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Original Research Article

Amphibian conservation using assisted reproductive technologies: Cryopreserved sperm affects offspring morphology, but not behavior, in a toad

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ABSTRACT

With increasing rates of global biodiversity decline, strategies such as reintroduction or supplementation, have become increasingly important in conserving our remaining biodiversity. To sustain reintroduction programs, insurance colonies are established and bred in captivity. Captive-bred offspring are then released to augment wild populations or establish new populations. A key issue determining the success of reintroduction programs, therefore, is the fitness of captive-bred individuals and their ability to survive once released. Unfortunately, little is known about the quality of captive-bred offspring produced using assisted reproductive technologies, such as gamete cryopreservation. To fill this gap in scientific knowledge and conservation practice, we examined differences in tadpole morphology, tadpole behavior, metamorph morphology, and duration of larval stage between Fowler's toad (*Anaxyrus fowleri*) offspring produced using cryopreserved sperm (experimental, cryo-derived individuals) and offspring produced by amplectant adults (control individuals). Results indicated cryo-derived individuals were smaller as tadpoles and emerged as smaller metamorphs. However, predator-avoidance behavior was not significantly different between the two treatment groups. Smaller body size in cryo-derived individuals can negatively affect their post-release survivorship and reproductive output, thus limiting the potential success of reintroduction programs. This pioneering study provides insights into the quality and competency of individuals produced using cryopreserved sperm across two distinct life-stages. We show that although cryopreservation has often been proposed as a promising way of contributing to wildlife conservation, more detailed examinations are needed to assess the quality of offspring produced for it to be an effective conservation tool.

1. Introduction

The loss of biodiversity across taxa in recent years has necessitated the development of cross-disciplinary strategies to prevent or decrease the rate of further species declines. Within applied conservation biology, reintroduction biology forms a bridge between *in situ* and *ex situ* conservation. Though the idea of reintroduction can be traced back for over 100 years, it is a subject that has garnered more attention in recent decades (reviewed in Armstrong and Seddon, 2008; Kleiman, 1989). By

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using captive-bred, head-started, or translocated individuals, reintroduction programs can augment existing populations or establish new populations (Fischer and Lindenmayer, 2000; Mendelson et al., 2019). These programs aim to improve species recovery and, ultimately, to restore ecosystem function and equilibrium (Armstrong and Seddon, 2008). To sustain reintroduction programs, captive insurance colonies are often established to serve as source populations. These insurance colonies are charged with producing offspring that can be released into natural habitats as part of the reintroduction effort (Kissel et al., 2014).

A novel area of research within reintroduction biology is the use of assisted reproductive technologies, specifically gamete cryopreservation, in captive-breeding. Assisted reproductive technologies are particularly crucial for threatened and endangered species, which often have small population sizes or are unable to breed naturally in captivity. Gamete cryopreservation can extend the genetic lifespan of individuals (Ballou, 1992), resulting in an increase in breeding success of adults and in genetic diversity of captive-bred offspring. Propagation using cryopreserved gametes has been reported in mammals (Amstislavsky et al., 2017), birds (Saint Jalme et al., 2003), fish (Asturiano et al., 2017; Martinez-Paramo et al., 2017), amphibians (Shishova et al., 2011), and invertebrates (Hagedorn et al., 2017; Liu et al., 2014). In corals, for example, success in gamete cryopreservation and propagation (Hagedorn et al., 2017) illustrates the potential impact such methods can have by not only conserving the targeted species, but helping to restore an ecosystem that supports a range of different taxa. Thus far, however, studies on cryopreservation have been largely skewed towards developing and optimizing laboratory protocols to increase fertilization success, with limited follow-up beyond fertilization. While it is necessary to investigate and refine such protocols to increase the number of offspring produced, the success of reintroduction programs can be more dependent on the quality of these offspring rather than the quantity (Mendelson and Altig, 2016). Without a systematic evaluation of the growth and behavior of cryo-derived offspring, we cannot predict or determine how they will fare once they are released into the wild. In other words, though cryopreservation is increasingly proposed as a means of species conservation (e.g. Amstislavsky et al., 2017; Asturiano et al., 2017; Martinez-Paramo et al., 2017), its effectiveness in producing high-quality offspring, and therefore its applicability to reintroduction programs in most taxa, has yet to be determined.

