understanding of hibernation is still limited for most species (Browne and Paszkowski 2010; Gao *et al.* 2015). This gap in knowledge hinders our ability to predict how wild populations will respond to changes in climatic conditions (e.g. Williams *et al.* 2014) and poses a challenge for the management of captive breeding programs that serve as a genetic reservoir for assurance and reintroduction (Santana *et al.* 2015). In captivity, animals are often exposed to artificially shortened periods of hibernation in an attempt to minimise the physiological stress of low temperatures (Concannon *et al.* 1989; Roth *et al.* 2010; Santana *et al.* 2015). However, exceeding or underestimating the amount of time needed for hibernation can negatively affect the health and reproductive success of an individual. Staying in a prolonged state of hibernation can

lead to reduced immunocompetence (Burton and Reichman 1999; Prendergast *et al.* 2002) and increased oxidative stress (Carey *et al.* 2000), while curtailing hibernation before adequate gonadal development can diminish reproductive success (Concannon *et al.* 1989). Ideally, captive programs should determine an optimal length of hibernation that will maximise reproductive success while minimising the erosion of body condition (Humphries *et al.* 2003).

In amphibians, reproductive phenology is strongly temperature dependent (reviewed in Wells 2007), with prolonged exposure to low temperatures being essential to the regulation of reproductive activity in multiple species, e.g. *Acris crepitans*, *Lithobates clamitans* (Brenner 1969) and *Rana muscosa* (Santana *et al.* 2015). However, for many amphibians, the relationship between hibernation and reproductive output is poorly known, with the few studies that have been conducted producing inconsistent results. For instance, using exogenous hormones to induce oviposition in captive females, Calatayud *et al.* (2015) reported that female fecundity was higher in Boreal toads (*Anaxyrus boreas boreas*) that have gone through hibernation, while Roth *et al.* (2010) reported the opposite effect in the same species. In males of the same species, following hormonal stimulation, Roth *et al.* (2010) found that hibernation elicited more amplectic behaviour but had no effect on sperm production. Consequently, it remains unclear whether captive hibernation is necessary for, or contributes to, reproductive success in this and other amphibian species. The paucity of information signals the need for further efforts to elucidate the relationship between hibernation and reproductive physiology in amphibians. This knowledge is particularly relevant to conservation efforts for threatened or endangered amphibians, which rely on captive breeding programs for survival.

Captive breeding programs are an important resource that protect species from extinction and provide a source of animals for reintroduction efforts (Robert 2009). Of the 20 threatened or endangered amphibians in North America (IUCN 2018), reintroduction efforts are ongoing for seven species (*Anaxyrus baxteri*, *Anaxyrus houstonensis*, *Lithobates onca*, *Lithobates sevosus*, *Lithobates subaquavocalis*, *Rana muscosa* and *Rana sierra*). Within these, the Wyoming toad (*A. baxteri*) breeding program has been a pioneer in captive breeding and release and provides a valuable opportunity to examine the effects of hibernation on reproductive output. This federally endangered anuran is listed as Extinct in the Wild (IUCN 2018) and is endemic to the floodplains of the Laramie Basin in Albany County, Wyoming (Baxter *et al.* 1982), where below-freezing temperatures persist from October through April. Wild populations experienced a marked decline in the mid-1970s (Odum and Corn 2005) and 1980s (Lewis *et al.* 1985), presumably due to mortality from chytridiomycosis (Hammerson 2004). In response, a captive breeding program was established in 1989 using wild individuals from Mortenson Lake National Wildlife Refuge, Albany County, Wyoming (US Fish and Wildlife Service 2015). Although ~10 000 tadpoles have been released annually since 2005 with the help of assisted reproductive technologies (US Fish and Wildlife Service 2016), natural breeding in the wild is seldom documented and wild populations are still dependent on captive releases. Given both the rarity of this species, its cryptic behaviour during winter months and its federally protected status, it has thus far been difficult to gain insights into its natural history and reproductive physiology in the context of hibernation. Information on the effects of hibernation will have direct and immediate benefits to conservation and management efforts, as there is an established need for applied research to improve the efficacy of the species' recovery program (US Fish and Wildlife Service 2015). Because of its long-standing *ex situ* (captive) breeding program, *A. baxteri* provides an excellent model for understanding how hibernation influences reproduction in temperate amphibians.