In fact, despite proposals to use gamete cryopreservation as a tool for wildlife conservation (Williams and Hoffman, 2009), its use is still largely restricted to aquaculture and livestock (reviewed in Comizzoli, 2015; Morrell and Mayer, 2017). Many species of amphibians are ideally suited for studies of cryopreservation not only because of their life-history characteristics (high fecundity, short generation time, and small sizes), but also because of the wealth of empirical studies documenting how growth, development, and behavior in early life stages can affect fitness and survivorship in later life stages (see Discussion). Moreover, as a taxon with one of the fastest rates of species declines (Wake and Vredenburg, 2008), amphibians are becoming increasingly dependent on the assistance of conservation efforts, including captive-release programs (Mendelson and Altig, 2016). Given these considerations, we used the Fowler's toad (*Anaxyrus fowleri*), a common and widespread species in the eastern U.S. (IUCN, 2018: Least Concern; Powell et al., 2016), as a model species. By integrating techniques for gamete cryopreservation with morphological and behavioral assessments, our goal was to determine how the use of cryopreserved gametes can affect the growth and performance of the resulting offspring.

Herein, we examined the morphological and behavioral differences in Fowler's toad offspring produced through cryopreserved gametes compared to those produced through natural mating (amplexus) of adults. We hypothesized that given the sensitivity and plasticity of early embryonic development in amphibians, offspring produced with cryopreserved sperm would be smaller in body size as tadpoles and as metamorphs. Moreover, we hypothesized that cryo-derived tadpoles would exhibit less adaptive predator-avoidance behaviors when exposed to threatening cues. Findings from this study will serve as an example of the effects of gamete cryopreservation on offspring fitness and shed light on how it may affect the capacity and impact of reintroduction programs.

2. Methods

2.1. Study species

We conducted this study from April to August 2018. All procedures were approved by the Memphis Zoo Animal Care and Use Committee (Approval 17−101) and the Tennessee Wildlife Resource Agency (Permit 1315). The Fowler's toad was chosen as a proxy for the six threatened *Anaxyrus* and 233 threatened Bufonid species worldwide (IUCN, 2018). It shares similar reproductive biology, larval environment, and life history strategies with endangered congeneric species in the U.S., such as the Houston toad (*Anaxyrus houstonensis*) and the Wyoming toad (*Anaxyrus baxteri*). Additionally, it has a well-established history as a model system (e.g. Green, 2015; McDonough et al., 2016; Poo and Hinkson, 2019; Venesky et al., 2009) and a stable conservation status (IUCN, 2018). We collected 30 Fowler's toads from Shelby County, Tennessee and maintained all individuals in 10 gallon glass aquaria (51 cm L × 25 cm W × 31 cm H), with up to four toads per aquaria. We fitted enclosures with coconut shavings, cover, and water, and cleaned enclosures daily. We fed adults a variety of insects ad libitum, including crickets, mealworms, and superworms, with similar ratios of insects given per toad.

2.2. Sperm collection, cryopreservation, and thawing

Toads were randomly assigned to either the experimental (cryo-derived) group or control group. For the experimental group, we conducted *in vitro* fertilization using cryopreserved sperm and fresh eggs, following established methods (e.g.

Kouba et al., 2012). To obtain sperm from male toads, we administered 7.5 IU hCG/g body weight to each male (hCG: human chorionic gonadotropin; Sigma-Aldrich, St. Louis, MO) through intraperitoneal injection using a 0.3 mL syringe and 29 gauge ½" needle. Toads were then transferred to individual 2.4 L plastic boxes filled with 1 cm of aged tap water to promote urine production. Spermic urine was collected 4 h post-injection via natural urination or urination facilitated by a medical-grade plastic catheter tubing (0.86 mm inner diameter × 1.32 mm outer diameter; Scientific Commodities, Inc.). To cryopreserve the samples, we prepared a cryoprotectant solution with 10% DMFA (N,N-Dimethylformamide; Sigma-Aldrich, St. Louis, MO) and 20% trehalose (Sigma-Aldrich, St. Louis, MO) in distilled water and gradually diluted it 1:1 with fresh spermic urine to create a sperm cryosuspension with a final concentration of 5% DMFA and 10% trehalose. This particular cryoprotectant was shown to produce the highest sperm recovery rate for Fowler's toads (Poo and Hinkson, 2019). Samples were loaded into 0.25 cc cryostraws (Reproduction Resources, Walworth, WI) and placed at 4 °C and then −90 °C for 10 min each, after which they were immersed into liquid nitrogen for long-term storage (−196 °C). Samples remained frozen for at least 48 h prior to thawing. To thaw, cryopreserved sperm samples were removed from liquid nitrogen, placed in room temperature (23 °C) and then in a 40 °C water bath for 10 s each, and diluted 1:10 with distilled water.

2.3. In vitro fertilization and natural breeding

To produce cryo-derived offspring, we induced ovulation in female toads and fertilized the eggs with cryopreserved and thawed sperm. To induce oviposition, we administered two priming doses of 2.5 IU hCG/g body weight 72 h apart, followed by an ovulation dose of 12.5 IU hCG +0.5 μg GnRH/g body weight 24 h after the second priming dose (GnRH: gonadotropin-releasing hormone; Sigma-Aldrich, St. Louis, MO) (Kouba et al., 2012). Once oviposition began, females were held over cell culture dishes (150 × 25 mm), so that eggs could be deposited directly on to the dish. Immediately after eggs were deposited, thawed sperm samples were pipetted onto eggs for fertilization. After 5 min, the Petri dish was filled with aged tap water to submerge the fertilized eggs. From a concurrent study on Fowler's toads, on average cryopreserved sperm cells were able to produce 27 fertilized eggs per 1,000,000 sperm cells applied (Poo and Hinkson, 2019). For the control group, we placed male-female pairs in individual plastic boxes (37 cm L × 20 cm W × 12 cm H) filled with 2 cm of aged tap water. For each box, one male and one female were randomly selected, paired, and given the same hormone injections as the experimental group, but allowed to lay their clutches naturally through male-female amplexus.

2.4. Rearing and assessments

From each male-female pairing, we randomly selected 50 tadpoles upon hatching and placed them in a 5 L buckets filled with aged water and fitted with an air pump. Tadpoles were fed a mixture of plankton food, alfalfa powder, and rabbit pellets ad libitum, and water was changed daily. Tadpole morphology and behavior were assessed for 16 randomly-selected individuals per bucket at 30 days post-oviposition. For tadpole behavior, we recorded their activity level following established methods (e.g. Chivers and Ferrari, 2013; Ferrari et al., 2008). Specifically, individuals were placed into 0.5 L opaque plastic cups and allowed to acclimate for 15 min. After acclimation, tadpole activity, in the form of the number of times an individual crossed the midline of the cup, was recorded for 4 min prior to and 4 min following the introduction of 5 mL of either a non-lethal predator cue (injured conspecific scent) or a control cue (aged water). The injured conspecific scent was obtained by filtering a solution of five homogenized tadpoles with 20 mL of aged water. Cues were introduced via a blunt headed syringe to minimize disturbance. Typically, an adaptive predator avoidance response in tadpoles is represented by a decrease in activity when sensing predator cues (Chivers and Ferrari, 2013; Ferrari et al., 2008). After behavioral assessments, we took dorsal photos of the same tadpoles and measured their total length, tail length, and body width using ImageJ (Rasband, 1997–2018). Tadpoles were returned to their original buckets after morphology photos were taken and reared until metamorphosis (Stage 46, Gosner, 1960). Upon metamorphosis, we recorded the snout–vent length (SVL), weight, and age (larval stage duration) of each metamorph.

2.5. Statistical analyses

Effects of cryopreservation on offspring morphology and behavior were tested using generalized linear models (GLMs). Clutch identity was included as an explanatory variable in all models to account for potential clutch effects. We tested the effects of cryopreservation on total length, tail length, and body of width of tadpoles using generalized linear models (GLMs) with underlying Gamma distributions and inverse link function. Similarly, we tested the effects of cryopreservation on SVL and weight at metamorphosis and larval stage duration using GLMs with underlying Gamma distribution and inverse link function. For tadpole behavior, we tested effects of cryopreservation on baseline activity (line crosses) using GLMs with underlying log-Normal distributions and identity link function. We then tested effects of cryopreservation and cue type on the change in activity (proportional change in line crosses) again using a GLMs with underlying log-Normal distributions and identity link function. Change in line crosses was calculated as the number of line crosses after cue introduction divided by the number of line crosses prior to cue introduction. We conducted all statistical analyses in the R programming environment (v. 3.6.0) using a significance level of $\alpha = 0.05$. Means are presented with standard errors.