Herein, we experimentally investigated the effects of hibernation length on male sperm output in captive *A. baxteri* and provide comparisons with wild toads that have hibernated under natural conditions. Given the necessity for long periods of hibernation due to harsh winter temperatures in the species' native range, we hypothesised that hibernation was essential for optimal reproductive output of captive toads. Specifically, we predicted that (1) sperm quality and quantity (i.e. total number of cells, concentration, motility and viability) would be greatest in toads following a longer (60 day) captive hibernation, compared with a shorter (30 day) captive hibernation or the absence (0 day) of a hibernation period. We administered either exogenous hormones or control saline injections to captive toads to confirm the necessity of using exogenous hormones to facilitate sperm collection and assessment in *A. baxteri.* We further predicted that (2) captive hibernation would have a negative effect on toad body condition, given the absence of prey intake during this period and the potential energy tradeoff between reproduction and physiological maintenance. Finally, comparing captive toads to wild toads after a natural period of hibernation, we predicted that (3) sperm quality and quantity in wild toads would be greater than that of captive toads, regardless of captive treatment.

Methods

Captive toads

Animal husbandry

Adult male *A. baxteri* ($n = 45$) were maintained indoors at the Leadville National Fish Hatchery (Leadville, CO). Individuals were identified by dorsal wart patterns and kept in groups of three to five in acrylic enclosures (98 cm × 46 cm × 34 cm, length × width × height) on a 9.5 : 14.5 h light : dark cycle (lights on at 0600 hours). Ambient temperature in the toad enclosures ranged from 25 to 30°C. Enclosures were cleaned daily and included rock hides and continuously filtered water. Each toad was fed three medium-sized crickets or cockroaches per day. All animal procedures were approved by the Memphis Zoo Animal Care and Use Committee (Approval 17–102) and the US Fish and Wildlife Service (Permit TE704930–1).

Experimental hibernation

Indoor hibernation of toads was conducted from March to June, 2017. Captive-bred, 1-year-old males, with snout–vent lengths (SVL) ranging from 42.8 to 54.8 mm and weights ranging from 16.0 to 26.5 g, were randomly assigned to one of three treatments: (1) no hibernation, (2) 30-day hibernation or

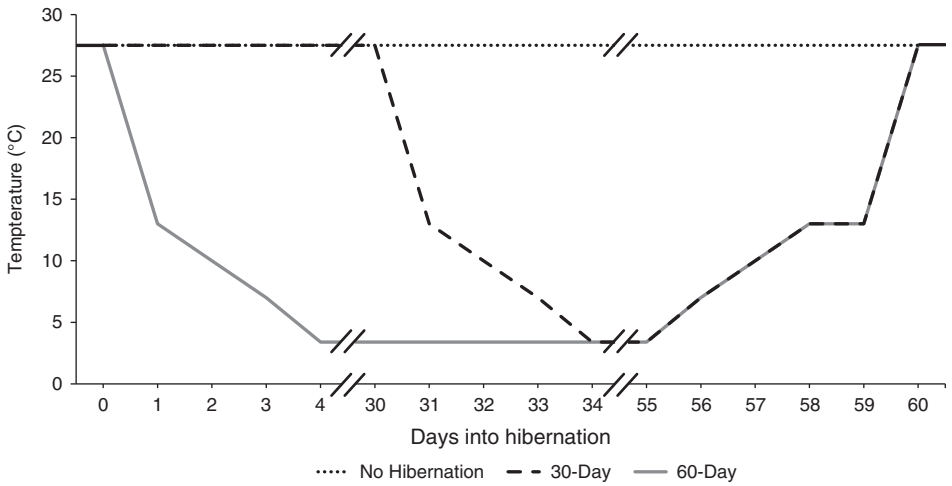

Fig. 1. Temperature regimes for captive *Anaxyrus baxteri* individuals undergoing no hibernation (dotted line), a 30-day hibernation (dashed line) and a 60-day hibernation (solid line).

(3) 60-day hibernation ($n = 15$ each). In the 30-day and 60-day hibernation treatments, toads were fasted for 7 days before and during the hibernation period. At the onset of hibernation, toads were transferred to 12-quart clear plastic boxes with air holes (42 × 46 × 16.5 cm). Toads were housed in groups of five per box. Substrate in boxes included, from bottom to top, one layer of carbon pad (Complete Filtration Services, Inc.), 7.5 cm of coconut shavings and 2.5 cm of sphagnum moss (Zoo Med Laboratories, Inc.). Toads in 30- and 60-day hibernation treatments were cooled to 3.4°C in two separate refrigerators (Gibson RT14F3WL) following a stepwise procedure (Fig. 1), with hibernation ending on the same calendar day for both treatments. Non-hibernating toads were maintained at ambient temperature (range 25–30°C) without any changes to housing or husbandry. Temperatures were controlled and monitored daily (Digital Temperature Controller Outlet Thermostat Itc-308; Inkbird) to ensure that refrigerators were functioning properly.

Spermiation

We evaluated the quality and quantity of spermatozoa produced by males in all three captive hibernation treatments. Sperm collection started 1 day after the end of experimental period (starting Day 61; Fig. 1), with three males from each treatment group assessed per day. To induce spermiation, toads were injected intraperitoneally with either 0.2 μg g^{-1} body-weight gonadotrophin-releasing hormone analogue (GnRH-a; Sigma-Aldrich; $n = 10$ toads per treatment) or an equivalent volume of saline as a control (0.9% sterile saline; Sigma-Aldrich; $n = 5$ toads per treatment). Injections were administered using a 0.3 mL syringe and 29 gauge 0.5 inch needle. GnRH-a stimulates the pituitary gland to release luteinising hormone and follicle-stimulating hormone to promote spermatogenesis and spermiation in males (Kouba *et al.* 2012*a*). Exogenous hormones were selected because hormonal induction of spermiation is currently the only method available for collecting anuran spermatozoa in a nondestructive manner. The hormone dosage used is based on and consistent with what is used across major *A. baxteri* breeding facilities (e.g. Saratoga National Fish Hatchery, Red Buttes Environmental Biological Laboratory and Detroit Zoo) and has been proven to be successful in annual captive breeding efforts (US Fish and Wildlife Service 2015, 2017). Exogenous hormone treatments are an established method of inducing spermiation in anurans (Kouba *et al.* 2009), although hormone protocols may vary across studies and across different species. Control injections of saline were used to account for effects of intraperitoneal injections on toads and to assess spontaneous sperm release without the aid of exogenous hormones.

Immediately after injection, each toad was placed in an individual 2.4 L plastic box filled with 1 cm of filtered water to promote urine production. Urine was collected 4 h after injection by gentle abdominal massage, with toads held above a 150 × 25 mm cell culture dish. If urine was not obtained within 5 min, collection was facilitated by inserting medical-grade, plastic catheter tubing (0.86 mm inner diameter × 1.32 mm outer diameter; Scientific Commodities, Inc.) into the cloaca. If an adequate urine sample (≥40 μL) was not collected within 5 min of catheterisation, the toad was returned to its box and a second collection was attempted 30 min later.