3. Results

Offspring from three cryo-derived clutches and seven control clutches were reared in the lab. At 30 days post-oviposition, total length and tail length of cryo-derived tadpoles were significantly shorter compared to control tadpoles (total length = 23.1 ± 0.5 and 26.5 ± 0.5 mm, tail length = 14.2 ± 0.3 and 16.7 ± 0.4 mm, respectively, $p < 0.001$ for both, N = 114, Fig. 1), though body width was not significantly different (6.0 ± 0.1 and 6.2 ± 0.1 mm, respectively, $p = 0.052$, Fig. 1). Baseline activity was not significantly different between cryo-derived and control tadpoles ($p = 0.955$, N = 113). Furthermore, change in activity level was not significantly different between cryo-derived and control tadpoles ($p = 0.384$, Fig. 2) or between those exposed to non-lethal predator cues and control cues ($p = 0.416$, Fig. 2). Upon metamorphosis, cryo-derived offspring had shorter SVLs and lighter weights compared to control offspring (SVL = 9.4 ± 0.1 and 11.1 ± 0.01 g, weight = 84.9 ± 3.0 and 124.4 ± 3.1 mg, respectively, $p < 0.001$ for both, N = 235, Fig. 3). Duration of the larval stage was longer in cryo-derived offspring (40.2 ± 0.6 and 38.9 ± 0.4 days, respectively, $p = 0.011$, Fig. 3). Clutch identity had a significant effect on all response variables examined ($P < 0.005$) except for metamorph weight ($P = 0.061$).

4. Discussion

By comparing cryo-derived individuals to their natural counterparts, we found a significant negative effect of the use of cryopreserved sperm on morphology, but not behavior, in an amphibian species. Specifically, Fowler's toad offspring produced using cryopreserved sperm were smaller as tadpoles and emerged as smaller, lighter metamorphs, despite having a longer tadpole stage. Given the paucity of studies on cryo-derived offspring across life stages, our findings provide a critical piece of information for amphibian conservation and reintroduction programs. While in captivity, small body size may not pose an immediate threat, but if introduced into natural environments, reduced body size can have negative implications for the fitness and survival of cryo-derived tadpoles. For instance, smaller tadpoles are known to be less efficient in prey consumption (Crossland, 1998) and more susceptible to predation (Formanowicz, 1986). Additionally, body size as tadpoles can affect post-metamorphic size and morphology (Relyea, 2001), which may have additional implications for survivorship in subsequent life stages.

Our data showed a carry-over effect, from larval to juvenile stages, resulting in not only smaller tadpoles, but also smaller metamorphs. Size at metamorphosis can have lasting effects on the fitness of juveniles and adults. In amphibians, larger body size has been linked to higher rates of growth and survival (Altwegg and Reyer, 2003; Cabrera-Guzmán et al., 2013; Morey and Reznick, 2001). Specifically, individuals that are larger at metamorphosis are known to have increased fecundity (Fontenot, 1999; Scott, 1994), earlier sexual maturity (Berven, 1990; Scott, 1994), better foraging abilities (Cabrera-Guzmán et al., 2013; Flowers and Graves, 1995; Newman, 1999), greater locomotive abilities (Beck and Congdon, 2000; Goater et al., 1993), and higher mating success (Howard and Young, 1998). It is possible that the effects of cryopreservation can be overcome through compensatory or postmetamorphic growth (e.g. Beck and Congdon, 1999; Smith, 1987), whereby early disadvantages of growth and development become less apparent over time. However, these differences can still persist for multiple years (Altwegg and Reyer, 2003), which could significantly stunt the reintroduction success of small, new populations. Additionally, stage-based matrix models and sensitivity analyses in various frog and toad species suggest that postmetamorphic survival rates are the most influential to population growth (Biek et al., 2002; Govindarajulu et al., 2005). As such, the morphological consequences cryo-derived tadpoles and especially metamorphs face can pose a significant obstacle to the successful establishment of amphibian species through reintroduction programs.

Surprisingly, the negative effect of cryopreservation on body size found in our study contradicts the majority of findings in aquaculture, which show that fry produced from cryopreserved sperm are not significantly different from those produced

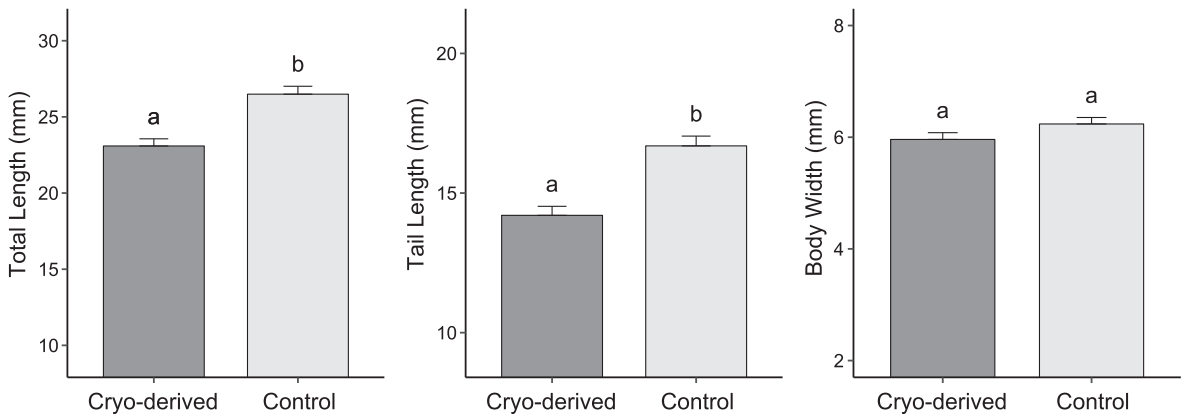

Fig. 1. Total length, tail length, and body width of *Anaxyrus fowleri* tadpoles produced using cryopreserved sperm (cryo-derived) and natural amplexus of adults (control). Values represent mean and standard error. Letters represent statistically significant groupings ($p < 0.05$).

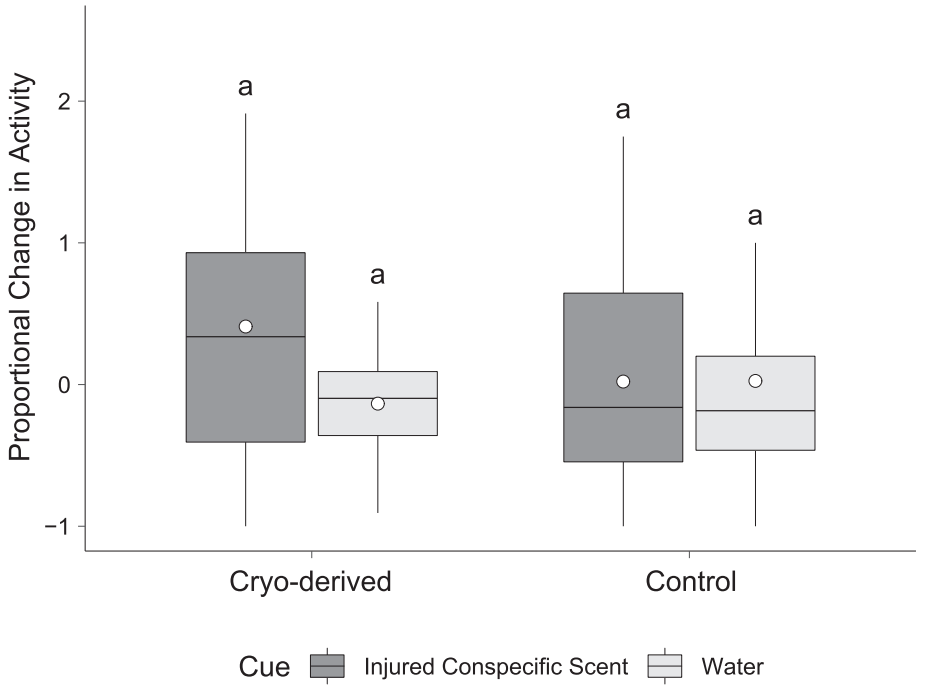

Fig. 2. Boxplot of porportional change in activity (line crosses) of *Anaxyrus fowleri* tadpoles produced using cryopreserved sperm (cryo-derived) and natural amplexus of adults (control). Lower and upper hinges of box plot correspond to the first and third quartiles, horizontal line denotes median, and white dot denotes mean. Letters represent statistically significant groupings ($p < 0.05$).