Sperm assessment

Each urine sample was immediately assessed for both the presence and quality of spermatozoa. For samples containing spermatozoa, the percentage of motile spermatozoa were evaluated at 400× using a manual differential cell counter and an Olympus CX41 phase-contrast microscope. Sperm motility was determined by counting all cells with flagellar movement within 100 cells, following Della Togna *et al.* (2017). Visual counts are a common method used in amphibian sperm analysis (Shishova *et al.* 2011; Kouba *et al.* 2012*b*; Germano *et al.* 2013; McDonough *et al.* 2016; Della Togna *et al.* 2017, 2018). All observations were conducted by the same observer to reduce potential biases between different observers. However, due to logistical constraints, the observer was not blind to the

experimental treatments. Alternative methods, such as computer-assisted sperm analysis, were not selected in this study because of the scarcity of low-cost methods developed specifically for anurans (but see Silla *et al.* 2017). Sperm concentration was determined using a Neubauer ruled chamber haemocytometer. Specifically, sperm cells within all four sets of the 16 corner squares were counted and multiplied by a dilution factor of 10 and a scaling factor of 2500 for each sample. Urine volume was determined using an adjustable-volume pipette.

Sperm samples were stained using 0.5% eosin Y stain in aqueous NaCl and nigrosin-saturated solution (Fisher Scientific) within 3 min of collection to evaluate cellular viability (i.e. plasma membrane integrity). For each sample, 5 µL of spermic urine was mixed with 10 µL eosin solution; 30 s later 15 µL nigrosin-saturated solution was added to the sample. The sperm solution was smeared onto a glass slide, air-dried and analysed under 400× magnification. Cells with intact plasma membranes displayed white heads, whereas those with non-intact membranes displayed pink heads. A total of 100 cells were counted per male.

Body condition

Body condition was assessed by weighing individuals using an electronic scale (LBK6a; Adam Equipment) and measuring SVL using a digital caliper (Carbon Fibre Composites Caliper; YKS) at the onset of the study (Day 1) and again after the experimental period was complete (Day 60).

Wild toads

Study site

Wild *A. baxteri* males ($n = 5$) were sampled in June 2018 at Mortenson Lake National Wildlife Refuge, Laramie, WY (41.211018, −105.843795). Males with SVL ranging from 47.3 to 55.6 mm and weights ranging from 18.0 to 23.9 g were injected with 0.2 µg g^{-1} bodyweight GnRH-a and placed in individual 2.4 L plastic boxes filled with 1 cm of filtered water to promote urine production. Urine collection and sperm assessment followed the methods used for captive toads, as described above. Due to sample size limitations when working with wild individuals of a critically endangered species, all males were injected with exogenous hormones to facilitate spermiation and control saline injections were not administered to a subset of wild males.

Statistical analyses

Statistical analyses were performed using R Statistical Software (Version 3.4.1; R Core Team 2017). Assumptions of data normality and homogeneity of variances were tested using the Shapiro–Wilk test and Bartlett's test respectively. Data are presented as mean ± standard error.

Captive toads

An ANOVA was performed to test for differences in sperm motility across treatment groups. To assess differences in sperm concentration and sperm viability across treatments, Kruskal–Wallis H tests were performed. Total sperm count was calculated by multiplying spermic urine volume by sperm concentration. These data were then square-root transformed and an ANOVA was performed to assess differences across treatment groups. A regression of log-transformed toad weight and log-transformed SVL was performed and the residuals were used as an index of body condition (i.e. length-independent body mass, see Schulte-Hostedde *et al.* 2005; Harris 2008). An ANOVA was performed to ensure that initial body condition across treatments was not significantly different. Differences in percentage change in body condition across treatment groups were assessed using a Kruskal–Wallis H test. Percentage change in body condition was computed as the difference in body condition at Day 1 and Day 60, divided by the body condition at Day 1. In addition, differences between initial and final body condition within treatment groups were assessed using paired *t*-tests. A Pearson's correlation was used to assess relationships between sperm metrics (motility, concentration and viability) and percentage change in body condition.

Wild toads

Welch's *t*-tests were performed to compare sperm motility and total sperm count between captive and wild toads. Total sperm count data were square-root transformed to meet the assumption of homogeneity of variances. To assess differences in sperm concentration and sperm cell viability, Mann–Whitney U tests were performed. Captive data were pooled and averaged across all three treatment groups (no hibernation, 30-day hibernation and 60-day hibernation).

Results

Sperm metrics

Across captive treatments, all males injected with GnRH-a ($n = 30$) produced spermic urine, whereas all males injected with saline as controls ($n = 15$) produced urine without spermatozoa. As such, all further comparisons and discussions of sperm quality and quantity for captive males use data collected from GnRH-a-injected males only. Within captive toads, there was no difference in any sperm metric (Table 1, Fig. 2). Overall, captive GnRH-a-injected toads produced urine samples with $1.08 \pm 0.16 \times 10^6$ total spermatozoa (range 0.01–3.51×10^6 spermatozoa), $2.47 \pm 0.36 \times 10^6$ spermatozoa mL^{-1} (range 0.02–9.10×10^6 spermatozoa mL^{-1}), $58.2 \pm 3.3\%$ motility (range 23.0–90.0%) and $71.5 \pm 3.3\%$ viability (range 38.0–92.0%). In comparison, wild-caught toads, which were all injected with GnRH-a, produced urine samples with $0.56 \pm 0.29 \times 10^6$ total spermatozoa (range 0.02–1.52×10^6

Table 1. Summary of statistics comparing metrics of sperm quality and quantity in captive *Anaxyrus baxteri*

Metrics compared individually across three treatments (no hibernation, 30-day hibernation and 60-day hibernation)

Sperm metric	Test type	*df*	N	Test statistic	*P* value
Total number (10^6 cells)	ANOVA	2	30	1.83	0.18
Concentration (10^6 cells mL^{-1})	Kruskal–Wallis H	2	30	1.44	0.49
Motility (%)	ANOVA	2	30	1.74	0.19
Viability (%)	Kruskal–Wallis H	2	30	0.17	0.92

spermatozoa), $1.53 \pm 0.89 \times 10^6$ spermatozoa mL^{-1} (range $0.16–4.83 \times 10^6$ spermatozoa mL^{-1}), $75.4 \pm 10.52\%$ motility (range 36.0–94.0%) and $57.2 \pm 4.05\%$ viability (range 42–65%). Average sperm quality and quantity in captive toads was not significantly different from wild toads that hibernated under natural conditions (Fig. 3; total spermatozoa: $T_{4.49} = \_0.25$, $P = 0.81$; sperm concentration: $U = 93$, $P = 0.42$; motility: $T_{4.79} = \_1.56$, $P = 0.18$; viability: $U = 112$, $P = 0.09$).