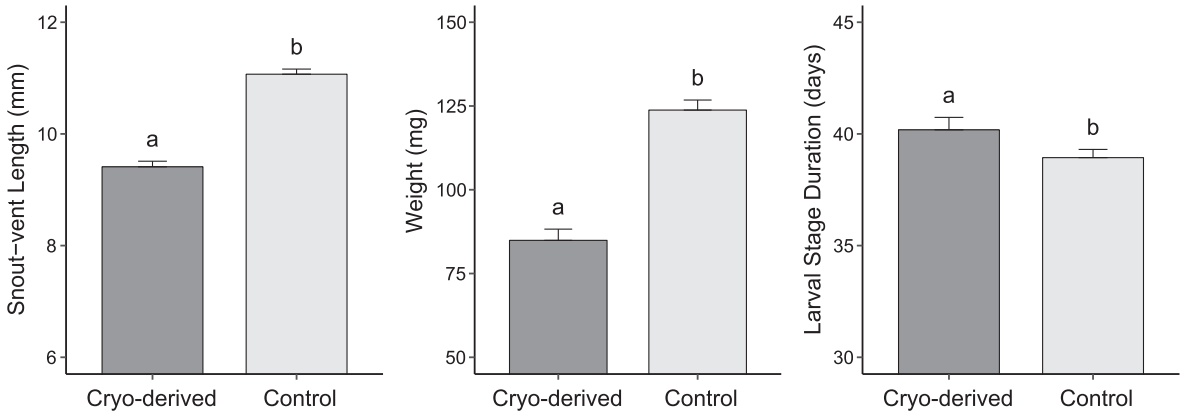

Fig. 3. Metamorphic snout-vent length, metamorphic weight, and duration of larval stage of *Anaxyrus fowleri* tadpoles produced using cryopreserved sperm (cryo-derived) and natural amplexus of adults (control). Values represent mean and standard error. Letters represent statistically significant groupings ($p < 0.05$).

with fresh sperm during early developmental stages (Akter et al., 2016; Labbe et al., 2001; Sarder et al., 2013; Viveiros et al., 2012). Our findings, however, are supported by studies in the African catfish (*Clarias gariepinus*) and spiny eel (*Mastacembelus armatus*) (Rahman et al., 2016; Van Der Walt et al., 1993), which show a slower growth rate in cryo-derived fry. Despite their findings, both of these studies dismiss the differences as artifacts of environmental variations within the rearing tanks of the fishes instead of considering it as effects of cryopreservation. In contrast, the smaller body size of cryo-derived tadpoles in our study is unlikely to be due to environmental variation because all tadpoles were reared under the same environmental conditions (e.g., equal enclosures, densities, temperature, food availability, etc.).

Alternatively, we suggest that structural damage to both the sperm and embryo are the most likely mechanisms for the differences in size between treatment groups. For instance, the process of cryopreservation and thawing is known to result in sperm DNA fragmentation in amphibians (Morrow et al., 2017) and has been shown to affect embryo viability in both amphibians and fish (Gosálvez et al., 2014; Pollock, 2015). In a previous study, decreased sperm quality after cryopreservation has been observed for both Ranidae and Bufonidae species, with a low fertilization ability of Fowler's toad sperm after the

cryopreservation process (Poo and Hinkson, 2019). During *in vitro* fertilization, it is possible that the oocytes were exposed to a residual toxicity from the cryoprotectants in the sperm solution. Adam et al. (1995) show that cryoprotectants can significantly decrease enzyme activity in developing fish embryos, which could explain the lasting effects we see on development. As such, it is important to examine the growth and development of offspring in addition to traditional studies that focus on the initial stages of fertilization. While our findings establish a relationship between cryopreservation and offspring growth, additional research is needed to elucidate exactly how the process of cryopreservation is affecting the offspring produced.