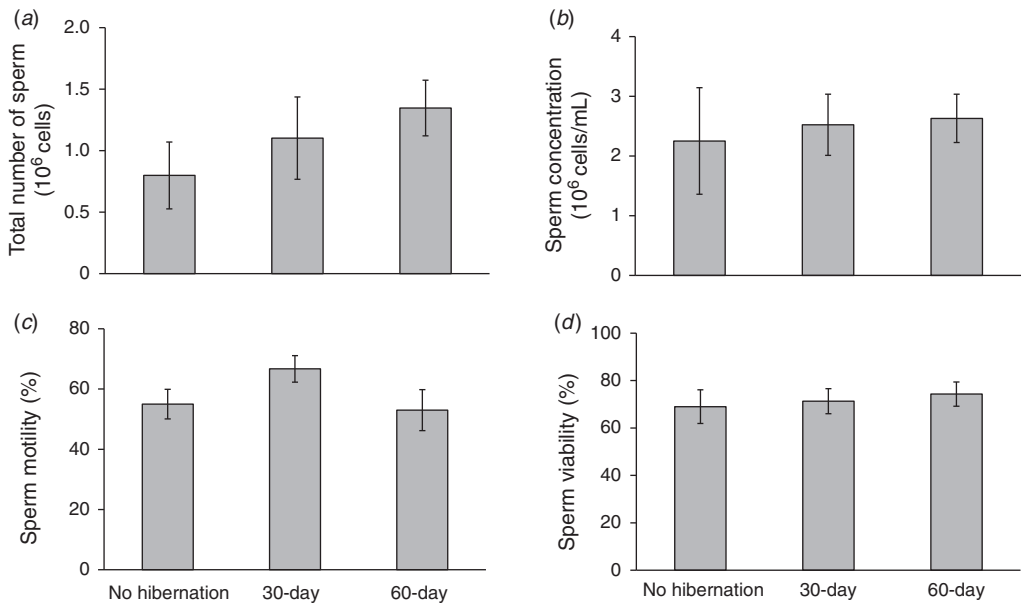

Fig. 2. (*a*) Total number of spermatozoa, (*b*) sperm concentration, (*c*) sperm motility and (*d*) sperm viability of *Anaxyrus baxteri* across the three captive hibernation treatments: (1) no hibernation, (2) 30-day hibernation and (3) 60-day hibernation ($n = 10$ per treatment). Data are mean ± standard error.

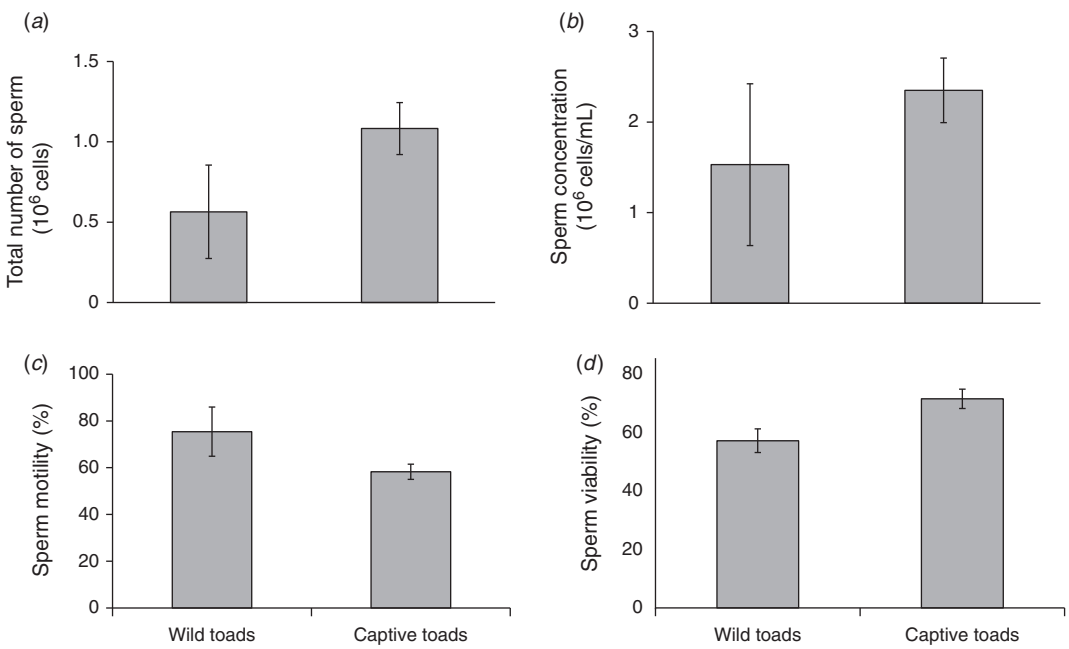

Fig. 3. (*a*) Total number of spermatozoa, (*b*) sperm concentration, (*c*) sperm motility and (*d*) sperm viability of *Anaxyrus baxteri* that have experienced a full winter hibernation under natural conditions (wild toads; $n = 5$) compared with those that were kept in captivity, regardless of hibernation treatment (captive toads; $n = 10$). Data are mean ± standard error.

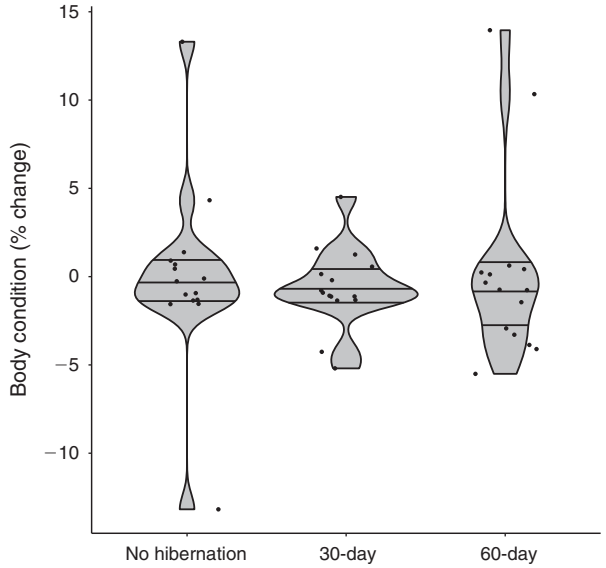

Fig. 4. Violin plots of percentage change in *Anaxyrus baxteri* body condition (length-independent body mass) across the three captive hibernation treatments ($n = 15$ per treatment). Dots represent raw data points, lines represent quartiles and shaded areas represent probability density.

Body condition

Initial body condition and percentage body condition change of individuals were similar across all treatment groups ($F_{2,42} = 1.59$, $P = 0.22$ and $X^2_2 = 0.39$, $P = 0.82$ respectively; Fig. 4). Moreover, body condition within all three treatment groups showed no significant change over the study period (no hibernation: $T_{14} = 0.80$, $P = 0.44$; 30-day hibernation: $T_{14} = -0.41$, $P = 0.69$; 60-day hibernation: $T_{14} = -0.13$, $P = 0.90$). Across all individuals, percentage body condition change (-0.15 ± 0.65, range -13.18–13.95) was not correlated with total number of spermatozoa ($r = -0.12$, $P = 0.51$), sperm concentration ($r = -0.03$, $P = 0.85$), motility ($r = -0.12$, $P = 0.54$) or viability ($r = -0.09$, $P = 0.64$).

Discussion

Hibernation is often assumed to play an important role in the reproductive cycle of temperate species. However, there is little empirical evidence on how essential hibernation really is to the different behavioural and physiological processes that lead to reproductive success. Consequently, it is difficult to predict the response of animals hibernating under captive conditions or in changing climatic environments. Our study was the first to investigate the influence of hibernation on sperm output in both captive and wild *A. baxteri*, a federally protected species that serves as a valuable research model for other hibernating amphibians. Contrary to our predictions, the presence and duration of hibernation did not affect sperm quality or quantity. Following hormonal stimulation, regardless of treatment group, both captive and wild toads produced similar sperm qualities and quantities. Additionally, only individuals receiving hormonal stimulation produced spermic urine. Finally, captive toads that hibernated for a longer period of time did not exhibit a reduced body condition. These unexpected findings raise intriguing questions about the role of hibernation in optimising reproductive success, an issue with direct relevance to *ex situ* amphibian conservation efforts.

Comparatively, two previous studies, while not specifically testing the effects of artificial hibernation on sperm output, have assessed *A. baxteri* sperm quality in captive individuals. Sperm concentration and motility of captive toads in our study ($2.47 \pm 0.35 \times 10^6$ cells mL^{-1} and $58.2 \pm 3.2\%$ respectively) were similar to findings in these studies that did (1.9×10^6 cells mL^{-1} and ~60%, $n = 5$; Obringer *et al.* 2000) and did not (~2×10^6 cells mL^{-1} and ~45%, $n = 8$; Browne *et al.* 2006) impose an artificial period of hibernation. Sperm viability in our captive toads was less than that found by Obringer *et al.* (2000; 71.5 ± 3.3 and 95.3% respectively), although this variation could be attributed to differences in staining methods (eosin–nigrosin and Hoechst 33258 respectively). Nevertheless, even though methods vary across studies, our findings, in conjunction with previous research, demonstrate that the production of healthy, high-concentration spermatozoa is possible in both the presence and absence of captive hibernation. Moreover, despite the complete absence of prey intake for up to 60 days, hibernation did not have an adverse effect on the body condition of captive males. Collectively, findings from sperm metrics and body condition indicate that hibernation in *A. baxteri* may be less related to a reproductive physiological need and potentially more driven by other factors, such as prey availability, predation threat and harsh environmental conditions during winter months.