Beyond growth and development, another critical factor determining the survivorship and reproductive output of individuals is their behavior. Here again, there is a lack of previous research on how the behavior of cryo-derived individuals differs from their natural counterparts that can be compared to our study. However, maladaptive behavior exhibited by captive-bred individuals in general can have a negative impact on the success of reintroduction programs (e.g. Jule et al., 2008). For instance, studies on captive-bred animals have indicated a reduced behavioral response to predators in swift foxes and oldfield mice (Bremner-Harrison et al., 2004; McPhee, 2004), lower foraging efficiency in bank voles (Mathews et al., 2005), and lower mating success in Chinook salmon (Berejikian et al., 2001). Given the importance of adaptive behavior to post-release survival, our findings provide the first insight into the behavior of cryo-derived individuals in an amphibian. Contrary to our predictions and previous findings on predator avoidance behavior in aquatic systems (reviewed in Ferrari et al., 2010), we did not see a decrease in activity in response to the threat of predation in either cryo-derived or control tadpoles. Moreover, behavior of cryo-derived tadpoles was not significantly different from that of the control tadpoles. The absence of a predator avoidance response may be due to a lack of sensitivity to these cues when tadpoles were assessed. Evidence from gray treefrog (*Hyla versicolor*) tadpoles suggest that predator avoidance can be limited to a specific stage of their development, when tadpoles are at a vulnerable size (Bridges and Gutzke, 1997). Similarly, in Atlantic salmon (*Salmo salar*), there is a window of time when predator recognition is heightened (Hawkins et al., 2008). Therefore, it would be important for future studies to examine behavior of cryo-derived individuals at multiple time points to better understand the ontogeny of these responses. Similarities in behavior between cryo-derived and control tadpoles could be encouraging for reintroduction programs, although the lack of any predator avoidance behavior in both groups could signal to conservation managers that releasing captive-bred tadpoles, regardless of treatment, could result in lower survivorship overall.

One thing to note when considering the effects of cryopreservation on the growth, development, and behavior of larval and juvenile amphibians is that these characteristics are known to be plastic and are shaped by the environmental conditions they experience. For example, changes in temperature, food availability, and predation threats can lead to differences in tadpole morphology (Carabio et al., 2017; Touchon and Warkentin 2008, 2011), tadpole behavior (Barnett and Richardson, 2002; Jara et al., 2019; Kurali et al., 2018), and larval stage duration (Barnett and Richardson, 2002; Goldstein et al., 2017; Gomez-Mestre et al., 2010; Vonesh and Warkentin, 2006). These variations in larval environment can have carry-over effects on the following life stage, resulting in variations in metamorphs and juvenile frogs (Goater, 1994; Gomez-Mestre et al., 2010; Hagman et al., 2009; John-Alder and Morin, 1990; Tejedo et al., 2000). Given these effects, it is possible that alternations of the environmental conditions during the larval stage can be employed to mitigate the negative effects of cryopreservation. However, phenotypic plasticity in amphibians can be species-specific and time-sensitive, with limited windows during development where an individual is more susceptible to change (Leips and Travis, 1994). Consequently, further studies are needed to determine how these methods can be applied to increase the fitness and survivorship of cryo-derived individuals that are intended for release.

In conclusion, although gamete cryopreservation has the potential to support conservation efforts for endangered species in theory (Lermen et al., 2009), to be a viable tool for wildlife conservation, it must also produce fit and healthy individuals in practice. Life history characteristics, such as morphology and behavior, are seldom measured or considered during reintroductions, and as a result, it is largely unknown if techniques used in captivity are producing individuals of good quality (Mendelson and Altig, 2016). Our study focuses on how the use of cryopreserved gametes in breeding influences key characteristics that can determine the growth and performance of the offspring produced and therefore the success of reintroduction programs. We show that although cryopreservation has often been proposed as a novel and promising way of contributing to wildlife conservation, more detailed examinations are needed to understand and maximize its effectiveness as a conservation tool.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gecco.2019.e00809.

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