For most amphibians, the relationship between hibernation and reproductive success remains poorly understood, in part due to the challenges of observing hibernation under natural conditions. The effects of hibernation on reproductive output in amphibians is further complicated when looking at the use of exogenous hormones. Though the use of exogenous hormones, such as the GnRH-a used in the present study, has been well established in captive breeding programs (Kouba *et al.* 2009), the interplay between hibernation and hormone use requires further investigation. Indeed, it is possible that the use of exogenous hormones in the present study, as well as previous studies examining captive hibernation, may have induced an effect on gamete release that either enhances, conflicts with or even overrides the underlying response to captive hibernation conditions. For example, Santana *et al.* (2015) found that only hibernated *R. muscosa* males were able to successfully fertilise eggs without the use of exogenous hormones. However, spermiation in captivity can be induced without hibernation through the use of exogenous hormones in several amphibians, including *A. baxteri* (Browne *et al.* 2006), *A. boreas boreas* (Roth *et al.* 2010), *A. fowleri* (McDonough *et al.* 2016) and the southern Gulf Coast toad (*Incilius valliceps*; Rowson *et al.* 2001). Although our study was not focussed on examining the possible effects of exogenous hormones or their interactions with hibernation, our findings provide support that hormone-induced spermiation can be achieved at similar qualities both in captive and wild toads, regardless of the hibernation regime.

The maintenance of sperm quality and quantity after year-round exposure to warm (≥25°C) temperatures raises the

question of whether the effects of hibernation on the reproductive cycle of *A. baxteri* are more plastic and facultative rather than a critical part of its life history. Although the potential for facultative hibernation has not been directly investigated in any amphibian species, our findings suggest that the effects of hibernation could be more flexible and dependent on environmental conditions rather than the physiological necessity for reproduction. It is important to note that the alterations in hibernation length could exert a stronger effect through multiple reproductive cycles. Moreover, a shortened hibernation could affect spermiation in subsequent breeding seasons, as spermatogenesis can be seasonal and occur before hibernation (Glass and Rugh 1944; Atherton 1974; Quinn and Mengden 1984). Unfortunately, due to the limitations in our knowledge of the relationship between hibernation and the ontogeny of spermatogenesis in most amphibians, several further studies are required before additional comparisons and discussions can be made. Potential areas that merit future research efforts include: (1) examining multi-year effects of shortened hibernation in captivity, (2) investigating the duration and timing of spermatogenesis, (3) documenting the effects of hibernation on reproductive behaviour and (4) exploring the interplay between hibernation, oogenesis and ovulation.

Overall, our study provides empirical evidence suggesting that, although there are several caveats to consider, hibernation may not be an essential process for achieving reproductive success in a captive temperate amphibian. This unexpected finding provides insight into the species' ecophysiology and potential response to hibernation in captivity and to changing climatic conditions in the wild. Specifically, our data suggest that with the use of exogenous hormones *A. baxteri* have the potential to yield viable spermatozoa both with and without exposure to colder environmental conditions. Moreover, toads maintained in a captive environment have the ability to produce spermatozoa that are of comparable quality and quantity to those produced by toads that are found in their natural habitats. However, this ability will only translate to increased reproductive success and fitness if other aspects of reproductive behaviour and physiology are maintained. To gain a more complete picture of the overall effects of hibernation on reproductive success, future research is needed in the areas we suggested above, in addition to quantifying mating success, percentage fertilisation and offspring survivorship in hibernated versus non-hibernated individuals. Collectively, research on hibernation in threatened and endangered amphibians will not only benefit conservation efforts but may yield new insights into the mechanisms of cold adaptation in ectothermic vertebrates.

Conflict of interest

The authors declare no conflicts of interest.

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